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and

SILPAKORN UNIVERSITY

PREFACE

This book, as far as we know, marks the first publication of a collection of scientific papers prepared by USAID Science Research Award Grantees. Certainly, it is the first publication of a collection prepared by Thai grantees. As such, we think it is a notable milestone.

The 45 papers included in the document were prepared for presentation at a Conference of Thai USAID science research award grantees who participated in a Conference held in Nakorn Pathom, Thailand, in July 1987. Although, at the Conference, the presentations were grouped by area of scientific emphasis, in this document it has been decided to group them by the particular USAID funding award program under which they were supported (i.e. PSTC, CDR, or BOSTID) and, within those groupings, to present them chronologically (earlier grants being presented before later grants).

We gratefully acknowledge the active involvement and support of those various officials of the Ministry of Science, Technology and Energy (particularly Mr. Sajja Boonthittanont, Ms. Anocha Noodharmcho and Ms. Thanarak Khateepacanond and his colleagues), of Silpakorn University (particularly Dr. Vivat Chantarapornchai), and of USAID (particularly Dr. Jazoon Kumnuanta, Dr. Gordon Hiebert, Mrs. Wannee Vardhanabhuti and Ms. Busaba Tejagupta) who worked so hard to make the Conference, and this publication, possible. We also would like to express our appreciation for the assistance in editing this document provided by Ms. Lara Howley.

We know that the publication of this book will provide clear and convincing evidence of the wide-ranging and excellent capabilities of Thai scientists. We are hopeful and confident that its publication will also help us to achieve one of the major objectives of the USAID science research award programs - that of stimulating even more new and innovative research on problems confronting developing countries.

The Editors

FOREWORD

We are very pleased to have this opportunity to express some thoughts about the papers included in this volume. These papers discuss projects being carried out by Thai scientists participating in the MOSTE/USAID "Fifth Cycle Commemorative Conference of USAID Science Award Grantees", which is being held to commemorate the 60th Birthday of His Majesty King Bhumibol Adulyadej.

Over the more than 36 years of association with Thailand, USAID has co-operated with Thai agencies and organizations in literally hundreds of useful and important development efforts. One of the efforts we can be most proud of is the currently ongoing "Program of Cooperation in Science and Technology". One of the most important elements of that program, we believe, is the worldwide competitive science research grants programs. These programs - the Program of Science and Technology Cooperation, the U.S.-Israel Cooperative Development Research Program, and the BOSTID Grants Program - are different and special. They are unlike any other programs mounted by AID in that financial support from these programs is made available not primarily on the basis of need, but rather, on the basis of potential for making a real contribution toward solving real problems facing the developing world. Support is provided not only because it is needed, but because the grantee has convincingly demonstrated that he or she has greater potential for making a contribution than the literally scores of other scientists who are seeking the grants.

Thailand has done spectacularly well in the worldwide competition for grants under these programs. More than 20% of all the grants made available around the world have been won by Thai scientists. This accomplishment says much for the quality of Thai science and Thai scientists.

The research project reports included in this volume provide excellent testimony to the quality of Thai science, an to its relevance to problems facing the developing world. They demonstrate conclusively, we believe, that Thai science has come of age, that "world class" science is being carried out in Thailand. We congratulate and commend the contributors.

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PART I

PROGRAM IN SCIENCE AND TECHNOLOGY COOPERATION

(PSTC)

FY 1983

"COMPARISON OF TWO IN VITRO TECHNIQUES FOR CULTURE OF PLASMODIUM FALCIPARUM" (3H-33)

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The Malaria Center, Region II, Chiang Mai, under the CDC Department, Ministry of Public Health, Thailand, in collaboration with the Department of Parasitology, Faculty of Medicine, Chiang Mai University established a malaria culture laboratory two years ago. One of the objectives in setting up this laboratory was to provide malaria-slide antigens to the Center, the Department, and the Malaria Division to be used for carrying out the Indirect Fluorescent Antibody Test (IFAT). IFAT is used in detecting the anti-plasmodium antibodies for monitoring and evaluating malaria control measures. Mass production of the slides was periodically undertaken at the laboratory from erythrocytic stages of malaria parasites obtained from the culture. Although there are considerable difficulties in comparing the methods used to culture the parasites from different laboratories, an attempt was made to compare multiplication rates obtained by a small-scale culture with those obtained by large-scale culture methods.

Three methods of cultivation were employed. One, a candle-jar method (Trager and Jensen, 1976) using a Petri dish of 35 mm diameter. The parasites, with a starting parasitaemia rate of about 0.12%, were cultured in a thin static layer of human red blood cells of 5% suspension with a total volume of 1.5 ml. Two, a large-scale method, as described by Siddiqui and Plamer (1981) was carried out by using 125-ml Erlenmeyer flask. Plasmodium falciparum isolates with a starting parasitaemia rate of about 1%, were grown in a thin settled layer of human erythrocytes. The flask contained 0.5 ml packed cells, 8.5 ml of complete RPMI-1640 medium, and 1 ml of human serum. The flask was supplied with a mixture of 2% O_2, 8% CO_2, and 90% N_2 at a rate of 10 ml per minute. The third method was similar to the second one except that 2% hematocrit was used and the flask was automatically rotated 15 minutes every four hours.

Over 150 malarial infected blood specimens were obtained from the Malaria Clinics of the Malaria Center, Region II, Chiang Mai province and from various provinces of the Malaria Zones under the supervision of the Malaria Center, Region II. A few infected blood specimens were obtained from the University Hospital (Maharaj Nakorn Chiang Mai) and from field collections. At the present time 18 strains are considered as well-established and culture-adapted isolates. For this presentation, nine isolates namely: CM-1 (Chiang Mai), CR-4 (Chiang Rai), IN-11 and LN-23 (Lamphun), MS-7 (Mai Hong Son), PB-4, PB-7 (Petchaboon), PL-22

(Pitsanuloke), and PR-2 (Prae) were used for comparative studies. As far as the establishment of a culture-adapted isolates was concerned, it appeared that the modified Siddiqui and Palmer method was superior to the candle jar method. In the former method, a large volume of completed RPMI-1640 medium and a continuous flowing gas mixture were used and the flask was shaken every four hours, whereas in the latter method, a smaller volume of the medium was used, a gaseous condition was obtained from smoke from a candle, and the red blood cells were static. The establishment of new isolates by the classical Siddiqui and Palmer technique was found to be unpredictable.

To compare the percentage of parasitaemia obtained by each method, percentiles of parasitaemia were calculated from counting 2000 red blood cells. Triplicate counts were performed by candle jar method and duplicate counts by large-scale methods. The percentages of parasitaemia obtained from the candle jar ranged from 2.42 (PR-2) to 15.13 (PB-7) with an average of 10.32. In the classical Siddiqui and Palmer method, parasitaemia ranged from 2.38 (PR-2) to 5.53 (PB-4) with an average of 3.76. In the modified Siddiqui and Plamer method, it ranged from 7.90 (PR-2) to 12.98 (PB-4) with an average of 9.98. It appeared that the amount of parasitaemia obtained from the candle jar and the modified Siddiqui and Plamer methods were not significantly different; however, it was slightly lower in the classical Siddiqui and Palmer method.

Multiplication rates (Final parasitaemia/Initial parasitaemia) of each isolate obtained from each culture method were also compared. It was found that the highest multiplication rate was obtained by the candle jar method with an average of 9.88, ranging from 4.92 (PR-2) to 12.30 (PB-7). Averages of multiplication rates of 3.15, ranging from 2.81 (PR-2) to 3.60 (PB-4); and 1.01 ranging from 1.54 (CR-4) to 2.35 (PB-4) were obtained by the modified and classical Siddiqui and Palmer methods, respectively.

From a known final parasitaemia rate of each isolate obtained by each culture method, a total yield of infected red blood cells could be calculated as per plate or flask. The results showed that the yields of infected red blood cells were more than 2.68 times, by the modified method: and 2.52 times, by the classical Siddiqui and Palmer methods than the yield of the candle jar method. The amount of parasitaemia obtained from large-scale methods was lower, but a larger volume of red blood cells was used. Therefore, this would reflect a higher yield of parasitized cells.

For daily routine maintenance of culture-adapted isolates in our laboratory, it was concluded that the candle jar method was the most convenient and practical technique as compared to large-scale culture methods. The large-scale culture methods required larger amount of medium used and larger incubating space. Transferring the medium was also a laborious task. In addition, maintaining parasites was 4.5 times more expensive in the large-scale culture methods than in the candle jar method.

Table 1 Comparison of expenses of material used and labor costs for cultivation of *Plasmodium falciparum* using three different culture techniques.

ITEMS	EXPENSE (IN BAHT)		
	CANDLE JAR METHODS	CLASSICAL SIDDIQUI METHOD	MODIFIED SIDDIQUI METHOD
Material used			
1. Glassware	10.00 (Culture Dish, Disposable)	47.00 (Flask, 125 ml; Resterilizing)	47.00 (Flask, 125 ml; Resterilizing)
2. Complete RPMI Medium	1.00 (1.5 ml)	5.00 (10 ml)	5.00 (10 ml)
3. Red Blood Cells	1.00 (0.075 ml)	6.00 (0.5 ml)	6.00 (0.5 ml)
4. Gas Mixture	1.00 (Candle)	13.00 (10 ml/min)	13.00 (10 ml/min)
5. Other	10500 (Dessicators)	-	39500 (Shaker)
Labor Cost			
Scientist	4.00 (10 minutes)	6.00 (15 minutes)	6.00 (15 minutes)
Total	17.00	77.00	77.00

Table 2 Summary of a Comparison of Small-Scale and Large-Scale Culture Techniques for the Culture of *Plasmodium falciparum*

	Items	Candle Jar Method	Classical Siddiqui Method	Modified Siddiqui Method
1.	Parasitaemia	10.32	3.76	9.98
2.	Multiplication Rate	9.88	1.91	3.15
3.	Yield	1.00	1.52	2.68
4.	Expense	1.00	4.50	4.50
5.	Establishment of New Isolates	Fair	?	Good
6.	Maintenance of Established Isolates	Excellent	?	Good

FY 1983

"SOIL SALINITY CONTROL FOR THE GROWTH OF PLANTS WHICH CAN SERVE AS AN ENERGY SOURCE" (3A-02) : A FIELD EXPERIMENT ON RECLAIMING SALINIZED - MARINE SOIL FOR THE GROWTH OF PHYSIC-NUT TREES

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ABSTRACT

A field experiment was conducted to compare methods for controlling the salinity of heavily-salinized-marine soil to prevent further spread of salinization to nearby cultivated land in the coastal region, and to determine if the soil would be suitable for growing physic-nut trees. The experiment included a determination of properties of problem - soil and deep-well water, and an assessment of the effects of leaching after adding rice husks and gypsum on the E.C. values of both the soil and the leachate, and on infiltration and seed yields. It was found that the addition of rice husks and gypsum in a 1:2 ratio decreased the E.C. values of the amended soil. The EC_d - leaching curve increased steeply initially, but then reached a plateau. Addition of rice husks and gypsum to the field soil before leaching tended to improve infiltration but seemed to reduce seed yields of physic-nut trees.

INTRODUCTION

Salinized-marine soil in the coastal cropland is a consequence of man's excessive utilization of water resources in nearby areas. A case in point is salt-farming in Thailand's Smut Songkram Province, whereby paddy fields are flooded with sea-water, allowing salt precipitation on the surface of the soil through sun drying. As the production of raw salt on coastal land increases, the extent of salinized soil grows. The problem is aggravated by drought in the dry season and by waterlogging in the wet season. Information on the prevention of further spread of salinization in coastal land by salinity-control procedure is limited. Beyce (1972), in his work on reclaiming peat soils in Turkey by continuous and intermittent ponding and sprinkling, reported that a depth of about 1.5 m of water had to be leached through a 1-m deep profile to remove 70% of the soluble salts. Prichard et al. (1985) found that a reclamation of saline, organic soils in the Sacramento-San Joaquin Delta of California could be accomplished by both sprinkling and continuously ponding water on the soil surface, especially sprinkling, through which 70% of the salt could be removed from the soil profile to a depth of 1.2 m. The experiment of Tyagi (1986) showed that the salinity of the soil in the area irrigated by the Bhakra Canal System could be controlled effectively by reducing the sub-surface irrigation return flow (IRF) that came in contact with the sub-surface sources of salts and reducing the later availability. Abrol and Bhumbla (1973) indicate that under conditions of poor

soil permeability, leaching of highly saline sodic soil with gypsum area would be best accomplished by continuous ponding. In the present study, a field experiment was conducted in a salinized in order to find a suitable method to limit soil salinity to a level, at which physic-nut trees could be grown. The specific objectives include:

1. An assessment of the effects of soil leaching with and without the addition of 3% rice husks plus 6% gypsum on changing the salinity of the soil and the leachate.

2. A determination of the effects of these treatments on infiltration and physic-nut yields.

MATERIALS AND METHODS

Determination of Soil and Water Properties

The site for this study was an area at a distance of 20 kms from the coast of Smut Songkram Province. Field samples of the top 30 cm of soil in the studied area were taken from twelve locations, at 20 m intervals, at the end of July 1983, and were then analyzed to determine soil pH, E.C. of a 1:5 soil paste at 25°C, moisture content, E.S.P., clay content, bulk density, and porosity. Ten samples of water, each taken weekly from a deep well, were analyzed for pH, E.C. at 25°C and SAR. Methods used for the analysis of soil and water were obtained from the Handbook of U.S. Salinity Laboratory (1954). Results of the analysis are shown in tables 1 and 2 respectively.

Field Experiment of Soil Salinity Control

A field experiment was conducted in late November 1985 with physis-nut trees grown in the marine soil, which was controlled with respect to salinity by two different treatments. (The field was installed with a 1.50 m-deep PVC-pipe subsurface drainage system, and a surface drip irrigation system). All treatments were replicated four times using the CRD method with equal plants. Each replication covered a plot size of 40 x 8 m^2. Methods used for controlling soil salinity were : leaching, and leaching with the addition of 3% rice husks and 6% gypsum (7 tons of rice husks + 14 tons of gypsum were mixed with the soil in each subplot by ploughing to a 0.5 m depth). Water was pumped from a deep well into each subplot in order to wash salts from the soil profile between the depth of 0 to 30 cm. The volume (Q) of water required to leach soil in each subplot, covering an area of 40 x 8 m^2, was calculated by a basic equation of dilution with the assumption that the salinity of deep-well water was negligible. The calculation formula was:

$$Q = \frac{E.C._w}{E.C._h} \left[\frac{E.C._f}{E.C._c} - 1\right] \cdot \theta_m \cdot \frac{P_b}{P_w} \cdot A \cdot h \cdot p$$

where $E.C._w$, $E.C._h$, $E.C._f$, $E.C._c$, θ_m, P_b, P_w, A, h, and p are the salinity of the deep-well water (table 2), salinity at an intermediate of low-salinity-hazard irrigation water (0.18) $mscm^{-1}$),

salinity at field condition (11 $mscm^{-1}$ from table 1), salinity at desired control level (8 $mscm^{-1}$), soil moisture content (0.4 from table 1), bulk density (1,180 kgm^{-3} from table 1), water density (1000 kgm^{-3}), area of each subplot (320 m^{-2}), depth of root zone (1.5 m), and average porosity (0.56), respectively. The calculated Q was about 200 m^3/subplot. Leaching was applied to each subplot four times at a rate of 50 m^3 per week. Samples of the topsoil were taken from every subplot a day after each leaching treatment for determining the soil moisture content, $E.C._f$ values, and pH. The $E.C._f$ values were plotted against leaching increment (fig. 1). In the meantime, the leachate was collected from each drain pipe to monitor $E.C._d$ values which were plotted against degree of leaching (fig. 2). Healthy physic-nut trees were then transplanted from pot soil to the field in late January 1986. There were 100 trees located 1.0 m apart in each subplot. All plants were irrigated twice a day at 7.30 a.m. and 5.30 p.m. by drip irrigation alternating with overhead sprinkling. Drip irrigation was applied in order to prevent growth depression caused by uptake of Na or Cl^- to toxic concentrations, osmotic effects, or restriction of the size of the root system (West et al. 1979), while overhead sprinkling was used to reduce effects of drip irrigation in saline soil or to prevent an uneven pattern of distribution of the salts present in the root zone (Goldberg et al. 1976). The depth of irrigation water was estimated from mean monthly temperature in the first six months (January to June 1986). The method used for this calculation was modified from the Blaney-Criddle formula, which is:

$$W = 0.14\ K\ .\ \sum_{m=1}^{6} \frac{t\ .\ P}{100}$$

Where W, K, T, and P are depth of irrigation water per day (mm/d), crop coefficient (0.75 from Anukulampi et al. 1981), mean monthly temperature in ^oF, and percentage of day-length for each month at 13.4 ^oN (Climatological data for the period 1951-1980, Meteorological Dept. 1982), respectively. Results of this calculation are shown in table 3.

Duplicate determinations of infiltration, soil moisture, E.C. of a 1:5 soil paste at 25 C, and the E.C. of the leachate at the same temperature were done every month during summer after the completion of daily irrigation at 7.30 a.m. Infiltration was determined by measuring the head of water placed in duplicate iron cylinders, each 33 cm in diameter and 40.5 cm high, which were pressed into the soil, at the distance of 20 m apart along N-S middle line passing through each subplot, to a depth of 30.5 cm below the surface. In the meantime average rate of rainfall and evaporation were investigated throughout the year from pan data. The variation of infiltration over six months in the hot season is illustrated in fig. 3. Differences in E.C. of the field soil and the leachate, as well as in infiltration among the two treatments were tested by using F-statistical method.

Ripe fruits of physic-nut trees in each plot were harvested and hulled manually in early December 1986. Seed yields were weighed after air drying for a few days (table 3). Input output variables in the controlled systems by two different methods were as formulated in table 3.

RESULTS AND DISCUSSION

The results of this study are presented in two parts. In the first part, properties of the salinized-marine soil and of the deep-well water at research site are described. The second part describes the results of the field experiment designed to control soil salinity and the resultant seed yields.

Properties of Salinized-Marine Soil

Table 1 Properties of Salinized-Marine Soil at the Research Site (n = 12)

Properties	Range	Mean	s	Unit
pH	7.4-8.0	7.7	0.2	
E.C. (1:5) at 25°C	7.0-20.0	11.8	3.4	$mscm^{-1}$
Moisture Content (Oven dry basis)	33.6-50.5	41.4	5.1	%
ESP	> 50			100gsoil/l
Clay	43-63	56	5.9	%
Bulk density	1140-1250	1178	36.7	kgm^{-3}
Porosity	54.2-57.8	56.1	1.2	%

Data shown in table 1 indicates that the soil had a saline-alkali nature, and a high clay content. The ESP was more than 50, which reflects an adversely high concentration of sodium ions resulting in damage soil structure. Rimwanich and Suebsiri (1984) explained that soil structure was destroyed by high concentration of monovalent sodium ions replacing divalent calcium and magnesium in the exchange complex; the substitution of sodium ion for calcium and magnesium ions in the soil solution caused a reduction of premeability and hydraulic conductivity, with the result that when rain came, the salt was uneasily washed out.

Properties of Deep-Well Water

Table 2 Properties of Deep-Well Water (n = 10)

Properties	Range	Mean	s	Unit
pH	8.0-8.1	8.0	0.07	
E.C. at 25°C	0.74-0.81	0.78	0.02	$mscm^{-1}$
Cl^-	2.38-2.60	2.43	0.06	mel^{-1}
SAR	3.35-3.43	3.39	0.02	me

Data shown in table 2 indicates that the deep-well water was only slightly saline. This implies that it is suitable to be used as a substitute for rainwater during a period of scarce rainfall.

Effects on Changing Salinity of the Field Soil and the Leachate

The $E.C._f$ values of the soil in both treatments declined as the leaching treatment increased at the rate of 0.48 $mscm^{-1}$/week for plot soil without rice husks and gypsum as against a rate of 0.68 $mscm^{-1}$/week for plot soil containing such materials (fig. 1). The $E.C._d$ of the leachate for both treatments increased steeply with initial leaching, and then both reached plateaus with increasing leaching treatments (fig. 2). Figure 1 shows that soil mixed with rice husks and gypsum reduced soil salinity more than leaching alone. Soil leached after applying these materials in the ratio 1:2 had E.C. values about 20% less than those of soil without any additions (table 3).

Table 3 Depth of Irrigation, Rate of Rainfall, Soil Moisture Content by Volume, Infiltration Rate, Evaporation Rate, Salinity of Soil and the Leachate, and the Physic-nut Yields in Soil Salinity Control System.

Treatment	Irrig. W mm/d	Av.rainfall $\bar{P}$ mm/d $n_P=12$ $s_P=5.9$	Av.Infilt. ac 30.5 cm $\bar{I}$ mm/d $n_I=24$	Av.Evap. $\overline{ET}$ mm/d $n_E=12$ $s_E=2.3$	Soil Moist θ_v % v/v $n_\theta=24$	$\overline{E.C._f}$ (1:5) at 25°C mscm^{-1} $n_S=24$	$\overline{E.C._d}$ at 25°C mscm^{-1} $n_d=24$	Seed yields kg/acre
Leaching	4.3	15.2	6.8 $s_I^*=1.6$	7.1	30.0 $s_\theta^*=6.9$	7.30 $s_S^*=1.6$	17.9 $s_d^*=3.7$	28.41
Leaching + 3% Rice Husks + 6% Gypsum	4.3	15.2	7.8 $s_I^{**}=2.7$	7.1	33.0 $s_\theta^{**}=8.2$	5.90 $s_S^{**}=1.3$	14.7 $s_d^{**}=4.2$	25.08

s^*, s^{**} Standard deviation for treatments 1 and 2 respectively.

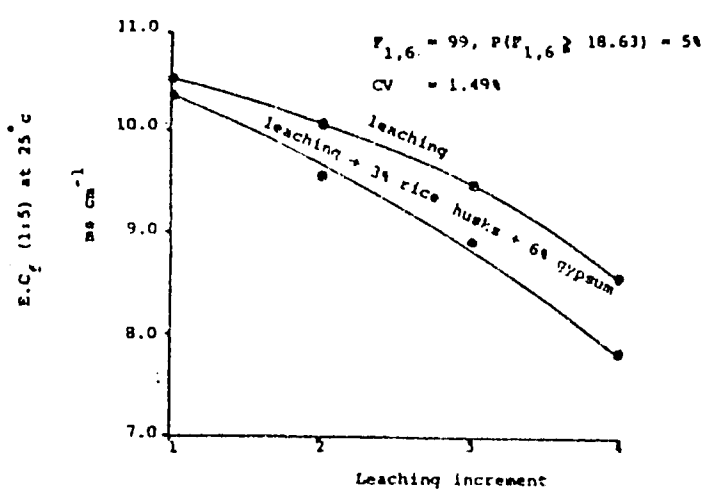

Figure 1 Change of $E.C_f$ with leaching increment (50 m^3 increments)

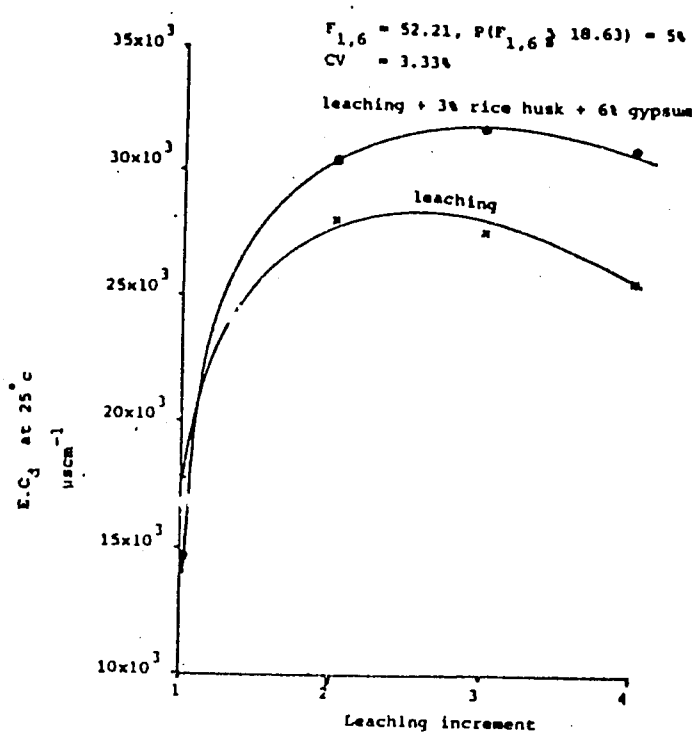

Figure 2 Change of $E.C_d$ with leaching increment

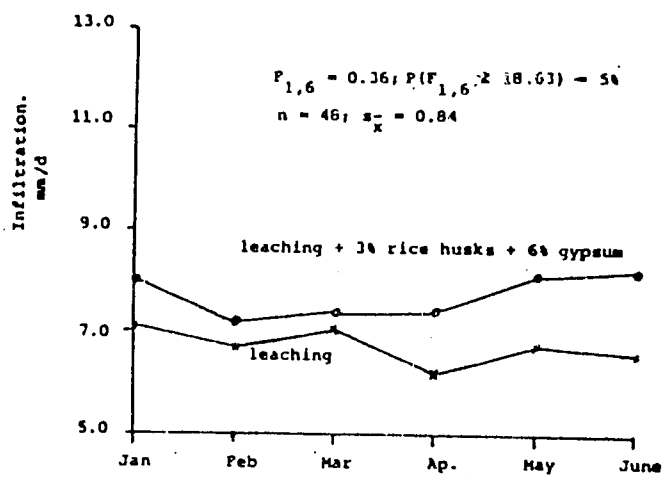

Figure 3 Variation of infiltration over six months

Effects on Infiltration and Yields

Soil treated by leaching alone had 15% less infiltration than that of soil treated with leaching plus 3% rice husks and 6% gypsum (table 3). However plants treated by leaching alone produced 12% higher seed yields than did plants in the other treatment (table 3).

According to figures 1-3, they imply that leaching with the addition of rice husks and gypsum increased the efficiency of salt leaching by improving the infiltration rate. Allison (1973) reasoned

that organic matter mixed with the soil aided greatly to increase infiltration by providing better aggregation and structure, and consequently lower bulk density and increased ease of water movement. Abrol and Bhumbla (1973) reported that the addition of gypsum resulted in greater salt leaching due to the changes in pore size distribution in the clay soil. In other words the addition of gypsum to a clay soil and the subsequent replace- ment of sodium by calcium in the exchange sites improve the air-filled pore space which increases the hydraulic conductivity (Bridge and Tunny, 1973), resulting in a decrease in the time required for reclamation and also a reduction in the period of contact between soil and water (Muhammed et al, 1969).

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AN EXPERIMENTAL STUDY ON THE USE OF PHYSIC-NUT OIL AS A FUEL SUBSTITUTE FOR SMALL DIESEL ENGINES[1/]

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ABSTRACT

A bench-dynamometer test of a 7.5 HP diesel engine was carried out using physic-nut (or Sabu Dam) oil as fuel. Engine performance parameters at varying loads were evaluated for two constant engine speeds of 1200 and 1600 rpm, and a comparison was made between the performance of physic-nut oil and that of diesel fuel. In general, very similar performance characteristic curves were obtained for the two fuels, with only slightly lower (1-2%) thermal efficiency being recorded when the engine was run on physic-nut oil. Thus, the use of physic-nut oil as a direct substitute for diesel in small engines appears to be technically feasible.

INTRODUCTION

In the wake of the world energy crisis in the 1970's, much interest has been aroused in the use of indigenous, biomass-derived engine fuels as alternatives to imported petroleum fuels in Thailand. Among the many alternatives considered are a variety of vegetable oils which may be used either singly or in combination (blended) with diesel oil in diesel engines and particularly in small farm engines. As the Thai nation strives to attain a higher level of farm mechanization in the face of falling agricultural commodity prices worldwide, this biomass option becomes increasingly more attractive.

The types of vegetable oils which have been proposed as substitutes for diesel fuel include coconut oil, corn oil, rubber seed oil, physicnut (Jatropha curcas) oil, caster oil, soybean oil and others (1). These oils generally possess fuel properties similar to diesel but are more easily processed. Some of the oil types have an added feature the crop species is tolerant to poor soil and climatic conditions. A case in point is the physic nut, which has been shown to be not only drought resistant, since the crop is commonly found in the drought-stricken area of the Northeast, but also tolerant to saline soils (2).

Although some experimental studies on the use of vegetable oils such as rubber seed, corn and physic nut have already been reported (3, 4, 5), further investigations on the various aspects surrounding such use have yet to be conducted in order to establish a broad data base for drawing definitive conclusions. In the case of physic nut oil (4, 5), for instance, experimental results have been reported for only one load. This report discusses the results of further studies on the use of physic-nut oil as a direct substitute for diesel fuel. In this study,

bench dynamometer tests were carried out to determine the performance of physic-nut oil as a fuel for a 7.5 hp/2200 RPM diesel engine at varying loads.

Fuel Characteristics

The physic-nut oil used for this experiment was extracted from physic nuts grown at the Samut Songkram Research Site of the USAID-sponsored project on "Soil Salinity Control For The Growth Of Plants Which Can Serve As An Energy Source", of which the present study is an integral part. The nut itself was blackish in color with a thin shell, measuring approximately 1.8 cm in length and 1 cm in diameter. When crushed and subsequently compressed in a 10-cm diameter cylinder using a simple hydraulic jack of 10 ton capacity, yellowish liquid oil was extracted at a rate of 0.221 litres of oil/kilogram of physic nut (or 20.1% oil on a mass basis). The major fuel characteristics such as viscosity, specific gravity and heating value, and sulfur content were then obtained through standard measuring techniques and are shown against those of diesel, rubber seed oil and physic nut-oil from the Agricultural Engineering Division, Department of Agriculture (Table 1).

It is observed from Table 1 that the properties of physic-nut oil obtained from this study are comparable to those of oil obtained by the Agricultural Engineering Division, although the viscosity values differ. However, since viscosity decreases with increasing temperature in liquids, the viscosity values of the two studies are actually closer than indicated. When compared to rubber seed oil, the property values are in close agreement. However, when compared to diesel the physic-nut oil about 8% heavier and the heating value about 8% lower, while the viscosity is about 10 times higher, representing the most significant difference between the two fuels. The sulfur content of the physic-nut oil is lower than for diesel oil.

Experimental Apparatus and Procedures

Bench dynamometer tests were carried out to determine the performance of physic-nut oil as a substitute fuel for a Yanmar TA80L diesel engine (Figure 3). The engine characteristics are shown below.

Type :	Horizontal 4 stroke
Number of cylinders :	1
Bore X stroke :	84 mm x 88 mm
Displacement volume :	0.487 litres
Continuous power rating :	7.5 HP/2200 RPM
Maximum power rating :	8.5 HP/2400 RPM
Compression ratio :	22

The engine was used to drive an EA-7C air-cooled eddy current electrodynamometer with a maximum power-absorbing capacity of 5 kW. Braking-force control of the dynamometer was through a D.C. power controller. The shaft-torque was determined by force balance using a spring balance. The tachometer was of a non-slip, directly - connected, power--generator type.

Fuel flow rate was measured by timing the volume of fuel consumed from a 100 ml. calibrated glass tube. Air flow rate was determined using a standard nozzle-plenum installation, whereby the resulting pressure differential was measured using an inclined manometer.

Temperature measurements were performed using K type thermocouples with a multi-input digital indicator.

Exhaust gas conditions (O_2 and temperature) were measured using a Neutronics combustion analyzer (Figure 6) with digital display and printout.

The overall measurement arrangement is shown in Figure 1.

Due to the extremely limited quantity of physic-nut oil confined to run only at two controlled speed of 1200 and 1600 rpm, while loads in the range of 0.5 to 4.8 kW were applied through the dynamometer power controller. Engine injection timing was set for optimum when using diesel fuel only.

RESULTS AND DISCUSSIONS

The main performance parameter evaluated in the bench dynamometer tests was the thermal efficiency, which was calculated using the relation:

$$n_{th} = \frac{3.6}{BSFC \times HHV} \times 100\% \qquad (1)$$

where 3.6 is a conversion factor (3.6 MJ is equivalent to 1 kW-hr), BSFC is the brake specific fuel consumption (kg/kW-hr) and HHV is the higher heating value of the fuel in MJ/kg. Engine exhaust temperature at the discharge value exit was also measured during the experimental runs. Other parameters measured include the air/fuel ratio and the O_2 content of the exhaust gas.

Figure 2 shows the thermal efficiency at varying loads for the two engine speeds of 1200 and 1600 rpm. It is clear that higher thermal efficiency was obtained when the engine was run on diesel, the difference being approximately one to two percentage points. The drop in efficiency could be attributed to the fact that injection timing had not been adjusted to optimum for physic-nut oil and that the physic-nut oil is much more viscous than diesel. Nevertheless, the engine exhibited similar efficiency-load relations for the two fuels.

Figure 3 shows the comparative brake-specific fuel consumption (BSFC) of the engine at varying loads. As expected, the BSFC for physic-nut soil is higher, since its heating value is lower, than for diesel. However, the difference in BSFC for the tow fuels is only about 15% (or 7% in terms of volume), which does not warrant a larger fuel tank for the physic-nut oil. Again the BSFC-kW relations for the two fuels are similar.

Engine exhaust temperatures at varying loads are shown in Figure 4, which indicates no significant difference between the two fuels.

Figure 5 shows the air/fuel ratio (kg/kg) for the two fuels. It is clear the higher air/fuel ratio was needed for diesel combustion at all load conditions because of the fuel's lower density.

A comparison of the O_2 content in the exhaust gas for the two fuels is shown in Figure 6, which indicates that a slightly higher O_2 content was observed for physic-nut oil than for diesel. Again, this could be attributed to injection timing which was set a optimum for diesel and not for physic-nut oil.

CONCLUSIONS AND RECOMMENDATIONS

Oil derived from the physic-nut grown on saline soil appeared to possess very similar fuel characteristics to those obtained from other sources. On the basis of the foregoing discussion, it appears that use of physic-nut as a direct substitute for diesel in a small diesel engine is technically feasible since there were no major differences in the performance parameters measured. However this conclusion is only suggestive and by no means definitive, since the available experimental evidence is extremely limited. Future experiments on the subject should cover a wider range of engine speeds and should include long-term observations of the effects on engine parts as well as detailed investigations on engine emissions. To compare the performance on an equal basis, injection timing for physic-nut oil should also be set for optimum operation.

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Table 1

Comparison of Fuel Characteristics

Fuel Type	Viscosity (Centipoise)	Specific Gravity	Heating Value (kJ/kg)	Sulfar Contnet (%)
Physic nut oil (present study)*	29.5 (40°C)	0.910	39,193	0.0
Physic nut oil (4, 5)	40.4 (31°C)	0.919	36,647	0.13
Rubberseed oil (3)	34.0 (40°C)	0.914	39,224	NA
Diesel	2.8 (40°C)	0.845	42,500	$<$ 1.2

* Test conducted in collaboration with the Petroleum Authority of Thailand (PTT)

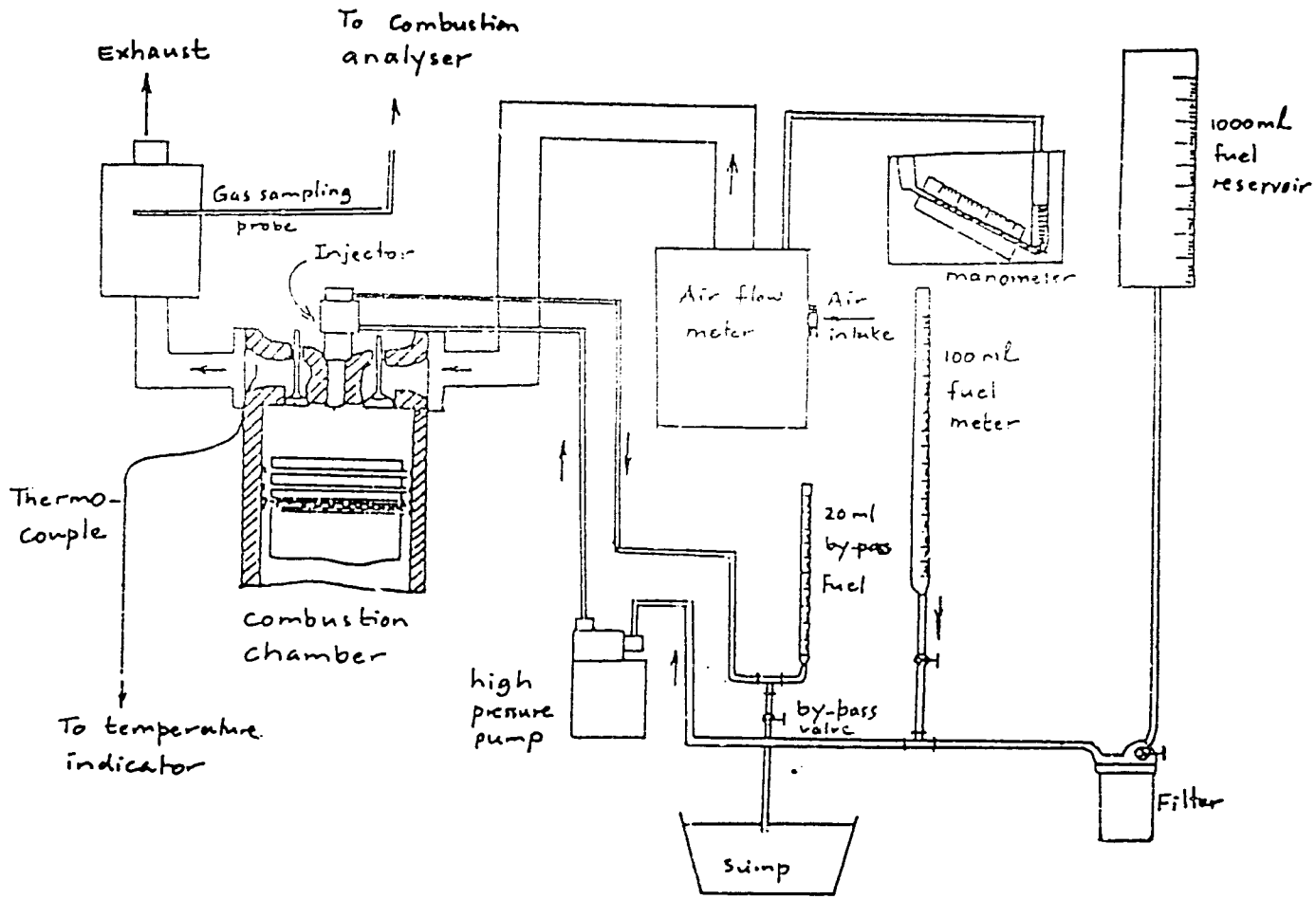

Figure 1 : Overall measurement set-up (excluding the dynamometer)

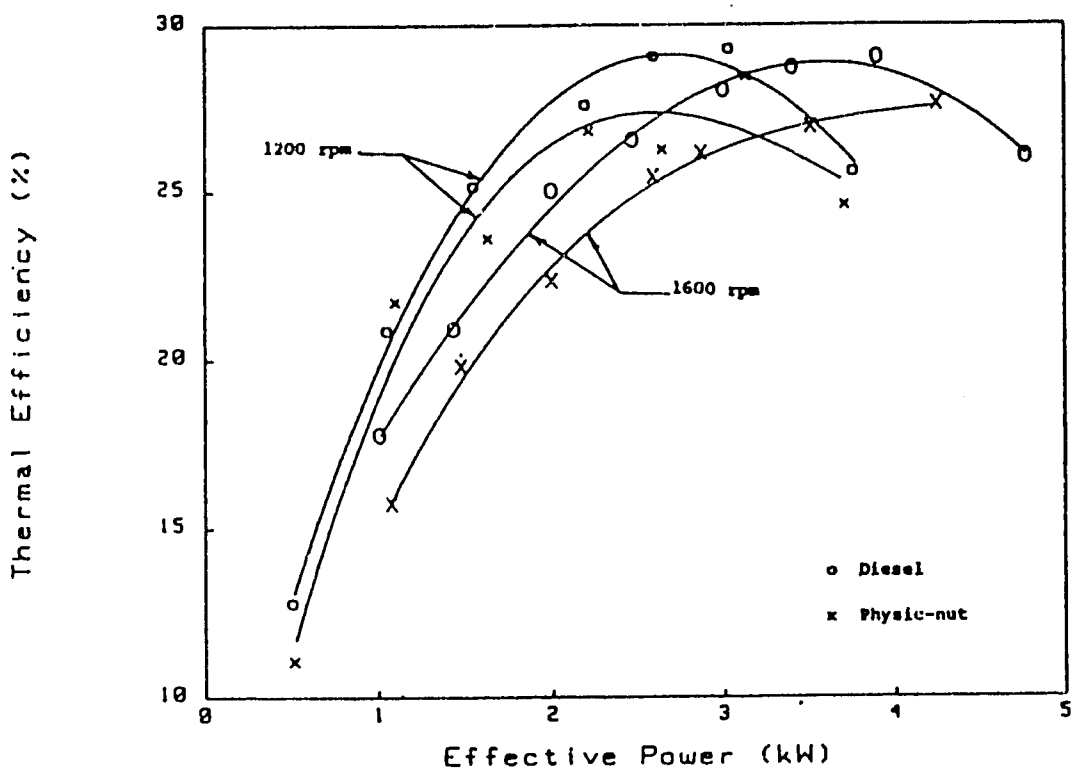

Figure 2 : Thermal efficiency at varying loads.

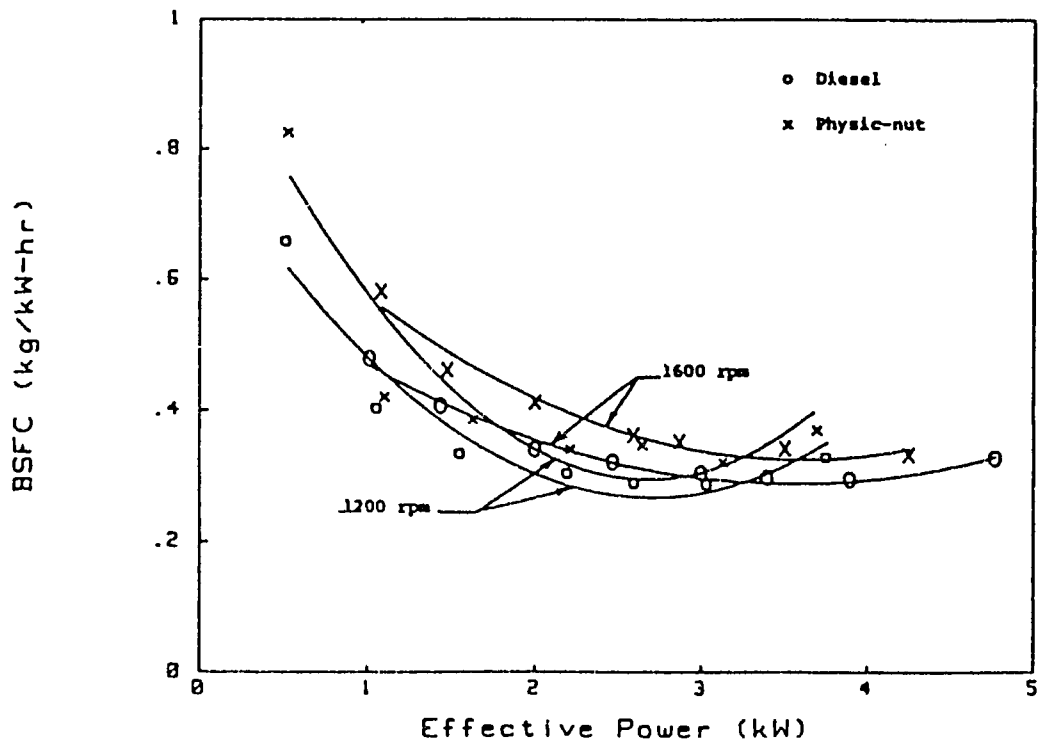

Figure 3 : Brake specific fuel consumption at varying loads.

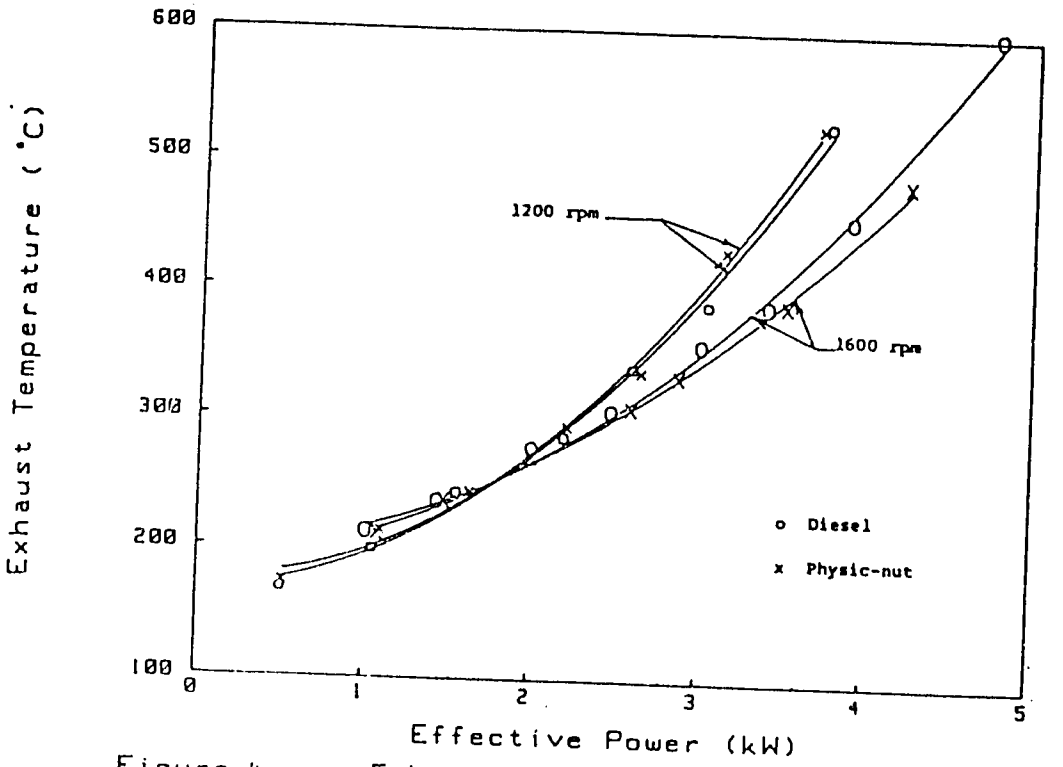

Figure 4 : Exhaust temperature at varying loads.

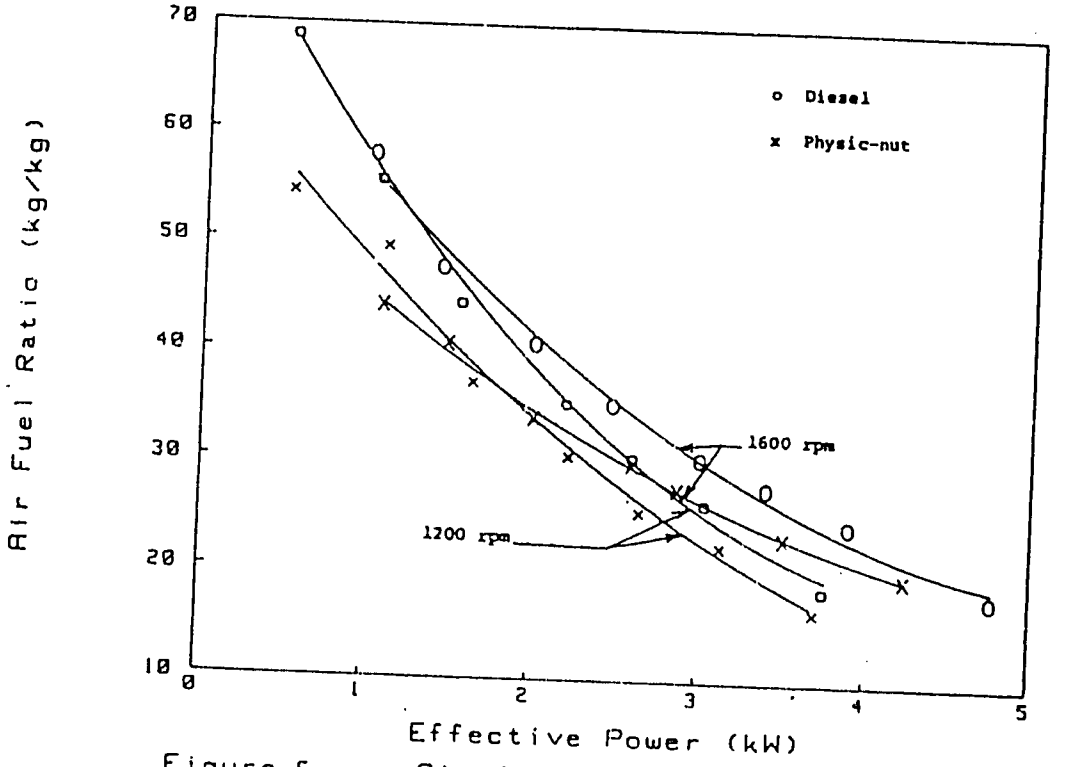

Figure 5 : Air fuel ratio at varying loads.

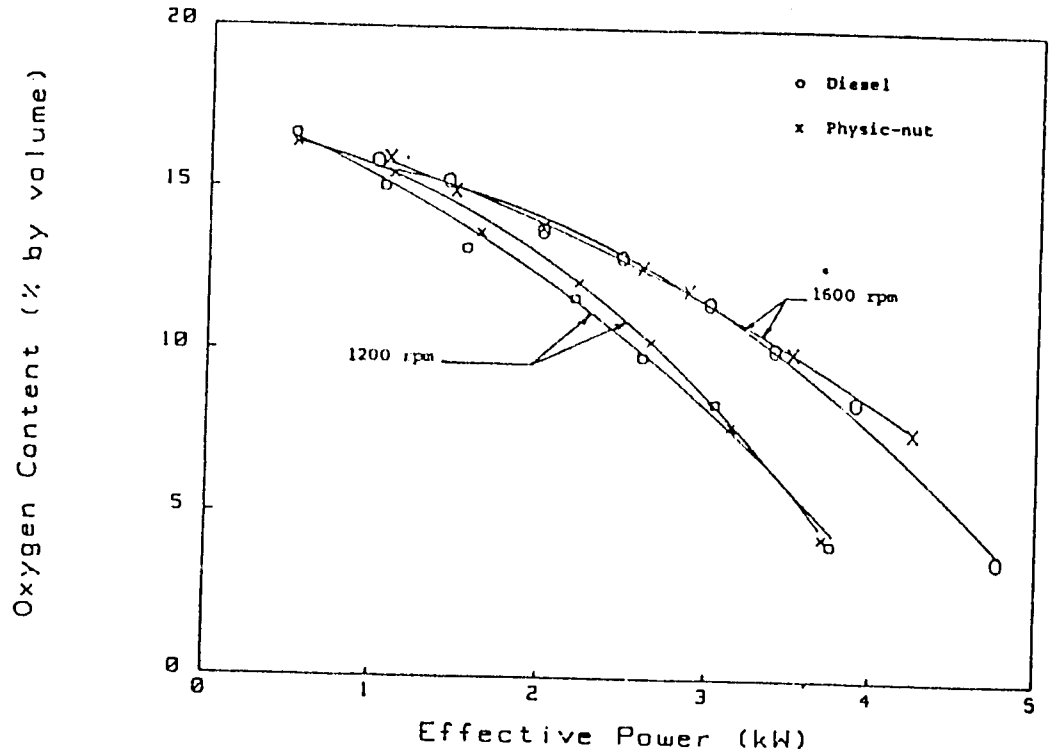

Figure 6 : Oxygen content in flue gas.

FY 1983

"NEW VARIETIES OF RICE FOR SALINE AND ACID SOIL THROUGH TISSUE CULTURE" (2A-15)

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ABSTRACT

An attempt was made to find salt and acid tolerant rice varieties through elevated frequency of somatic mutation occurring in tissue culture.

Selection in vitro was made by incorporation of 0.5 to 4% NaCl to the culture media or by lowering the pH to 4.0 during regeneration of the plants from one-month old embryo calli. Only 0 to 0.5% of the calli were able to develop and continue to grow under stress, whereas 5 to 30% of the controls regenerated into complete plants. The survivors were grown in natural condition to maturity for further tests.

Salt tolerance screening was also done on the seedlings of plant regenerated in vitro with or without stress. Three to five-leaf seedlings were subjected to nutrient solution with 0.5% NaCl added for one month. Most of the seedlings died during the third and fourth weeks; if the number of the survived seedlings were more than 2% only the healthiest 2% were saved for growing.

Some seedlings of the selected plants showed definite increase in tolerance over those from the untreated ones. These still is need further selection in later generation to establish the salt or acid or acid tolerant lines which retain all other characteristics of the original varieties.

INTRODUCTION

Rice is the main agricultural product of Thailand. This is indicated by the fact that out of a total arable land area of 59 million acres, rice is grown in 24 million acres. As a result, Thailand produces over 17.8 million tons of rice annually, with 2.8 million tons left for export, which makes Thailand the second biggest rice exporter. This represents the largest income (over one billion dollars) for any single agricultural product. (Anonym., 1979 a, Anonym., 1979 b, Anonym., 1982).

However, Thailand is not without problems in rice production. A variety of problems result in low yield, i.e. the average yield in Thailand is 780 kilograms per acre, compared to the average yield of 1430 kilograms per acre in China, Indonesia and Malaysia, whereas the United States, Japan and Korea produce 2296 kilograms per acre. (Anonym., 1979b, Anonym., 1982). Some of the problems that causes the low yields

are : Irrigation, grower seed production, management practices, availability of suitable agricultural land.

From the problem listed above it may become apparent that there are several causes several plausible solutions. In some of these cases, the solutions must be applied concomitantly since the problems are interrelated. For example, the new high-performance varieties usually demand high fertilizer input and irrigation and are less tolerant to adverse conditions. This makes it impractical for farmers in poor soil area who depend solely on the monsoon rains and who may not have the financial resources to pay for fertilizers and pesticides, to plant these varities.

The present project intends to deal only with the two world wide problems related to saline soil and acid soil. According to Carter (1975) the world arable land subject to salt and drought stress is around 25 percent. And another 25 percent of soil is affected by high acidity which bring large amount of toxic metal into solution in the soil (Christiansen and Lewis, 1982).

The method employed in obtaining new varieties will be plant tissue culture selection which has already been successfully used to obtain salt tolerant cereal and other crop plants (Nabors, 1982). This technique is based on findings of several tissue culturists that the nuclei of the cells often change during culturing which results in increased frequency of variability in whole plants (Murashige and Nakano, 1967; Vajrabhaya and Vajrabhaya, 1974; Vajrabhaya, 1977; Heinz et a., 1977). The chances of mutations and segregations are normally slim but the tissue culture method deals with multibillion actively dividing cells in a limited space and time. It is believed that the resulting valuable variants when come through screening will include several which are really promising. There is an alternative method to increase rice production by liming the acid soil, or using intensive drainage system in saline soil, both of which are extremely expensive.

New varieties obtained by this method will also be valuable for future rice breeding program.

MATERIALS AND METHODS

Eight rice varieties, RD23, RD25, KDML, KTH, LPT, NMS, RD8 and NSPT (the last two being glutinous rices) were used in the experiments; these are among the recommended varieties of the Rice Division, Department of Agriculture.

Calli were initiated from mature embryos cultured in Linsmaier and Skoog's (1965) basic salts and vitamins with 3% sucrose, 0.8% agar, 1 ppm 2,4-D and supplemented with either 0.3 ppm kinetin or 10% V/V coconut water.

Regeneration medium is based on White's (1943) macro-elements supplemented with Murashige and Skoog's (1962) micro-elements, 0.8% agar, and 10% V/V coconut water. One to three percent of NaCl was added for salt selection; or the pH was adjusted to 4.0 for acid selection medium.

Selection under natural condition was made on seedlings of the plants regenerated from callus under stress conditions, and some seedlings were from second generation of the selected parents. When the seedlings reached 2-leaf stage they were transplanted to water culture trays with a modified WP nutrients formula (Bentley, 1959). When most of the seedlings attained 5-leaf stage in approximately 7 to 10 days, nutrients solution was drained off and replaced by a new solution with 0.5% NaCl. Electrical conductivity of the solution at the beginning was around 9 to 10 mMhos/cm at 25°C and tended to rise when day temperature was high due to rapid evaporation and transpiration. Whenever the conductivity was 10 mMhos/cm or higher the level of nutrient was adjusted by adding tap water and if the conductivity was still lower than 10mMhos/cm a nutrient solution with 0.5% NaCl was then added. Every week the dead plants were removed and the solution was changed.

Termination of treatments was determined by the number of plants killed. When 90% or more plants treated died, which happened mostly during the third and the fourth week of treatment, all plants were removed from the trays and only the healthiest ones were selected for planting. In practice, 2% of the plants treated were saved and planted in sand culture for seed production.

RESULTS AND DISCUSSION

Salt Selection in vitro A total of 14,133 calli from over 50,000 embryos of seven varieties, namely, KDML, KTH, LPT, NMS, RD8, RD23, and RD25 were used in the treatment. Two-week old calli were cultured in the regeneration medium with 1.5% NaCl for four weeks. The results are shown in Table 1.

Table 1 Shoot formation of callus of seven rice varieties after treated with 1.5% NaCl for four weeks

VARIETY	No of callus treated	Callus survival after 4 weeks		Callus with shoot		Shoot	
		No.	%	No.	%	No.	%
KDML	2639	1597	60.52	0	0	0	0
KTH	690	396	57.39	0	0	0	0
LPT	3352	1633	48.72	4	0.12	17	0.51
NMS-4	1140	540	47.37	0	0	0	0
RD 8	2050	1022	49.58	1	0.05	2	0.10
RD 23	2138	951	44.48	1	0.05	4	0.19
RD 25	2124	994	46.80	1	0.05	1	0.05
ALL VARIETY	14133	7133	50.47	7	0.05	24	0.17

It is interesting to note that upto fifty percent of the calli treated still survive at the end of the fourth week. The living calli were transferred to the regeneration medium, many of them ceased to grow any further and died afterward. Some continued to grow and form green-spots but very few produced shoots.

The LPT is the best for regeneration in media with or without NaCl; four shoots were produced while others produced only one or none. The total number of calli of all varieties produced shoots were 7 from the total of 14,133 calli which was only 0.05% regeneration. Normally, the LPT has rate of regeneration from 15 to 30%, after being treated with salt only 4 out of 3352 calli had shoots which is 150 to 300 times different. However a total of 17 shoots were obtained from these four calli. Some varieties, RD8, RD23 and RD25, produced one shoot per 2,000 calli while KDML, KTH and NMS did not produce any shoot during this period.

The results of selection show variability in the response of calli to salt treatment at the cell level. However, it is interesting to note that the whole process of regeneration occurs after the treatment. This presents us with a problem that the whole plant obtained by this method would still be tolerant to salt like the cells in callus form or not. Further investigation is needed.

Acid Selection in vitro A total of 7115 calli of the some seven varieties as in salt selection were used for acid selection. Two-week old calli were cultured in the regeneration medium adjusted to pH 4.0 by buffer acetate for four weeks. The results are shown in Table 2.

Table 2 Shoot formation of callus of seven rice varieties after treated with pH 4.0 buffer acetate for four weeks.

VARIETY	No of callus treated	Callus survival after 4 weeks		Callus with shoot		Shoot	
		No.	%	No.	%	No.	%
KDML	1076	73	6.78	0	0	0	0
KTH	284	117	41.20	0	0	0	0
LPT	1840	205	11.14	14	0.76	14	0.76
NMS-4	948	49	5.17	2	0.21	2	0.21
RD 8	1151	152	13.21	0	0	0	0
RD 23	584	26	4.45	4	0.69	4	0.69
RD 25	1232	117	9.50	0	0	0	0
ALL VARIETY	7115	739	10.39	20	0.28	20	0.28

Only ten percent of calli survive until the end of the fourth week which is five times lower than those in salt treatment. During treatment most calli turned white or very pale yellow instead of the normal light yellow in the first two weeks. After transferring calli onto regeneration medium considerable number of calli recovered and formed greenspots and later produced shoots. In spite of high mortality during the treatment more shoots were formed than calli in the salt treatment. A total of 0.28% of the treated calli of all varieties initiated shoots. This figure is 5.6 times higher than in the salt treatment.

The LPT, again, is the variety that gives best regeneration whether they are in normal condition or in salt or acid stress conditions.
In this case, 14 out of 1840 calli produced shoots, which is 0.76% or 20 to 40 times less than in reference medium. The KDML and KTH calli still gave no regeneration in both salt and acid treatment during this period, these proved to be difficult varieties. The RD8 and RD25 gave no regeneration while they barely came through salt treatment, on the contrary, the NMS surprisingly produced two shoots in this acid treatment but never in salt treatment.

The acid selection reported here is a preliminary work following the established method for stabilizing the pH. One should be aware that acid soils in nature are of several types among which acid is the only common factor. Other components of the soil vary to a great extent according to soil formation and subsequent changes either by nature or by man. Deficiency of phosphorus and other nutrients are more prevalent which can possibly by corrected by adding phosphate fertilizer, but toxicity due to aluminum, iron or other ions at low pH are probably the most difficult to deal with. However, acid selection method should be the first step to be established before tackling more complex treatment.

Salt Selection in Natural Condition A total of 12,181 seedlings at four to five leaf stages were grown in a modified WP nutrient formula with 0.5% NaCl added. These comprise three varieties (KDML, RD23, and RD25) some of which are seedlings of the first or second generation of the regenerated plants and some are seedlings of the plants regenerated under salt stress.

Table 3 demonstrates survival of the plants each week. During the first week, most of the plants did not show any symptom of salt stress, only the weakest ones died during this period. The common salt injury symptom appears in the mature leaves from distal to proximal end. In severe cases, younger leaves show same symptom and finally the whole plant die. A dead plant is defined as the one in which the leafblades and leaf sheath have lost their green color altogether.

At the end of the second week 63.5% of the plants still survived, and some grew profusely. It is worth noting that the characteristics of some plants appeared to be fairly normal like the ones in the control.

Table 3 Survival of five-leaf stage seedlings of a regenerated plant (TC 1) treated with 0.5% NaCl in nutrient solution for four weeks

Variety : KDML		Electrical Conductivity : 9-10 m Mhos/cm							
Treatment No.	No. of Plant	Plant Surviving (%)							
		1st wk		2nd wk		3rd wk		4th wk	
		No.	%	No.	%	No.	%	No.	%
1	156	146	93.6	81	51.9	30	19.2	5	3.2
2	375	336	89.6	214	57.1	117	31.2	62	16.5
3	364	351	96.4	247	67.8	173	47.5	116	31.9
4	400	371	92.7	235	58.7	152	38.0	83	20.7
5	400	380	95.0	299	74.7	207	51.7	70	17.5
Total	1695	1584	93.4	1076	63.5	679	40.0	336	19.8

During the third and the fourth the weeks, the weak plants with fever symptom died leaving the ones with lesser symptom growing. At the end of the fourth week 336 plants (19.8%) were still living, these included the plants with both mild and severe symptoms. Only the healthiest plants were saved for further investigation, these were approximately two percent of the treated plant.

KDML is a variety selected for salt tolerance which has been recommended by the Department of Agriculture for 28 years to be grown in several locations in the country where soil salinity is high. Seedlings of the regenerated plants showed variability in tolerance. It is hoped that if favorable somatic mutations should occur during callus growth and development they can be screened effectively by the above method. Such variations may arise partly as a result of variation of environment during screening and some, may be a remote chance, from somatic variations. If there is no new gene occurring, only some reorganization or activation of certain genes will, by chance, lead to favorable characteristics. But, if there is any gene for salt tolerance should arise *de novo*, it will persist and await further recombination in later generations. In these cases the advantage of tissue culture becomes apparent and it will play a major role in plant improvement in the future.

Table 4 Survival of four-leaf stage seedlings of 21 regenerated plants treated with 0.5% NaCl in nutrient solution for four weeks.

Variety : RD 23 Electrical Conductivity : 9-10 m Mhos/cm

Treatment		No. of plant	Plant Surviving							
			1st wk		2nd wk		3rd wk		4th wk	
No.	Stock No.		No.	%	No.	%	No.	%	No.	%
1	R_1-Tc4	46	41	89.1	33	71.7	23	50.0	13	28.3
2	R_1-Tc6	150	150	100.0	99	66.0	[illegible]	37.3	4	2.7
3.	R_1-Tc8	45	29	64.4	29	64.4	0	0.0	0	0.0
4.	R_1-Tc9	200	107	53.5	7	3.5	4	2.0	0	0.0
5.	R_1-Tc10	100	48	48.0	3	3.0	1	1.0	0	0.0
6.	R_1-Tc11	225	176	78.2	20	8.9	9	4.0	0	0.0
7.	R_1-Tc12	100	86	86.0	4	4.0	0	0.0	0	0.0
8.	R_1-Tc16	379	379	100.0	208	54.9	71	18.7	0	0.0
9.	R_1-Tc17	375	256	68.3	45	12.0	8	2.1	0	0.0
10.	R_1-Tc18	398	398	100.0	369	92.7	264	66.3	5	1.3
11.	R_1-Tc22	416	416	100.0	283	68.0	37	8.9	2	0.5
12.	R_1-Tc23	600	569	94.8	437	72.8	175	29.9	98	16.3
13.	R_1-Tc24	250	220	80.0	145	58.0	49	19.6	16	6.4
14.	R_1-Tc25	225	222	98.7	188	83.6	69	30.7	32	14.2
15.	R_1-Tc26	150	126	84.0	74	49.3	41	27.3	29	19.3
16.	R_1-Tc27	75	75	100.0	4	5.3	0	0.0	0	0.0
17.	R_1-Tc29	300	282	94.0	125	41.7	2	0.7	2	0.7
18.	R_1-Tc30	40	39	97.5	26	65.0	0	0.0	0	0.0
19.	R_1-Tc31	120	119	99.2	24	20.0	0	0.0	0	0.0
20.	R_1-Tc33	309	285	92.2	37	19.9	0	0.0	1	0.3
21.	R_1-Tc34	255	238	93.3	60	23.5	0	0.0	0	0.0
Total		4758	4261	89.6	2220	46.7	809	17.0	202	4.2

A total of 4758 seedlings of 21 plants regenerated under *in vitro* condition were screened. The results were interesting because the variation was very high, most of the treated seedling died at the end of the experiment. Seedlings from four mother plants had high survival rates over 14 percent, the highest being 28.3 percent. These are the plants that will be grown for second and third screenings. It is interesting to note that the treatment No. 1, which has the highest survival rate of 28.3%, demonstrates its ability to survive under salt stress in the second and third weeks as well. Others seem to follow suit (Treatments 12, 14 and 15) except treatment No. 2 and 10 for which the plant surviving rates drop sharply during the fourth week to only 2.7 and 1.3% respectively.

Table 5 Survival of four-leaf stage seedlings of three plants regenerated under salt stress *in vitro** when treated with 0.5% NaCl in nutrient solution for four weeks.

Variety : RD 23 Electrical Conductivity : 9-10 m Mhos/cm
*Na 1 = 1% NaCl in culture medium *Na 2 = 2% NaCl in culture medium

Treatment		No. of plant	Plant Surviving							
			1st wk		2nd wk		3rd wk		4th wk	
No.	Stock No.		No.	%	No.	%	No.	%	No.	%
1	R_1-Tc7 Na1	247	242	98.0	204	82.6	129	52.2	82	33.2
2	R_1-Tc22 Na2	150	144	96.0	122	81.3	69	46.0	44	29.3
3.	R_1-Tc142 Na2	250	242	96.8	176	70.4	100	40.0	36	14.4
Total		647	628	97.1	502	77.6	298	46.0	162	25.0

Seedlings of the plants under salt stress regenerated *in vitro* are of interest. Generally, RD23 callus gives regeneration rates ranging from 8 to 10% but under 1% NaCl stress the regeneration rate is reduced to 0.14%. Furthermore, most of the plants regenerated under produced no viable seeds. Only three mother plants produces viable seeds which could be used for screening.

The result of screening is quite satisfactory (Table 5), the plant surviving rates are 14.4, 29.3 and 33.2 respectively. The average surviving rate of the seedlings from stressed mother plants is only 4.2% (Table 4) which is only 1/6 of the figure obtained from the above population.

It is interesting to note that survival of the plants after the third week was at least 40%, which is a very high value for the RD23. This reflects the growth and development of calli during the salt stress. Under salt stress condition, the cells which are sensitive will be come malfunction and die which affect the tolerant cells. If the population of tolerant cell cover large area, they are likely to form shoot apex and finally become a shoot. Therefore any shoot arising from such callus under stress should be tolerant.

The method of salt stress in vitro seems to be promising for the selection of salt tolerant plants directly, this gives positive result in the first generation. Further gene recombination will give rise to more tolerant individuals in later generations.

Table 6 Survival of four-leaf stage second generation regenerated plants, treated with 0.5% NaCl in nutrient solution for four weeks.

Variety : RD 23 Electrical Conductivity : 9-10 m Mhos/cm

Treatment		No. of plant	Plant Surviving							
			1st wk		2nd wk		3rd wk		4th wk	
No.	Stock No.		No.	%	No.	%	No.	%	No.	%
1	R_2-Tc 1a	1386	1386	100	934	67.4	564	40.7	340	24.5
2	R_2-Tc 1b	1386	877	79.7	532	48.4	249	22.6	116	10.5
3	R_2-Tc2	1386	662	85.4	178	23.0	19	2.4	2	0.2
4	R_2-Tc3	1386	420	84.0	119	23.8	36	7.2	5	1.0
Total		3761	3345	88.9	1763	46.9	868	23.1	463	12.3

Second generation (R2) of seedlings collected from four mother plants which had been grown in 0.5% NaCl for four weeks during four leaf stage in natural condition. The pattern of responses was not different from the R1 during the first two weeks. In the third week the number of survivors were rather high and none of the groups died completely. At the end of the fourth week the TC1a and Tc1b showed tolerant trait clearly while the other two populations demonstrated sensitivity like the ones in the RI. However the percentage of the survivors were not better than the best of the RI population (Table 6).

It is still too early to compare the two methods of salt stress selection, one in vitro and the other in natural condition. The mother plants regenerated in vitro under salt stress seemed to give more seedlings with high salt tolerance than the unselected ones but only few fertile plants were obtained. On the contrary, more mother plants were obtained after selection under natural condition which gave a wide variation. However, the genetic of stress tolerance is rather complex and the chance to have just a simple gene responsible for a given characteristics is remote. If this is the case, many useful variants with favorable recessive genes occurring during callus growth would be lost through stressing when the surrounding cells are killed. Furthermore, if the gene in question is a recessive one, it would not have the chance to express itself, or if an array of genes responsible for a specific character are of a multiple type, this would further reduce chance of occurrances essentially to zero by selection in the callus stage. By allowing regeneration to take its normal course (without stressing) and let the plants grow to maturity, gene recombination through sexual reproduction is facilitated. Selection of seedlings from regenerated plants would be the combined result of gene mutation during tissue culture and gene recombination which finally is expressed in the organism. This seems logical and has a direct application. However, subsequent selections are still needed to fix gene combination in the case of multiple genes.

Since both methods have their own merits and drawbacks, we are working for both.

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"THE ROLE OF VA MYCORRHIZA IN PHOSPHORUS NUTRITION OF ECONOMIC LEGUMINOUS CROPS IN THAILAND" (2A-04)

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ABSTRACT

The experiment was started with spore collection from soybean and mungbean fields in the North and Northeastern Regions of Thailand. Twelve species of selected VA mycorrhiza (VAM) were inoculated to soybean variety S.J.5 and mungbean variety U-Thong 1 to compare their effectiveness on legumes growth (1 st Crop). Three of the twelve species showed highly significant increased yield in the greenhouse study. Under field conditions in soil low in phosphorus content and indigenous VAM population, some superior species enhanced growth and yield. These isolates were studied further for their survivals in flooded condition by growing rice (2nd Crop) after legume with no reinoculation, followed by soybean and mungbean planting again in 3rd Crop. The number of VAM decreased in flooded condition. Colonization was found in 1st Crop (legumes) but not in 2nd Crop (rice). The evidence of influence of VAM was also found in the 3rd Crop.The method for producing VAM inoculum has been studied and it has been found that the number of spores and infectivity were high when using sand + clay, peat and coconut coir dust as the media for plant growth. Soybean, corn and sorghum were the best as host plants. The secondary were peanut and kudsu.

INTRODUCTION

Mycorrhizal fungi are a symbiotic association between the fine feeder roots of green plants and highly specialized root inhabiting fungi. The establishment of vesicular-arbuscular mycorrhizae (VAM) usually increase plant growth, especially on infertile soils (Mosse 1973; Gerdemann, 1968). VAM can markedly stimulate plant growth by increasing phosphorus uptake (Mosse 1973; Tinker 1975). Since some of the endophytes which form VAM are more effective in increasing plant growth than others (Mosse 1972; Powell 1975; Sanders et al. 1977), it may be possible to reduce the rate of application of phosphate fertilizer to agricultural soils by introducing superior strains of VA endophytes. Although a survey on diversity of VAM in the leguminous plantation in Thailand showed that VAM could enhance growth and yield of soybean and peanut (Nopamornbodi et al, 1981), the mechanisms of infection, their influence on phosphorus absorption and their survival in flooded conditions were still not known. The study described in this paper were designed to determine A). The effect of selected species of effective VAM strains on growth, yield and phosphorus uptake of soybean and mungbean B). The survival of mycorrhizae following flooding conditions C). The improvement of methods for producing the mycorrhizal inoculum for field trial.

MATERIALS AND METHODS

Inoculum Preparation

VAM spores collected from soil cultivated soybean and mungbean were used as inoculum in this experiment. The number of spores were multiplied by Menge pot culture technique (Menge et al 1977). Sorghum vulgare was selected to be the host plant. It was grown first in paper cups for one week then transferred to grow in pot culture. Spores of the collected VAM were applied at the bottom of the transplant hole. At the flowering stage, root and soil samples were collected to examine for percent root infection and number of spores produced. After harvesting the infected root and soil in the culture pot were used as inoculum for further study both in greenhouse and field experiments.

Determination of Percent Root Colonization and Spore in Soil

After the plants germinated for 30, 60 and 90 days the number of VAM spores in soil were determined. The spores were extracted from the soil by the wet seiving and decanting method (Gerdemann & Nicolson 1963). Spores with 250, 100 and 63 microns were separated from other debris by the sucrose floatation technique (Jenkins 1964). The number of spores was counted under stereo microscope. The percent of root colonization was examined by stained root with trypan blue followed by Phillips and Hayman 1970 to observe the hyphae arbuscule or vesicle colonization.

Effect of VAM on Growth and Phosphorus-absorption

Twelve isolates of VAM identified by technique of Nicolson and Schenck 1979 were inoculated to each cultivar of legumes grown in pots containing 10 kgs of sterile low phosphorus (7 ppm) soil in the greenhouse. Fourty spores per pot of each isolates of Glomus etunicatum (ETU #31), Glomus deserticola (DES #39), Gigaspora margarita (MAR #40), Gigaspora gregaria (GRE #33), Enthrophospora sp.(ENTRO #8), Gigaspora margarita (MAR #57), Glomus sp. (Gl. #56), Glomusmosseae (MOS #6), Glomus sp. (Glomus #17) and Acaulospora scrobiculata (ASCR #22) were applied 3 cms below soil surface. This was a Randomized Complete Block design experiment composed of 12 VAM isolates and uninoculated control treatment with 6 replications. Four seeds of soybean S.J. 5 (Glycine max (L.) Merr.) and mungbean U-Thong 1 (Vigna radiata(L.) Wilzeek) were planted per pot and thinned to two plants five days after emergence. All pots were inoculated with proper rhizobia for each legumes and applied 1 gram of rock phosphate per pot. Plant growth, yield, percent root infection and number of spores were collected every 30 days.

Six isolates of VAM selected from greenhouse screening MOS# 6, Glomus #17, INT #26, ASCR #22, ETU #31, DES #39 were used in the experiment. Two hundred spores of each isolate were used as inoculum placed at the bottom of soybean and mungbean plant hole grown at Chiang Mai and Kalasin fields. The experiment was conducted in split plot design with 4 replications and 7 treatments. Main plots were no phosphate plot and the rock phosphate(3% P2O5) plot at the rate 625 Kg/ha. Sub plots were the

uninoculated control plot and the inoculated plot with 6 VAM isolates. The plot size of treatment was 3x4 metres with spacing between rows of 50 cms and between hole of 25 cms, then thinned to two plants per hole. The harvested area was 2x4 metres. All data was recorded the same as in greenhouse experiment.

Survival of VAM from Flooded Condition

After first crop of comparison the effectiveness of VAM species on legumes had been harvested, rice (Oryza sativa L.) variety RD 23 were planted as the second crop followed soybean and mungbean crops in the same plot with no reinoculation. Ditches were made around each plot to prevent contamination when flooding. The third crops were repeated with soybean and mungbean, planting with no inoculation after the rice. Growth, yield, number of spores in soil and percent root colonization were also recorded.

Method for Producing VAM Inoculum

The experiments were conducted in the greenhouse. Glomus mosseae was inoculated with peanut grew in sterilized peat, sand, clay, loamy sand, vermiculite, rice husk, coconut coir dust,sand + peat, sand + clay. Then cowpeas, Nepia grass, Sudan grass, corn sorghum and onion were used to compare effectiveness as the host plant grown in sand + clay inoculated with Glomus fasiculatum. Each experiment was conducted in Randomized Complete Block design with 6 replications. The treatments were similar to the plant growth medium in the former experiment and the plant host species in the latter experiment. Once a week, treatment was applied with the standard Hoagland's solution minus phosphorus.

RESULT AND DISCUSSION

A. Effect of VAM on Growth, Yield and Phosphorus-absorption

For soybean, the result in greenhouse showed that the most effective species in height were GRE #33 and ASCR #22, in shoot dry weight was MOS and in yield were INT #26, MAR #40 and ASCR #22. In the field at Chiang Mai, the effective species in dry weight was DES #39, in yield ASCR #22. VAM ASCR #22 also produced the highest quantity of spores in soil and obtained the highest percent root colonization. (Tables 3 and 5)

For mungbean, the result in greenhouse showed that no effective species encountered in height, the most effective species in shoot dry weight are INT #26 and MAR #40, and most in yield MOS #6, INT #26, and MAR #40. In the field at Kalasin the effective were INT #26, DES #39 and ASCR #22. ASCR #22 produced the highest amount of spores while INT #26 obtained the highest root colonization. (Tables 4 and 6)

No significant difference was found between the main photo-applied and non-applied phosphate in both locations. At Chiang Mai where

phosphorus content was higher than 15 ppm, VAM was shown to have no effect on phosphorus content in shoot while at Kalasin where the phosphorus content in soil was about 7 ppm the VAM was affected. VAM isolates INT #26, MOS #6 and ASCR #22 were the effective species in increasing yield when grown in low phosphorus soil applied only with small amount of available phosphorus. (Tables 5 and 6)

The result indicated that the phosphorus absorption in VAM inoculated plant grown in soil with high phosphorus content (15 ppm) did not change when extra phosphate fertilizer was applied, but in soil low in phosphorus content (10 ppm) the ability of phosphorus absorption in plants was increased when phosphorus fertilizer was applied.

B. Survival of VAM from Flooded Condition

The survival incidences of inoculated VAM and indigenous species in this experiments were similar in both soybean and mungbean field. The spores quantity in the soil from 1st Crop was the highest and decreased with time. The number of spores was lowest in the second crop when rice was grown and the fields were covered about 20-30 cm. of water for four months. Then the level increased again in 3rd Crop when beans were grown. (Figure 1)

Root colonization was also highest in 1st Crop. The inoculum which was placed below the seed provided a high inoculum potential for the introduced fungi, giving them an advantage over the indigenous fungi which were spread throughout the soil. So at both fields where soil became colonized by the indigenous fungi at the same level of inoculation, plant growth and yield responded significantly to inoculation with some of the introduced fungi in the first crop. Most sample found no colonization of VAM in the rice root. (Figure 1)

Yields of inoculated soybean and mungbean are significantly higher than uninoculated plants in the 1st Crop but no changes were shown on 2nd Crop & 3rd Crop. The total infection and spore number after 3rd Crop showed that VAM fungi could survive flooding in the spore stage but not in the colonized roots stage because no colonization was found in rice of the 3rd Crop. The number of VAM spore decreased because of flooded conditions and micro fauna in the soil. According to this phenomena, the crop after rice must be reinoculated with VAM. (Figure 1.)

C. Improving the Method of Pot Culture for Producing VAM Inoculum for Field Trials

The results after studying the suitable plant growth medium revealed that sand+clay and sterilized peat were the best plant growth media for producing VAM spores in pot culture. As for the studies of the appropriate host plant, the results showed that soybean, corn and sorghum were the most satisfactory hosts. Peanut and Kutsu plants came in second. The poorest host was chinese cabbage.(Table 7 and 8)

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Table 1 Soil characteristics of samples from paddy field surveys in different locations with the number of VA mycorrhizal

Location	Date Collected	Soil order	Soil texture	Soil pH	Available P by Bray II (ppm)	No. of spores per 100 g soil
1. Ayudthaya 1	12.5.83	Inceptisols	Clay	6.30	20	246
2. Ayudthaya 2	12.5.83	Inceptisols	Clay	6.05	20	389
3. Chiengmai 1	10.5.83	Alfisols	Sandy clay loam	6.20	36	190
4. Chiengmai 1	10.5.83	Alfisols	"	6.57	39	160
5. Kabinburi 1	11.4.83	Ustults	"	7.30	15	116
6. Kabinburi 2	11.4.83	"	"	6.80	9	63
7. Kabinburi 3	11.4.83	"	"	6.72	14	79
8. Kabinburi 4	20.7.83	"	"	7.50	13	132
9. Kabinburi 5	20.7.83	"	"	6.90	14	263
10. Kalasin 1	6.7.83	Ustults/ Aqaults	Loamy sand	4.60	13	340
11. Kalasin 2	6.7.83	"	"	4.99	13	104
12. Kalasin 3	6.7.83	"	"	4.88	17	54
13. Kalasin 4	13.3.84	"	"	5.70	15	120
14. Kalasin 5	13.3.84	"	"	6.00	12	174
15. Kalasin 6	13.3.84	"	"	6.20	16	363
16. Khonkaen 1	22.3.14	Utisols	"	6.30	11	27 (dead)
17. Khonkaen 2	22.3.84	"	"	6.20	12	18 "
18. Khonkaen 3	22.3.84	"	"	6.60	7	21 "
19. Mahasarakarm 1	23.3.84	"	"	5.69	5	230
20. Mahasarakarm 2	23.3.84	"	"	5.50	3	170 (dead)
21. Mahasarakarm 3	23.3.84	"	"	5.60	16	260
22. Mahasaramarm 4	23.3.84	"	"	4.80	8	175
23. Nakonrajsima 1	16.5.83	Inceptisols	Clay	6.30	13	145
24. Nakonrajsima 2	16.5.83	"	"	6.50	20	180
25. Pisanuloke 1	8.5.83	Alfisols	"	4.49	36	20
26. Pisanuloke 2	8.5.83	"	"	4.36	35	208
27. Pisanuloke 3	8.5.83	"	"	4.58	42	120

Location	Date Collected	Soil order	Soil texture	Soil pH	Available P by Bray II (ppm)	No. of spores per 100 g soil
28. Pisanuloke 4	12.8.83	"	"	4.70	35	1235 (dead)
29. Pisanuloke 5	12.8.83	"	"	4.90	30	674 "
30. Prae 1	14.6.83	Ustults	Sandy clay loam	6.20	29	382
31. Prae 2	14.6.83	"	"	6.54	32	528
32. Prae 3	14.6.83	"	"	6.83	31	223
33. Prae 4	14.6.83	"	"	6.50	28	120
34. Prae 5	20.7.83	"	"	6.50	28	893
35. Prae 6	20.7.83	"	"	6.78	27	1029
36. Roiet 1	25.3.84	Utisols	Loamy sand	5.30	34	260
37. Roiet 2	25.3.84	"	"	4.90	12	145
38. Roiet 3	25.3.84	"	"	5.60	12	470
39. Saraburi 1	12.5.83	Vertisols	Clay	7.49	25	147
40. Saraburi 2	12.5.83	"	"	6.80	22	568
41. Saraburi 3	12.5.83	"	"	6.90	25	983
42. Saraburi 4	12.10.83	"	"	6.85	25	27,200 (dead)
43. Saraburi 5	23.10.83	"	"	6.70	15	4,200 "
44. Saraburi 6	23.10.83	"	"	6.80	23	1,500 "
45. Saraburi 7	15.01.84	"	"	6.83	12	23 "
46. Saraburi 8	15.01.84	"	"	6.50	17	none
47. Saraburi 9	15.1.84	"	"	7.74	24	none
48. Saraburi 10	15.3.84	Vertisols	Clay	6.53	26	79 (dead)
49. Saraburi 11	16.3.84	"	"	7.33	23	830
50. Saraburi 12	16.3.84	"	"	7.08	22	1,523
51. Saraburi 13	16.3.84	"	"	6.80	21	868
52. Saraburi 14	16.3.84	"	"	6.83	19	357
53. Saraburi 15	16.3.84	"	"	7.70	18	240
54. Saraburi 16	16.3.84	"	"	6.15	27	330

Table 2. Number of 14 common species of VA mycorrhizal fungi collected from paddy field in 1983-1984.

No*	Species
1.	*Acaulospora morrowae*
2.	*Acaulospora laevis*
3.	*Gigaspora gregaria*
4.	*Acaulospora mellea*
5.	*Gigaspora heterogama*
6.	*Glomus intraradices*
7.	*Gigaspora magarita*
8.	*Glomus etunicatum*
9.	*Glomus clarum*
10.	*Acaulospora spinosa*
11.	*Gigaspora gigantea*
12.	*Acaulospora scrobiculata*
13.	*Gigaspora calospora*
14.	*Gigaspora auriglobe*

* number listed in order of spores prevalence.

Table 3. Effects of VAM species on height, shoot dry weight, yield, number of spores in soil and percent root colonization of soybean in the greenhouse.

Species	Height (cm)	Shoot dry wt (gm/pl)	Yield (gm/pl)	spores/ 100 gm soil	percent root colonization(%)
ETU # 31	28.8 ab	9.8 b	1.6 b	38 d	24 c
DES # 39	28.9 ab	13.5 ab	3.8 ab	443 b	72 ab
MAR # 40	28.3 abc	11.9 ab	4.9 a	128 c	73 ab
GRE # 33	30.6 a	11.3 ab	1.7 b	15 d	9 d
ENTRO # 8	24.6 c	10.8 b	3.3 ab	518 b	23 c
GRE # 57	27.5 abc	10.8 b	2.7 ab	7 d	25 c
GI # 56	27.0 abc	12.2 ab	3.8 ab	40 d	30 c
ASCR # 22	30.0 a	16.0 a	4.6 a	1837 a	60 b
MOS	27.6 abc	16.5 a	3.4 ab	202 c	80 a
INT # 26	27.9 abc	14.5 ab	5.0 a	207 c	98 a
MOS # 6	29.2 ab	12.0 ab	3.8 ab	155 c	31 c
GLOMUS # 17	28.9 ab	11.8 ab	4.4 ab	5 d	36 c
Control	26.7 bc	11.5 ab	2.0 b	0 d	1 d
% C.V.	6.2	21.7	18.4	98.7	87.7

In a column, means followed by a different letter are significantly different at the 5% level using DMRT.

Table 4. Effects of VAM species on height, shoot dry weight, yield, number of sproes in soil soil and percent root colonization of mungbean in the greenhous.

Species	Height (cm)	Shoot dry weight (gm/pl)	Yield (gm/pl)	spores/ 100 gm soil	Percent Root colonization(%)
ETU # 31	20.4	5.3 ab	3.7 bc	92 d	50 c
DES # 39	20.1	4.2 b	1.7 c	150 c	94 b
MAR # 40	23.7	6.6 a	6.0 ab	37 e	97 a
GRE # 33	20.2	4.9 ab	2.6 c	15 f	16 d
ENTRO # 8	21.6	4.4 b	2.7 c	4 f	84 ab
GRE # 57	21.7	5.2 ab	2.2 c	15 ef	68 b
GI # 56	21.1	5.7 ab	-	50 e	14 d
ASCR # 22	23.7	5.5 ab	5.6 b	1,005 a	70 b
MOS	22.5	5.9 ab	3.3 c	213 c	68 bc
INT # 26	23.0	6.2 ab	6.0 ab	96 d	98 a
MOS # 6	21.7	4.9 ab	7.7 a	182 c	98 c
GLOMUS # 17	20.3	4.2 ab	6.4 a	360 b	49 c
Control	21.8	4.3 ab	3.8 bc	0 f	19 d
% C.V.	8.6	19.9	11.8	91.4	72.5

In a column, means followed by a different letter are significantly different at the 5% level using DMRT.

Table 5. Effects of VAM species and phosphate application on height, shoot dry weight, yield, number of spores in soil, percent root infection and phosphorus content of soybean at Chiangmai.

Species	Height (cm)		Shoot Dry weight (gm/pl)		Yield (kg/ha)		Spores/ 100 gm soil		Percent root colonization(%)		phosphorus content inplant(%)	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
MOS # 6	45.7 a	42.3 ab	10.9 a	10.0 ab	1250 b	1489 ab	118	104	76	81	0.093	0.107
GLOMUS # 17	41.1 b	40.1 b	7.7 a	9.5 ab	1191 b	1241 b	218	144	45	48	0.088	0.091
INT # 26	45.7 a	42.1 ab	10.2 a	6.2 b	1342 b	1236 b	177	164	50	63	0.100	0.089
ETU # 31	44.1 ab	42.0 ab	9.9 a	17.8 a	1411 b	1230 b	124	89	71	70	0.099	0.076
DES # 39	46.6 a	41.4 ab	8.8 b	6.8 b	1603 ab	1275 ab	235	279	69	79	0.890	0.117
ASCR # 22	46.8 a	44.9 a	6.1 c	3.9 b	1834 a	1653 a	323	169	82	78	0.078	0.087
Control	46.0 a	43.1 ab	8.1 a	12.2 ab	1575 ab	1244 b	144	178	74	85	0.094	0.095
Means	45.1 a	42.3 a	8.8 a	9.5 a	1458 a	1334 a	191	161	67	72	0.092	0.095
C.V.P.(%)	11.1		58.8		9.6		7.7		20.5			
C.V. Species(%)	5.1		61.8		16.0		8.3		12.0			

In a column, means followed by a different letter are significantly different at the 5% level using DMRT.

Table 6. Effects of VAM species and phosphate application on height, shoot dry weight, yield, number of spores in soil, percent root infection and phosphorus content of mungbean at Kalasin.

Species	Height (cm)		Dry weight (gm/pl)		Yield (kg/ha)		Spores/ 100 gm soil		Percent root Colonization (%)		Phosphorus content in plant(%)	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
MOS # 6	25.1 b	26.0 bcd	6.5	4.6	618 bcd	643 ab	157	170	44	29	0.214	0.183
GLOMUS #17	28.4 ab	28.7 bc	4.7	5.1	589 a	618 ab	189	200	31	26	0.204	0.198
INT # 26	31.8 a	30.9 ab	6.1	4.4	701 a	622 ab	197	189	37	39	0.211	0.158
ASCR # 31	33.2 ab	24.8 d	6.6	6.9	605 bcd	589 b	217	165	22	30	0.248	0.200
ETU # 39	28.0 ab	34.6 a	6.5	6.1	668 ab	668 b	288	206	38	34	0.216	0.200
DES # 22	28.0 ab	26.0 bcd	4.1	3.4	664 abc	664 a	212	255	37	34	0.235	0.225
Control	27.9 ab	30.9 ab	3.7	6.7	601 cd	605 ab	219	216	17	23	0.257	0.190
Means	28.9 a	28.4 a	5.5	6.5	635 a	630 a	211	187	32	31	0.226	0.193
C.V.P.(%)	12.9		16.9		4.1		7.2		33.9			
C.V. Species (%)	12.9		48.8		6.5		5.2		29.5			

In a column, means followed by a different letter are significantly different at the 5% level using DMRT.

Table 7. Average height, root dry weight, number of spores, and percent root colonization of mycorrhizal inoculated peanuts grown in different media.

	Height (cm)		Root dry weight/pl(gm)		No.of spores/100 g.soil		% root colonization	
Treatments	60 days	90 days	60 days	90 days	60 days	90 days	60 days	90 days
1. Sand	29.7 de	34.5 e	1.82 cd	1.38 c	105 b	160 a	6 b	27 b
2. Clay	48.3 abc	58.0 abc	4.72 ab	3.49 abc	284 a	315 a	8 b	35 b
3. Peat	54.9 a	66.5 a	2.98 bcd	4.73 a	345 a	351 a	17 a	58 a
4. Loamy sand	28.5 e	34.0 e	1.08 d	1.50 c	21 b	164 a	6 b	25 b
5. Rice husks	38.8 cde	39.8 de	2.24 bcd	1.67 bc	2 c	12 b	11 ab	40 ab
6. coconut coir dust	42.7 bc	49.0 bcde	3.06 abc	2.34 bc	139 a	140 a	14 a	48 a
7. Sand + Peat	40.7 bc	43.5 cde	3.57 abc	2.59 bc	245 a	294 a	9 b	39 b
8. Sand + clay	51.9 ab	63.3 ab	5.20 a	5.03 a	313 a	370 a	12 a	60 a
9. Vermiculite	49.6 abc	52.7 abcd	3.86 abc	3.72 ab	144 a	156 a	4 b	16 b

-Means followed by different letter are significantly different at the 5% level using DMRT.

Table 8. Root dry weight, spores on soil, and percent root colonization of different kinds of plant hosts inocualted with *Glomus fasiculatum* in sand + clay.

Plants host	Root dry weight/Pl(gm)				Spores/100 g.soil			Root colonization(%)		
	30 days	60 days	90 days	120 days	60 days	90 days	120 days	60 days	90 days	120 days
1. Onion	6.85 a	6.94 a	4.92 b	3.80 c	253 c	342 c	312 c	46 a	80 a	80 a
2. Sorghum	6.60 a	8.38 a	8.29 a	8.11 b	293 b	413 b	410 a	12 bc	60 a	80 a
3. Corn	4.87 a	1.30 c	3.91 b	9.01 a	269 bc	365 bc	415 a	20 b	76 a	80 a
4. Soybean	1.67 c	3.59 b	3.60 b	3.68 c	333 a	421 a	431 a	32 a	64 a	84 a
5. Mungbean	1.54 c	0.51 c	0.34 c	1.28 d	205 d	313 d	329 c	6 c	10 c	14 cd
6. Peanut	2.56 b	3.80 b	1.72 c	1.20 d	253 c	372 c	379 b	10 bc	46 b	20 c
7. Sabi	0.42 d	0.72 c	0.71 c	1.20 d	137 f	284 c	310 c	6 c	6 cd	8 d
8. Bahia	1.17 c	0.46 c	0.25 c	1.18 d	170 e	319 c	325 c	8 c	10 c	10 c
9. Kudsu	1.37 c	2.42 b	2.89 b	4.59 c	250 c	368 c	390 ab	10 bc	27 b	56 b
10. Chinese Cabbage	0.61 d	0.78 c	1.23 c	1.36 d	130 f	139 e	120 d	0 d	0 d	0 d

In a column, means followed by a different letter are not significantly different at the 5% level using DMRT.

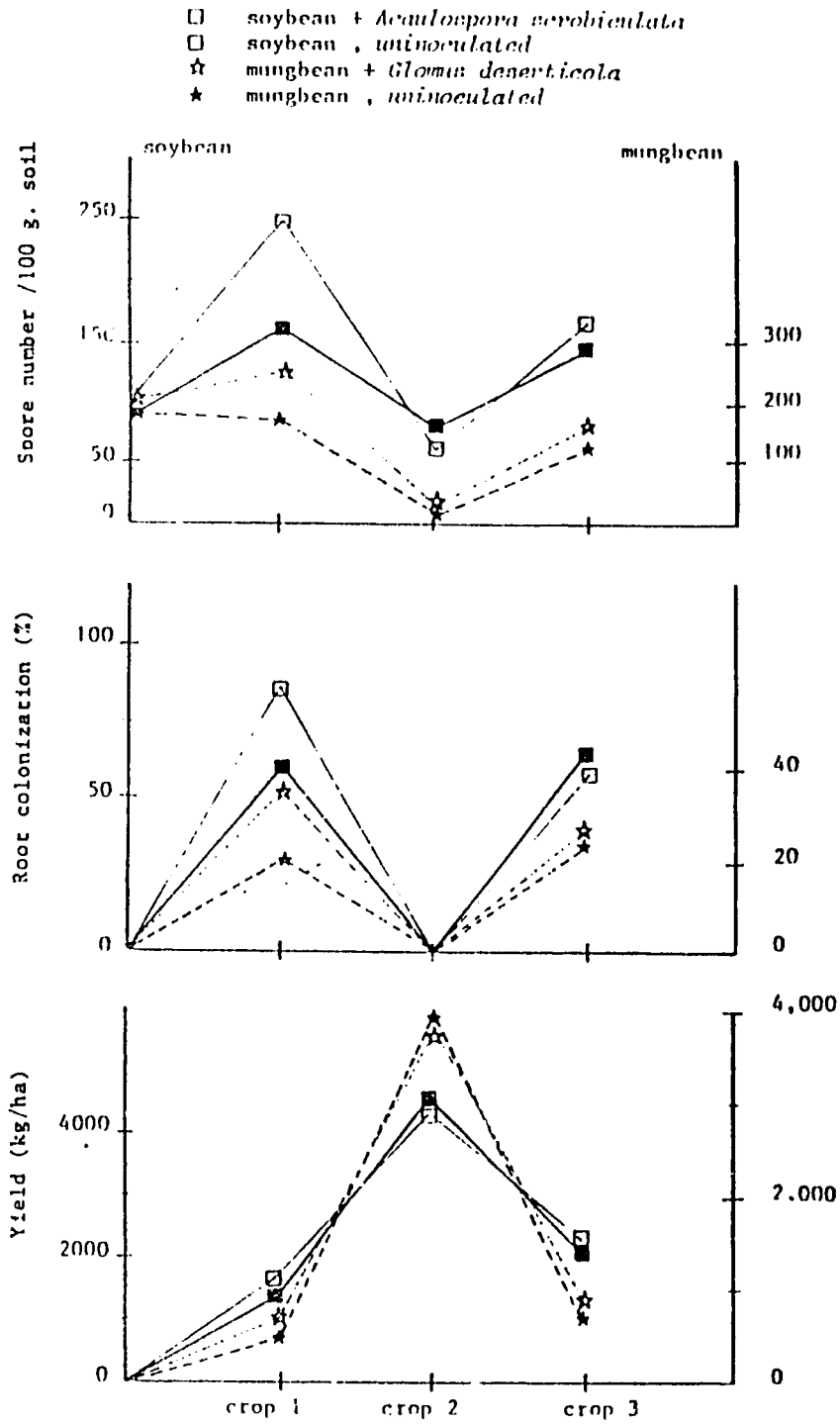

Figure I. Comparison of VAM spores, root colonization and yield in the field of soybean/mungbean (crop I), rice (crop II) and soybean/mungbean (crop III).

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"BIOLOGICAL CONTROL OF SCHISTOSOMIASIS-TRANSMITTING SNAILS IN SOUTHEAST ASIA" (3F-50)

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ABSTRACT

A Biological control study of schistosomiasis-transmitting snails in Southeast Asia was conducted in two parts: testing of the efficacy of biological control of snails which transmit schistosomiasis by using biological agents, such as insects and molluscs; and study of the genetic variability of snails which transmit schistosomiasis in relation to degrees of parasite compatibility and susceptibility. The results of the biological control study of snails by using insects as control agents revealed that both sciomyzid and lampyrid fly larvae were effective control agents in killing snails. The sciomyzid fly larvae caused higher mortality in pulmonate snails while lampyrid fly larvae were more effective in controlling both pulmonate and operculate snails. The results of the biological control study of snails by using molluscs as control agents revealed that Pila ampullacea appeared to be the most effective control agent for both pulmonate and operculate snails due to its large size and voracious habits. The genetic variation study of Asian Schistosoma had revealed that all strains of Asian schistosomes examined were genetically invariant; there were more variations within S. japonicum species; and the Malaysian schistosome was related to S. mekongi, not S. japonicum. The genetic variation in the snail, Oncomelania hupensis revealed that the Chinese O.h. hupensis and Philippines snail, O.h. quadrasi were so different from one another as to require their recognition as separate species.

INTRODUCTION

Schistosomiasis is one of the great endemic diseases of warm climates. It affects nearly 200 million people. The four most important species of schistosomes infecting man are Schistosoma haematobium, Schistosoma mansoni, Schistosoma japonicum and Schistosoma mekongi.

Asian schistosomiasis is comprised of 2 species: S. japonicum and S. mekongi. The disease is one of the major health problems in Asia. S. mekongi is prevalent in Laos and Cambodia along the north and northeastern borders of Thailand (Schneider, 1976; Sornmani et al., 1980). S. japonicum is endemic in China and many Southeast Asian countries especially the Philippines and Indonesia. Recently, a new species of schistosome has been reported in Malaysia (Malaysian schistosome).

The basic life cycle of a schistosome has an alternation of generations with the sexual generation of adult schistosomes in the definitive vertegrate host and the asexual stage in a molluscan host, which is a species of freshwater snail. Transmission of schistosome infections requires contact with water infested with snails, intermediate hosts in an area where sanitation is poor and a large population of infected people.

In the last decade, schistosomiasis control was synonymous with snail control, which was generally considered the most effective and rapid means of reducing transmission (Faroog et al., 1966). The efficiency of snail control can be enhanced if combined with other methods of control. Snail control can be performed in many areas such as environmental control, water management, agricultural practices, chemical control (Molluscicidng) and biological control.

Regarding bio-control of snail intermediate hosts, several hundred species, ranging from fish to fungi, have been considered potential competitors or predators, but their efficacies have very rarely been tested outside laboratory model systems (Michelson, 1957; Ferguson, 1972; Hairston et al., 1975).

The great majority of successful examples of biological control of undesirable species has involved the use of insects. Among snaileating insects, the most interest has focused on schiomyzid flies, which are obligatory feeders on mollusc larvae, mainly gastropods. (Berg, 1953; 1964; Berg and Neff, 1959; Neff, 1964; Knutson and Berg, 1963; Fontana, 1972). In addition, the introduction of possible competitive species of snails has received the most attention to date. Hence, one of the objectives of this investigation was to study the efficacy of biological control of snails which transmit schistosomiasis in Southeast Asia using biological agents, the insects and the molluscs.

A genetic analysis of molluscs by allozyme electrophoresis provides a convenient basis for the characterization of populations and species and assessment of the degrees of variation between such groups of animals. A study of the genetic variations of Asian blood flukes and the relationship between the fluke and its snail host is very important since an understanding of the genetic variations between species and populations of the parasites and hosts would be an important key to understanding epidemiology of the disease as well as to control the spread of schistosomiasis in Asia.

The results would not only facilitate a greater understanding of the complex evolutionary divergences of S. japonicum and other Asian schistosomes but would also lead to the evaluation and development of immunological diagnostic tools and vaccines against the parasites. Moreover, the study of genetic variations of Oncomelania snails would help clarify the evolution of the host-parasite relationship, and benefit those who work on disease control. Hence, another objective of this investigation was to study genetic variability of snails transmitting schistosomiasis in Southeast Asia in relation to degrees of parasite compatibility and susceptibility.

MATERIALS AND METHODS

I. Biological Control of Snails

A. Insects

Adults of Sepedon plumbella (Diptera : Schiomyzidae) were collected from their natural habitats and reared in the laboratory. They were cultured in a 2000-ml glass jar containing a batch of rice plants which were used as resting and oviposition sites. Crushed flesh of various species of snails was given as food to these sciomyzid flies.

Sciomyzid fly larvae and lampyrid fly larvae were used as biological control agents against pulmonate and operculate snails in the ratios of 4:0, 3:1, 2:2, 1:3 and 0:4, under laboratory conditions, simulated field conditions, and field conditions.

B. Molluscs

Four species of snails, Pila ampullacea, Tarebia (Thiara) granifera, Melanoides tuberculata and Brotia costula costula were used to test their efficacies against various species of schistosomiasis-transmitting snails. Both biological agents and target species of snails were cultured in the laboratory. The experiments were divided into 3 stages : laboratory, simulated field conditions and small scale field trials.

II. Genetic and Susceptibility Studies

We were accordingly and particularly interested in estimating the relative degrees of interpopulation genetic differentiation between Asian schistosomes and intermediate host snails. Starch gel electrophoresis was used to distinguish allozyme patterns between species and populations of Asian schistosomes and Oncomelania snails. Different genotypes were identified by differences in positions of the colored bands on the gel. Unbiased estimates of genetic distances between species and strains were calculated using Nei's revised formula (Nei, 1978) and the BIOSYS computer program (Swofford and Selander, 1981). Nei's D is a measure of the number of codon substitutions per locus; it can vary between 0 (when two samples are genetically identical) and infinity. Studies of over a thousand species of animals have revealed a clear relationship between D and relative divergence and taxonomic status of a group of related organisms (Avise and Aquadro, 1982; Thorpe, 1983). D values between conspecific populations were typically less than 0.06, on the other hand, interspecific genetic distances were being in the range of D = 0.10-1.05. It is these values in mind that we used in this study for Asian schistosomes and their intermediate host snails.

RESULTS AND DISCUSSION

I. Biological Control of Snails

The biological control study of snails by mollusc and insect control agents was conducted in three phases: (1) experimental work in

the laboratory, (2) experimental work under simulated field conditions, and (3) experimental work under a small scale field trial.

A. Insects

The biological control study between sciomyzid fly larvae and the snails in the laboratory revealed that the third stage sciomyzid fly larvae were effective control agents for pulmonate target snails as previously mentioned; the ratio of three sciomyzid fly larvae to one snail rendered the best results.

The experimental work on biological control of snails by insects under simulated field conditions revealed that the sciomyzid fly larvae were effective control agents for the pulmonate snails in the ratio of 1:3 (snails: sciomyzid fly larvae). They did not have much effect on operculate snails due to the closing of the opercula of these snails when disturbed. The lampyrid fly larvae were more effective in killing pulmonate and operculate snails than the sciomyzid fly larvae were, but were more successful with pulmonate than with operculate snails. Unlike the sciomyzid fly larvae, the lampyrid fly larvae were seldom killed when attacking operculate snails.

The experimental work on the biological control of snails by insects under a small scale field trial showed that lampyrid fly larvae were more effective as control agents in killing both pulmonate and operculate snails than sciomyzid fly larvae. The larvae, especially those of the sciomyzid fly, caused higher mortality to pulmonate snails rather than to operculate snails due to the closing of the opercula of these snails when disturbed. The lampyrid fly larvae were more effective in killing pulmonate and operculate snails than the sciomyzid fly larvae were, but were more successful with pulmonate than with operculate snails. Unlike the sciomyzid fly larvae, the lampyrid fly larvae were seldom killed when attacking operculate snails.

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B. Molluscs

From the experimental work on biological control of snails by mollusc control agents in the laboratory, it was concluded that the biological control agents, *Brotia costula costula*, *Tarebia granifera*, and *Melanoides tuberculata*, even though they had higher reproductive potential than the target pulmonate snails, *Biomphalaria glabrata*, *Indophanorbis exustus* and *Radix rubiginosa*, had higher mortality rates and required a much longer time for the young molluscs to become mature and did not seem to be appropriate control agents for the pulmonate snails. As for the prosobranch target snails, *Tricula aperta*, *Robertsiella*

kaporensis, Oncomelania hupensis quadrasi, and Bithynia siamensis goniomphalos and there seemed to be no relationships between the control molluscs and the target snails due to differences in habitat. However, the three species of control agents certainly had higher reproductive and survival rates than the target species. Pila ampullacea, the other biological control agent, appeared to be an effective control agent for B. glabrata, the pulmonate snail. The ampullacea B. glabrata could be an accidental predator of the egg masses of B. glabrata in the laboratory.

The experimental work on the biological control of snail by mollusc control agents under simulated field conditions was performed at a small field station for 8 months. The results showed that B.c. costula. T. granifera were effective control agents for I. exustus and R. rubiginosa due to higher reproductive and survival rates. M. tuberculata seemed to be effective for R. rubiginosa, but not for I. exustus. These three species of control agents could not control B. glabrata had been established longer and was more well adapted to cultivation than the control agents. However, P. ampullacea was effective in controlling the population of all three target species of pulmonate snails including B. glabrata. The competition for food, physical interference between snails or predation on egg masses of the target species. All four species of control agents did not seem to have many effects on the prosobranch target snails, T. aperta, O.h. quadrasi and B.S. goniomphalos.

The experimental work on the biological control of snails by molluscs in a small scale field trial was performed in natural canals outside Bangkok. P. ampullacea was chosen as the control agent to be tested against the local species of target snails, I. exustus, R. rubiginosa and B.s. goniomphalos. Under natural conditions, P. ampullacea appeared to be effective in killing snails and to have better survival rates than the small target snails due to their large survival rate and voracious habits. At the end of the eighth month, the numbers of target snails had decreased drastically. Even though the target snails laid large numbers of eggs, only a small number of offspring survived. This stage of experimentation is still in progress and may take longer than one year to complete. The population densities of the control agents and the target species must be studied regularly until the target species disappears or is replaced by the snail control agents.

II. Genetic and Susceptibility Studies

A. Susceptibility study

The study of the susceptibility of Oncomelania hupensis quadrasi from different geographical locations to infect with geographic strains of Schistosoma japonicum revealed that the strain of S. japonicum from Mindoro was able to develop in all geographical strains of the snail host O.h. quadrasi, with the best fluke development in O.h. quadrasi from Irosin; the snail species from Mindoro and Mindanao was next, and finally the Leyte snails. S. japonicum from Leyte developed best in Irosin snails, and poorly in snails from Leyte, Mindanao, and Mindoro, respectively. S. japonicum from Mindanao developed best in snails from Mindanao, and very poorly in snails from Irosin, Mondoro, and Leyte.

B. Genetic variation of Asian Schistosoma

Our research has resulted in 3 significant discoveries.

1. All strains of Asian schistosomes examined were genetically invariant.

2. There is more variation than previously suspected within S. japonicum species, which indicates that they may be made up of several sibling species. The Philippines' schistosomes (from Leyte, Luzon, and Mindoro islands) were genetically very different from strains from China, Taiwan, and Japan.

3. The still undescribed Malaysian schistosome is related to S. mekongi and not S. japonicum.

C. Genetic variation in the snail Oncomelania hupensis

Our results indicate that;

1. Nei's genetic distances among the Philippines snails (Oncomelania hupensis quadrasi) from various islands were D = 0.001-0.118, well within expectations for conspecific comparisons.

2. Nei's genetic distances between O.h. hupensis in mainland of China and O.h. quadrasi in the Philippines were D = 0.63±0.20. This indicates that the Chinese and Philippines snails are so different from one another as to require their recognition as separate species; O. hupensis in China and O. quadrasi in the Philippines.

The systematic revision of these biomedically important animals will facilitate their control and the improved treatment and prevention of schistosomiasis.

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FY 1984

"HEAT STERILIZATION AND ACCELERATED DRYING OF HIGH-MOISTURE RICE FOR SAFE STORAGE" (4.488)

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ABSTRACT

High temperature conduction heat treatments reduced fungal growth and prevented spoilage in freshly harvested high-moisture rice leading to its safe storage prior to drying. In addition, heat treatments resulted in accelerated drying of rice with an improvement in its milling and as well as cooking qualities, depending upon the initial moisture content of the rice and time-temperature combinations used to reduce the fungal activity.

The prototype rice husk-fired rotary heating unit developed specifically for the heat sterilization of rough rice showed good mechanical and thermal performance. Such rotary heaters could be effectively used for sterilization and accelerated drying of high moisture rice.

INTRODUCTION

In Southeast Asia, immediate drying of freshly harvested rough rice is essential to prevent its quality deterioration under warm and humid climatic conditions. Rapid growth of microorganisms in high-moisture rough rice being held for drying especially during the rainy season, results in loss of seed viability, reduced milling yield, discoloration of milled rice grains, altered texture and potential hazards from toxins[1, 2]. The deterioration in the quality of high-moisture rice leads to a direct economic loss. A practical solution of this problem will consist of either avoiding drying delays or extending the safe storage period of high-moisture rice. Various methods of holding high moisture rice prior to drying, which have been attempted in the past, include the aeration of high-moisture grain[3], airtight storage[4] and the use of chemical preservatives[5]. These methods have been used only with limited success.

Heat sterilization-cum-partial drying of high-moisture rice could prove to be innovative and practical in holding the rice safely for short periods prior to drying without damage to grain quality. This paper describes the results of the use of rotary dryers specifically designed and developed for heat sterilization and accelerated drying of freshly harvested high-moisture rice.

MATERIALS AND METHODS

The overall experimental plan included the following: (i) heat treatment of high-moisture rice in a batch type rotary conduction heating unit to prolong its safe temporary storage, and (ii) performance evaluation of a prototype rice husk-fired rotary heating unit for heat sterilization and as well as accelerated drying of high-moisture rice. The first part of this study included a detailed investigation of the following: (a) thermal inactivation of fungi in freshly harvested rough rice, (b) quality effects of high temperature heating of rice, and (c) storage tests for heat-treated rough rice.

Heating surface temperatures ranging from 75 to 200 °C were used to sterilize freshly harvested rice having 18 to 30% moisture content. The effects of heat treatments were investigated in terms of heat rice yield, milled rice whiteness and cooked rice texture. Subsequently, safe holding periods of heat-treated rough rice samples having moisture content ranging from 22 to 26.5% were determined based upon an acceptable level of reduction in head rice yield and an increase in the proportion of discolored kernels.

Heating of Rough Rice Samples

The experimental heating unit consisted mainly of a rotary cylinder, 0.35 m in diameter and 0.85 m long, with six equidistant straight axial flights extending 4 cm from the inside surface. The temperature of the cylinder surface could be raised up to 300 °C. The cylinder rotation speed could be adjusted from 10 to 40 rpm by a variable speed electric motor. Rice samples were heated in batches of 3 kg using heating surface temperatures of 75, 100, 150 and 200 °C. The details of the experimental procedure are given by Reyes and Jindal[6].

Storage Experiments

After the heat treatment, rough rice samples were allowed to cool to room temperature in plastic buckets. Subsequently the samples were transferred into 2 liter capacity glass bottles with provisions for carbon dioxide to escape through the cotton plugs into the atmosphere during storage[7].

Rough Rice Quality Evaluation

Measurement of fungal activity. The number and proportion of fungi present in the rice samples were determined by dilution plating assay as described by Flannigan[8].

Milling quality evaluation. All rough rice samples (heat-treated and control) were air-dried to 14% moisture content prior to milling. The milling quality of rice samples was evaluated in terms of the percentage of the head rice representing 3/4 of whole kernels size or larger and total milled rice using standard laboratory procedure[9].

Determination of discolored kernels. The discolored kernels present in milled rice samples weighing about 20 to 30 g were separated manually and expressed later as a percentage of the total sample weight.

Other details of the equipment and experimental procedure are given by Reyes and Jindal[6, 7].

The schematic diagram of the prototype rotary dryer heated by the burning of rice-husk is shown in Fig. 1. In short, the unit consisted mainly of an inclined grate-type furnace and a slowly rotating cylinder, 0.6 m in diameter and 2.0 m long. As the cylinder rotated, the helical flights pushed the grain causing it to slide, roll or tumble along the inside wall of the cylinder. The high moisture rice got heated and dried simultaneously during its conveying to the other end of the cylinder until discharged. The cylinder rotation speed could be varied from 1 to 45 rpm. The furnace served as the heat source to the dryer by burning of rice husk. The husk feed rate depended upon the desired heating surface temperature which was calibrated in terms of the flow gas temperature. Other design details and experimental procedure are reported elsewhere[10, 11].

RESULTS AND DISCUSSION

Thermal Inactivation of Fungi in High Moisture Rice

The heat-treated rice samples exhibited reduced fungal activity as measured by dilution plating and carbon dioxide production. The reduction in fungal activity depended directly upon the heating surface temperature and exposure time. The fungal mortality in high-moisture rice was expressed in terms of bulk grain temperature and equivalent exposure times for general application (Fig. 2). Subsequently a procedure was developed for estimating the exposure times required for reducing the fungal activity to a desired level based on the time-temperature history of the grain in a given heating system[6].

Temporary Storage of Heat-Treated High-Moisture Rice

Heat treatments reduced the fungal growth and prevented the spoilage in newly-harvested high moisture rice leading to its safe temporary storage. The fungal mortality levels close to 90% or slightly higher attained as a result of heat treatments were found to be satisfactory for general use.

An increase in the proportion of discolored kernels in milled rice due to the temporary storage of high moisture rice appeared to be a more sensitive criterion than the reduction in head rice yields for developing the limits for safe storage periods. In general, heat treatment of high moisture rice using exposure times corresponding to 90-95% fungal mortality levels resulted in increased safe holding periods of about 9 to 20 days based on an acceptable limit of 1.5% increase in discolored kernels in the milled rice. A relationship for estimating the

safe storage periods of freshly-harvested rice was developed based on the moisture content and the fungal mortality level achieved with various heat treatments at the beginning of temporary storage[7].

Effects of Heat Treatments on Rice Quality

High temperature heat treatments on rough rice having moisture contents in the range of 18% to 30% produced milled rice showing better head yield and improved cooked-rice texture. A slight reduction in milled rice whiteness was noticed depending upon the experimental conditions[11]. The heated samples produced firmer and less sticky cooked rice in comparison with the control sample. Based on the time-temperature history of bulk grain, reaction kinetic parameters and F_C and Z_C - values were determined for specified changes in heat rice yields for general application. The estimated isothermal heating times (F_C-values) were related to the reference bulk grain temperature (T_{ref}) and initial moisture content of the rice samples (IMC). The plots of F_C- value and the T_{ref} led to the determination of Z_C-value and its dependence on the IMC of rough rice[12].

Conduction Heating for Accelerated Drying

The prototype rotary unit heated by the burning of rice husk was found suitable for heat sterilization and accelerated drying of high-moisture rice with additional advantage of improved head yield[10, 11]. For maximum head rice yield, heating surface temperatures in the range of 100 to 180 °C were found to be suitable for continuous drying of rough rice from 26% to about 18-21%. Whereas in rough rice having about 22% initial moisture content the final moisture content for maximum head yield ranged from 19 to 20%. Optimum time-temperature combinations and final moisture contents for obtaining maximum head rice yield were also established. In general, an alternate second stage drying procedure would be desirable to bring the moisture content of paddy down to safe storage level while at the same time maintaining the head rice yield gained during high-temperature short-time conduction heating.

Further work on the performance and economic aspects of rotary conduction dryers is currently in progress.

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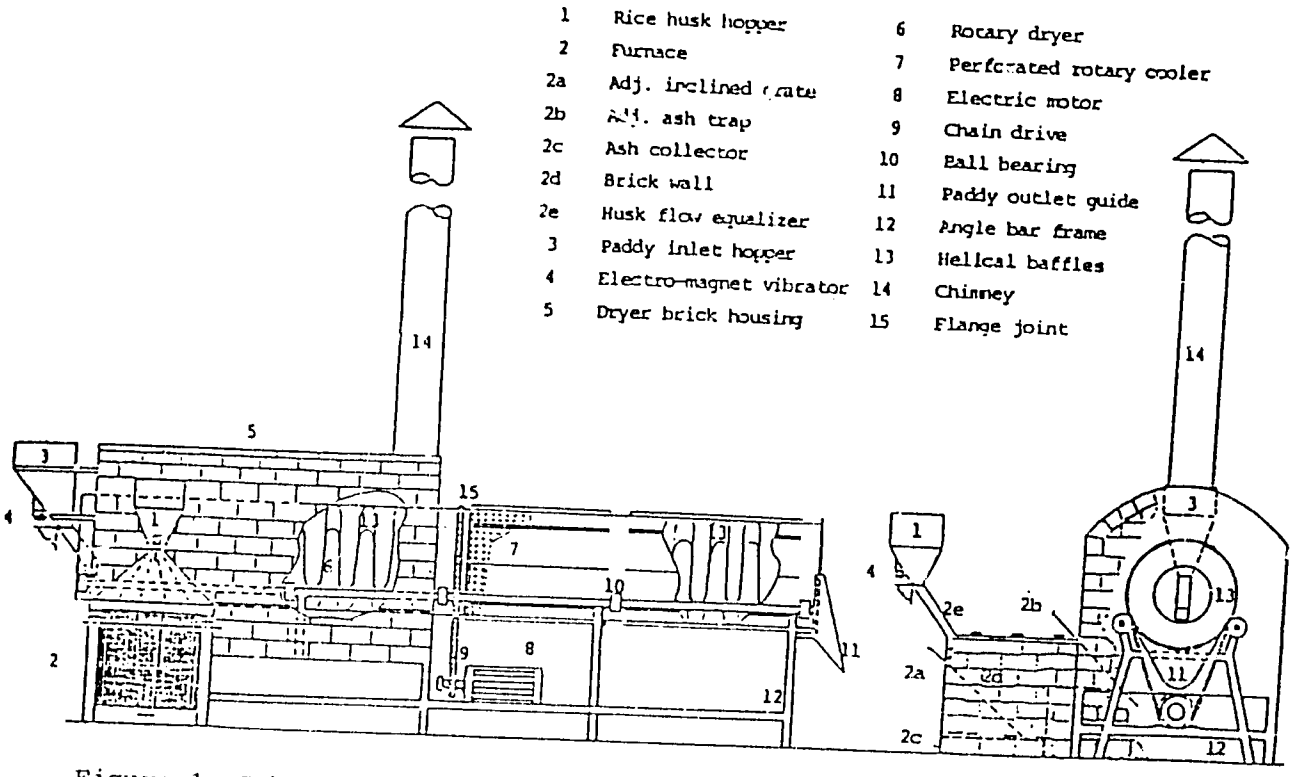

Figure 1 Schematic Diagram of Rotary Dryer and Cooler

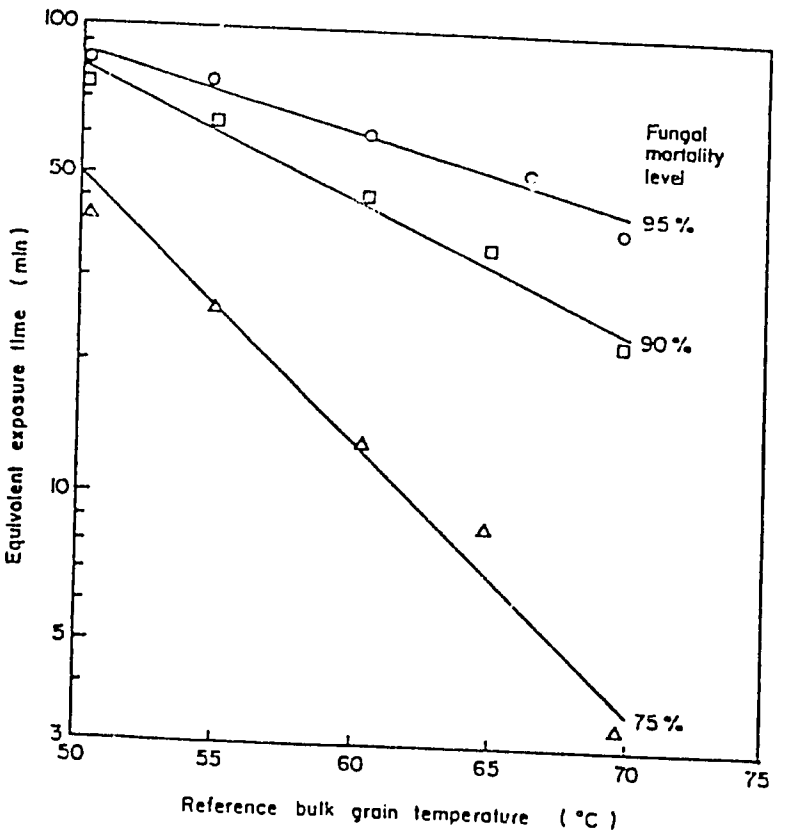

Figure 2 Equivalent Exposure Time as a Function of Reference Bulk Crain Temperature for Various Fungal Mortality Levels.

FY 1984

"IMPROVEMENT OF BACTERIAL AGENTS FOR CONTROL OF MOSQUITO VECTORS" (4.528)

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ABSTRACT

Recombinant plasmids containing delta-endotoxin genes were constructed by ligating Sau 3AI fragments of B. thuringiensis H-14 5 kb fragments into plasmid vectors pUR290, pUR291 and pUR292. One of the clones had about 0.5 kb insert and produced β-galactosidase fused protein which reacted specifically to the antibody specific for 25 kDa protein. This clone had transient toxicity against A. aegypti larvae. Thirty-two out of 800 clones derived from recombinant plasmids constructed from XbaI fragments of 110 kb plasmid of B. thuringiensis H-14 ligated to pUC12 were obtained. Sixteen out of these 32 clones gave immunopositive reaction against the delta-endotoxin antibody and the sizes of DNA inserts were of 7.5 kb, 2.4 kb or 1.9 kb. Seven out of 16 clones were found to contain mosquito toxins against A. aegypti larvae. Two of them were transiently toxic whereas five clones showed stable toxicity. These two clones contained 5.1 and 0.5 kb inserts, respectively and hybridized to the 72 mDa plasmid of B. thuringiensis H-14. The bifunctional recombinant plasmid constructed from 130 kDa delta-endotoxin gene could replicate and code for endotoxin production in both B. subtilis and E. coli. Both clones exhibited toxicity towards A. aegypti larvae, but protein crystals were observed only in E. coli cells.

INTRODUCTION

Since the discovery of Bacillus thuringiensis var. israelensis in 1977, B. thuringiensis var. israelensis has become one of the most promising potential bioinsecticides against larvae of mosquitoes. A number of field trial studies are in progress to utilize this bacterium for the biological control of mosquito vectors. There are three properties which are foreseen as limitations to the effectiveness of B. thuringiensis var. israelensis for mosquito control. The first is its genetic instability to toxin production, the second is its limited persistence in natural environments and the final property is its narrow host range; the dosage required to control malaria-carrying mosquitoes (Anopheles spp.) is rather high. On the other hand, B. sphaericus, another famous biological control agent, demonstrates a good potential for recycling and persistence in certain condition[1-4]. However, recycling does not always occur; it seems to be favored by organic pollution. In general, Anopheles are more susceptible to B. sphaericus than they are to B. thuringiensis var. israelensis but this susceptibility varies according to species and

even strains of the same species. Particulate matter, strong ultraviolet radiation, low temperatures and alkalinity decrease larvicidal activity[5-6]. Improvement in larvicidal activity and broadening of the host range is a more appropriate alternative to combat malaria-carrying mosquitoes.

Gene manipulation techniques have brought a new dimension to applied genetics. The initial step in developing a biotechnological process is generally a search for suitable organisms. Such organisms will be expected to create products or services that will generate a financial return to the industry in which they are utilized. Selection of improved organisms is tedious and time demanding and most of the methods available to geneticists up until recently have involved trial and error. However, new genetic technologies, i.e. protoplast fusion, conjugation, and the use of recombinant DNA techniques, are allowing new approaches by which useful genetic traits can be inserted directly into the chosen organisms. Thus, totally new capabilities can be engineered and micro-organisms in particular may be made to produce substances beyond their naturally endowed genetic capabilities.

The objective of this study was to increase the toxicity of bacterial pathogens of malaria-carrying mosquitoes through genetic engineering.

MATERIALS AND METHODS

Chemicals

All chemicals used throughout this study were of analytical grade or the purest grade available.

Microorganisms

Bacillus thuringiensis var. *israelensis* serotype H-14 was obtained from Institut Pasteur and used as a source of delta-endotoxin gene. *Escherichia coli* strain JM 107 and *B. subtilis* were used as recipients in gene cloning.

Plasmid vectors used for the construction of recombinant DNA plasmids were pUC12, pUR290, pUR291, pUR292, pUN121 and pUB110.

Restriction enzymes used for the construction of recombinant DNA plasmids, restriction enzyme mapping and DNA restriction analysis were Acc I, Acy I, Bam HI, Bel I, Bst NI, Cla I, Dde I, EcoRI, Hae III, Hind III, Hpa II, Msp I, Pst I, Pvu II, Sau 2AI, Sau 961, Xba I, and Xho II.

Mosquitoes

The mosquito used in the assay for toxicity was *Aedes aegypti*. *A. aegypti* was from stocks reared for several generations at the Department of Microbiology. The colonies were started from field-collected larvae from Bangkhen, Bangkok. The colonies were maintained using standard mosquito rearing techniques in a temperature and humidity-controlled rearing room to assure maximum vigor of the progeny selected for testing.

Preparation of B. thuringiensis H-14 protein crystals

Bacillus thuringiensis H-14 cells were grown in a nutrient broth supplemented with mineral salts (NBS) at 30°C in a rotary shaker at 200 rpm for 5 days or until fully sporulated cells were obtained. The fully sporulated cultures were harvested by centrifugation at 10,000 rpm for 10 min in a sorvall RC-5 super speed refrigerated centrifuge. The pellets, which contained spores and crystals, were resuspended in a solution containing 1 M NaCl and 0.01% Triton X-100, and the suspension was dispersed by sonication. The sonicates were centrifuged on continuous renografin gradients of 40-75% at 4,800 rpm for 30 min using a HB-4 rotor. The band containing crystals was located by sampling aliquots of 0.5 ml and examining each fraction with a phase-contrast microscope. The fractions containing crystals were collected and pooled together. The renografin was eliminated from the purified crystals by dilution of the suspension and centrifugation.

Identification of delta-endotoxin gene

The purified delta-endotoxin was solubilized and analyzed by SDS-polyacrylamide gel electrophoresis to determine the major components that are responsible for the larvicidal activity of the delta-endotoxin crystals. The larvicidal activity was done by using 18 hr old A. aegypti larvae exposed directly to the suspension of the solubilized component and observed 24 to 48 hr after exposure. Individual protein components obtained from the solubilization of delta-endotoxin were tested against purified rabbit antibodies which were prepared from soluble delta-endotoxin proteins and rabbit antibodies which were prepared from the 25 kDa protein purified from Sephadex G-100. The mixtures of individual protein components that reacted with the antibodies were analyzed by SDS-PAGE followed by Western blot transfer, probing with the antibody and subsequent ^{125}I-protein A coupled with autoradiography.

Construction of recombinant plasmids and gene cloning

Plasmids from B. thuringiensis H-14 were isolated by using the alkaline-phenol extraction procedure of Kado and Liu[7]. Recombinant plasmids containing delta-endotoxin genes were constructed by ligating Sau 3AI fragments of B. thuringiensis H-14 plasmids into expression vectors, pUR290, pUR291 and pUR292. The ligated product was used to transform E. coli strain JM 107 by the DMSO method described by Hanahan[8] and the transformants were selected on L-agar containing ampicillin at the concentration of 50 µg/ml. The colonies of transformant were lifted and transferred onto a nitrocellulose filter, induced with IPTG for 3 hr at 37°C and lysed with 0.5 M NaOH. These colonies were probes for deltaendotoxin production using rabbit antibody raised against the delta-endotoxin coupled with ^{125}I-protein A following the technique described by Kemp et al.[9].

Recombinant plasmids were also constructed from 110 kb plasmid of B. thuringiensis H-14 digested with XbaI and ligated to XbaI digested pUC12. The recombinant clone was selected and screened for delta-endotoxin mRNA-enriched RNA. The recombinant clone was further screened by

the delta-endotoxin antibody. The DNA inserts of the protein products which kill A. aegypti larvae were analysed.

The 5 kb fragments were further subfragmented by partial Sau 3AI and ligated to Bam HI digested pUC12, followed by transformation using E. coli as a recipient. The transformants were screened for products using antibody to the total crystal proteins and larvicidal activity.

The positive clones were used for characterization of the DNA insert and the protein product in order to determine whether this endotoxin gene was related to the 130 kDa endotoxin gene. DNA restriction analysis of the insert delta-endotoxin gene was done with a series of restriction enzymes, i.e., Acc I, Acy I, Bel I, Bst NI, Cla I, Dde I, EcoRI, Hae III, Hind III, Hpa II, Msp I, Pst I, Pvu II, Sau 961, XbaI, and Xho II.

Attempts to introduce the delta-endotoxin gene into Bacillus subtilis were also done by conjugation. The 130 kDa delta-endotoxin gene was inserted into pUB110 by joining the pUB110 plasmid vector with the recombinant E. coli plasmid containing the 3.8 kb gene encoding 130 kDa delta-endotoxin. The joining was done at Bam HI site. This recombinant plasmid was transferred into B. subtilis. The production of the delta-endotoxin gene was determined using bioassay against A. aegypti larvae and observed for the crystal formation under the phase-contrast microscope.

RESULTS AND DISCUSSION

Isolation and Identification of the delta-endotoxin from B. thuringiensis H-14

The delta-endotoxin isolated from B. thuringiensis H-14 consisted of 3 major proteins and 3 minor ones. The molecular weights of the 3 major ones were 130, 65 and 25 kilodaltons (kDa) when solubilized and analyzed by SDS-PAGE coupled with sensitive silver staining technique. Solubilization of the delta-endotoxin required strong denaturalising agents such as SDS plus mercaptoethanol, urea, guanidine hydrochloride, strong alkaline solution, all of which resulted in loss of mosquito larvicidal activity. The larvicidal activity of solubilized delta-endotoxin could be maintained at conditions of 50 mM EDTA, pH11.7, for 2-3 hr. In such conditions the major protein band on the SDS-PAGE was the 25 kDa.

It was found that two major proteins of MW 65 and 25 kDa solubilized with 50 mM NaOH, 10 mM EDTA pH 11.7. The insoluble proteins found were those of 130 kDa and some 65 kDa. Either 130 kDa or 25 kDa protein alone demonstrated larvicidal activity to A. aegypti. However, the 65 kDa protein alone did not show toxicity against A. aegypti larvae. Recently, Wu and Chang[10] demonstrated that neither 65 kDa nor 25 kDa alone was significantly toxic to mosquito larvae but that a combination of the two proteins showed synergistic toxicity. Thus, these purified proteins were used to prepare specific antibodies in rabbits. Results of

analyses by SDS-PAGE, Western blot transfer, probing with specific antibody, and autoradiography indicated that gene (genes) coding for the delta-endotoxin crystal resided on plasmids of B. thuringiensis H-14.

Cloning of delta-endotoxin gene into E. coli

One recombinant clone designated as pUN-1/5 was obtained from over 20,000 clones of transformed E. coli JM 107. These transformants were derived from B. thuringiensis H-14 plasmids digested with EcoRI and ligated to the EcoRI-cut pUN121. Once the inserted DNA of pUN-1.5 was analyzed, it was found that the DNA insert was 5 kb long. Analysis of the gene product by Western blot analysis using the available antibodies showed a weak protein band around 25 kDa. When the recombinant DNA of pUN-1.5 was used to transform E. coli P678-54 which was a minicell producing strain. The minicells containing pUN-1.5 were obtained and protein products were assayed using ^{35}S-methionine. A very faint protein band of approximately 25 KDa was observed. However, this transformant containing pUN-1.5 did not show toxicity towards A. aegypti larvae.

Transformants derived from recombinant plasmids obtained from Sau 3AI fragments of B. thuringiensis H-14 5 kb fragments ligated to pUR 290, pUR291 and pUR292 and introduced into E. coli JM 107 were produced as fused proteins. One of the clones which was designated as clone pUR-O_3 had about 0.5 kb insert and produced B-galactosidase fused protein which reacted specifically to the antibody specific for 25 kDa protein. The larvicidal activity of clone pUR-O_3 against A. aegypti demonstrated transient toxicity in which the toxicity disappeared in the later bioassays. This result probably due to the fact that 0.5 kb is too small to code for 25 kDa protein.

Eight hundred transformants derived from recombinant plasmids constructed from XbaI fragments of 110 kb plasmid of B. thuringiensis H-14 ligated to pUC12 were obtained. After they were screened for delta-endotoxin-mRNA-enriched RNA, approximately 32 positive clones were found and expected to contain the delta-endotoxin gene or its fragments. These 32 clones were further screened by the delta-endotoxin antibody and 16 clones gave positive reactions. The analysis of DNA inserts of the antibody positive clones showed that the sizes of DNA inserts were 7.5 kb, 2.4 kb or 1.9 kb. Seven out of 16 clones were found to contain mosquito toxin against A. aegypti larvae. Two of them were transiently toxic whereas five clones showed stable toxicity. DNA restriction analysis of the insert delta-endotoxin genes revealed that the clones had a common DNA insert of 3.8 kb. A detailed restriction enzyme mapping showed recognition sites by enzymes EcoRI, Pvu II, Pst I, XbaI, Hind III, Hae III, Bel I, Xho II, Acc I, Msp I, Hpa II, Acc I, Msp I, Hpa II, Acy I, Sau 961, Dde I, Cla I, and BstNI. Other E. coli clones which were immunopositive but did not show toxicity to A. aegypti larvae had inserts of either 7.5, 2.4 or 1.9 kb. Further analysis of the protein products of these clones by Western blot immunoassay showed that all of the E. coli clones that contained mosquito toxicity to A. aegypti larvae produced proteins with 130 kDa as the major component. The minor

components apparently were degraded products of the 130 kDa proteins. Those clones that gave no toxicity with the inserts of 7.5, 2.4 and 1.9 kb produced proteins with 90 kDa as the major component. It was found later that recombinant plasmids obtained from XbaI which cut mosquito toxin genes joined with XbaI digested plasmid vector pUC12 were not stably maintained in E. coli JM 107 after successful transformation.

Plasmids from recombinant clones pUN-1.5 and pUR-O_3, which were deemed to contain delta-endotoxin genes, were extracted and characterized. It was found that clones pUN-1.5 and pUR-O_3 contained 5.1 and 0.5 kb insert, respectively. These two DNA inserts hybridized to the 72 megadalton (mDa) plasmid of B. thuringiensis H-14. This result indicated that these DNA inserts were derived from a common plasmid which had been postulated to contain delta-endotoxin genes.

Introduction of the delta-endotoxin gene into B. subtilis

In an attempt to introduce the delta-endotoxin gene into Bacillus spp., the 130 kDa delta-endotoxin gene was used to construct the recombinant plasmid and introduced into B. subtilis. As a result, the bifunctional plasmid could replicate the code for endotoxin production in both B. subtilis and E. coli. Both clones exhibited toxicity towards A. aegypti larvae. The protein crystal was observed only in E. coli cells under the phase-contrast microscope as shown in Figure 1. The bifunctional plasmid was found to be stably maintained in B. subtilis as well as in E. coli.

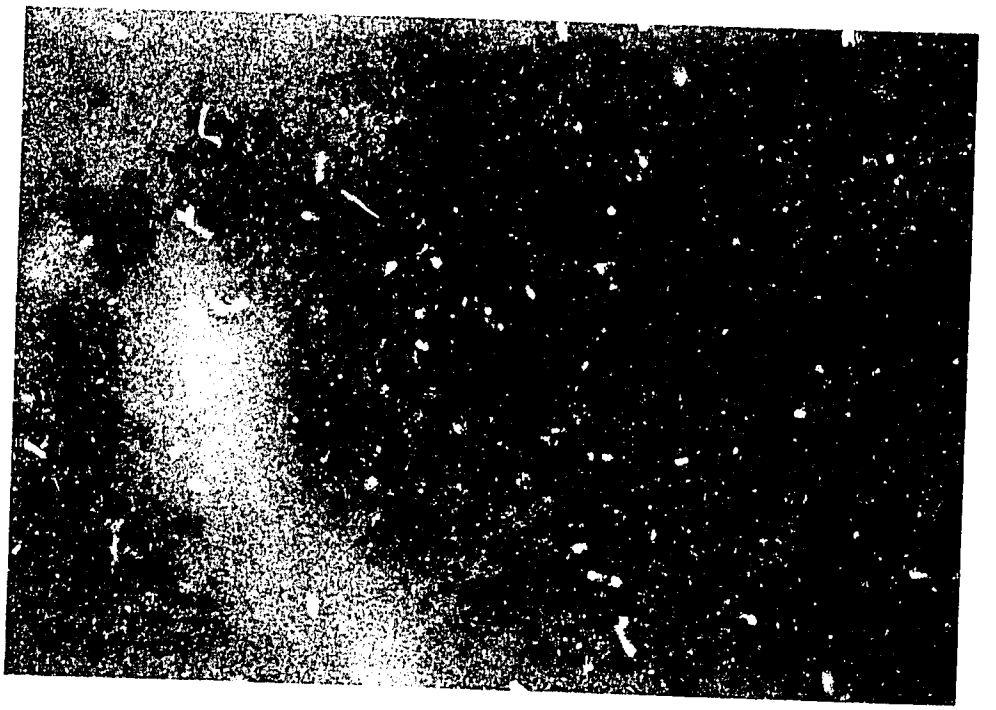

Figure 1 Characteristics of E. coli clones that contain endotoxin protein crystal

A successful transfer of mosquito toxin gene from B. thuringiensis H-14 into E. coli in which 2 clones exhibited the larvicidal activity towards A. aegypti was conducted. This result goes along very well with results reported by other investigators[11-12]. A recombinant clone, pUN-1.5, was found to contain a portion of the delta-endotoxin gene from B. thuringiensis H-14. This clone was derived from recombinant plasmids constructed from the digestion of 72 mDa plasmid with restriction enzyme EcoRI. The size of DNA insert was found to be about 5,000 base pairs which were responsible for the production of proteins which reacted to rabbit antibodies prepared against crystal proteins of B. thuringiensis H-14 as demonstrated by colonies immunoassay. However, the ability to produce proteins of this clone was very poor, and thus proteins and toxicity of this clone were difficult to characterize. Another recombinant clone pUR-O_3 which expressed the B. thuringiensis H-14 delta-endotoxin gene was found to harbour a 500 basepair Sau 3AI fragment of B. thuringiensis H-14 DNA and the clone could produce proteins detectable by a specific antibody prepared to 25 kDa polypeptide derived from B. thuringiensis H-14. The lack of larvicidal activity of clone pUR-O_3 is probably due to the ability of the inserted DNA of 500 basepairs that could not encode the full length of the delta-endotoxin polypeptide that was sufficient to exert toxic activity against the mosquito larvae. This incomplete polypeptide may have lost its biological activity via the absence of the active residue. When the relationship between DNA of clone pUN-1.5 and pUR-O_3 was determined, it was found that the clones contained homologous sequences of inserted DNA. Thus, it was believed that clone pUN-1.5 should carry the delta-endotoxin gene as clone pUR-O_3 does.

It is now believed that success in transferring mosquito-toxin genes from B. thuringiensis H-14 into other organisms can be done and it relies on the selection of an appropriate plasmid vector that can be expressed in the recipient cell. The development of methods and gene transfer systems will lead to the successful transfer of an individual gene coding for the mosquito-toxin into a host micro-organisms and the organism can be grown in volume to yield the product. However, this objective seems to be very difficult to obtain. This is due to the fact that different organisms have different properties even within the same species that consists of several strains or mutants. The most difficult part of the transfer is the matter of the selection of an appropriate plasmid vector for the construction of a recombinant plasmid. This is based on the fact that a cloning vehicle should be as small as possible to reduce the possibility of including undesirable genes and prevent self-transmission by conjugation to another cell or organism. Moreover, the yield and purity of cloned genes and the efficacy of ligation and transformation are also greater with smaller DNA molecules.

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FY 1984

"UTILIZATION OF CASSAVA AND CASSAVA WASTE THROUGH FERMENTATION TECHNOLOGY" (4.031)

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ABSTRACT

Based on both socioeconomic and technological consideration, the project was proposed with the aim to more effectively utilize cassava and its waste to produce fuel alcohol and nutritionally balanced silage. Attempts have been made to produce enzymes efficient in the hydrolysation of raw cassava and root fiber to provide sufficient fermentable sugars prior to, or simultaneous with, alcohol fermentation. Purification and characterization of the enzymes involved were also carried out. Competitive yeast strains were selected, so that they could be applied both for alcohol and silage fermentation under conditions unfavorable to contaminants in the raw substrates. Fermentation kinetics was studied. A mixed culture fermentation was proposed for ensilaging. At present, microorganisms with desirable characteristics are being selected.

INTRODUCTION

Cassava is one of the major cash crops for Thailand and involves over 8 million farmers. Exporting of cassava product in the form of pellets, mainly to the European Economic Community (EEC) nations, has ranked second in foreign exchange earnings. With the imposition of import restrictions by the EEC since 1982, an excess of cassava roots has resulted and the income of the farmers involved has decreased. A Cassava Action Plan has, therefore, been set out by the Thai Government.
Included among other objectives of the plan, are the aims to increase and diversify the in-country utilization of cassava.

Based on its comparatively low protein content, cassava as such is not competitive with other grains available for animal feed in Thailand. Although studies on enrichment of cassava by fermentation are not new, more research is still needed to improve both the process and the product.

In terms of diversifying industrial uses, fuel alcohol has proven to have a great potential as evidenced from success in Brazil and establishment of a pilot plant at Thailand Institute of Scientific and Technological Research. However, more research is required to reduce the production cost. This project is, therefore, proposed with an aim to

1. produce enzymes effective in digestion of raw cassava
2. develop and/or improve technology for alcohol fermentation using raw cassava
3. produce nutritional balanced cassava silage.

METHODOLOGY, RESULTS AND DISCUSSION

Selection of fungi effective in hydrolysation of raw cassava

Effective strains were selected according to the scheme shown in Figure 1.

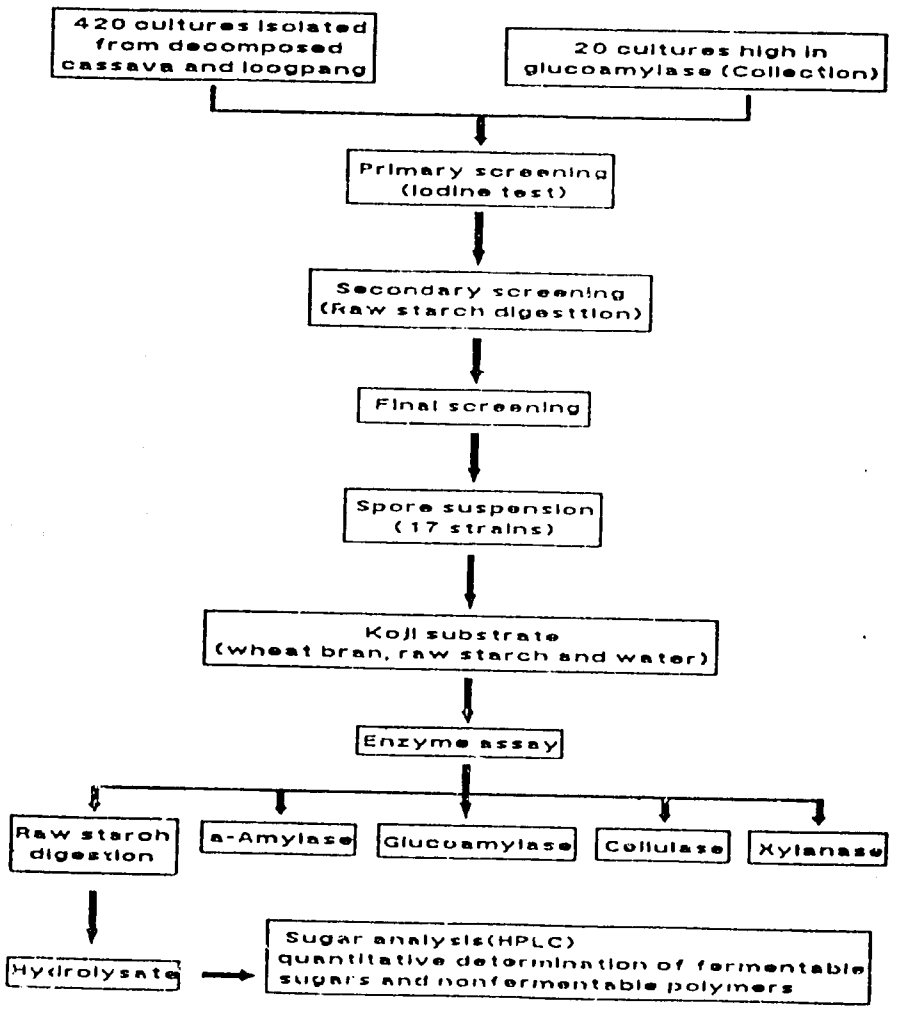

Figure 1. Selection of Fungi Effective in Hydrolysation of Raw Cassava.

Based on their activities in raw cassava digestion, (Figure 2 and 3) *Aspergillus sp.* J8, J4, J10, and *Rhizopus sp.* N37 and 102R were selected. Besides being active in the hydrolysation of raw starch,

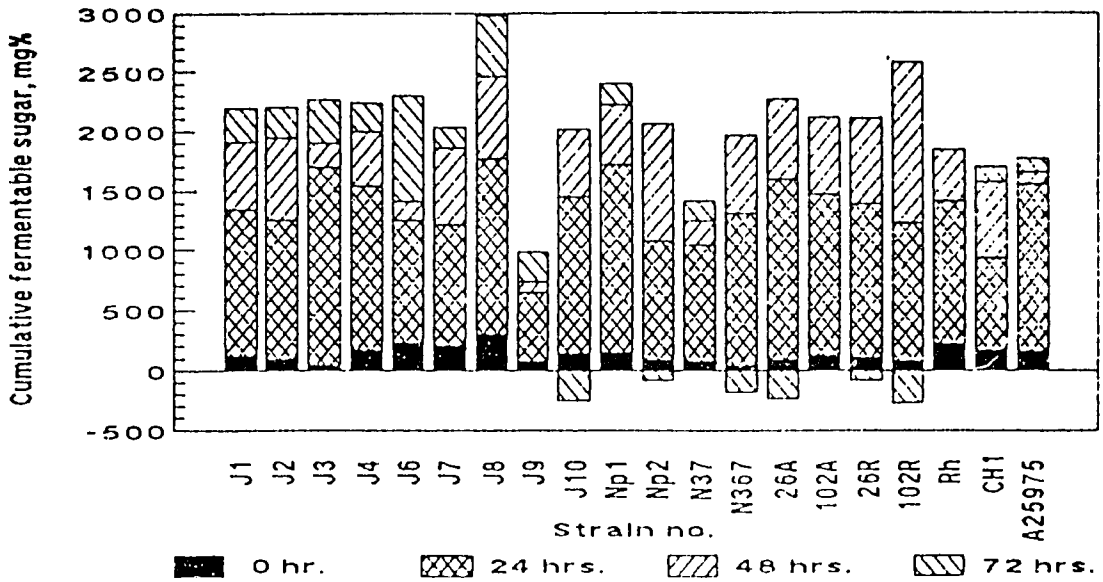

Figure 2. Time Course of Hydrolysis of Raw Cassava Starch by Koji Extract of 20 Potent Strains.

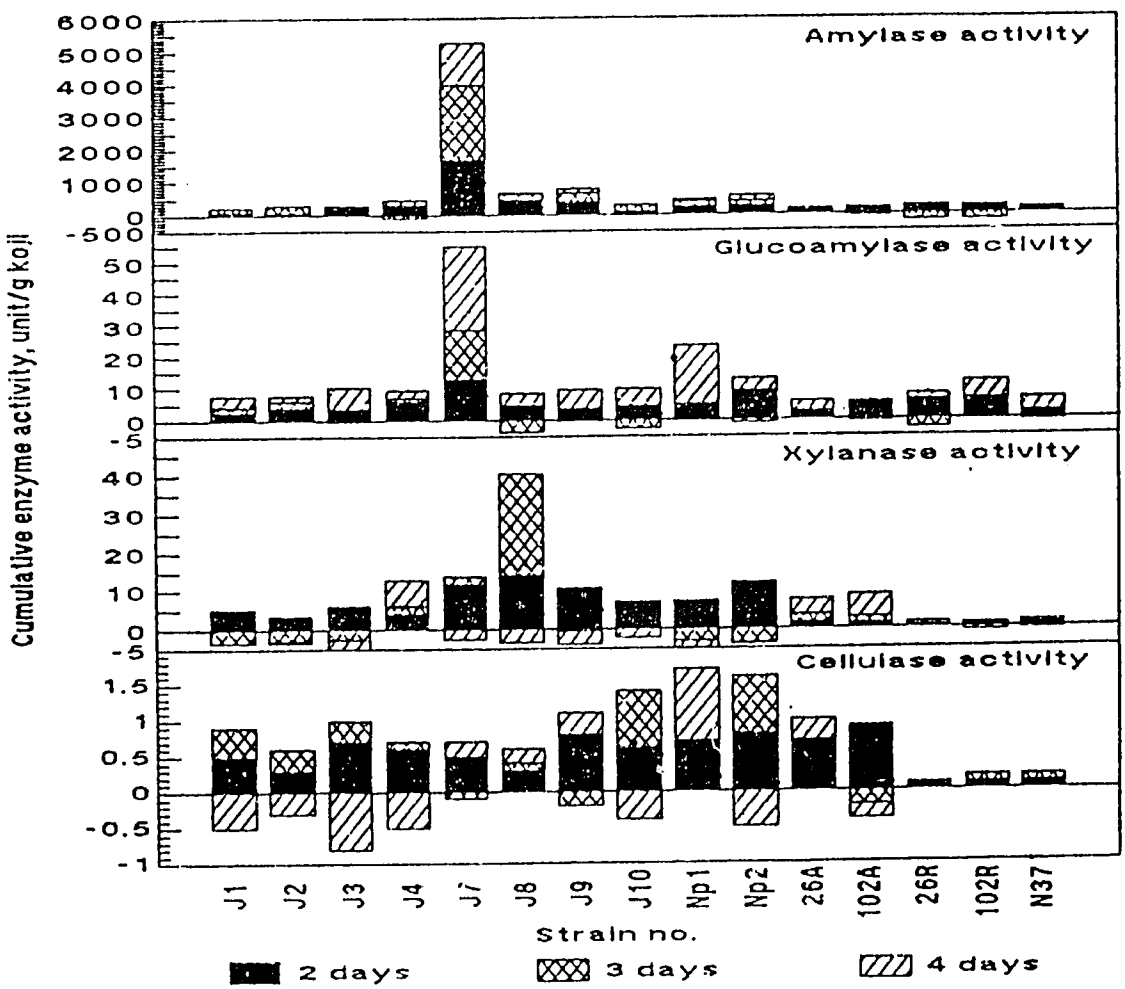

Figure 3. Enzyme Activities of Potent Strains.

strain J8 also produced the highest amount of xylanase, while J4 was strong in α-amylase production and J10 was superior in cellulose activity.

Enzyme Production and Enzyme Properties

Optimization of culture conditions for maximum enzyme production and determination of some physicochemical properties were made, so that the strains could be compared under optimal conditions (Table 1 and 2). Information obtained was also required for the scaling up of enzyme production and applications.

Table 1 Optimal Conditions for Production on Raw Starch Digesting Enzymes.

Condition	Strain		
	Aspergillus sp.		*Rhizopus* sp.
	J8	N37	102R
Substrate	Wheat bran or wheat bran + 15% cassava waste		
Initial moisture (%)	47 - 63	47 - 50	50 - 60
pH	5.0	5.5	5.5
Temperature (C)	30 - 35	35 - 37	35 - 37
Incubation Time (Hr)	80	72	72

Table 2 Properties of Raw Starch Digesting Crude Enzyme.

Condition	Strain		
	Aspergillus sp.		*Rhizopus* sp.
	J8	N37	102R
Optimal pH	3.5 - 4.0	4.5	4.5
Optimal temperature (C)	45	45	45
pH stability for 48 hrs.	5.5	5.0	5.0 - 5.5
Temperature stability (C) for 48 hrs.	35	45	40

Enzyme Purification

In order to elucidate roles of each enzyme contained in the crude extract on raw starch digestion, enzyme purification and characterization were carried out. Figure 4 represents purification scheme and results obtained at the present stage of work.

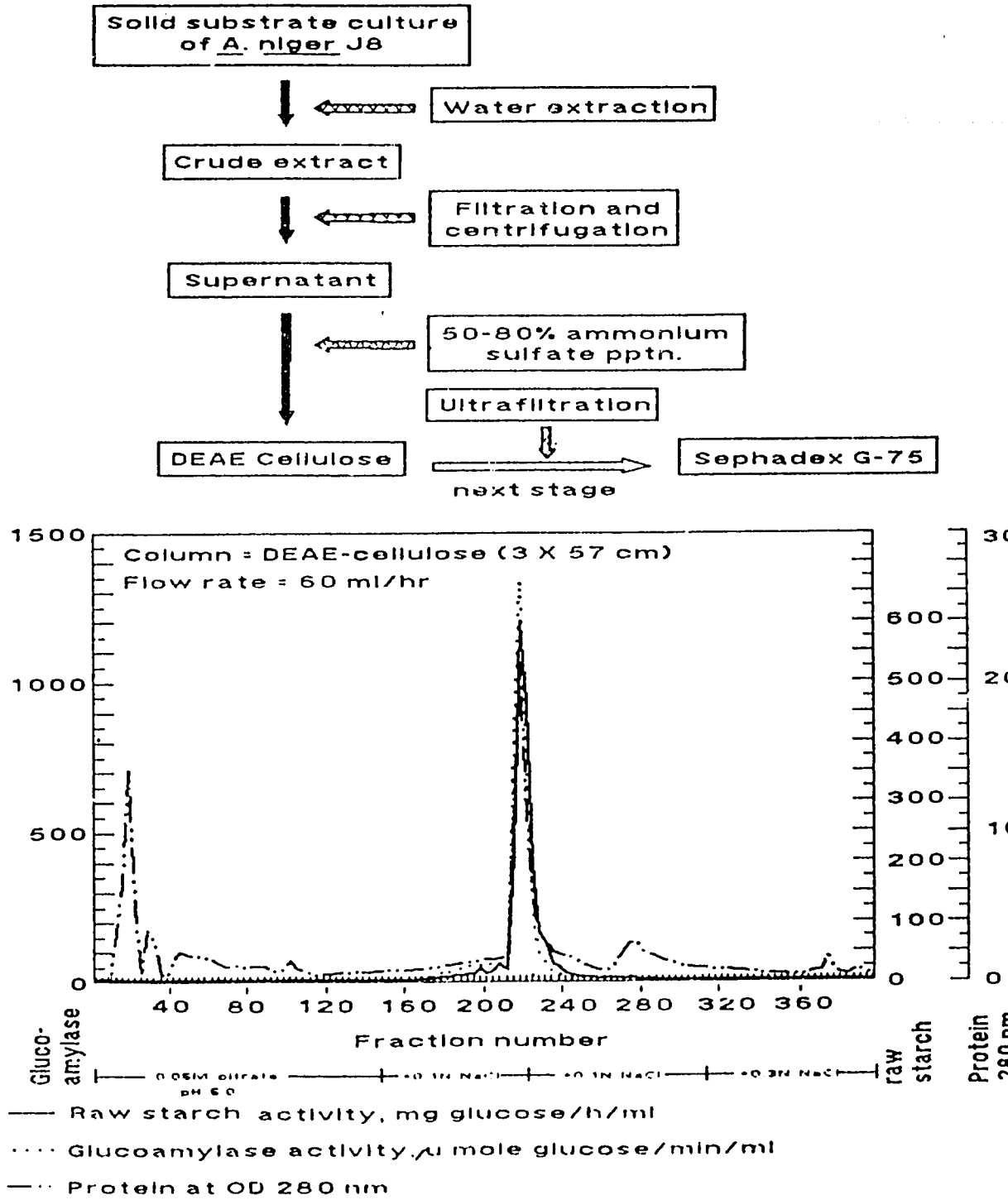

Figure 4. Flow Chart of Enzyme Purification and Chromatogram.

Yeast for Alcohol and Silage Fermentation

A number of cultures previously selected as being efficient in ethanol production from molasses were screened for their activity in cassava hydrolysate following the scheme shown in Figure 5. Two thermotolerant strains which produced high alcohol concentrations at comparatively low pHs were obtained. One of these was a flocculating yeast.

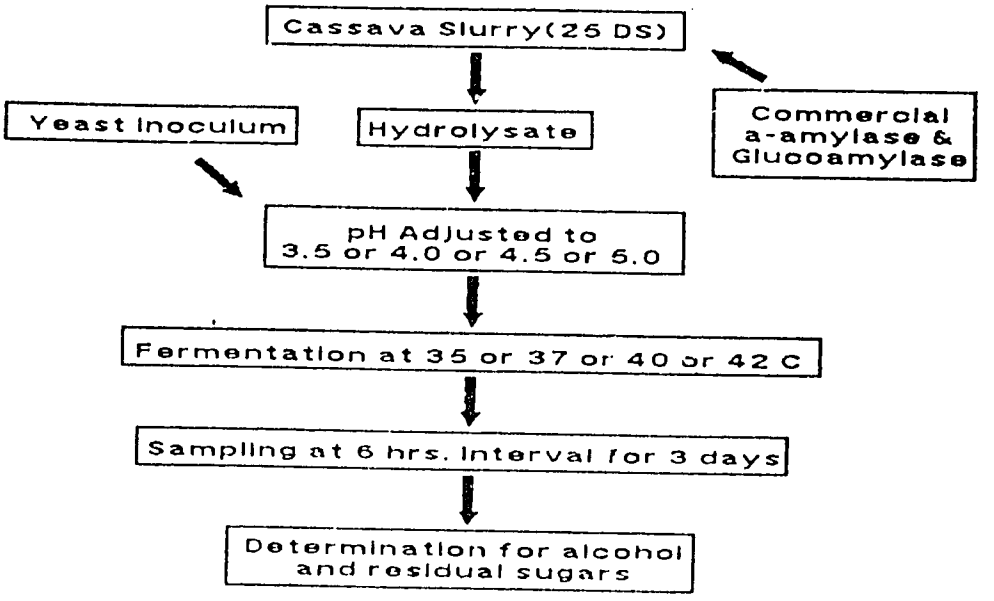

Figure 5. Selection of Yeast for Alcohol and Silage Fermentation.

Silage Fermentation

Mixed culture fermentation following the flow chart shown in Figure 6 is proposed to produce cassava silage of the following characteristics : 8-10% protein content, sufficient vitamin B12, the presence of certain strains of lactic acid bacteria having inhibitory effects over enteropathogens. Strains of microorganisms to be used have been selected.

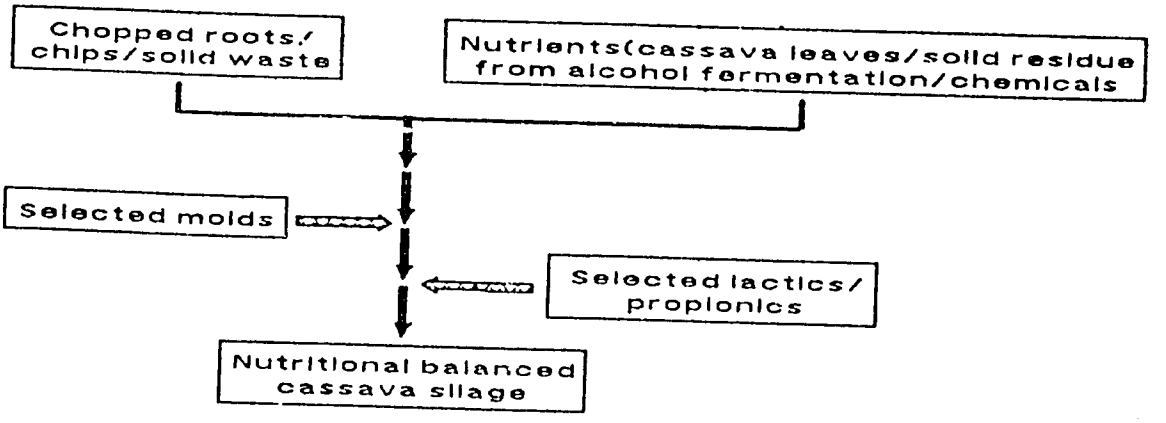

Figure 6. Flow Chart of Silage Fermentation.

FY 1985

"NATURAL INSECTICIDES FOR SPODOPTERA LITURA FABR." (5.240)

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ABSTRACT

Over 100 plant species of reputed insecticidal properties were screened for their toxicity on Spodoptera litura Fabr. The three most promising species were further investigated for their active ingredients. The most active ingredient in Zingiber purpureum was proved to be 4(3′, 4′-dimethoxyphenyl) but -1, 3-diene(1), a synthetic analogue of which, 4(3′, 4′-methylenedioxyphenyl) but -1, 3-diene(2), showed 2-3 fold increase of activity. Ageratum conyzoides, a common weed, was shown to contain the active ingredients precocene I (3), and precocene II(4) easily obtained by steam distillation. An Aglaia species showed a strong antifeeding activity; a possible active component was found to be the bis-amide(5). Preliminary use of this plant extract for protecting crops from S. litura in the field gave a satisfactory result.

INTRODUCTION

S. litura Fabr. is a widespread insect pest. It is polyphagous and regarded as one of the most damaging pests in world agriculture. Pandy and Srivastava (1967) reported that the insect attacks 112 species of cultivated crops all over the world. In Thailand, at least 30 varieties of plants are found to be host plants of this insect[1]. These include vegetables, floral plants, ornamental plants, economic field crops and weeds.

The time required for egg, larval and pupal development are 2-3, 14 and 7-8 days respectively[2]. The adult longevity is 8-10 days in the male and 10-12 days in the female. The larva molts 5 times. It is at this stage that the insect is most damaging. Since the early larval instar is quite vulnerable to external damage, it has been chosen as the target organism for this work.

For self-defence, it is not surprising that plants would contain substances which are insect antifeedants, insect repellents and insecticides. Some naturally occurring antifeedants which have been documented are: glycosides of steroidal alkaloids, demissine[3], solacauline[4], tomatine[5], leptines I and II[6], ring A enone and/or ring D aromatic steroids, the nicandrenones and others[7,8,9,10]; juglone (5-hydroxynaphthoquinone)[10]; the isoquinoline alkaloid isoboldine[11], phenylpropanoids[12], germacranes sesquiterpenes shiromodiol and shiromol[13,14]; ent-clerodane and clerodane diterpenes, clerodendrin[15]; caryoptin and

others[16]; the hydroxylated steroid meliantriol[17]; azadirachtin[18]; xylomolin[19]; ajugarins[20]; harrisonin[21]; polygodial ugandensidial and warburganal[22]; inflexin[23]; and isodomedin[24]; crotepoxide[24] and unedoside[25]. However, there is no report of any of these substances being used effectively in agriculture.

A number of naturally occurring compounds have been found to exhibit insecticidal activity. These include alkaloids, terpenoids, phenolics, amines, chromenes, phenylated guinones and sesquiterpenes esterified with phenolic acids[26]. Commercially, the substances which have found their way to agricultural applications are nicotine, derris, pyrethrum and the newer product, ryania[27]. Crude extracts of a plant which produces these substances, as well as dried and powdered plant tissue have a long history of use in SE Asia as insecticides. However, none of these natural products is a universal insecticide : Tabacco leaf containing nicotine is subject to attack by several insects the root of derriscontaining plant is attacked by the larvae of a species of beetle, the flower from which pyrethrins are extracted needs synthetic insecticides for protection from insects in the fields. In addition, insects can develop resistance to a certain chemicals after a long period of exposure. New and better insecticides will, therefore, have to be constantly sought.

The work was undertaken in order to isolate and identify insecticides/antifeedants for S. litura from plants growing in Thailand and to extract/synthesize those compounds on large scale for evaluation of their applicabilities in the field.

MATERIALS AND METHODS

Plants investigated were collected in the southern region of Thailand. Samples were classified taxonomically and vouchers were deposited in the herbarium in the Department of Biology, Prince of Songkla University.

Ground plant materials were steam distilled or exhaustively extracted with solvents. The extracts/steam distillates were screened for insecticidal and antifeedant activity with S. litura and any active extracts resolved into fractions. Each fraction was screened for activity and the active fraction, again, separated into individual constituents using quick column and preparation layer chromatography on silica gel.

Each of the individual components was tested again for activity and the LC_{50} value of the active compound determined. The structure of the compound was elucidated using spectroscopic techniques.

The leaf dipping (LD) method was used for the detection of stomach poisoning and antifeeding activity. The third instars of Spodoptera litura larvae were left to feed on the leaves of Vigna radiata which had been immersed in the tested solution then air-dried. Ten larvae were used in each of the four replications (one as control). The number of dead organisms was recorded at the end of each day. The amount of leaf consumption and the larvae size were also estimated.

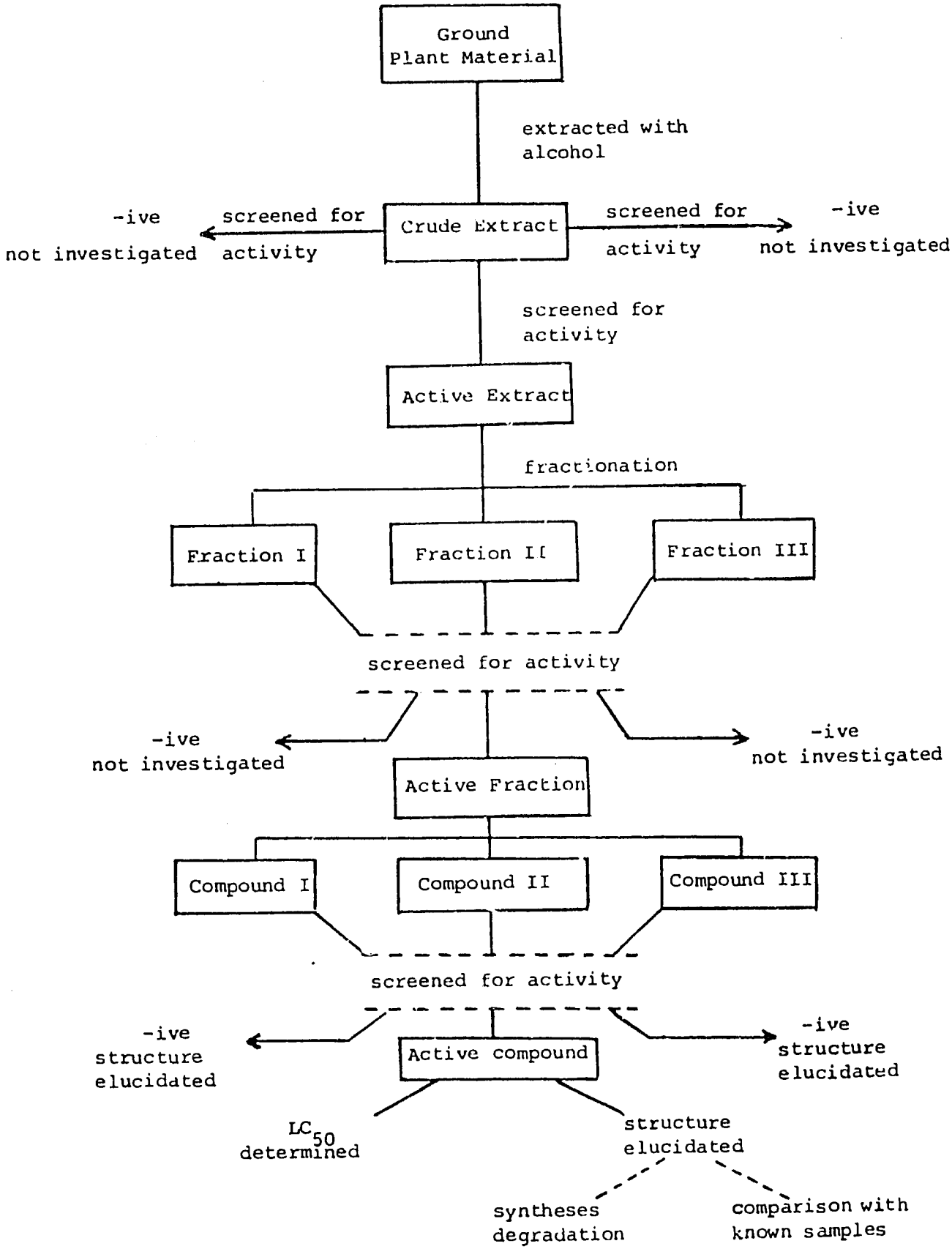


Diagram showing work plan

The topical application (TA) method was used for the detection of dermal toxicity. The tested solution was dropped on the thorax of their instar larvae. The organisms were then left to feed on untreated leaves of Vigna radiata in a plastic container. Ten larvae were used in each of the four replications (one as control). The number of dead organisms was recorded at the end of each day.

The LD coupled with TA method (TD) was used for detection of the combined effect of stomach poisoning and dermal toxicity. The tested solution was dropped on the thorax of third instar larvae. The organisms were then left to feed on the leaves of Vigna radiata which had been immersed in the same tested solution then air-dried. Ten larvae were used in each of the four replications (one as control). The number of dead organisms was recorded at the end of each day.

To prevent the loss of organisms from their carnivorous behavior, one larva was placed in each plastic container with a small leaf of Vigna radiata. Thirty treated larvae and ten control larvae were used for each test. The number of dead larvae and the size of the larvae alive were estimated at the end of each day.

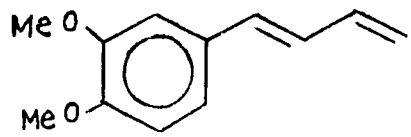
For more accurate and reproducible results, one larva was left to feed on a soybean artificial diet instead of Vigna radiata leave. Substances/fractions from plants were dissolved in ethanol and mixed with the artificial diets by vigorous stirring. The solvents were then evaporated under reduced pressure with a high vacuum pump. As a control, the artificial diet was mixed with ethanol without substances/fractions and treated in a similar manner.

RESULTS AND DISCUSSIONS

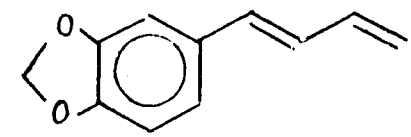
Using the basic references for plants which are reported to be toxic to lower forms of living organisms and insects[26,27], 100 plants were selected for testing. Ten plants were selected, on the basis of their availability and activity, for further investigation. Among them, three of most interesting plants were Zingiber purpureum, Ageratum conyzoides and an Aglaia species.

The active components of Zingiber purpureum appeared to be in the steam distillate which was found to contain a mixture of phenyl-butanoids in agreement with previous reports[28,29]. The most active component appeared to be 4(3′, 4′-dimethoxyphenyl) but -1, 3-diene(1). The compound was then synthesized and tested (TD). LC_{50} was found to be 0.70% (48 hr), 0.33% (72 hr), 0.12% (96 hr).

Previous work showed that compounds containing a methylenedioxyphenyl group can act as synergist[30] for pyrethrins. The compound 4-(3′, 4′-methylenedioxyphenyl)but-1-3-diene(2) was then synthesized and tested for activity, both alone and with selected compounds (1). While no synergism was observed, the activity of the compounds (2) was found to be 0.50% (24 hr), 0.21% (48 hr), 0.07% (72 hr), 0.04% (96 hr); and the LD_{50} value was 3.04 µg/insect (24 hr) in the TA test.

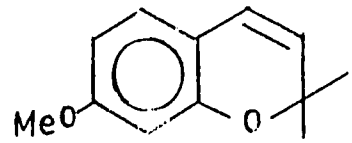


(1)

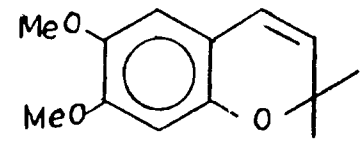


(2)

Another steam distillate which showed consistant activity was that of Ageratum conyzoides. Preliminary results showed that it contained precocene I (3) and precocene II (4) which were the antijuvenile hormone compounds of considerable interest[31]. The LC_{50} of the crude steam distillate was found to be 0.83% (48 hr), 0.36% (72 hr), 0.29% (96 hr).

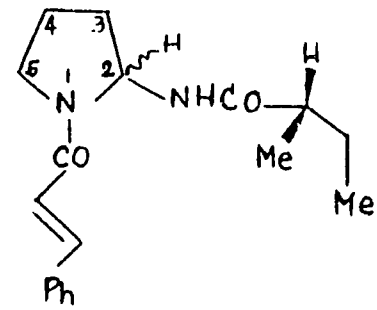


(3)



(4)

The most outstanding results obtained to date have been from the genus Aglaia. Six species of plants in the genus have been studied. In particular, one species showed a strong antifeeding activity. Among several compounds from the active fraction, the bisamide (5) has been isolated.



The compound is presently being evaluated for its specific activity. However, preliminary field test of the crude extract of this Aglaia gave a satisfactory result. Further attempts are being made to improve the formulation and the spraying techniques, and to evaluate the costeffectiveness in the field.

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FY 1985

"IMMUNODIAGNOSIS AND SEROEPIDEMIOLOGY OF TUBERCULOSIS" (5.224) : MYCOBACTERIUM TUBERCULOSIS I. EVIDENCE OF SPECIES-SPECIFIC LIPID ANTIGEN FROM MYCOBACTERIUM TUBERCULOSIS

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ABSTRACT

An attempt to isolate and purify specific antigens or haptens from Mycobacterium tuberculosis has been carried out in our laboratory. A glycolipid substance was isolated from M. tuberculosis H37Rv (TMC 102) by chloroformmethanol (2:1) extraction. After passing through a silicic acid/celite column, the glycolipid was eluted with 2% methanol in chloroform in any one or two fractions of 25-35 fractions consisting of 10 ml/fraction. This glycolipid was evaluated for specificity by ELISA test using 19 different antisera to mycobacteria. This glycolipid reacted only with antiserum to M. tuberculosis while the other 18 antisera to different species of mycobacteria were negative. When the glycolipid was analysed by thin layer chromatography using diisobutylketone: acetic acid: water (85:50:7) as the developing agent, it gave a red spot with orcinol in sulphuric acid with a Rf value of 0.63 similar, but not identical to, phenolic glycolipid I (PGL-I) of M. leprae. When it was studied by infrared spectrophotometry, it was found to contain an aromatic nucleus and a prominent hydroxyl group.

INTRODUCTION

Tuberculosis is one of the oldest and most widespread of the life-threatening infectious diseases still affecting humans throughout the world. The diagnosis of tuberculosis is important both to the individual patient and to the community. There is no simple diagnostic test for tuberculosis. Clinical and radiological signs and bacterial staining by smear are notoriously non-specific; and culture techniques, although providing a definitive diagnosis, are expensive and time-consuming, and more seriously, they vary considerably in their reliability from laboratory to laboratory. There is no doubt that tuberculosis is often misdiagnosed; many cases are not recognized until a postmortem examination is performed, and cases of renal, meningitis, bone and intestinal tuberculosis are often only diagnosed late in the course of the disease. (1,2,3)

Extensive studies have established beyond doubt that M. tuberculosis induces humoral immune response in the infected host. There is also no doubt that some antibodies, capable of binding to antigens in this organism, are present in both patients with other diseases and in healthy individuals. Until the present time, no investigator has developed a completely reliable test for serodiagnosis of tuberculosis. Many researchers have shown that there is a high degree of shared antigens between the various mycobacterial species and that some of the antigens are also shared with other bacterial genera (4).

A serologic test that would indicate active tuberculosis with reasonable precision would be of great value in numerous conditions in which tuberculosis is part of a differential diagnosis, and in the diagnosis of diseases of internal organs that are difficult to assess, such as those of the spine or the gut. A serologic test would also save the time required for culture of specimens containing too few tubercle bacilli to yield a positive direct smear examination. The development of an effective serologic test to detect an antibody in preclinical or clinical tuberculosis would also greatly aid the effectiveness of tuberculosis control programs. Such an assay could both facilitate early diagnosis of the disease and could decrease incidence of the disease by early detection in multibacillary individuals.

Several laboratories have developed immunoassays for diagnosis of tuberculosis. These assays include: fluorescent antibody test (5), double diffusion gel precipitation test (6), enzyme-linked immunosorbent assay (ELISA) (7), hemagglutination test (8), and solid phase radioimmunoassay (9). Howver, the antigens used in each method are different and none are specific antigens of M. tuberculosis. For detection of a specific antibody against M. tuberculosis, a specific antigen from M. tuberculosis or a specific antibody to this bacilli is necessarily required. Species specific glycolipids have been isolated from M. leprae (10) and various other species of mycobacteria (11); however, species specific antigens or haptens still have not been isolated form M. tuberculosis, nor have specific monoclonal antibodies to M. tuberculosis been produced to date (12). We have successfully isolated a species specific antigen of M. tuberculosis.

MATERIALS AND METHODS

Preparation of Mycobacterium Culture.

M. tuberculosis H37Rv (TMC 102) and 15 atypical mycobacteria namely, M. fortuitum (ATCC 6841), M. smegmatis (TMC 1546), M. avium (NIH 724), M. gordonae (TMC 1324), M. marinum (TMC 1218), M. kansasii (TMC 1204), M. vaccae (TMC 1526), M. intracellulare (TMC 1403), M. bovis (BCG, TMC 1030), M. gastri (ATCC 15754), M. nonchromogenicum (ATCC 15930), M. terrae, M. chelonei, M. triviale and M. scrofulaceum were cultured for antigen preparation. Each species of bacilli was cultivated at 37° C for 2-6 weeks in Sauton's liquid media, autoclaved and centrifuged at 8,000 G 30 min., washed twice with NSS and resuspended in NSS containing

60 mg bacilli/ml. The cell suspension was sonicated 4 times for 5 min. each by a Braun Sonic 1510 Sonicator with an effect of 100 W. One portion of sonicated material was mixed with an equal volume of incomplete Freund adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.) for immunization of rabbits. The other portion was centrifuged at 8,000 G 30 min., and the supernatant was collected and stored at -20°C for use as crude antigen in ELISA test.

Production of Antisera.

New Zealand white rabbits were given intramuscular injections of 0.5 ml of each antigen emulsion in the hind leg. The animals were further immunized intracutaneously with 0.1 ml of each antigen emulsion in the neck region every 14 days. Bleeding was done every 7 days, the sera separated and assayed for antibodies by ELISA test using corresponding crude antigen. Blood was taken from rabbits that gave an appropriate titer of antibodies, the serum separated and stored at -20°C. Rabbit antisera to M. bovis (Bacillus Calmette-Guerin, BCG), M. duvalli and M. paratuberculosis (bovine) were purchased from Dakopatts (42, Produktionsvej, P.O. Box 1359, DK-2600 Glostrup Denmark).

Extraction and Purification of Glycolipid from M. tuberculosis.

One gram of dried M. tuberculosis H37Rv (TMC 102) was extracted with 40 ml chloroform-methanol (2:1) mixture by stirring at 50°C for 18 hours. After centrifugation at 300 G for 15 min., the residue was re-extracted with a half volume of chloroform-methanol (2:1) at 50°C for 18 hours. Pooled supernatants were washed with water, then evaporated to dryness and redissolved with chloroform. The crude lipid in chloroform was further purified by pouring through a column 3.5 x 6 cm containing a silicic acid/celite (2:1) mixture which was successively irrigated, first with 10 bed volumes of chloroform, and then eluted with 2% methanol in chloroform approximately 40 Bed volumes. Ten millilitres of eluate were collected for each fraction. The chloroform-methanol mixture of each fraction was evaporated by nitrogen gas and each fraction tested for specific glycolipid of M. tuberculosis by ELISA using various different rabbit antisera to mycobacteria.

Thin Layer Chromatography and Biochemical Procedure.

Thin layer chromatography

Pre-coated silica gel G 60 thin-layer plates (20 x 20 cm, 0.2 mm thick, E.Merck, Darmstadt) were activated at 110°C for 30 min. before use. 15 ul samples were applied as small discrete spots on the plate at a distance of 1 cm from the bottom end of the plate. Diisobutylketone: acetic acid:water (80:50:7) were used as the developing solvent. The developed chromatograms were dried at room temperature for at least 30 min. and then lightly sprayed with 0.1% (w/v) orcinol in 40% H_2SO_4. They were heated at 110°C for 30 min. in a hot air oven resulting in the characteristic red spot color.

Infrared absorption spectra analysis

Samples were injected into a Unicam SP1000 Infrared spectrophotometer at a concentration of 0.02 mg/ml of chloroform using air as the blank.

ELISA TEST FOR SPECIFICITY OF GLYCOLIPID ANTIGEN.

100 microlitres of $CHCl_3$:CH_3OH (2:1) were added to each desired sample. The samples were mixed and diluted in 2 ml absolute ethanol. 50 microlitres of diluted samples were applied to a flat--bottomed well of 96-well microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). The plates were dried at 70°C for 30 min, followed by 10 min wetting with phosphate buffered saline pH 7.4. Each rabbit antimycobacterial antiserum was diluted to 1:100 in PBS containing 1% bovine serum albumin fraction V (Gibco, Laboratories, Grand Island, NY.), 0.1% Tween 20 (Sigma Chemical Company, St. Louis, MO.) and 0.02% NaN_3. 50 microlitres of each diluted antiserum were added to each well. Plates were incubated for 30 min in a humid chamber at room temperature. Antisera were aspirated and the plates were washed five times with PBS. Goat anti-rabbit immunoglobulins (IgG+IgM+IgA) conjugated with peroxidase (Cappel Laboratories, Cochranville, PA.) were diluted to 1:5000 in PBS containing 0.1% Tween 20. 50 microlitres of the diluted conjugate were added. After 30 min incubation, the unbound conjugate was discarded and the plates were washed five times with PBS. 50 microlitres of substrate mixture (40 mg of orthophenylenediamine in 100 ml of citrate phosphate buffer) were distributed into wells and the plates were incubated in the dark for 30 min. The reaction was terminated by the addition of 50 microlitres of 2.5 N H_2SO_4 and the optical density was read at 492 nm by Titertek Multiskan spectrophotometer (Flow Laboratories, California, U.S.A.).

RESULTS

Isolation of *M. tuberculosis* specific glycolipid and evidence of specificity by ELISA test.

Crude lipids from chloroform-methanol (2:1) extraction of *M. tuberculosis* H37Rv (TMC 102) were fractionated by passing through silicic acid/celite mixture in a column. The eluates of 2% methanol in chloroform were collected in 10 ml/tube, and the chromatograms shown in figure 1. Each fraction of eluate was tested by ELISA using 19 different rabbit antisera to mycobacteria. The results of ELISA test for each fraction are shown in table 1. Most of the glycolipids came out from the column very quickly.

Some biochemical properties of the specific glycolipid.

The specific glycolipid fraction from silicic acid/celite column was studied by thin layer chromatography using Diisobutylketone: acetic acid: water 80:50:7) as a developing solvent. The eluate was sprayed with 0.1% orcinol in 40% sulphuric acid, heated, and a red spot appeared with a Rf value of 0.63 as shown in Figure 2.

A study of this specific glycolipid by infrared spectrophotometry is shown in the IR spectrum compared to phenolic glycolipid I of M. leprae (Figure 3). This specific glycolipid should contain an aromatic nucleus and a hydroxyl group identified by the characteristic absorption bands at 1,510 and 3,000 - 3,500cm^{-1} respectively.

DISCUSSION

A sensitive and specific serological test for serodiagnosis of tuberculosis has long been sought by many investigators (13). However, until now no one has succeeded in developing such a serologic test. One of the major problems has been the presence of large numbers of shared antigens between the various mycobaterial species and some of these antigens are even shared with other bacteria. Therefore, when any serological test was developed using crude mycobacterium antigen, these antigens cross-reacted with other mycobacterium species. Such tests can never be specific for serodiagnosis of tuberculosis. A specific antigen or hapten of M. tuberculosis is, therefore, very essential for developing a specific serologic test for tuberculosis.

Many investigators have attempted to separate specific antigenic components from cells of M. tuberculosis (13). The separating techniques ranged from simple precipitation with different solvents to more sophisticated techniques, including gel filtration, ion-exchange chromatography, and preparative electrophoresis. However, all of the results were disappointing (14). Some studies even suggested that fractionation of soluble antigens from M. tuberculosis may not prove to be the way to develop a more specific test for tuberculosis (15).

In our study, we tried to separate specific antigens or haptens from M. tuberculosis strain H37Rv using many different biochemical techniques. We have successfully extracted M. tuberculosis cells with chloroform-methanol (2:1) at 50°C for 18 hours, dried and redissolved the extracted materials in chloroform, and then passed them through a column of silicic acid/celite mixture (2:1). The column was eluted with chloroform first, then 2% methanol in chloroform. We have tested every fraction of the eluate without pooling it. One glycolipid showing specificity was obtained. At least 8 such separating procedures were performed and all confirm this result in our laboratory. When this glycolipid was tested with rabbit anti-M. tuberculosis and rabbit antiatypical mycobacteria species for 18 antisera, we found that this glycolipid is rather specific. It reacted only with rabbit anti-M. tuberculosis, but did not react with the other 18 atypical mycobacterial antisera. When this glycolipid was studied by thin layer chromatography along with PGL-I of M. leprae, it gave a red spot color with orcinol in sulphuric acid with a Rf value of 0.63 which is rather similar, but not identical, to PGL-I. When it was analysed by infrared spectrophotometry, the absorption spectrum was similar to PGL-I of M. leprae. The sugar constituents of this specific glycolipid will be further analysed by gas-liquid chromatography and nuclear magnetic resonance spectroscopy.

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FRACTION	AVI	BCG	BOV	CHE	DUV	FOR	GAS	GOR	INT	KAN	MAR	NON	PIB	SME	SCR	TUB	TER	TRI	VAC
1	1.385	0.008	0.000	0.000	1.193	0.000	0.176	0.102	0.000	0.120	0.129	0.060	0.418	0.069	1.433	0.120	0.280	0.038	0.161
2	0.000	0.000	0.000	0.000	0.192	0.000	0.000	0.000	0.000	0.041	0.000	0.000	0.067	0.055	0.194	0.602	0.689	0.096	0.113
3	0.503	0.003	0.035	0.114	0.334	0.205	0.136	0.540	0.269	0.341	0.278	0.156	0.017	0.529	0.265	0.447	0.396	0.381	0.413
4	0.895	0.006	0.159	0.190	0.971	0.391	0.260	0.908	0.023	0.420	0.423	0.113	0.561	0.806	0.786	0.716	0.560	0.584	0.527
5	0.000	0.000	0.000	0.000	0.169	0.000	0.000	0.325	0.000	0.001	0.013	0.000	0.167	0.119	0.044	0.319	0.285	0.093	0.131
6	0.000	0.000	0.000	0.000	0.170	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.011
7	0.136	0.003	0.000	0.000	0.673	0.000	0.038	0.021	0.000	0.051	0.001	0.038	0.462	0.037	0.065	0.302	0.314	0.046	0.080
8	0.416	0.001	0.000	0.000	1.203	0.000	0.102	0.205	0.000	0.057	0.049	0.036	0.068	0.039	0.014	0.027	0.041	0.000	0.025
9	0.000	0.000	0.000	0.000	0.205	0.000	0.041	0.000	0.000	0.049	0.000	0.018	0.447	0.000	0.024	0.043	0.018	0.000	0.001
10	0.000	0.000	0.000	0.000	0.263	0.000	0.082	0.000	0.000	0.081	0.000	0.054	0.017	0.044	0.060	0.046	0.016	0.011	0.013
11	0.000	0.000	0.000	0.000	0.239	0.000	0.068	0.014	0.000	0.065	0.000	0.015	0.020	0.031	0.069	0.058	0.335	0.007	0.080
12	0.330	0.005	0.000	0.000	1.958	0.004	0.090	0.401	0.000	0.062	0.189	0.079	0.052	0.216	0.368	0.726	0.372	0.030	0.048
13	0.529	0.008	0.000	0.000	1.529	0.013	0.117	0.556	0.021	0.090	0.638	0.078	0.136	0.131	0.243	0.400	0.220	0.051	0.077
14	0.000	0.000	0.000	0.000	0.093	0.000	0.072	0.008	0.000	0.079	0.000	0.037	0.000	0.035	0.060	0.049	0.019	0.000	0.011
15	2.000	0.018	0.018	0.018	2.000	0.180	0.291	1.546	0.272	0.825	0.823	0.113	0.377	0.097	0.418	0.184	1.090	0.314	0.154
16	0.000	0.000	0.000	0.000	0.318	0.027	0.143	0.059	0.007	0.129	0.000	0.086	0.030	0.083	0.143	0.063	0.037	0.018	0.031
17	0.031	0.000	0.000	0.000	0.219	0.000	0.134	0.021	0.000	0.145	0.000	0.073	0.195	0.065	0.163	0.103	0.130	0.023	0.041
18	0.053	0.001	0.000	0.000	0.533	0.000	0.123	0.031	0.000	0.130	0.000	0.062	0.089	0.048	0.127	0.135	0.172	0.035	0.022
19	0.000	0.000	0.000	0.000	0.158	0.000	0.124	0.008	0.000	0.130	0.000	0.096	0.066	0.047	0.099	0.114	0.069	0.006	0.010
20	0.045	0.002	0.000	0.000	0.670	0.000	0.120	0.047	0.000	0.093	0.000	0.067	0.093	0.047	0.069	0.061	0.121	0.000	0.020
21	0.000	0.001	0.000	0.064	0.345	0.000	0.122	0.000	0.000	0.116	0.000	0.097	0.046	0.046	0.071	0.084	0.074	0.007	0.010
22	0.096	0.002	0.000	0.000	0.943	0.003	0.111	0.062	0.047	0.107	0.000	0.065	0.154	0.045	0.124	0.183	0.326	0.048	0.071
23	0.122	0.001	0.000	0.004	0.961	0.116	0.146	0.148	0.034	0.136	0.070	0.125	0.277	0.105	0.203	0.494	0.491	0.107	0.125
24	0.225	0.014	0.009	0.023	1.844	0.136	0.180	0.198	0.035	0.151	0.054	0.107	0.130	0.096	0.125	1.028	0.232	0.060	0.128
25	0.699	0.006	0.000	0.067	1.040	0.127	0.147	0.135	0.023	0.129	0.058	0.074	0.108	0.139	0.227	1.822	0.259	0.040	0.143
26	0.524	0.011	0.000	0.178	1.549	0.237	0.156	0.452	0.037	0.128	0.253	0.088	0.348	0.332	0.831	1.949	0.694	0.112	0.253
27	0.094	0.002	0.000	0.400	0.407	0.253	0.098	0.105	0.005	0.092	0.054	0.086	0.131	0.106	0.283	1.655	0.186	0.015	0.054
28	0.008	0.003	0.000	0.384	0.559	0.180	0.082	0.152	0.010	0.080	0.014	0.067	0.054	0.043	0.150	1.531	0.125	0.000	0.035
29	0.114	0.002	0.000	0.287	0.895	0.099	0.080	0.198	0.000	0.083	0.038	0.070	0.244	0.064	0.179	1.466	0.290	0.029	0.081
30	0.000	0.000	0.000	0.317	0.082	0.010	0.062	0.000	0.000	0.060	0.000	0.057	0.250	0.011	0.087	1.491	0.110	0.000	0.000
31	0.309	0.002	0.000	[illegible]	1.634	0.037	0.100	0.149	0.000	0.095	0.108	0.062	0.487	0.069	0.240	1.856	0.582	0.044	0.106
32	0.006	0.000	0.000	[illegible]	0.024	0.011	0.077	0.000	0.000	0.085	0.000	0.069	0.176	0.020	0.076	1.513	0.038	0.000	0.000
33	0.174	0.002	0.000	0.220	0.461	0.023	0.075	0.003	0.000	0.139	0.001	0.076	0.333	0.037	0.093	1.508	0.160	0.003	0.000
34	1.783	0.016	0.000	0.778	2.000	0.071	0.105	0.273	0.000	0.098	0.131	0.070	2.000	0.192	0.383	1.871	1.264	0.056	0.210
35	0.315	0.003	0.000	0.111	0.131	0.000	0.093	0.000	0.000	0.081	0.000	0.056	0.571	0.020	0.071	1.334	0.076	0.000	0.007
36	0.886	0.008	0.000	0.131	1.017	0.000	0.060	0.045	0.000	0.069	0.048	0.053	1.150	0.052	0.120	1.589	0.700	0.011	0.114
37	0.956	0.008	0.000	0.100	0.122	0.016	0.057	0.000	0.000	0.066	0.026	0.043	1.255	0.035	0.072	1.393	0.073	0.000	0.000
38	1.442	0.009	0.950	0.073	0.357	0.038	0.101	0.056	0.000	0.067	0.073	0.068	1.686	0.041	0.107	1.488	0.330	0.000	0.091
39	1.625	0.010	0.000	0.099	0.047	0.096	0.123	0.000	0.000	0.117	0.111	0.087	1.688	0.082	0.121	1.536	0.110	0.027	0.014
40	2.000	0.011	0.000	0.179	0.129	0.115	0.120	0.012	0.000	0.111	0.124	0.077	1.778	0.102	0.167	1.545	0.106	0.041	0.029
41	2.000	0.020	0.000	0.144	1.816	0.110	0.126	0.069	0.000	0.125	0.140	0.083	2.000	0.142	0.264	1.814	0.683	0.059	0.123
42	2.000	0.013	0.000	0.127	0.513	0.093	0.145	0.011	0.000	0.117	0.135	0.082	1.872	0.122	0.169	1.746	0.177	0.034	0.008
43	2.000	0.014	0.000	0.687	0.921	0.115	0.153	0.051	0.000	0.120	0.119	0.082	2.000	0.133	0.196	1.770	0.341	0.038	0.063
44	2.000	0.018	0.000	0.110	1.054	0.129	0.187	0.291	0.000	0.115	0.261	0.065	2.000	0.240	0.621	1.919	1.608	0.203	0.034
SC	0.000	0.000	0.000	0.000	0.018	0.000	0.119	0.000	0.000	0.090	0.000	0.046	0.000	0.031	0.073	0.077	0.018	0.012	0.020

Table 1. Test for Specificity of Eluates from Silicic acid/Celite Column by ELISA Using Rabbit Antisera to Various Mycobacteria.

SC = Serum Control.

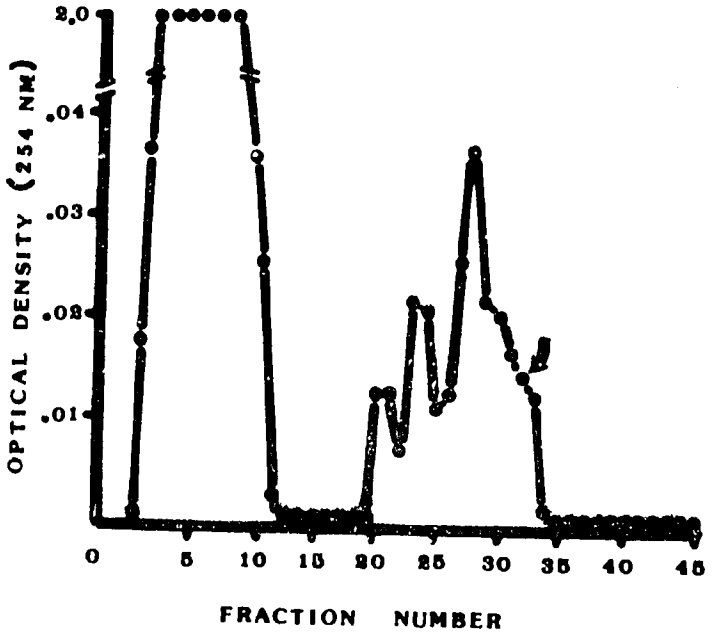

Figure 1. Chromatogram of crude lipid from M. tuberculosis passing through silicic acid/celite column, the specific lipid coming out in fractions around the arrow.

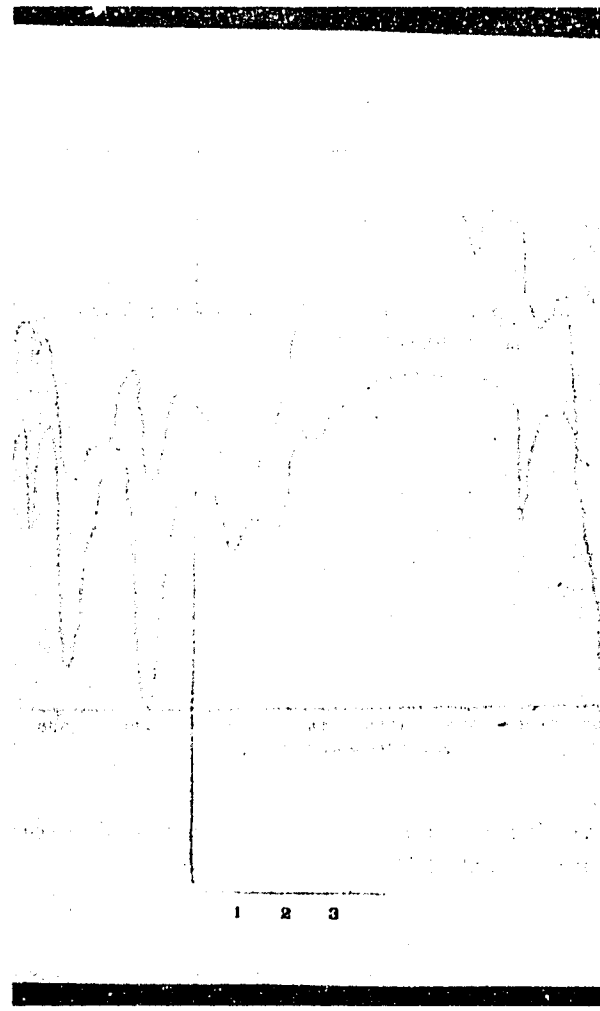

Figure 2 Thin layer chromatogram of crude lipid (1), specific lipid from M. tuberculosis (2) and PGL-I of M. leprae (3).

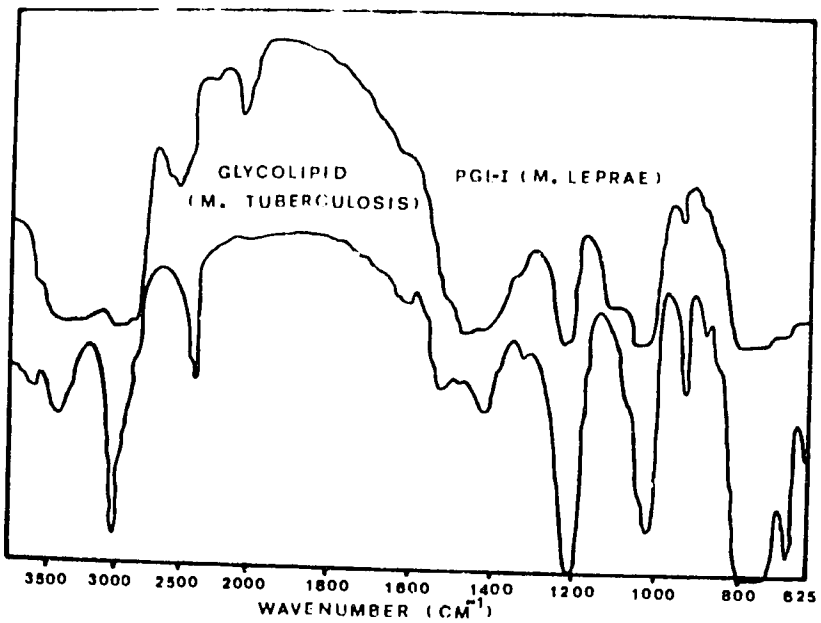

Figure 3 Infrared spectrum of specific lipid from M. tuberculosis compared with PGL-I of M. leprae.

FY 1985

"DEVELOPMENT OF AN IMMUNOPEROXIDASE TEST FOR EARLY DIAGNOSIS OF ACUTE REACTIONAL STATES IN LEPROSY PATIENTS" (5.141) : EVOLUTION OF IN SITU IMMUNE RESPONSES IN CLINICAL SUBGROUPS OF ERYTHEMA NODOSUM LEPROSUM REACTIONS OF LEPROSY: AN IMMUNOPEROXIDASE STUDY USING SUCTION-INDUCED SKIN BLISTERS

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ABSTRACT

Performing suction induced skin blisters at different periods of time and staining cells with monoclonal antibodies using the immunoperoxidase method, the numbers and ratio of T lymphocyte subsets in the lesions of two clinical subgroups of the ENL reactional state of leprosy (13 acute ENL and 17 chronic, recurrent ENL) were determined and compared with those found in stable, non-reactional patients (9 borderline lepromatous and 11 lepromatous). In acute and chronic, recurrent ENL, the percentages of the helper (inducer) phenotype were significantly higher than those found in non-reactional lepromatous lesions, but only chronic, recurrent ENL showed significantly larger helper/suppressor ratio. The increase in helper activity with or without the reversal of the helper/suppressor ratio in two clinical subgroups of ENL as compared to the non-reactional lepromatous disease suggests some role for cell-mediated immunity in the pathogenesis of ENL. The non-invasive blister technique may be used for monitoring immune status and improving treatment.

INTRODUCTION

Leprosy is a granulomatous disease caused by infection with *Mycobacterium leprae*. An immunologic spectrum exists in the disease, with lepromatous leprosy patients having defective cell-mediated immune responses to *M. leprae* as compared to patients with the tuberculoid type. (1, 2)

Erythema Nodosum Leprosum (ENL) reactions are found towards the lepromatous end of the spectrum of leprosy, usually when the patients are on anti-leprosy drugs (3). It is characterized by the presence of tender, painful, erythematous, subcutaneous crops of nodules together with malaise, fever, and arthralgia, and histologically the lesions show perivascular infiltration of polymorphonuclear (PMN) cells along with histiocytes which contain fragmented bacteria (4).

Characterization and semi-quantitation of mononuclear subpopulations in frozen sections of skin biopsies in leprosy have been performed extensively in the most recent years (5-11). However, these studies cannot be performed repeatedly and consecutively many times, thus limiting the studies in the cellular dynamics of the inflammatory cells in the skin lesion of leprosy. In addition to performing skin biopsies, we are now introducing a new method to overcome this obstacle by using induced suction blisters on the skin which provides an opportunity to study the disease in a consecutive time sequence without any pain and without leaving scars.

Performing this new blister technique in leprosy may yield new information concerning the pathogenesis of the observed differences in cell-mediated immune responses that exist in leprosy. Therefore, we studied the distribution and ratio of T helper and suppressor cells in ENL compared to non-reacting lepromatous patients in consecutive intervals directly in the leprosy lesions by using monoclonal antibodies with the immunoperoxidase technique.

MATERIALS AND METHODS

Patients

A total of 50 leprosy patients whose age group was 17-71 years were studied. The patients were selected from the McKean-Rehabilitation Institute, Chiang Mai, Thailand. The patients were classified according to the Ridley's criteria (12). Twenty patients who did not have any reaction at the time of examination were classified as Lepromatous (LL) or Borderline lepromatous (BL) leprosy patients. Fifteen patients of this group were fresh cases and had no previous history of antileprosy treatment, and 3 patients were treated with anti-leprosy for less than one year. 30 lepromatous patients with ENL reactions were studied. All of these patients were followed up subsequentially 2 to 8 times right from the lepromatous state to the post ENL state.

Suction blisters

For skin blister preparations, the dermis is exposed by unroofing blisters induced over 1-2 hours controlled suction, causing separation of the epidermis from the dermis at the basement membrane (13). Suction is applied via a small plastic template with 4 openings, each 8 mm. in diameter, resulting in 4 small, symmetrical blisters after 200-300 mmHg continuous vacuum suction. Approximately 0.1 cc. fluid can be aspirated from each blister, and cells are collected on a 13 mm. Millipore filter using conventional cytopreparatory techniques.

Monoclonal antibodies

Primary mouse monoclonal antibodies were used at concentrations predetermined by checkerboard titrations. Each specimen was stained with a panel of monoclonal antibodies against T lymphocyte subpopulations

including the pan T cell marker Leu 4 at 1:50 (Becton Dickinson, Rutherford, NJ), the helper/inducer T cell marker Leu 3a at 1:5, the suppressor/cytotoxic T cell marker OKT-8 at 1:10. Antibody controls consisted of the omission of the primary antibody or the use of an antibody of irrelevant specificity with the same protein concentration.

Immunoperoxidase staining

For all antibodies, a modified avidin-biotin-immunoperoxidase technique was used (14). Cells collected on the filters were placed in fresh formol-acetone for 10 min at room temperature and allowed to air dry. Filters were subsequentially incubated for 30 min with primary mouse monoclonal antibodies, biotinylated horse anti-mouse antibodies (20 µg/ml) (Vector, Burlingame, CA), and avidin-biotin-peroxidase complex (Vectastain kit, Vector). Five-minute washes with PBS were performed between all incubations.

A red-colored reaction product was obtained by incubating specimens with aminoethyl carbazole in the presence of hydrogen peroxide for 10 min. Filters were then washed in tap water for 10 min., counterstained with Meyer's hematoxylin for 1 min., washed again, and then mounted with glycerol jelly.

Enumeration of lymphocytes

The total number of mononuclear cells were counted per one filter. The percentage of each T-cell phenotype was then calculated from the number of positive cells per filter and the total number of mononuclear cells.

RESULTS

Clinical groups

Group I

Lepromatous leprosy patients without reaction (BL and LL). Twenty patients who did not have any reaction at the time of examination fulfilled Ridley's criteria (4,12). Eleven and nine out of twenty patients were classified as lepromatous (LL) and Borderline Lepromatous (BL) leprosy patients respectively. The age range of these patients was 18-71 years with the median age of 36 years as shown in TABLE 1.

Group 2

Erythema Nodosum Leprosum, acute. Thirteen out of thirty lepromatous with ENL patients were classified as an acute group according to the pattern of reaction which started abruptly and then subsided within 7 to 10 days after steroid treatment. No further reactions were observed during the one to two year follow-up. The age range of this group was 18-44 years with the median age of 30 years.

Group 3

Erythema Nodosum Leprosum, chronic or recurrent. Seventeen out of thirty lepromatous with ENL patients were found with intermittently waxing and waning episodes of ENL. Reactions flared up in few days when steroids were discontinued. Five patients in this group started with a reactional pattern of acute ENL and then developed recurrent episodes of ENL within two to four weeks after the last steroid dose. Their results are included along with chronic patients since both groups showed similar results. The age range was similar to the previous groups with the median age of 34 years. No significant differences in the median age were observed among the groups.

T cell marker staining

The distribution of the mean percentages among clinical groups is shown in TABLE 2. A striking difference in T lymphocyte subsets was observed in acute and chronic recurrent ENL as compared to non-reactional lepromatous patients. Greater numbers of helper phenotypes ($P<0.01$) were found in both acute and chronic recurrent ENL lesions. The overall helper/suppressor T cell ratio in chronic, recurrent ENL was 3.6, which was greater ($P<0.01$) than observed in non-reactional lepromatous specimens, while the specimens in acute ENL showed similar low ratio of helper/suppressor phenotypes as to the non-reactional lepromatous group. T suppressor lymphocytes were lewer in chronic, recurrent ENL but did not differ statistically among the groups.

DISCUSSION

We have introduced a unique technique, suction-induced skin blisters, in order to delineate the subsets of T lymphocytes using monoclonal antibodies against T helper (Leu 3) and T suppressor (OKT-8) cells with the ABC immunoperoxidase method in non-reactional lepromatous leprosy compared to subgroups of ENL reactions of leprosy. Any imbalance in the ratio of helper or suppressor subsets leads to excessive helper activity or suppressor activity (15). The balance between helper and suppressor activity is very important in the regulation of immune responses as shown by animal studies (16).

Using monoclonal antibodies for identifying suppressor and helper T cells, Bach et al., Wallach et al., Mshana et al. and Modlin et al. found reduced helper, increased suppressor T cell percentages with reduced helper/suppressor ratio in lepromatous patients whereas in patients with ENL, they observed increased helper, decreased suppressor T cell percentages with an increased helper/suppressor ratio (17,18,19,20). On the other hand, Van Voorhis et al and Rea et al observed that there were no differences in the helper and suppressor cell percentages and the helper/suppressor ratio between ENL patients and lepromatous patients (9,21).

Thus there were contradictory reports on the number of helper and suppressor T phenotypes. However, no reports separated clinical subgroups of ENL in the study. Our results of blister study in non-reactional lepromatous and lepromatous with ENL lesions confirm that the helper/suppressor ratio is indeed increased in chronic, recurrent subgroups of ENL but not in acute subgroup of ENL compared to non-reactional lepromatous patients. Beside increasing of helper/suppressor ratio, we also found that the amount of helper T cells significantly increased in both clinical subgroups of ENL than lepromatous patients. Increased helper activity either produces soluble T cell growth factors which help T cells to have functional capability to respond to antigenic stimulations, thereby increasing CMI and causing inflammatory responses, or helping B cell differentiation to produce antibodies. These antibodies bind with the fragmented bacilli formed due to the treatment leading to the formation of immune complexes which are either found in circulation or deposited in the tissues. Deposition of immune complexes in the tissues mediates PMN cell infiltration and leads to phagocytosis of the complex by the release of enzymes which destroy the complex as well as produce local tissue damage (19,22).

It thus appears that an alteration in helper T cell subset numbers is probably the main cause of the reactional episode of ENL, both acute and chronic, recurrent subgroups.

The higher helper/suppressor ratio (3.6 ± 1.83) in chronic, recurrent ENL as compared to acute ENL (1.4 ± 0.38) and lepromatous patients (1.1 ± 0.55) indicates a significant shift of the ratio causing a severe imbalance in the regulation of immune responses in chronic, recurrent ENL.

This report has presented on the information concerning sequential changes of immune status from the lepromatous state to acute and chronic, recurrent ENL in leprosy patients which provides an understanding of the pathogenesis of observed differences in cell-mediated immune responses that exist in leprosy. This unique blister technique may be used as non-invasive method of monitoring immune status and improving treatment.

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Table 1. Age Distribution and Steroid Responsiveness by Clinical Groups in BL, LL and ENL

Clinical group	No. of cases	Age range (yrs)	Median age (yrs)	Response to Steroid	Relapse when stop or taper off steroid
Lepromatous (BL and LL)	20	18-71	36	*-	*-
Acute ENL	13	18-44	30	yes	no
Chronic, recurrent ENL	17	17-57	34	yes	yes

* No role of steroid in this group

Table 2. Percentages and Ratios of T Lymphocyte Subsets in Leprosy Blister Fluid*

clinical group	n	% Leu 4^+ (mean ± s.d.)	% Leu 3^+ (mean ± s.d.)	%OKT-8^+ (mean ± s.d.)	%Leu 3^+/OKT-8^+ (mean ± s.d.)
Lepromatous (BL and LL)	20	29 ± 11	18 ± 7.5	20 ± 8.5	1.1 ± 0.55
Acute ENL	13	41 ± 17.9	30 ± 14.2@	24 ± 7.4	1.4 ± 0.38
Chronic, recurrent ENL	17	36 ± 19.6	30 ± 16.3@	11 ± 5.3	3.6 ± 1.83@

* The percentages of T lymphocytes were calculated as compared to the total mononuclear cells in the blister fluid. Leu 4, a pan T cell marker, Leu 3, a helper (inducer) marker and OKT-8, a suppressor (cytotoxic) marker were used.

@ Statistically significant data : Leu 3^+ : Lepromatous VS Acute ENL, $P<0.01$; Lepromatous VS Chronic, recurrent ENL, $P<0.01$, Leu 3/OKT-8: Lepromatous VS Chronic, recurrent ENL, P 0.001

FY 1986

"EVALUATION IN VITRO OF INTERLEUKIN 1 AND INTERLEUKIN 2 AS POSSIBLE IMMUNOTHERAPEUTIC AGENTS IN LEPROSY" (5.140)

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ABSTRACT

To study the capability of monocytes and lymphocytes from leprosy patients to produce IL1 and IL2, we measured the levels of IL2 from 25 BL/LL and 6 BT/TT and found that they were not statistically different from 11 normal subjects when PBML were stimulated with mitogens, PHA-P or Con A. However, when PPD was used as the stimulating agent, there was a significant difference between BL/LL vs normals, and BB/TT vs normals. We found that the level of IL1 in 23 BL/LL was significantly lower than in normal subjects. It was also demonstrated that macrophages are required for IL2 production.

INTRODUCTION

Leprosy is a chronic infectious disease characterized by a unique spectrum of human immune response to a single causative agent, M. leprae[1]. Lepromatous patients, at one extreme, are almost totally and specifically nonresponsive to M. leprae in vivo and in vitro; strong delayed hypersensitivity and cell-mediated immunity (CMI) mark tuberculoid patients at the other extreme. Various defects in CMI have been demonstrated both in vivo and in vitro, especially in lepromatous patients, but their underlying causes and pathogenesis are unknown[2-5].

Recent developments in immunology and molecular biology now make it plausible to hypothesize that the range of aberrant CMI in leprosy is due to inadequate or inappropriate production of IL1 and/or IL2 by host mononuclear cells, resulting in a range of immunoregulatory disturbances as is observed in the immunopathologic spectrum of leprosy[6-7]. If this is true, host immunity to M. leprae might be modified by immunotherapy to "normalize" IL1 and/or IL2 production or responsiveness; if false, it would exclude a number of presently known immunoregulatory mechanisms and re-direct work toward other immune cells and interactions.

MATERIALS AND METHODS

Production and Determination of IL1

Ten to twenty milliliters of heparinized blood was obtained from subjects. The blood was diluted 1:2 with sterile 0.01 M phosphate buffer

saline (PBS) pH 7.2 and centrifuged on Ficoll-Hypaque[8]. The peripheral blood mononuclear leukocytes (PBML) were collected and washed 3 times with RPMI 1640 media. The cell concentrations were adjusted with RPMI 1640 containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% heat inactivated fetal calf serum (FCS).

PBML $2x10^6$ cells in 2.0 ml media were added onto a 35x10 mm. plastic plate, incubated at 37°C, 5% CO_2 for 2-4 hrs. Non-adherent cells were washed off 3 times with 10% FCS-RPMI-1640. PBML, non-adherent and adherent cells were stained with nonspecific esterase stain (NSE). The exact number of monocytes adhering to the plate was calculated and the concentration of monocyte was achieved by adding a certain amount of 10% FCS-RPMI 1640 media.

The adhered monocytes of normal or leprosy patients to the plastic plate were adjusted to $2x10^5$ cells/ml by adding various amounts of 10% FCSRPMI 1640 media. Twenty µg/ml of lipopolysaccharide (LPS) was added, and incubated at 37°C, 5% CO_2 for 24 hours. Cell suspension were collected and centrifuged and the resulting supernatants assayed for IL1.

Thymocytes from eight week old BALB/C inbred strain mice were used as the indicator cells in IL1 assay. Animals were killed by cervical dislocation and the thymus gland removed under sterile conditions. the thymocyte suspension was prepared by gently homogenizing the thymus gland in a sterile mesh with a sterile rubber plunger. The thymocytes were washed twice with IL1 media, counted, and then adjusted to $3x10^7$ cells/ml.

Samples for the IL1 assay were diluted 1:2, 1:4, 1:8 and 1:16 by IL1 media with a final volume of 600 ul for each dilution. One hundred microliters of thymocyte suspension were added to each tube, and thoroughly mixed. Thirty microliters of PHA-P (conc.20 µg/ml) were added to each tube and mixed.

All IL1 sample dilutions were assayed in triplicate using 96 well tissue culture plates. Two hundred microliters of the above mentioned mixture were pipetted into each well. The microtiter tissue culture plate was incubated at 37°C, 5% CO_2 for 72 hours. Thymidine incorporated into DNA was measured by adding 25 ul of tritiated thymidine (8 uCi/ml) into each well, mixed, and incubated for 2 hrs.

The culture was harvested onto glass microfiber filters using a cell harvester. The glass microfiber filters were dried at room temperature overnight and transferred into scintillation counting vials containing 7.0 ml liquid scintillation fluid. All samples were counted in a liquid scintillation counter (LS 7000 Beckman Liquid Scintillation Counter, Beckman Scientific Instruments, Inc., Fullerton, CA 92634 U.S.A.).

Production and Determination of IL2

PMBL $1x10^6$ cells/ml were stimulated with 2 µg/ml PHA-P (Wellcome), or 40 µg/ml Con A (Sigma) or 10 µg/ml PPD (Connaught), incubated at 37°C 5% CO_2 18 hours for PHA-P and Con A and 48 hours for

PPD. Supernatants were harvested by centrifuge for Con A stimulation. Supernatants were added with 20 mg/ml of α-methyl-D mannoside, filtered until sterile, and stored at -70°C for the IL2 assay.

Tested samples of the IL2 assay were diluted in serial dilution 1:2, 1:4, 1:8, 1:16 to 1:256 with IL1 media in 100 ul volume in each well of a 96 well tissue culture plates. One hundred microliters of twice washed CTLL-2 containing $8x10^3$ cells/100 ul were added into each well, mixed, and incubated 24 hours. Fifty microliters of ^{3}H-TdR containing 0.2 uCi/50 ul were added into each well and incubated for 18 hours. Cultures were harvested onto glass fiber filters and 3H-TdR incorporation was determined by a liquid scintillation counter (Beckman LS 7000).

Determination of Cell Types Required for IL2 Production

1. PBML. Heparinized blood was underlaid with Ficoll Hypaque, centrifuged at 400xG for 30 min. Generally we achieved PBML with more than 95% purity.

2. Monocytes. Two milliliters of $1x10^6$/ml PBML were added onto a 15x60 mm plastic plate, incubated at 37°C, 5% CO_2 for 1 hours. Non-adherent cells were washed out twice with a warm RPMI medium. Adherent cells on the plastic plate were more than 90% positive for nonspecific esterase staining.

3. T cells. Ten milliliters of $2x10^6$/ml PBML were added into a Nylon Wool column, and incubated at 37°C 5% CO_2 for 1 hour. Non-adherent cells were then eluted with warm RPMI medium. The resulting non-adherent cells were rosetted with AET-SRBC overnight at 4°C. The rosetted T cells were separated from non-rosetted cells by Ficoll-Hypaque gradient centrifugation. AET-SRBC were lysed by hypotonic ammonium chloride solution. When these AETSRBC rosetting cells were mixed with AETSRBC, more than 95% gave rosette formation.

4. IL2 production. Two milliliters of $5x10^5$/ml T cells were added onto plastic plate containing autologous or heterologous monocytes. The cell mixtures were then stimulated with 2 μg of PHA-P for 18 hours. Supernatants from cell culture were assayed for IL2 activity by CTLL-2 proliferation.

RESULTS

Production and Determination of IL1

Results of separation and purification of normal and leprosy patients' monocytes are shown in table 1 and 2 respectively, and figure 1 shows levels of IL1 in unit/ml of normal volunteers and leprosy patients respectively. The mean±S.E. of IL1 in normal subjects is 622.4±121.4 units/ml, while in lepromatous (LL) is 235.8±52.0 units/ml.

Production and Determination of IL2

Figure 2 shows the level of IL2 from 11 normal and 25 BL/LL patients when $1x10^6$/ml PBML were stimulated with 2 μg/ml PHA-P

(Wellcome). Figure 3 illustrates the level of IL2 from $1x10^6$/ml PBML stimulated with 40 μg/ml Con A (Sigma), of 11 normal and 13 BL/LL patients. Figure 4 depicts the IL2 level, from $1x10^6$/ml PBML stimulated with 10 ug/ml PPD (Connaught), of 7 normal and 21 BL/LL patients. When the mean$\pm$S.D. of IL2 level of BL/LL patients were compared to normals, there was no significant difference in the IL2 level when stimulated either with Con A or PHA-P. However, when PBML was stimulated with PPD, there was a significantly difference ($p < 0.025$).

When IL2 levels in tuberculoid leprosy patients (BT/TT) were compared with normal subjects, there was no significant difference when Con A or PHA-P were used as stimulating agents (figure 5 and 6 respectively). However, when PPD was used for stimulation of IL2 production, there is a significant difference between IL2 of 5 BT/TT and 7 normal patients (figure 7).

Determination of Cell Types Required for IL2 Production

Interleukin 2 production by normal T cells, monocytes, T cells mixed with autologous or heterologous monocytes are shown in figure 8. It is demonstrated that macrophages are required for IL2 production.

CONCLUSION

The levels of IL2 in BL/LL and BT/TT patients are not statistically different from normal subjects when PBML is stimulated with the mitogens PHA-P or Con A. However, when PPD is used as the stimulating agent, there is a significant difference between BL/LL vs normal, and BT/TT vs normals subjects.

We have also determined the IL1 levels in 7 normal and 20 BL/LL patients. The level of IL1 in BL/LL are significantly lower than in normal subjects. It is also demonstrated that macrophages are required for IL2 production.

The study of IL1 production from both untreated and treated leprosy patients and normal volunteers will be continued. Determination of IL2 levels from PBML of both untreated and treated leprosy patients and normal subjects stimulated with different doses of Con A, PHA-P and PPD will also be continued. The immunotherapeutic effect of IL1 and/or IL2 shall be carried out. We will begin to evaluate PBML, monocytes, T cells, T helper and T suppressor cells from normal and leprosy patients in the regulation of IL2 production. Finally, the number of IL2- producing cells from the PBML of normal and leprosy patients using our new technique will be carried out.

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Table 1. Separation and Purification of Monocytes from Normal Subjects.

Subject Number	Percent positive nonspecific esterase staining cell		% cell adhere
	Non-adherent cell	adherent cell	
N.1	5	93	14.5
N.2	4	92	15.0
N.3	4	85	11.0
N.4	6	91	9.0
N.5	3	89	14.0
N.6	4	90	11.5
N.7	6	89	10.5
X	4.57	89.86	12.21
S.D.	1.13	2.61	2.29

Table 2. Separation and Purification of Monocytes from Leprosy Patients.

Subject Number	Type of Leprosy	Percent positive nonspecific esterase staining cell		% cell adhere
		Non-adherent cell	adherent cell	
U.1	LL	5	89	13.0
U.2	LL	8	78	15.5
U.3	LL	9	85	9.5
U.4	LL	3	83	10.5
U.5	LL	5	93	12.5
U.6	LL	3	85	9.0
U.7	LL	3	75	11.0
U.8	LL	3	82	9.5
U.9	LL	2	80	14.0
U.10	LL	1	89	9.0
U.11	LL	6	86	11.5
U.12	LL	7	85	14.0
U.13	LL	4	87	12.5
U.14	LL	7	91	12.0
U.15	LL	5	92	13.0
U.16	LL	4	87	11.5
U.17	LL	4	90	13.0
U.19	LL	3	92	9.5
U.20	LL	7	87	14.5
U.21	LL	2	93	15.0
U.22	LL	4	92	14.0
U.23	LL	5	91	12.0
U.24	LL	5	91	10.0
n = 23	X	4.57	87.09	12.00
	S.D.	2.04	4.95	1.99

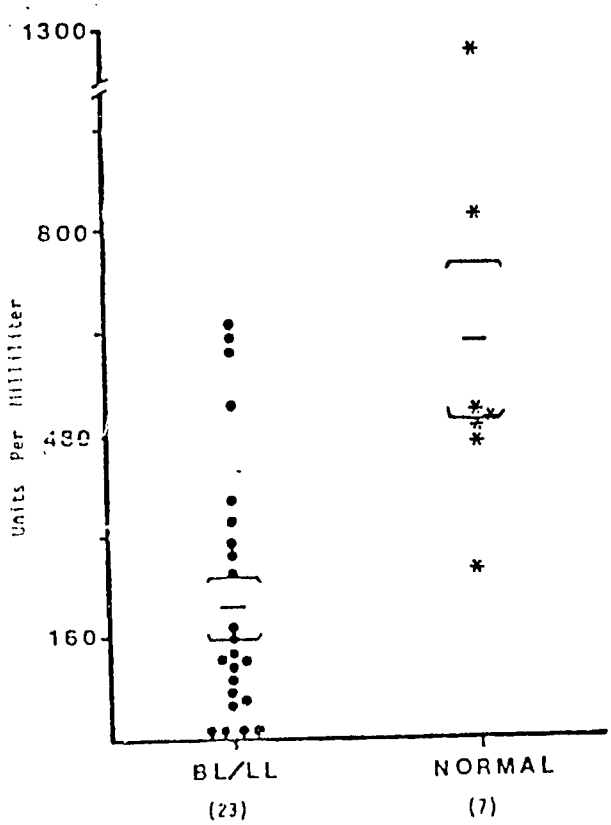

Figure 1. Interleukin 1 Level from Monocytes of Normals and Lepromatous Leprosy Patients in Term of Units Per Ml, ⊜ Mean ± S.E.

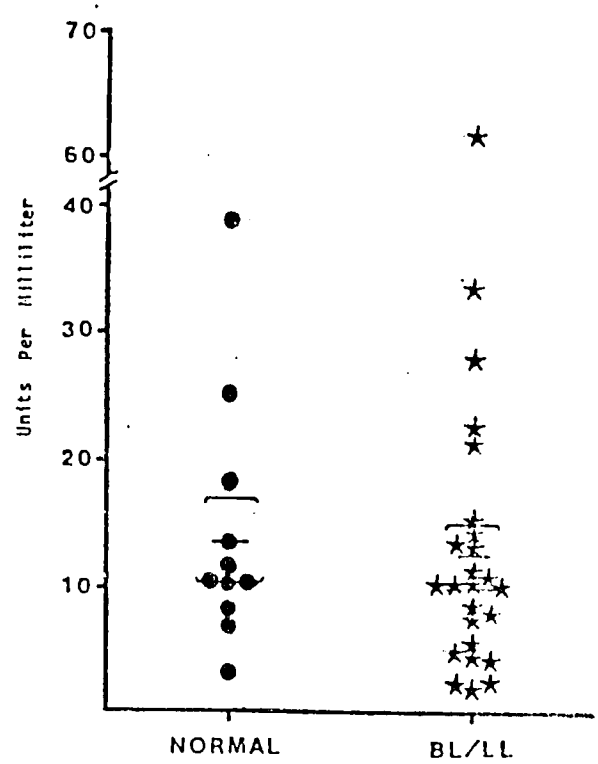

Figure 2. IL2 Level from PBML of BL/LL and Normal Subjec: by PHA Stimulation, ⊜ Mean ± S.E.

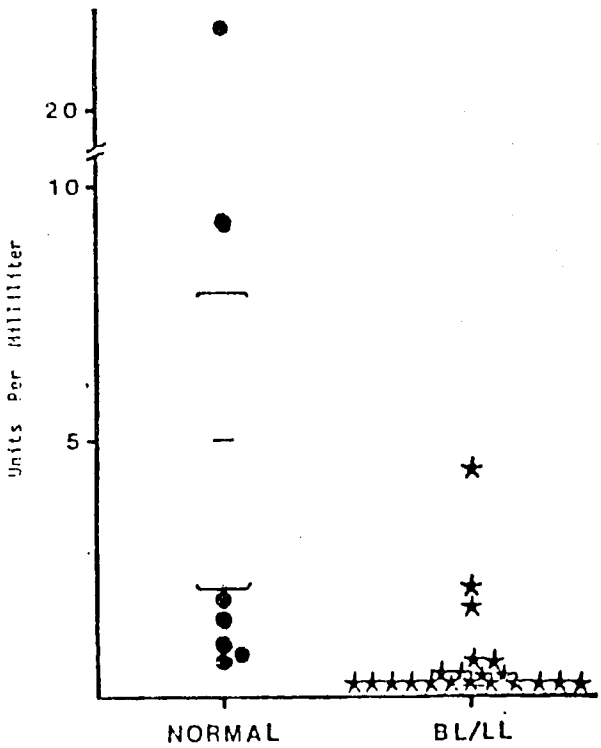

Figure 4. IL2 Level from PBML of BL/LL and Normal Subjects by PPD Stimulation, ⊜ Mean ± S.E.

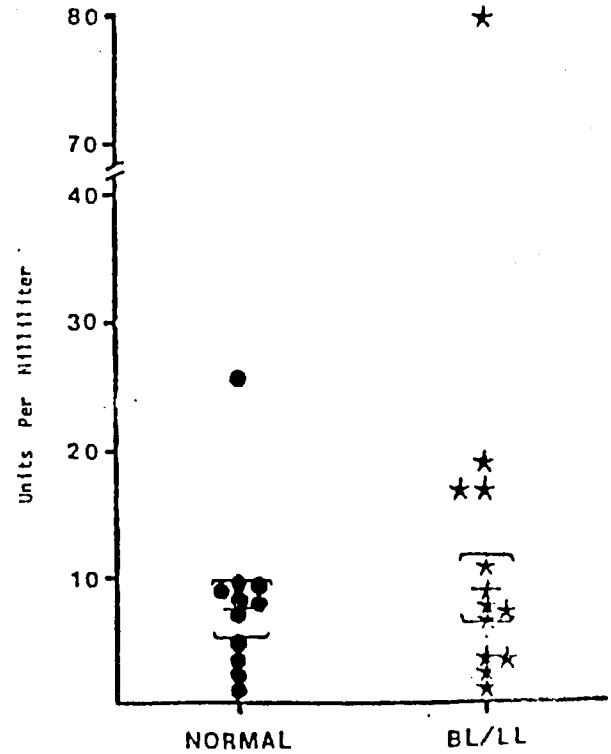

Figure 3. IL2 Level from PBML of BL/LL and Normal Subjec by Con A Stimulation, ⊜ Mean ± S.E.

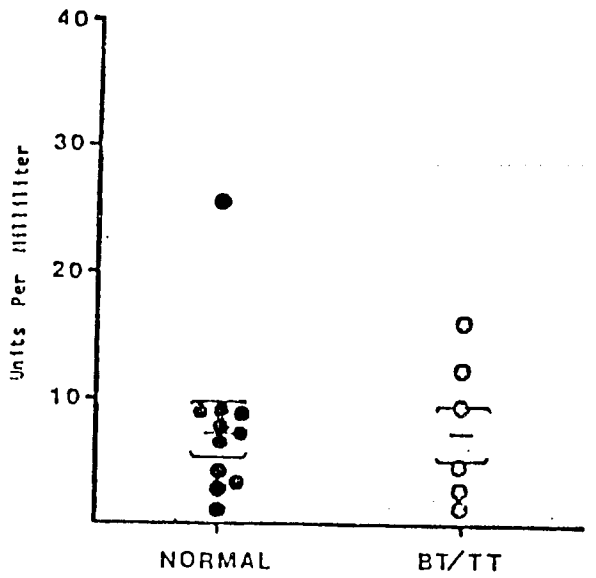

Figure 5. IL2 Level from PBML of BT/TT and Normal Subjects by Con A Stimulation, ⊜ Mean ± S.E.

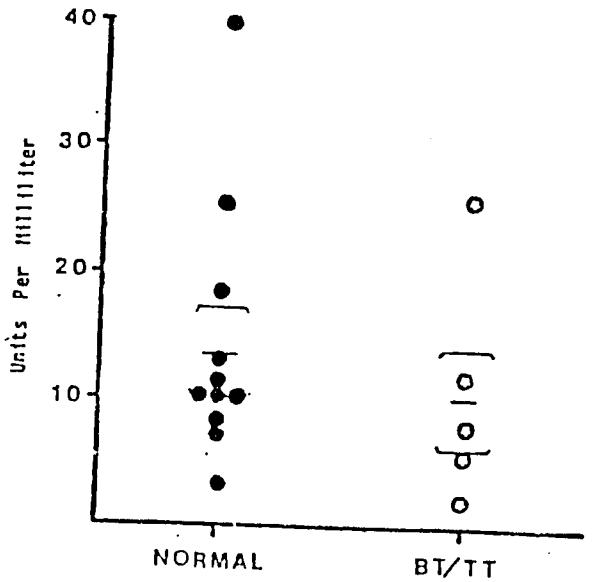

Figure 6. IL2 Level from PBML of BT/TT and Normal Subjects by PHA-P Stimulation, ⊜ Mean ± S.E.

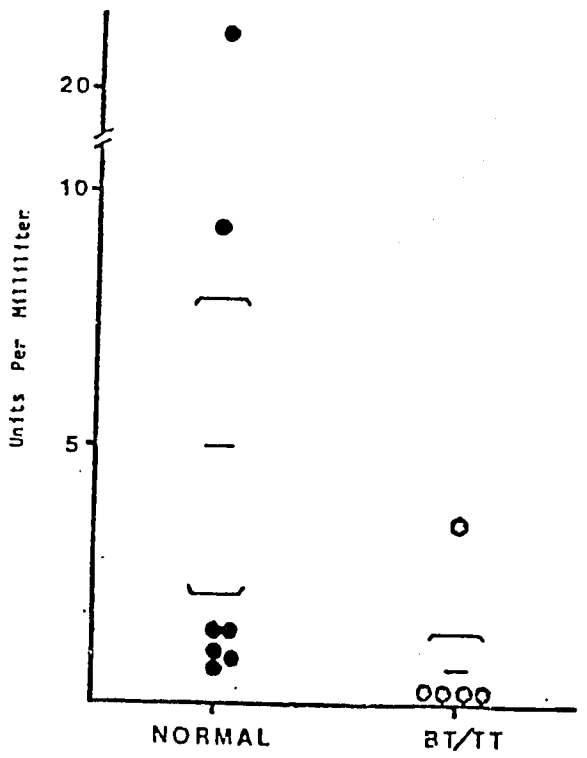

Figure 7. IL2 Level from PBML of BT/TT and Normal Subjects by PPD Stimulation, ⊜ Mean ± S.E.

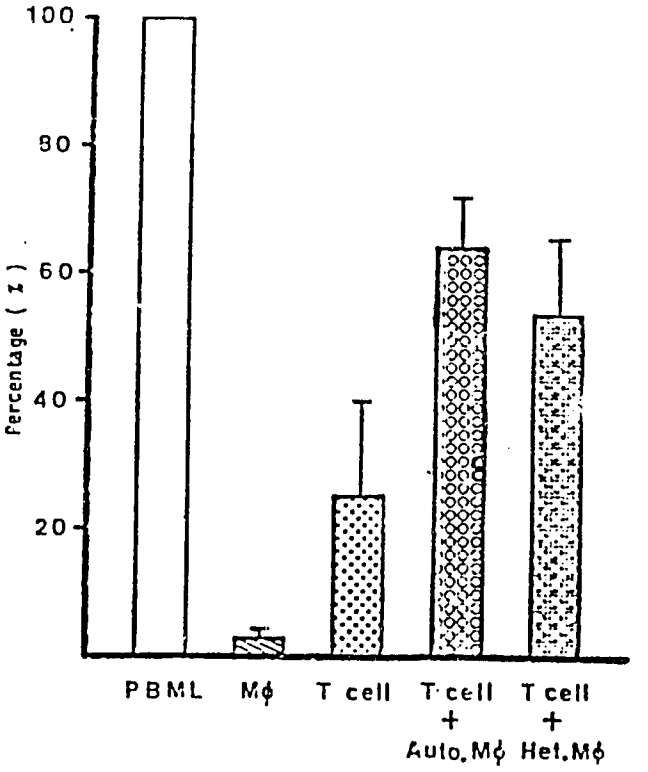

Figure 8. Production of IL2 by Various Cell Types and Cell Coculture (Compare with PBML), ⊜ Mean ± S.E.

FY 1985

"*IN VITRO* CONSERVATION AND GERMPLASM EXCHANGE OF BAMBOO" (5.247)

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ABSTRACT

Tissue culture of bamboo for conservation and germplasm exchange has been developed since 1985 at Kasetsart University, Thailand. Seeds, stem-buds segments and leaf cultures systems are being established. Tissue culture technological development has been successful in some species of bamboo. Conservation of Thai species and some introductions by using growth retardant i.e. daminozide and CCC and low temperature have been partially successful. Cryo-preservation and germplasm exchange are now being studied.

INTRODUCTION

Bamboo has been a very important crop throughout history. Human and wild life have been closely associated with bamboo for a long time. Though man has greatly utilized bamboo as food, fiber, building material and a biofuel source, still little is known about bamboo. It has been harvested from the forest year after year with, if any, only minimum conservation.

The objective of this project is to develop tissue culture technology for bamboo conservation and propagation.

Specific research objectives include:

1. Development of *in vitro* conservation techniques;
2. Collection and conservation of bamboo varieties grown in Thailand and Sri Lanka using tissue culture techniques;
3. Development of tissue culture techniques for mass propagation;
4. Development of techniques for international bamboo germplasm exchange using tissue culture.

MATERIALS AND METHODS

1. Development of Tissue Culture Techniques for Mass Propagation

1.1 Stem bud system: Experiments were conducted to study suitable media and methods for long term culture, multiplication of shoots and improvement of rooting efficiency.

1.2 Young leaf culture system: Experiments were performed in order to investigate suitable media and conditions for callus multiplication and regeneration.

1.3 Seed system: Experiments were repeated to multiply shoots in media and then to apply these techniques to other species.

2. Development of Tissue Culture Technique for In Vitro Conservation

2.1 The effect of low temperature on conservation was investigated.

2.2 The effect of growth retardants i.e. CCC and daminozide on conservation were studied.

2.3 The effect of cryo-preservation on conservation was determined.

3. Germplasm Collection

Experiments included a systematic plant material survey in Thailand, collecting and culturing tissue in septic conditions.

RESULTS AND DISCUSSION

1. Development of Tissue Culture Techniques for Mass Propagation

1.1 Stem bud segment system: The most suitable medium for stem bud segment was woody plant medium supplemented with BA (2 mg/l) and NAA (1 mg/l). The most suitable medium for multiple shoot formation was a MS (1962) supplemented with BA (10 mg/l). The excised explant from mature branches or shoots were the most suitable tissue because the explant survived at a higher percentage, higher bud and at higher number of shoots per bud, than explants taken from young ones.

1.2 Leaf callus system: Young leaf sheaths were the most suitable for culturing to induce callus formation. Calli occurred at the cut-edge and on the leaf surface. Calli started to form as fast as within one week. In most cases, if successful, callus formation occurred within 1 month. 2, 4-D was proven to be a suitable callus inducing agent. 2, 4-D at 3 mg/l incorporated into medium induced callus formations in several bamboo species. Calli could be subcultured and multiplied on the same medium.

1.3 Seed system: By using <u>in vitro</u> propagation, a high percentages of seed germination could be obtained. The most suitable medium was a woody plant medium supplemented with BA (4 mg/l) and NAA (2 mg/l). Kinetin and 2 iP were not as effective as BA, but they stimulated callus formation. It was found that shoots produced roots when they were cultured on NAA (1-2 mg/l) enriched media.

2. Development of Tissue Culture Technique for In Vitro Conservation

Research to develop tissue culture techniques for *in vitro* conservation was in parallel with the sub-culture techniques which were developed simultaneously with the tissue culture techniques for propagation. Finding a minimum growth technique was the objective. The following were the summary of research:-

2.1 Stem-bud segments system

Low temperature

Experiments have been conducted to evaluate the effect of low temperature on the growth and length of the incubation period of *Bambusa glaucescens* stem-bud segments. Experiments are in progress. Preliminary data suggested that tissue of *B. glaucescens* could be kept as low as 5°C. Height was reduced 24 times from the control. After 3 months, most of the plants survive at 5°C and 10°C but some die at 25°C.

2.2 Leaf callus system

During this report period, the number of available calli was inadequate for the conservation experiments. As soon as an adequate amount of calli is obtained, experiments similar to those with the stem-bud segments will be conducted.

2.3 Seed system

The effects of the growth retardants, CCC and Daminozide were further investigated. Seedlings, and 7 day old *Dendrocalamus membranaceus* Munro were used. The findings are summarized next.

3. Germplasm Collection

Up to date, eighty-one clones of forty-one species, representing 13 genera, were collected in Thailand. All of them were cultured in a defined medium. Thirty-two clones, representing twenty-two species and 7 genera have survived in culture. The rest have not been successfully cultured. Attempts to maintain these species of bamboo in culture and reestablish culture of those which did not survive earlier are in progress.

CONCLUSION

Research activities have been progressing well. Tissue culture development for mass propagation is progressing satisfactorily, though not all species could be cultured. Viable technique for bamboo propagation have been demonstrated. Several experiments are still needed to refine the composition of the media and techniques and to complete the tissue culture protocol.

Research to develop *in vitro* conservation techniques has progressed well and moved in the right direction. Several additional experiments are planned for the next period to bring this phase of the study to a successful conclusion.

Germplasm collection in Thailand is going very well. Almost all species have been collected, though not all survived. Bamboo habitats have been identified, so it is now easy to collect additional samples.

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FY 1985

"FIBER FARMING THROUGH WEED UTILIZATION" (4.525)

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ABSTRACT

The project will explore the potential for using a common and widely dispersed weed (Rux) Calotropis gigantea R.Br. for fiber that can be incooperated with cotton into yarn. The hypothesis is that this weed is able to grow on marginal land without irrigation and fertilization and its tolerance to pests would seem to provide advantages over conventional crops which can not grow under such conditions.

OBJECTIVES

The objectives of the project are as follows:

1. Collection of germplasm of Calotropis gigantea R.Br. from diverse locations;
2. Agronomic evaluation of germplasm;
3. Variation assessment and selection of superior varieties for further study;
4. Physico-chemical evaluation of Rux fiber;
5. Yarn properties evaluation; and
6. Fabric evaluation.

The technical work plan is divided into three main parts: agronomic evaluation and assessment for the selection of superior varieties of Calotropis gigantea R.Br. for further study, physico-chemical evaluation of rux fiber and yarn properties, and fabric evaluation. The first part will mainly be carried out for the first year and continue through the second and third year for management trials. The second part will be conducted after obtaining fiber samples from the experimental plots. Research for finding the appropriate technology which will be applied for yarn and fabric making will be included. The last part will include agricultural engineering aspects and an economic analysis.

BACKGROUND

Synthetic and Natural Product

Before the advent of synthetic fibers, man has used such natural products as cotton, wool, silk and flax as raw material to make textile

fabrics. From these fabrics, garments were made. Of these natural fibers, cotton is the most widely used because, perhaps, cotton has certain inherent characteristics that make it suitable for conversion into yarn and ultimately into fabric.

Although synthetic fibers are superior to their natural counterparts in many aspects such as lightness and crease-resisting properties, they also posess many undesirable qualities. They often are easily charged electrostatically and do not provide as good ventilation or absorption of perspiration as natural fibers, making them more uncomfortable to wear in hot weather.

The usual practice in textile fabric manufacturing is to mix natural fibers with synthetic fibers in a variety of proportions to obtain desirable qualities for different uses. Thus, natural fibers, especially cotton are still important in fabric processing (Archaraporn Silasuta, 1977).

Pesticide and Fertilizer

Cotton farming in Thailand has, however, suffered a severe setback due to cotton's vulnerability to pests. Large amounts of pesticides have to be applied with destructive consequences to the health of people and the quality of the environment.

The use of fertilizer in times of energy crisis means much higher cost and much less net income to the cotton farmer. Furthermore, cotton has to be grown on fairly good land which is becoming scarcer and scarcer due to the increasing pressure for land to grow food crops to satisfy the needs of the expanding population. Marginal land left over from shifting cultivation, mining and other destructive human activities, which is quite abandant in Thailand, cannot be utilized to grow cotton, unless there is excessive investment in improvement, irrigation, and fertilization. These high costs for land improvement can not be borne by the farmer.

Natural Fiber Source

The group of plants commonly referred to as weeds have been put into consideration because of their advantages over natural fiber crops like cotton.

1. They can grow in poor soil without the needs for irrigation and fertilization.

2. They are tolerant of pests; hence, pesticides are not required.

Kritsanaruk Theerarat, (1984) found that Rux or Calotropis gigantea had fibers in its pod and could be used to be blended with cotton with 50:50 weight percentage and be woven by machine and hand looms. The fabrics were found to be quite satisfactory, namely, they

looked more shiny than the fabrics made from cotton, the colour did not run when washed, and ironing was easy. This preliminary experiment has demonstrated that if Calotropis gigantea can be developed and its fiber can be used to supplement cotton as a raw material for textile fabric, three important benefits might arise;

1. The amount of energy input needed for mechanization, irrigation, and fertilizer would be reduced, resulting in higher net income from the lower costs and "value added" for the farmer.

2. Less pesticides would be required, which would reduce the health risk and possible damage to the environment.

3. Marginal land utilization at present could be put under cultivation, thereby considerably increasing the area of cultivated land.

METHODS AND TECHNICAL WORK PLAN

Agronomical, Biological Studies and Evaluation

1. Collection of Calotropis gigantea. Conduction of field surveys covering 6 physiographic regions of Thailand.

Collection of Rux plants and seeds according to the stratified random sampling method.

Compilation of detailed notes on location, soil type, insects and diseases, and other facets of the environment in which each plant is found.

2. Morphology and Taxonomy Studies

This was conducted under standard environmental conditions using the clone collection plot at Salaya Campus, Mahidol University. Three samples of Rux were selected randomly from the plots 45-50, 90-95, 180-190, 220-230 days respectively after planting. Samples were measured and observed for the characteristics of root systems, stem, leaf, flower, pod, seed. The following were the concerned subjects in the floral biology study.

(1) Type and morphology of flower by using plant taxonomy in herbarium.

(2) Floral structure by using plant taxonomy index, floral diagram and floral formula.

(3) Type and morphology of pollen and pollen viability by using light microscope and pollen samples which were prepared by standard acetolysis method (Erdman, 1952) for type and morphology study. Germination test which was suggested by Brewbaker and Knack (1960) with 5, 10, and 20% sucrose solution, was applied for a pollen viability study.

(4) Growth development and blooming day of flower by recording the number of blooming days for one inflorescence.

(5) Pollen shed and stigma reception to at suitable periods of fertilization by hand pollination at various times, 10 flowers per treatment.

3. Agronomical Evaluation

(1) Seeding Preparation of seed samples from selected populations will be made according to the ecotype of the main land form under each physiographic region as well as the climatic condition.

(2) Experimental sites will be selected to represent the marginal land form in each region.

(3) Experimental design.
Using split plot design with two treatments----high and low input, each treatment having 3 replications.

(4) Data collection and analysis
Yield and growth factors will be the studied variables. Dulcan's New Multiple Range Test will be used to perform statistical analysis.

(5) Selection of Rux
Results from the analysis will be used to determine the best selection in each experimental farm.

4. Management trials
Replicated management trials will be initiated in each region using seedlings from at least 3 of the best populations from each experimental farm. At least two treatments will be conducted with the same design.

Engineering, Physico-chemical Evaluation

1. Fiber extraction
Fibers and seeds will be extraced from pods by hand. Seeds will be separated by the following methods:
by hand
by ginning (simple gin roller type)
by Shirley Analyser

2. Fiber physical properties evaluation
Measurements will be taken of fiber properties such as length, fineness, strength, formity.

3. Fiber chemical properties and dyeing evaluation. The compositions for rux fiber eg. cellulose and dyeing property will be analyzed.

4. Yarn properties and woven fabrics.

Measurements of yarn physical properties will be taken, then the dyed yarn will be woven both by hand loom and loom.

5. Fabric properties evaluation

All strength properties as well as wash and wear properties will be measured.

Economic Studies

Studies will be done estimating the cost effectiveness of producing Rux fabric. Production scale may be a major factor of cost computation but it will be compared rigorously with those scales of cotton, silk, rayon and polyester.

RESULTS AND DISCUSSION

Half of the project still must be continued. This study examines sections 3.1.1-3.1.3 and 3.2.1-3.2.3 of the mentioned technical work plan. Thus, results demonstrated in this report are only parts of the study. The report covers only part of the whole set of data and information in summarized form. Discussion of the results also designates the work done and some expected outcomes.

Collection of Calotropis gigantea R. Br. (Rux)

Rux plants and seeds were collected and compiled at the Faculty for a total number of 120 samples. All samples were prepared for the stem cutting propagation method. Thereafter, the seedlings were selected according to the ecotype of the main land formed under each physiographic region as well as the climatic conditions. A total number of 55 accessions were selected and will be distributed to selected regions for planting. (Figure 1,2 and Table 1)

Morphological and Taxonomic Studies

General taxonomy and Floral Structure

Cigigantea is a bush reaching about 15 feet (4.5 metres) tall, although usually smaller, has young parts which are woolly and hairy, leaves which are pale green, reach above the middle of the plant, tip with a broad, very short point and have a leaf's base about 2.5 incles long which is heart shaped and somewhat wrinkled, and 1.5 inches wide to 10 inches long and 5 inches wide.

Both surfaces of the leaf are covered with white wool when young, the wool persisting on the undersurface, which has a broad and pale yellowish midrib, and 4-5 pairs of side vein. The Rux flower is classified as an umbelliform inflorescence, racemose blooming type. The flower inflorescence is a perfect flower. There are 40.4-41 flowers per inflorescence. The flower development period from beginning until flowering is 18-22 days. The average widths and lengths of the white and

purple flower are 17.85, 14.73 and 19.68, 17.37 mm. respectively. The flower will fall down in 5-7 days after flowering if it is not pollinated by insects. The suitable period for fertilization is at the flowering stage and lasts for 2 days. (Figure 3-6)

Selection of Rux

Data from 12 months of records were used for analyses of variance and Duncan's New Multiple Range Test. Three experimental stations -- at Nakornpathom, Lampang, and Khon Kaen -- were presented as examples results from the Rux clone selection. (Table 2)

Nakornpathom Experimental Station

From the experimental results, C-10 performed the best because it gave the highest fiber weight/tree, number of inflorescence/tree, and number of pods/tree than other clones. With regard to the growth rate, C-10 also gave a high rate growth too. When compared to high and low inputs the data showed that C-10 production was higher than any other clones in both treatments. (Figure 7)

Lampang Experimental Station

There were four clones which were selected from the northern region's clones N-7 N-6 N-3 and N-13. N-7 had a greater number of pods/tree than other clones but had a low fiber weight/pod which could be developed to increase fiber weight. N-6 clone had a lot of pod abortions although it could produce a satisfactory of rate fiber weight/pod and inflorescence. (Figure 8)

Khon Kaen Experimental Station

Three clones of Rux, NE-37 NE-38 and NE-29, were selected from Khon Kaen Experimantal station for the northeastern region's clones. Each clone gave some high production rates, numbers of pods/tree and numbers of inflorescence especially in clone NE-37 which gave a higher rate of inflorescence higher than others although it had a greater pod abortion rate than clone NE-38. (Figure 9)

Fiber Length Analysis

Fiber from three experimental stations both in low and high input ranges were selected randomly to be processed in a comb sorter to measure the effective length of the Abea. Effective length is a useful guide for roller setting in spinning. An effective length which is longer than 3 centimetres is suitable for the spinning process.

Fiber length analyses showed that the average effective length was 3.439 cm. for high input fibers and 3.309 for low input fibers. The percentage of short fiber was less than 20%. This result demonstrated that Rux fiber is suitable for yarn spinning. (Table 3)

Table 1 Selected clones from 6 physiographic regions

Region	Landform	Selected Clones
North	Flood Plain	4
	Low terrace	6
	High terrace	5
Northeast	Low terrace	5
	Natural levee	2
	Karst Topography	1
	Structural plain	2
	Sand dune	1
	High terrace	2
Central	Flood plain	2
	Low terrace	1
	High terrace	3
	Foothill slope	2
	Karst regina	1
East	Plain	1
	Low terrace	2
	Beach	2
	Karst topography	1
	Coastal plain	1
South	Foothill slope	1
	Former tidal flat	2
	High terrace	1
	Low terrace	4
	Beach	2

SUMMARY

AVERAGE OF TOTAL YIELD AND CHARACTERISTICS
IN 3 EXPERIMENTAL STATIONS

CLONE *	TOTAL YIELD/TREE	NO.POD/ TREE	FIBER WEIGHT/ POD	FRESH WEIGHT /POD	NO.OF INFLORESCENCE /TREE	POD SIZE (WIDTHxLENGTH)	TREE SIZE (HEIGTHxWIDTH)
C-10	16.94	16.49	1.07	14.54	519.5	2.91x10.28	226.2x242.10
C-04	6.65	8.44	0.80	15.70	387.4	3.26x9.66	216.7x250.30
C-12	5.79	6.09	0.87	17.45	277.3	3.22x9.66	225.9x248.90
C-03	5.44	6.42	0.85	16.11	268.0	3.10x9.44	224.0x215.30
N-07	51.78	109.75	0.49	13.21	326.58	2.64x8.95	262.06x259.25
N-06	46.47	50.08	0.88	15.30	401.11	2.55x8.79	275.03x298.00
N-03	45.42	57.72	0.70	12.84	217.70	3.19x7.68	227.92x207.61
N-13	44.20	74.72	0.55	12.67	338.33	2.47x7.87	276.92x274.95
NE-37	26.51	32.74	0.80	11.16	141.28	2.63x8.64	264.00x243.92
NE-38	24.85	36.00	0.67	11.26	117.47	2.80x8.75	282.55x204.92
NE-29	22.35	29.67	0.70	10.90	94.19	2.71x8.45	259.56x243.92

* selected from Rux clones which were colected from each region and planted in 3 experimental station.

Table 2. Average of Total Yield and Characteristics in 3 Experimental Stations.

Table 3. Fux Fuber Effective Length.

HIGH				LOW			
SAMPLE	MAXIMUM LENGTH (cm.)	EFFECTIVE LENGTH (cm.)	SHORT FIBER %	SAMPLE	MAXIMUM LENGTH (cm.)	EFFECTIVE LENGTH (cm.)	SHORT FIBER %
1	3.520	3.170	17.580	1	3.430	3.110	18.160
2	3.460	3.090	10.390	2	3.350	3.080	18.640
3	3.530	3.220	13.360	3	3.240	2.910	12.620
4				4	3.410	3.050	14.710
5				5	3.430	3.190	13.410
6	2.880	2.750	22.290	6	3.010	2.880	15.230
7	3.620	3.470	19.310	7	3.750	3.480	20.270
8	3.570	3.460	17.930	8	3.470	3.370	21.380
9	3.470	3.350	18.170	9	3.380	3.230	18.350
10	3.910	3.760	21.850	10	3.660	3.410	22.330
11	3.870	3.760	21.390	11	3.900	3.670	19.770
12	3.580	3.450	20.320	12	3.410	3.220	19.570
13	3.870	3.560	22.350	13	4.020	3.530	24.700
14	4.220	3.860	21.110	14	4.230	3.940	22.070
15	3.600	3.240	20.380	15	3.370	3.100	18.660
16	4.240	4.010	23.380	16	4.090	3.770	23.610
AVERAGE	3.667	3.439	19.272	AVERAGE	3.576	3.309	18.968

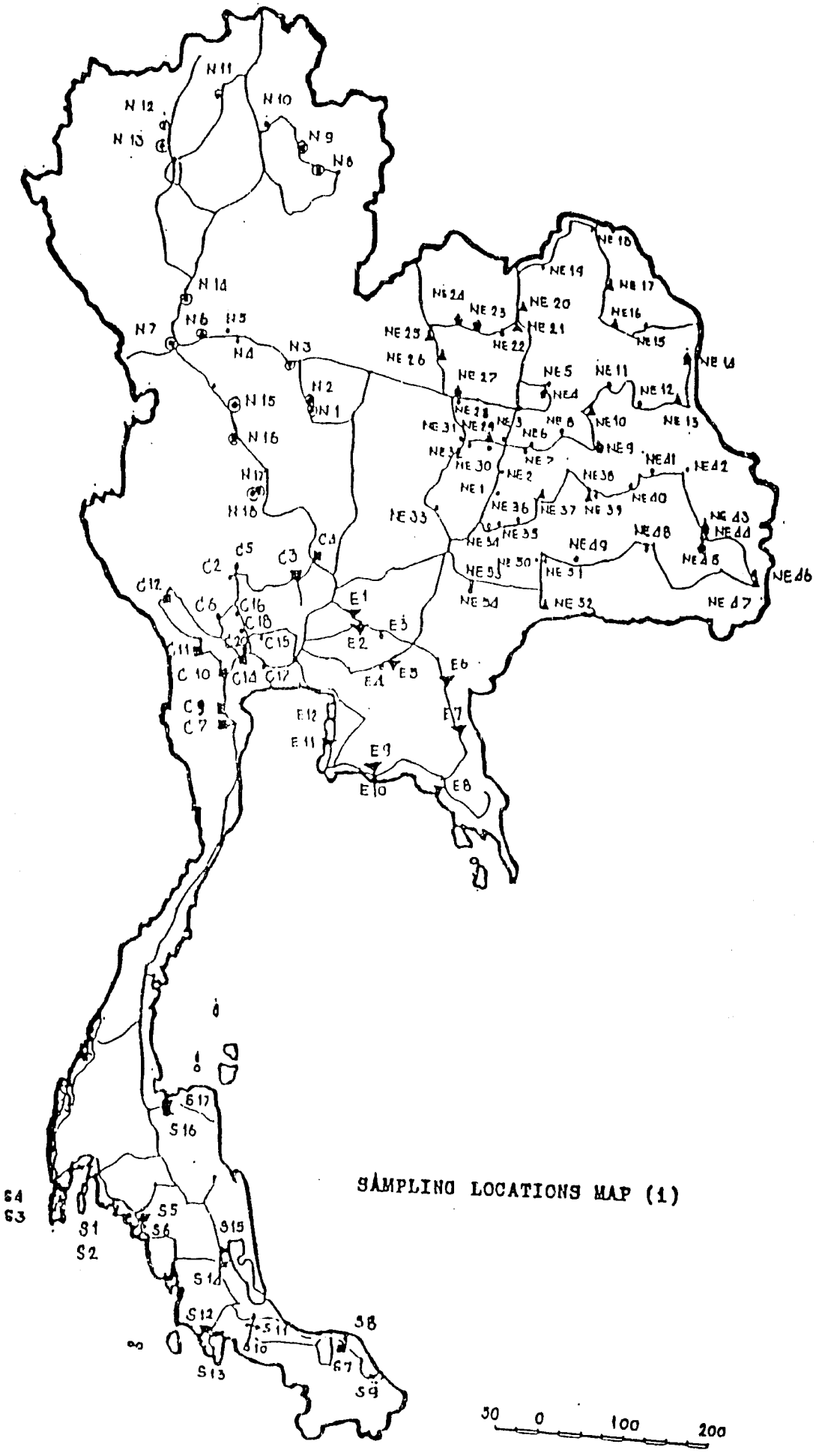


Figure 1. Sampling Locations Map (1)

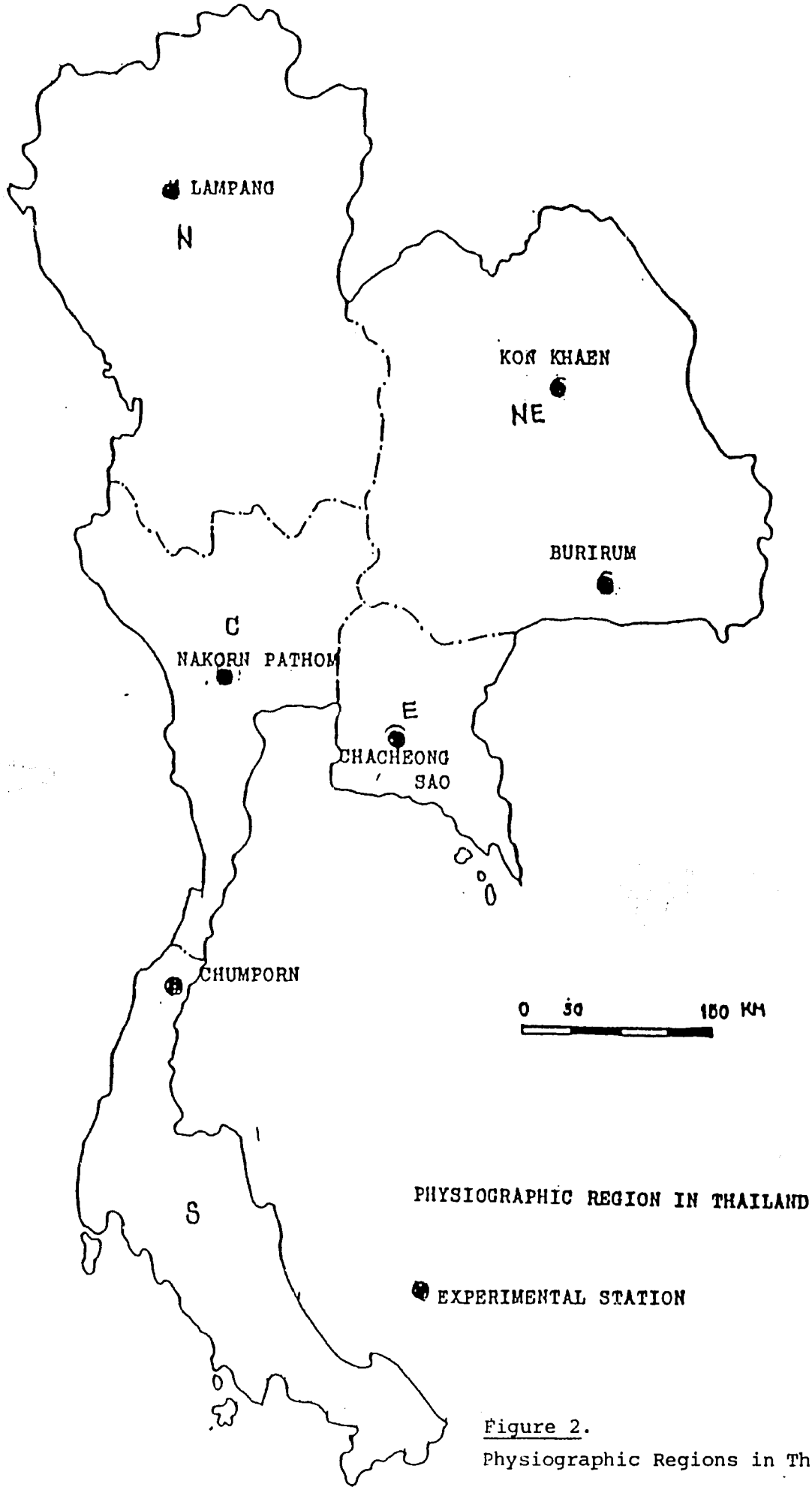
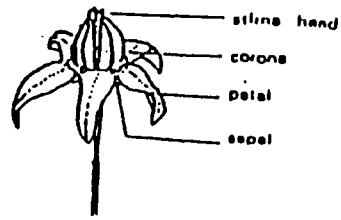
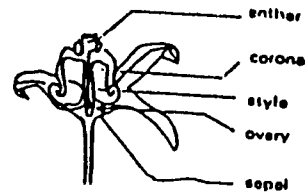


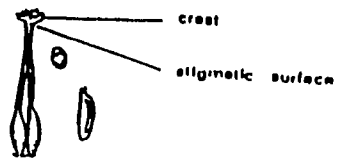
Figure 2.
Physiographic Regions in Thailand.



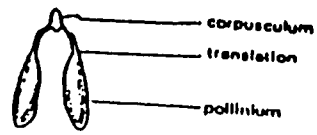
FLOWERS

HALF – FLOWER

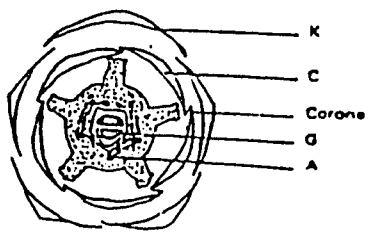




GYNOECIUM AND OVARY SECTIONS

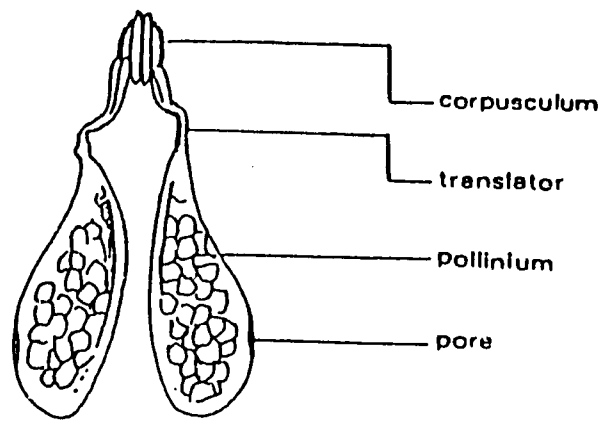


PAIR OF POLLINATION



FLORAL FORMULA

*, K(5), C(5), A(5), P(1), G̲, G(2), O(∞), Corona(5)



*	radial symmetry
K	sepal
C	petal
A	stamen
P	pistil
G̲	superior ovary, superior gynoecium
G	carpel
O	ovule

Figure 3

Figure 4.1 Width of the Flowers (White)

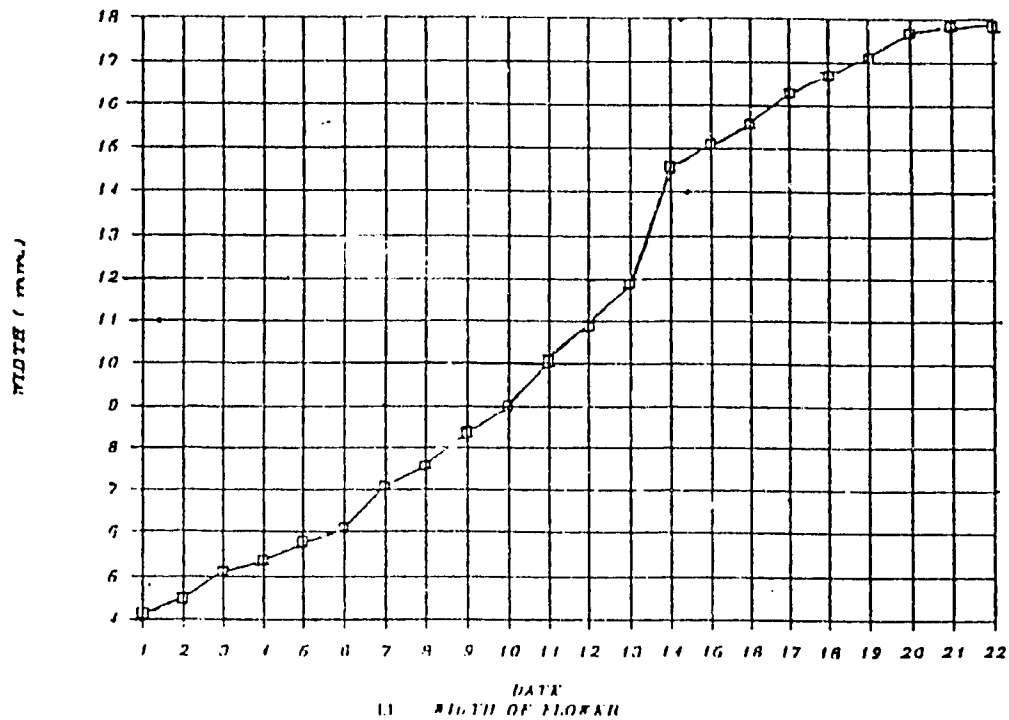


Figure 4.2 Width of the Flowers (Purple)

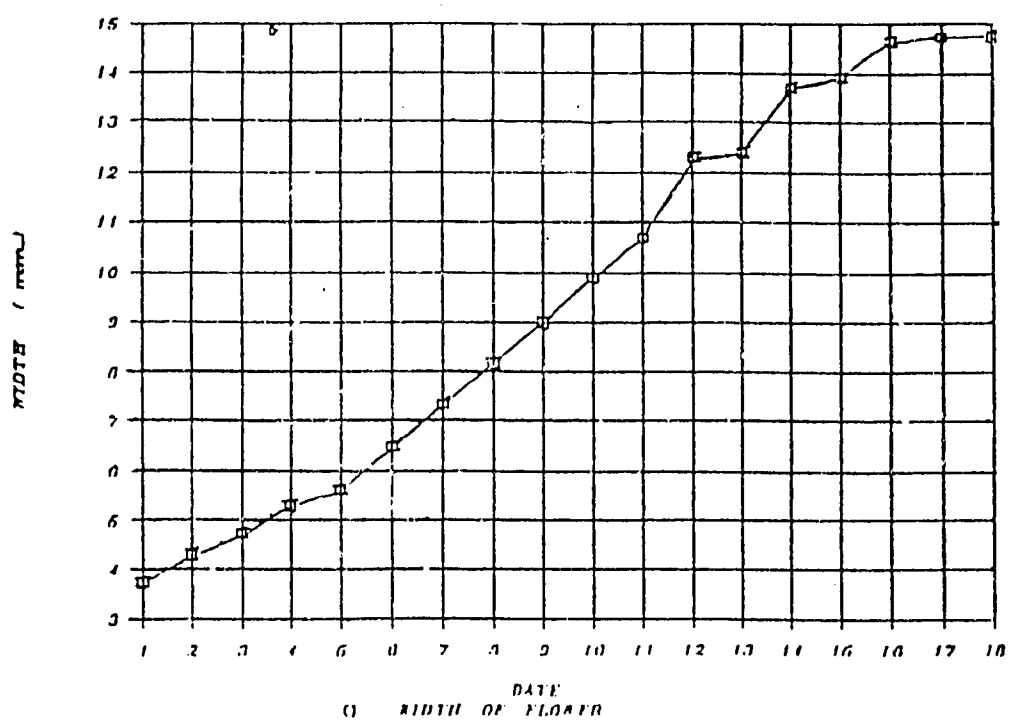


Figure 4

Figure 5.1 Length of the Flower (White)

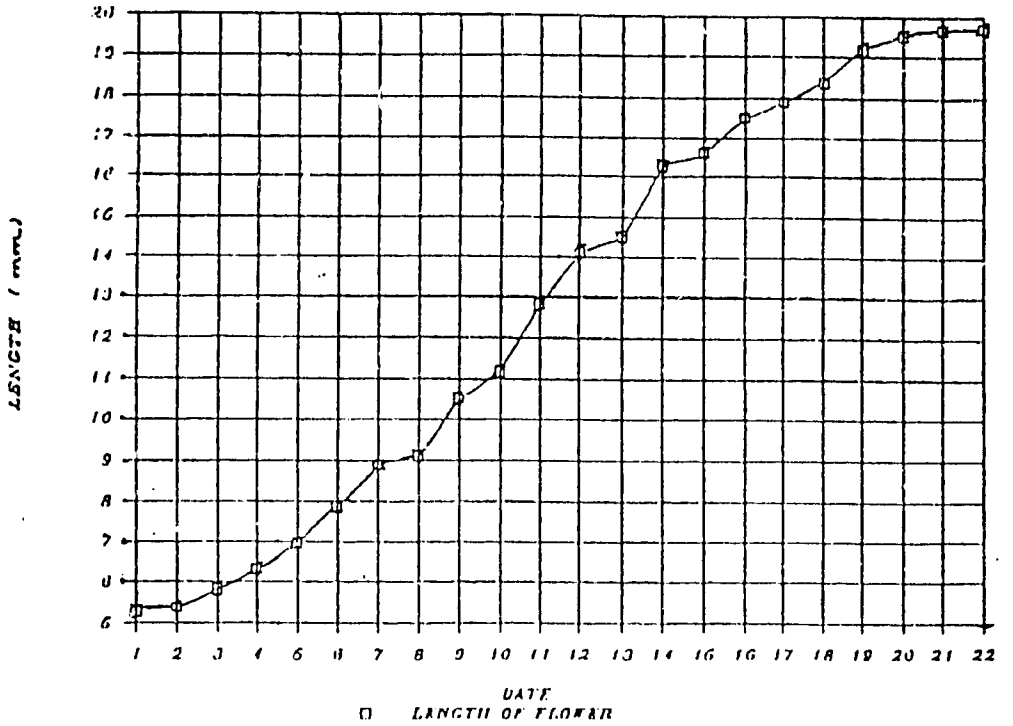


Figure 5.2 Length of the Flower (Purple)

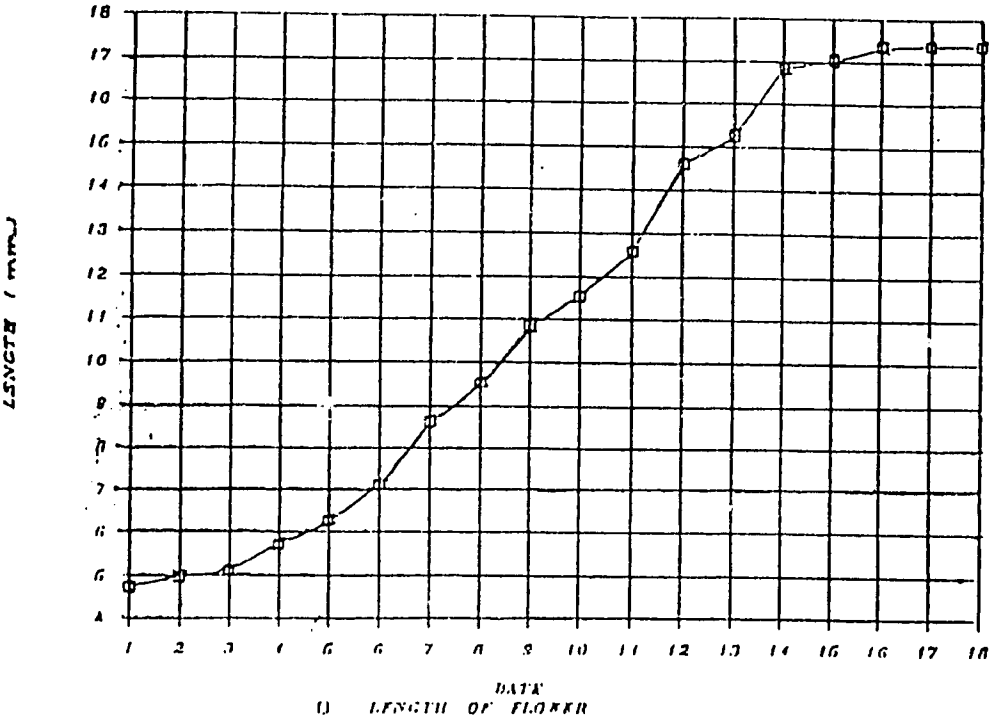


Figure 5

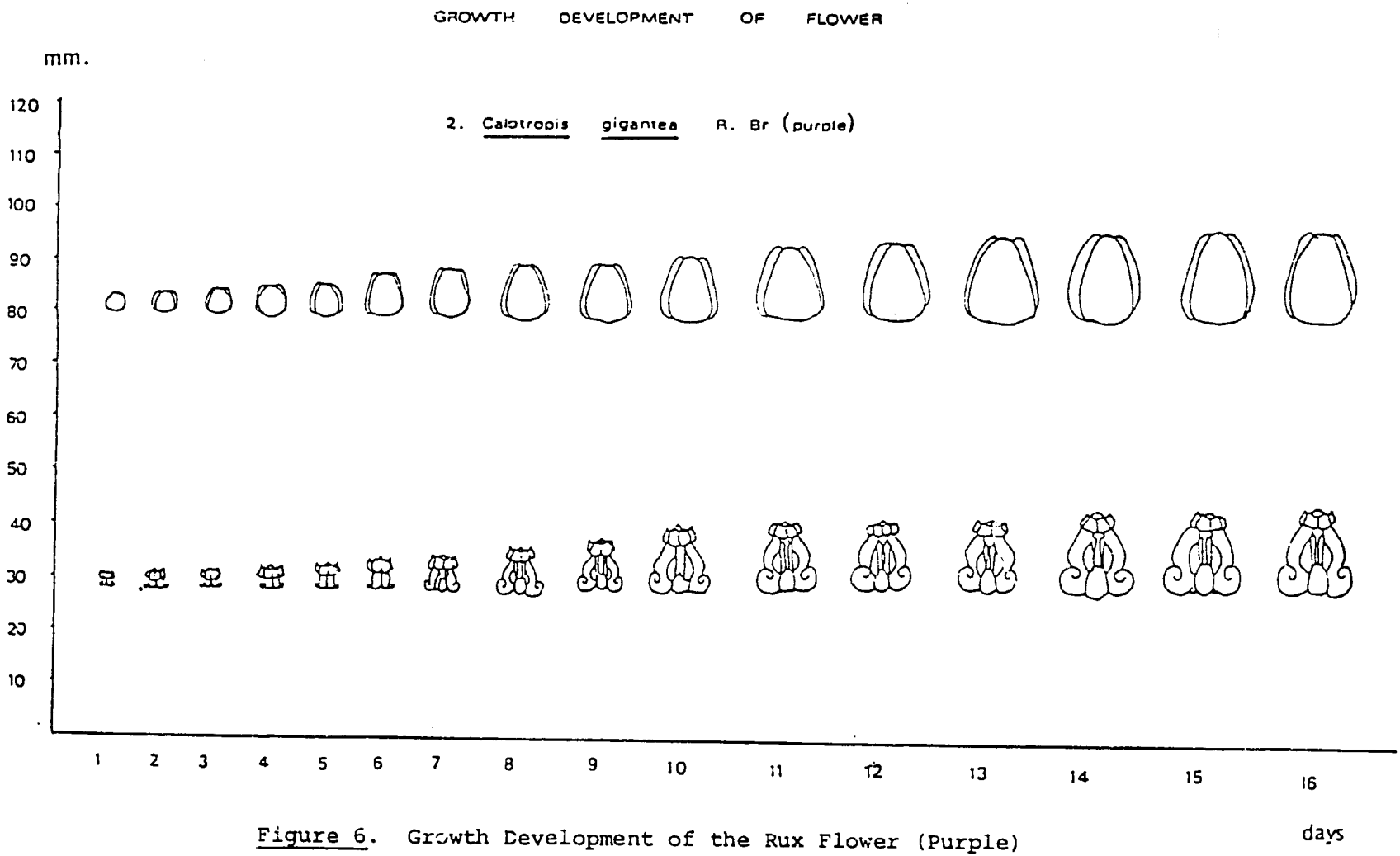


Figure 6. Growth Development of the Rux Flower (Purple)

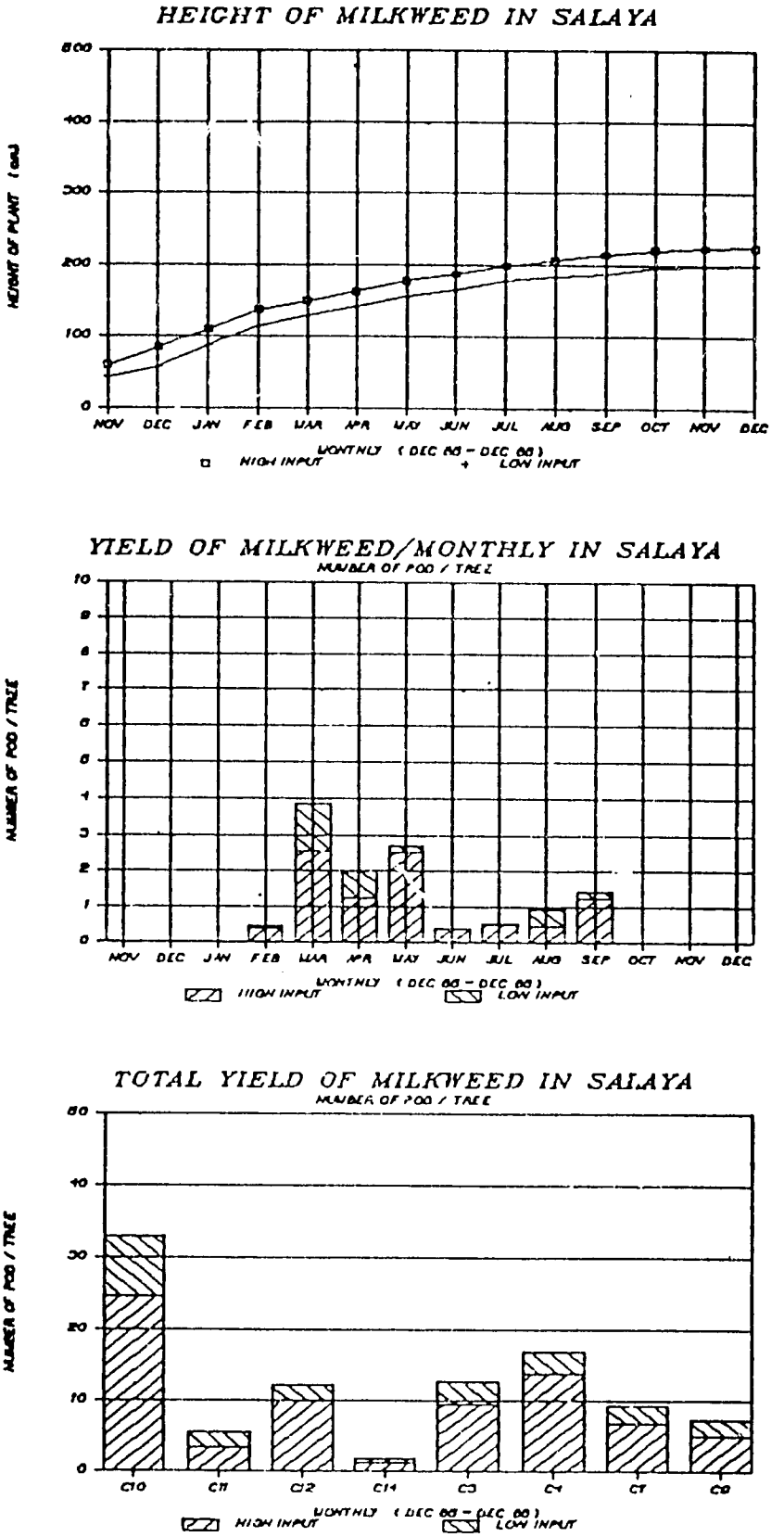
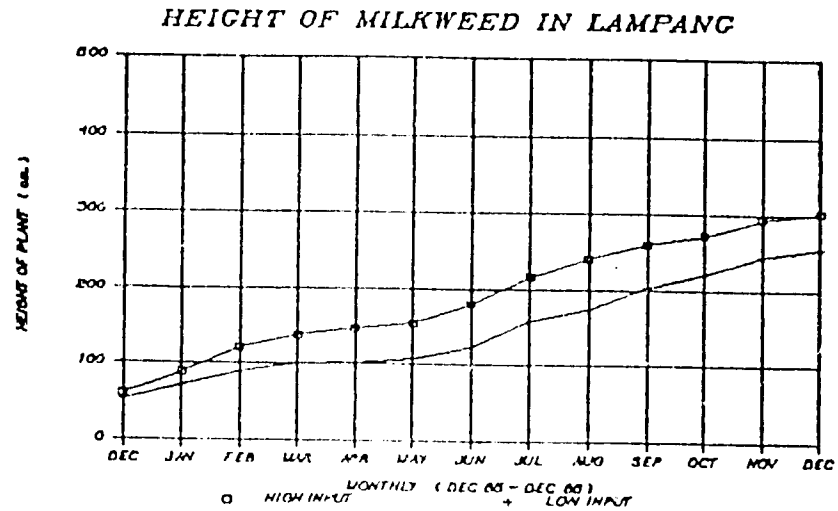
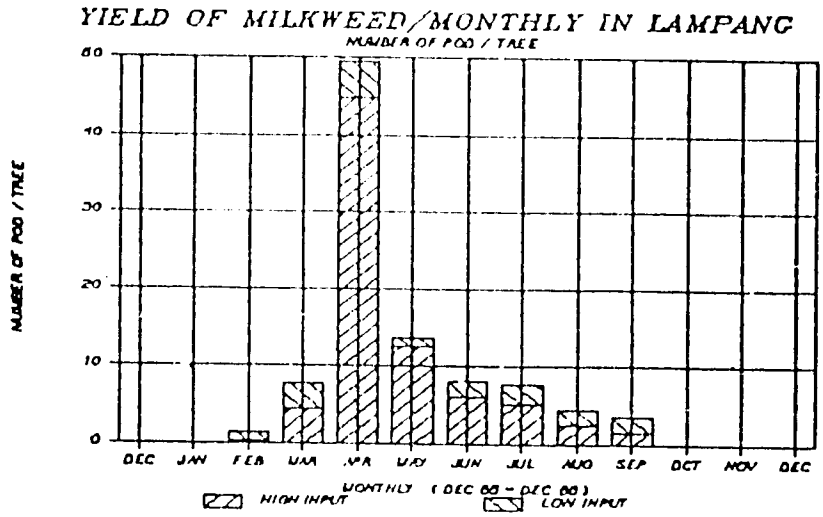

HEIGHT OF MILKWEED IN SALAYA
HEIGHT OF PLANT (cm)
0
100
200
300
400
500
NOV DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT
YIELD OF MILKWEED/MONTHLY IN SALAYA
NUMBER OF POD / TREE
NUMBER OF POD / TREE
0
1
2
3
4
5
6
7
8
9
10
NOV DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT
TOTAL YIELD OF MILKWEED IN SALAYA
NUMBER OF POD / TREE
NUMBER OF POD / TREE
0
10
20
30
40
50
C10 C11 C12 C14 C3 C4 C7 C8
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT

Figure 7


HEIGHT OF MILKWEED IN LAMPANG
HEIGHT OF PLANT (cm.)
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT


YIELD OF MILKWEED/MONTHLY IN LAMPANG
NUMBER OF POD / TREE
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT

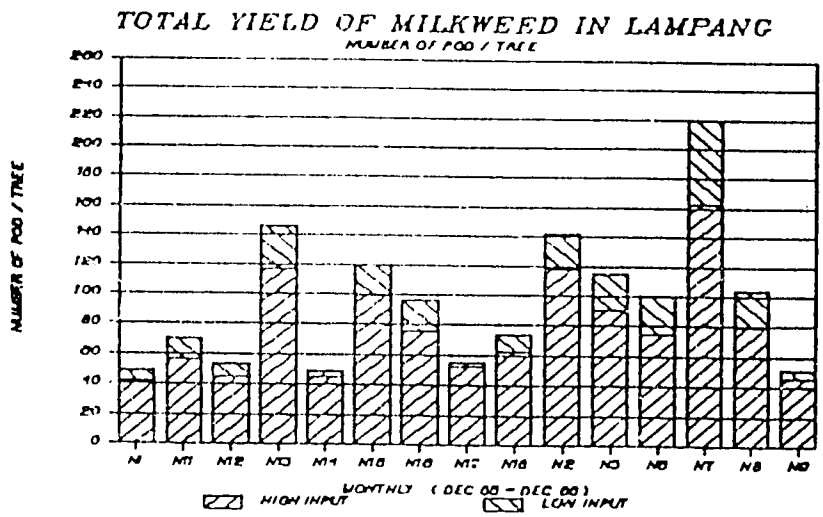

TOTAL YIELD OF MILKWEED IN LAMPANG
NUMBER OF POD / TREE
MONTHLY (DEC 88 - DEC 89)
HIGH INPUT
LOW INPUT

Figure 8

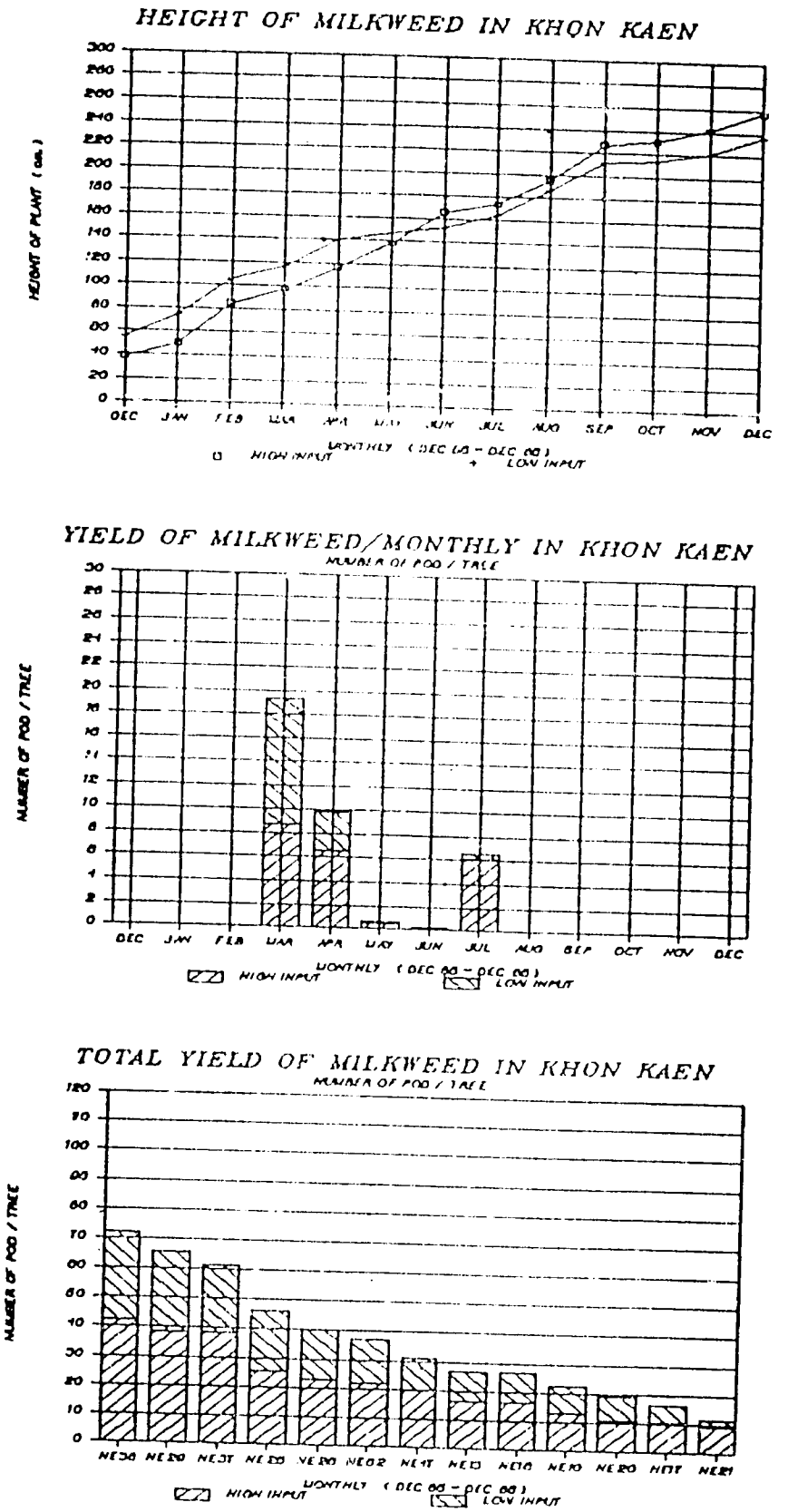

HEIGHT OF MILKWEED IN KHON KAEN
HEIGHT OF PLANT (cm.)
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 68 - DEC 68)
HIGH INPUT
LOW INPUT
YIELD OF MILKWEED/MONTHLY IN KHON KAEN
NUMBER OF POD / TREE
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTHLY (DEC 68 - DEC 68)
HIGH INPUT
LOW INPUT
TOTAL YIELD OF MILKWEED IN KHON KAEN
NUMBER OF POD / TREE
NUMBER OF POD / TREE
MONTHLY (DEC 68 - DEC 68)
HIGH INPUT
LOW INPUT

Figure 9

FY 1985

"PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER" (5.227)

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ABSTRACT

Five mouse monoclonal antibodies (mAbs) were established. Two mAbs (STP13, STP14) were specific to 34.0 Kilodalton (Kd) protein antigen of S. typhi, while one mAb (STVi14) was specific to Vi antigen of S. typhi, the other 2 mAbs, SPA20 and SPB20 were specific to 30-58.0 Kd and 30-56.0 Kd protein antigen of S. paratyphi A and S. paratyphi B respectively. The STP13 belonged to the IgG2aK subclass while the other mAbs could be assigned to the IgG1K subclass. The specificity of STP13, STP14, SPA20 and SPB20 was tested by immunoblotting using a panel of crude protein antigens from 12 bacteria which consisted of S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. cholerasuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, E. coli, Ps. pseudomallei, and Y. enterocolitica which cause enteric fever and enteric fever-like illnesses. By the modified double antibody sandwich ELISA, the STP13 and STP14 could detect protein antigens at the approximate concentration of 0.6 μg/ml ($p<0.005$), while STVi14, SPA20 and SPB20 could detect their antigens at concentrations less than 0.1 μg/ml ($p<0.001$). These mAbs should be of great value for use in diagnostic tests for the detection of Salmonella antigens in biological fluid of the patients with enteric fever, and for the study of the chemical structure and other immunological properties of their specific protein antigens.

INTRODUCTION

Enteric fever is caused by four species of Salmonellae namely Salmonella typhi, S. paratyphi A, S. paratyphi B and S. paratyphi C. Among these 4 species, S.paratyphi C has never been isolated from patients' specimens during the past 10 years. One of the diagnostic problems of enteric fever is differential diagnoses which do not distinguish between enteric fever and enteric fever-like illnesses caused by other Enterobacteriaceae. The most dependable way to establish a definitive diagnosis is by hemoculture. However, laboratory diagnosis by hemoculture suffers from certain disadvantages. Many cultures are incorrectly negative due to prior antibiotic therapy. Even when appropriate hemocultures are taken, the presumptive bacteriological results require at least 48 hours or longer for positive results.

Serological diagnosis of enteric fever by the Widal test has been found to be unreliable by some investigators (1,11) and the positive diagnosis requires both acute and convalescent sera which are rarely collected (11). Hence, the antibodies in single serum specimen are not very useful for serodiagnosis, since they can not distinguish current from previous infections.

Therefore, there is a need for a test that will permit a rapid laboratory diagnosis of enteric fever by a single specimen. That test should aim to detect specific Salmonella antigens from biological fluids of the patient. Many investigators have established such tests by using conventional polyclonal antisera in their tests but they have met problems of cross reaction with other members of family Enterobacteriaceae (2,6,8,13, 14). Attempts to reduce cross reactivities by absorption methods have not been able to eliminate all of the cross reactive antibodies (8). To overcome this problem, mAbs which are specific to antigens of the mentioned Salmonellae are required.

MATERIALS AND METHODS

Bacteria

All bacteria used in this study can cause enteric fever and enteric fever-like illnesses. They were as follows, S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. cholerasuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, Escherichia coli, Pseudomonas pseudomallei, Yersinia enterocolitica and Campylobacter jejuni.

Preparation of Antigens

The protein (Bp) antigens from all bacteria except C. jejuni were prepared by the method originally described by Barber (4). Briefly, washed and acetone dried bacterial cells were extracted with veronal buffer pH 8.4 and the protein was precipitated from veronal buffer extract (VBE) by trichloroacetic acid. The precipitate was washed, resolubilized, lyophilized and stored at 4°C until used. The protein content was assayed by the method described by Lowry and modified by Hartree (7). This antigen had a small amount of lipopolysaccharide contamination as confirmed by double immunodiffusion.

The C. jejuni was used as the acetone dried cells antigen in this experiment.

Production of Monoclonal Antibodies

Female BALB/c J mice 6 to 10 weeks of age (kindly provided by the Division of Veterinary Medicine, Armed Force Research Institute of Medical Science (AFRIMS), Bangkok were immunized with Bp antigen from S. typhi or S. paratyphi A or S. paratyphi B once every 3 days for 15 days subcutaneously into 2 footpads, 2 auxillary and 2 inguinal areas and also intravenously (9).

One day after the last administration of antigen, mice were sacrified by cervical dislocation. The auxillary and inguinal lymph nodes and one spleen were aseptically removed for hybridization with P3x63-Ag8.653 mouse myeloma cells by using 42% polyethylene glycol 4000 (Accurate Chemical and Scientific Co., Westbury, NY.). The fused cells were dispensed into 24 well tissue culture plates (Costar, Cambridge, Mass.) in the presence of syngeneic mouse peritoneal feeder cells and cultured at 37°C with 80% humidity and 7% CO_2. Hybridomas secreting specific antibodies were cloned by the single dilution method in the presence of syngeneic mouse peritoneal feeder cells. About 7 days after cloning, the hybridoma could be seen by microscopy and each well was scored for monoclones.

Indirect ELISA for mAb screening and specificity testing

Hybridoma-secreting specific antibodies against antigens of S. typhi, S. paratyphi A and S. paratyphi B were detected by indirect enzyme-linked immunosorbent assay (ELISA) as described by Banchuin et al (3) using panel protein antigens of S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. cholerasuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, E. coli, Ps. pseudomallei, Y. enterocolitica, dried whole cells of C. jejuni, LPS and Vi antigen.

Determination of mAb isotypes

Culture supernatants of hybridomas secreting specific antibodies were characterized for their isotypes by indirect ELISA. The procedure was the same as in mAb screening and specificity testing except that alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, K and (Southern Biotechnology, Birmingham, Ala) were used instead of the anti-mouse polyvalent Ig.

Immunoblot technique for mAb specificity testing

The protein antigens of 12 bacteria were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method originally described by Laemmli (10), in the LKB 2001 vertical electrophoresis unit (LKB-Produkter AB, Bromma, Sweden). After electrophoresis, the proteins were converted to nitrocellulose by a Transblot apparatus with a 15°C cooling system (LKB-Produkter AB, Bromma, Sweden) at 90 V overnight. The nitrocellulose membranes were washed with 1% (vol/vol) Tween 20-PBS (pH 7.2) at 37°C for 30 minutes and unoccupied binding sites were blocked by incubation in 3% (w/v) bovine serum albumin in Tween-PBS at room temperature for 60 minutes on a rotating platform. MAbs diluted in Tween-PBS were added to the membranes, and they were incubated at room temperature for 2 hours. After the membranes were washed with Tween-PBS, alkaline phosphatase-conjugated goat anti-mouse polyvalent Ig (Dakopatts, Glostrup, Denmark) was added. The incubation was performed for 60 minutes at room temperature. Red purple color development was accomplished by incubating washed membranes in a mixture of 4 mg B-naphthyl phosphate (Sigma Chemical Co., St. Louis, MO) and 60 mg O-dianisidine tetrazotized (Sigma Chemical Co., St.Louis, MO) (12,15) for 5 minutes, then the filter was rinsed with tap water to stop the reaction.

Molecular Weight Determination

The molecular weights of protein bands of S. typhi, S. paratyphi A and S. paratyphi B specific to mAbs on nitrocellulose filter membrane were determined by comparing their electrophoretic mobility with known protein markers as described by Weber and Osborn (16).

Antibody Production In Vivo

To obtain high yields of antibodies, the hybridomas were grown as ascite tumour in pristane-(2,6,10,14-tetramethylpentadecane, Aldrich chemical Co., Milwaukee, WI) primed BALB/c J mice. Ascitic fluid developed within 2 weeks after hybrid cells were injected intraperitoneally, and the total of 10-15 ml fluid per mouse could be withdrawn.

Modified Double Antibody Sandwich ELISA for Efficiency Testing of mAbs

All monoclonal antibodies in the ascitic fluid mixture were precipitated by the addition of saturated $(NH4)2SO4$ to arrive at a final concentration of 50%. The precipitate was dialysed against PBS, pH 7.2 for 48 Hrs with five changes of buffer and then stored at -20°C until used in ELISA.

The purified mAbs were diluted in 0.05 M carbonate buffer, pH 9.8, containing 0.1% sodium azide, to obtain the optimum protein concentrations. One hundred microlitres of the solution was added into each well of Microelisa Immulon^R plates (Dynatech Laboratories, Inc., Alexandria, VA), which were then incubated at 4°C overnight. After that, the plates were washed 3 times with phosphate buffered saline-tween 20 (PBST)and tapped dry. One hundred microlitres of Bp antigen or VBE at various concentrations in PBST were added to each well and the plates were incubated at 37°C for 3 hours. Each specimen was assayed in duplicate. After 3 washes with PBST, 100 ul of the second antibody, which was a rabbit anti-veronal buffer extract of the corresponding Salmonellae diluted in PBST, were added to each well. The plates were then incubated at 30°C for 2 hours. After washing, 100 ul of goat anti-rabbit Ig-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) diluted 1:2000 in PBST containing 1% BSA were added to each well and incubated at 4°C for overnight. The excess conjugate was washed out 3 times with PBST and 100 ul of substrate solution (p-nitrophenyl phosphate, Sigma chemical Co., St. Louis, MO) concentration 1 mg/ml were added to each well. The reaction was allowed to take place at 37°C for 1 hour and stopped by the addition of 25 ul 3 M NaOH. The absorbance value was read spectrophotometrically at 405 nm (Titertek Multiskan , Flow Laboratory, GmbH, Bonn, West Germany). In each assay, negative control, conjugate control and substrate blank were also included. One hundred microlitres of PBST were substituted for the antigen in the negative control, for both the antigen and rabbit anti-VBE Ig in the conjugate control and also for the conjugate in the substrate blank. Several assays were performed with each mAb and the Student's t test was used for statistical analyses.

RESULTS

By indirect ELISA using a panel of 12 Bp antigens, dried cell of C. jejuni, Vi antigen and LPS, two hybridomas producing specific antibodies to protein antigens of S. typhi (STP13, STP14) and one each of hybridoma specific to S. typhi Vi antigen (STVi14), and protein antigens of S. paratyphi A (SPA20) and S. paratyphi B (SPB20) (Table 1), were selected for further investigation. Their origins (lymph node or spleen) and isotypes were shown in Table 2.

Demonstration of Specific Protein Bands by Immunoblotting

The separated protein bands of S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. cholerasuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, E. coli, Ps. pseudomallei, Y. enterocolitica on nitrocellulose filter membranes were stained with immunoenzymes using mAbs STP13, STP14, SPA20 and SPB20 as the specific antibodies.

The STP13 and STP14 mAbs reacted only with one protein band of S. typhi (Fig. 1). The molecular weights of the reacting bands were determined from a standard curve of Coomassie blue dye stained protein markers on the same filter membrane which revealed that both bands had the same molecular weight of 34.0 Kd and had the same relative mobility of 0.51. The S. typhi protein band which reacted with STP14 was wider than the band that reacted with STP13. Both mAbs could react very weakly with bands of protein of approximately the same molecular weight from S. paratyphi C, S. cholerasuis and S. typhimurium (Fig.3,4A,4B), although these weak reactions could not be seen in indirect ELISA. This 34.0 Kd protein band also demonstrated that the separated protein bands of S. typhi reacted with the sera from patients with typhoid fever and vaccinated subjects who received parenteral acetone inactivated or heat inactivated-phenol preserved typhoid vaccines, or oral live attenuated S. typhi Ty21a vaccine.

The SPA20 and SPB20 could react with several protein bands of corresponding S. paratyphi A and S. paratyphi B (Fig.2) The range of molecular weight of protein bands specific to SPA20 was 30-58.0 Kd and for SPB20 was 30-56.0 Kd. Both SPA20 and SPB20 showed no reaction to other protein antigens in the panel (Fig.2,4C,4D).

Antigen Detection by Modified Double Antibody Sandwich ELISA

All mAbs were experimentally designed for detection of corresponding antigens at various concentrations. The results were shown in Fig.5,6 that both STP13 and STP14 could detect the protein antigen of S. typhi approximately at the concentration of 0.6 μg/ml ($p<0.005$), the STVi14, SPA20 and SPB20 could detect less than 0.1 μg/ml ($p<0.001$) of S. typhi Vi antigen, protein antigen of S. paratyphi A and S. paratyphi B respectively.

DISCUSSION

Five monoclonal antibodies, (STP13, STP14, STVi14, SPA20, SPB20) were established. STP13 and STP14 was shown to be specific to protein bands of S. typhi at a molecular weight of 34.0 Kd by the immunoblotting technique. The pattern of the stained bands was not exactly the same; the band which reacted with STP14 was wider than that which reacted with STP13, which suggests that both mAbs are not exactly specific to the same epitope of the protein antigen, since the band separated by SDS-PAGE may contain the same molecular weight protein but has a different amino acid composition. The SPA20 and SPB20 were specific to 34-58.0 Kd protein bands of S. paratyphi A and 30-56.0 Kd protein bands of S. paratyphi B respectively.

To confirm the specificity of these protein-specific mAbs, immunoblotting was performed with panel antigens of 12 bacteria. The STP13 and STP14 showed unexpectedly weak reaction with Bp of S. paratyphi C, S. cholerasuis and S. typhimurium. These cross reactivities were not found in indirect ELISA which suggests that the hidden epitopes of Bp-ST are exposed after the protein has been separated by the high resolving power of SDS-PAGE, then are enhanced by the sensitivity of immunoblotting in comparison with the indirect ELISA in the solid phase. However, the SPA20 and SPB20 showed no cross reactivity with other protein in the panel.

The detection of specific antigens by STP13, STP14, STVi14, SPA20 and SPB20 mAbs should be very useful for serodiagnosis of patients with enteric fever. The weak cross reactivities of STP13 and STP14 with protein antigens of S. paratyphi C, S. cholerasuis and S. typhimurium in the immunoblotting are so minimal that they should not interfere with other less sensitive conventional tests used in routine diagnosis and should not be considered great disadvantages. With regard to the effects of the test on patient treatment, these cross reactivities should not interfere the plan of treatment because antibiotic therapies among these 4 bacteria are very similar (5).

To determine the ability of the established mAbs for Salmonellae antigen detection, modified double antibody sandwich ELISA was performed with various concentrations of antigen. The ELISA could detect very small amounts of antigen i.e. 0.6 μg/ml for STP13 and STP14, $<$ 0.1 μg/ml for STVi14, SPA20 and STB20. In view of these results, the sensitive and specific established mAbs make more considerable potential reagents for use in tests for detection of specific Salmonella antigens in the specimens of patients with enteric fever than the conventional polyclonal antibodies.

The evidence of the 34.0 Kd protein band that was demonstrated when Bp-ST reacted with sera of typhoid patients and vaccinees who received different kinds of typhoid vaccines suggest that the 34.0 Kd protein antigen plays a role in the immune response against S.typhi. Hence, purified 34.0 Kd protein of S. typhi is required as an essential structural probe for dissecting the basic mechanisms of the immune

response to this organism and also should have a potential application as a specific skin-test reagent for testing the cell-mediated immune response to S.typhi in vivo. Further studies concerning the purification and characterization of this 34.0 Kd protein by using STP13 and STP14 mAbs as well as clinical trials of all mAbs with patients' specimens are in progress in our laboratory.

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Clone	*S. typhi*	*S. paratyphi* A	*S. paratyphi* B	*S. paratyphi* C	*S. cholerasuis*	*S. enteritidis*	*S. krefeld*	*S. panama*	*S. typhimurium*	*E. coli*	*Ps. pseudomallei*	*Y. enterocolitica*	*C. jejuni*	LPS antigen	Vi antigen	*S. paratyphi* A, B, C
STP13	1.13	0.15	0.11	0.15	0.13	0.12	0.12	0.11	0.13	0.12	0.14	0.15	0.17	0.17	0.13	-
STP14	1.61	0.16	0.13	0.17	0.13	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.15	0.14	0.17	-
STVi14	1.15	0.17	0.17	0.28	0.18	0.19	0.17	0.16	0.18	0.19	0.13	0.12	0.16	0.18	1.89	-
SPA20	0.15	1.71	0.13	0.15	0.12	0.12	0.13	0.13	0.13	0.15	0.14	0.13	0.16	-	-	0.50
SPB20	0.11	0.13	1.11	0.13	0.11	0.13	0.12	0.13	0.12	0.14	0.14	0.13	0.14	-	-	0.63

Table 1. Reactivities of Monoclonal Antibodies to Specific Panel Antigens.

Clone	Isotype	Specificity	Source of immune cells
STP13	IgG2aK	protein	lymph node
STP14	IgG1K	protein	spleen
STVi14	IgG1K	Vi	spleen
SPA20	IgG1K	protein	spleen
SPB20	IgG1K	protein	spleen

Table 2. Characteristics of Established Monoclonal Antibodies

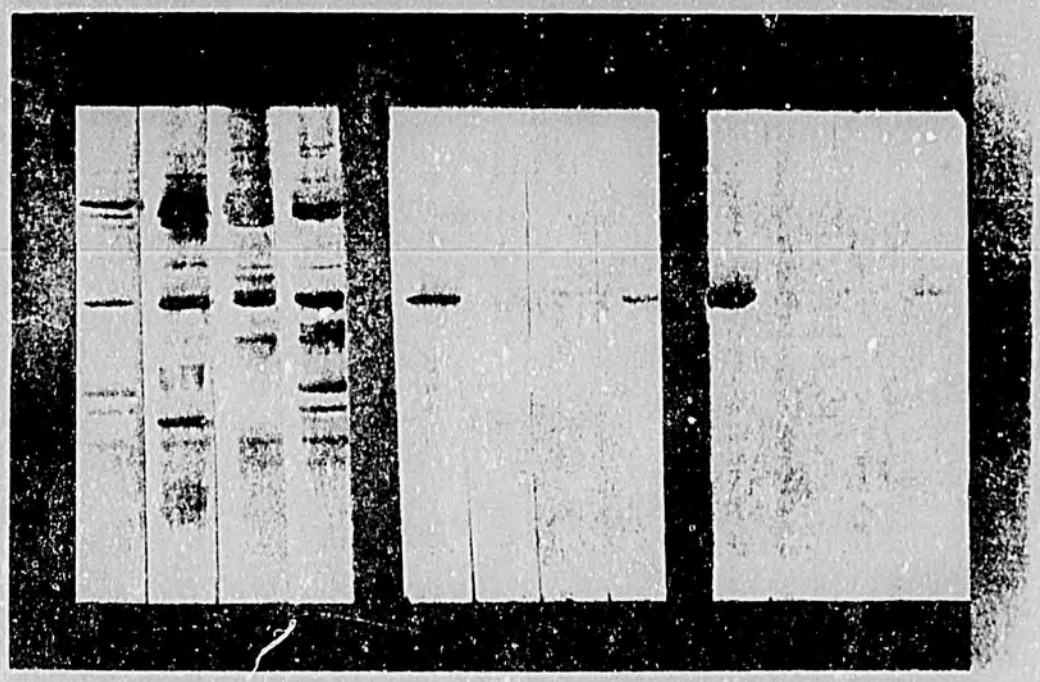

Figure 1 Immunoblot Analysis and Immunoenzyme Staining Patterns of Bp Antigen from S. typhi (1), S. paratyphi A (2), S. paratyphi B (3) and S. paratyphi C (4). The membranes were developed with the homologous antisera produced in mice (A), STP13 (B) and STP14 (C). The weak cross reactivities of both mAbs are seen in S. paratyphi C patterns.

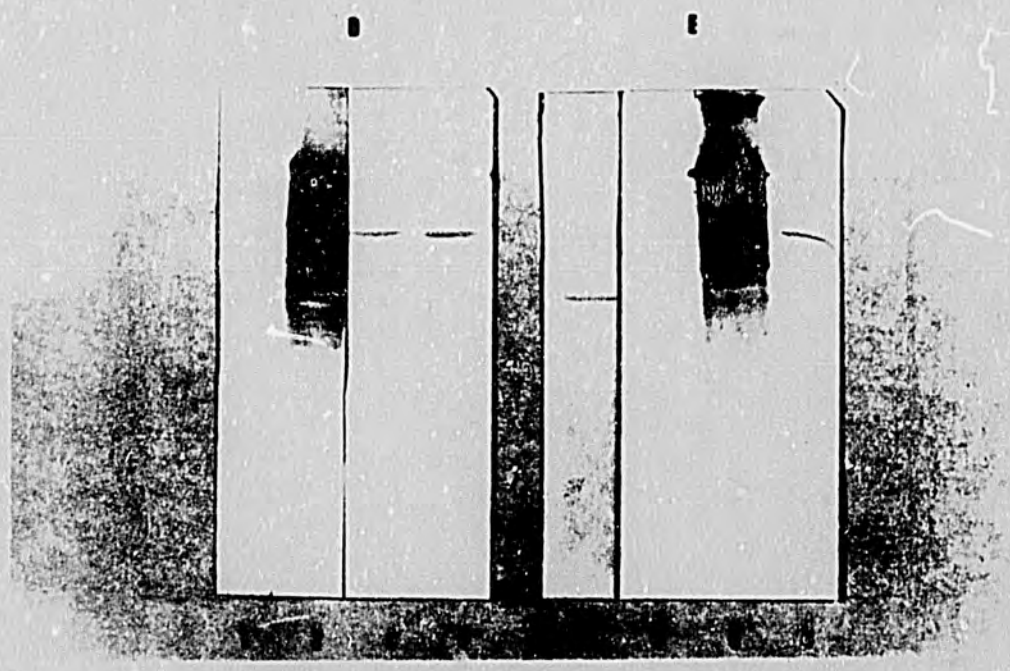

Figure 2 Immunoblot Analysis and Immunoenzyme Staining Patterns of Bp Antigen from S. typhi (1), S. paratyphi A, S. paratyphi B (3) and S. paratyphi C (4). The membranes were developed with SPA20 (D) and SPB20 (E).

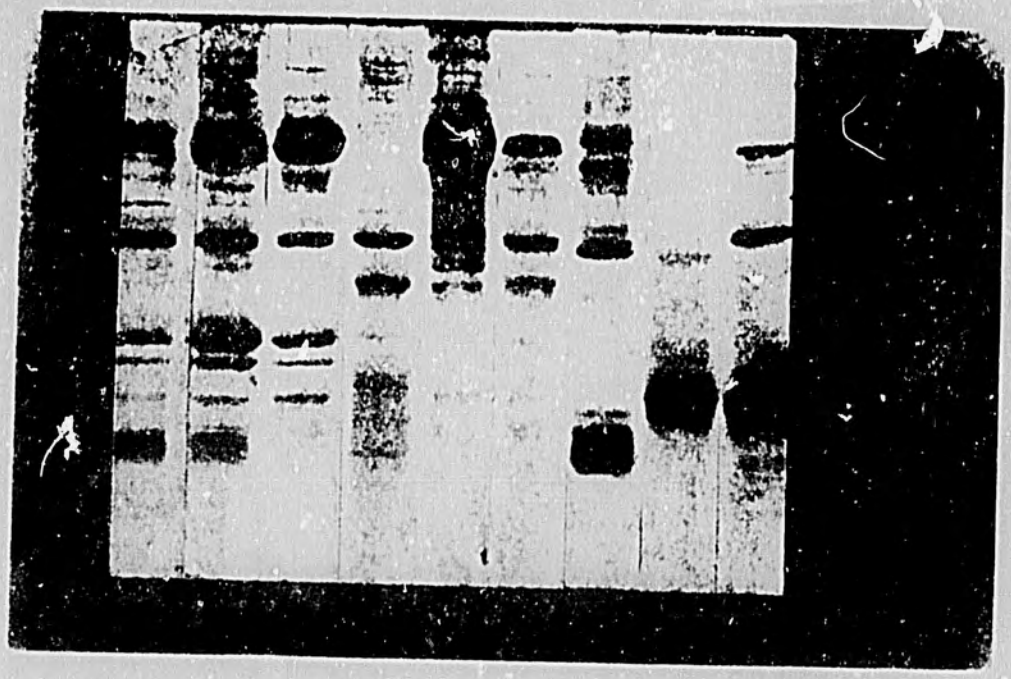

Figure 3 Immunoblot Analysis of Bp Antigen from *S. typhi* (1), *S. cholerasuis* (2), *S. enteritidis* (3), *S. krefeld* (4), *S. panama* (5), *S. typhimurium* (6), *E. coli* (7), *Ps. pseudomallei* (8) and *Y. enterocolitica* (9). Each antigen (50 or 100 ug per land) was run in an SDS-PAGE and transferred to nitrocellulose membrane and reacted with their homologous antisera produced in mice. The alkaline phosphatase-labelled goat anti-mouse Ig was used as a second antibody.

Figure 4 (A, B, C, D) Immunoblot Analysis and Immunoenzyme Staining Patterns of the Same Antigens Shown in Figure 3, except S. paratyphi A was replaced for S. typhi in (C) and S. paratyphi B for S. typhi in (D). All antigens reacted with mAb STP13 (A) STP14 (B) SPA20 (C) and SPB20 (D). In A and B, the weak cross reactivities are observed in S. cholerasuis (s) and S. typhimurium (6) strains.

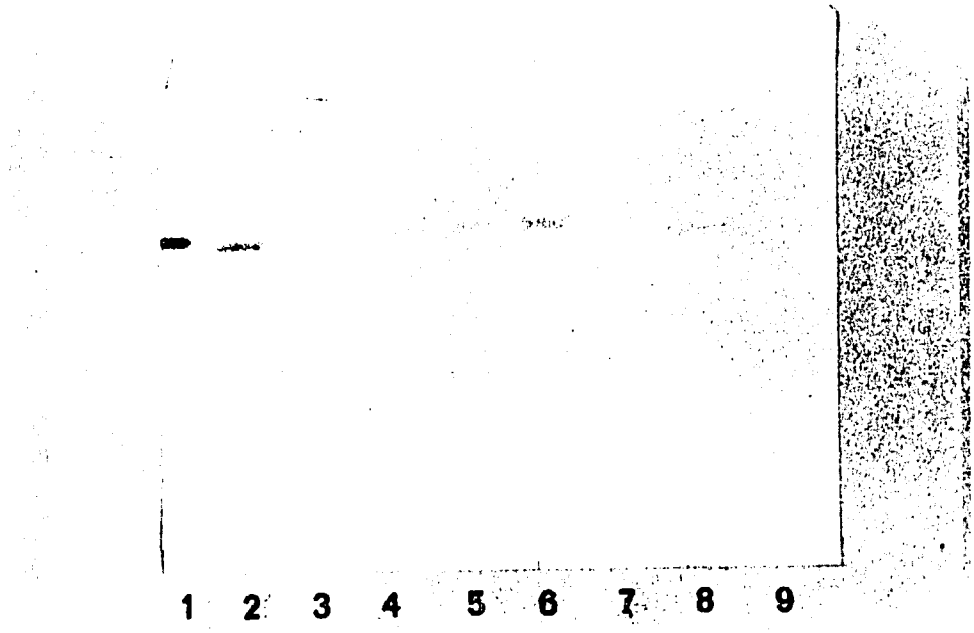

Figure 4 A

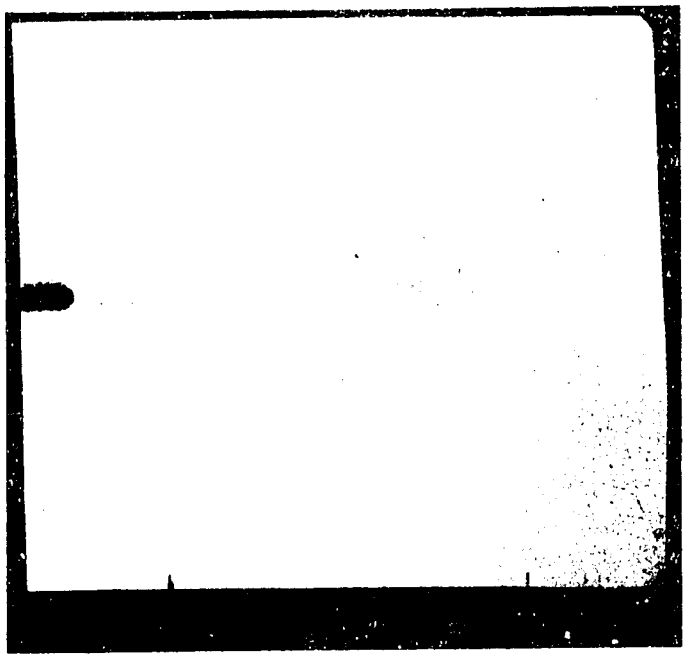

Figure 4 B

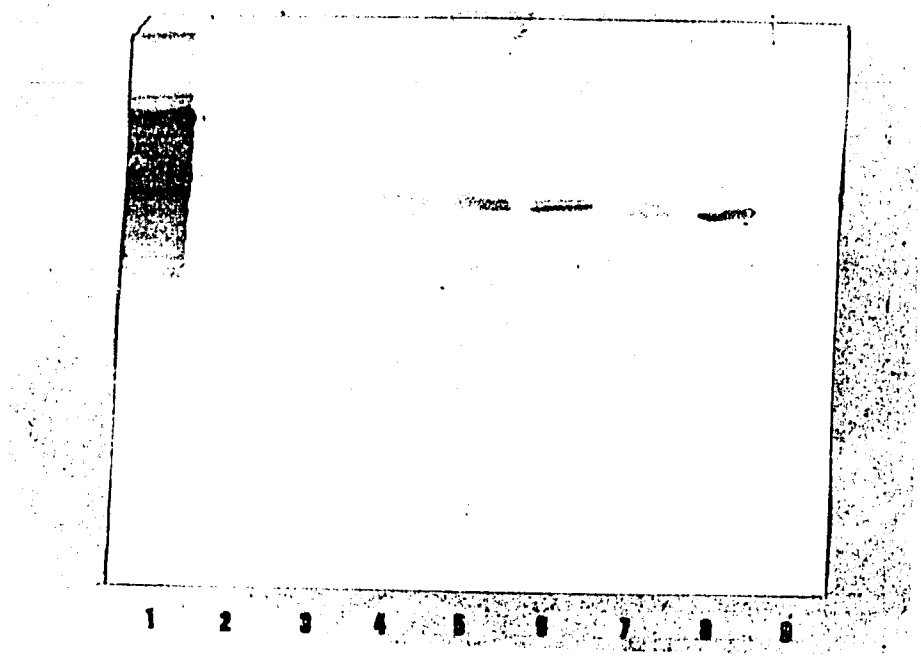

Figure 4 C

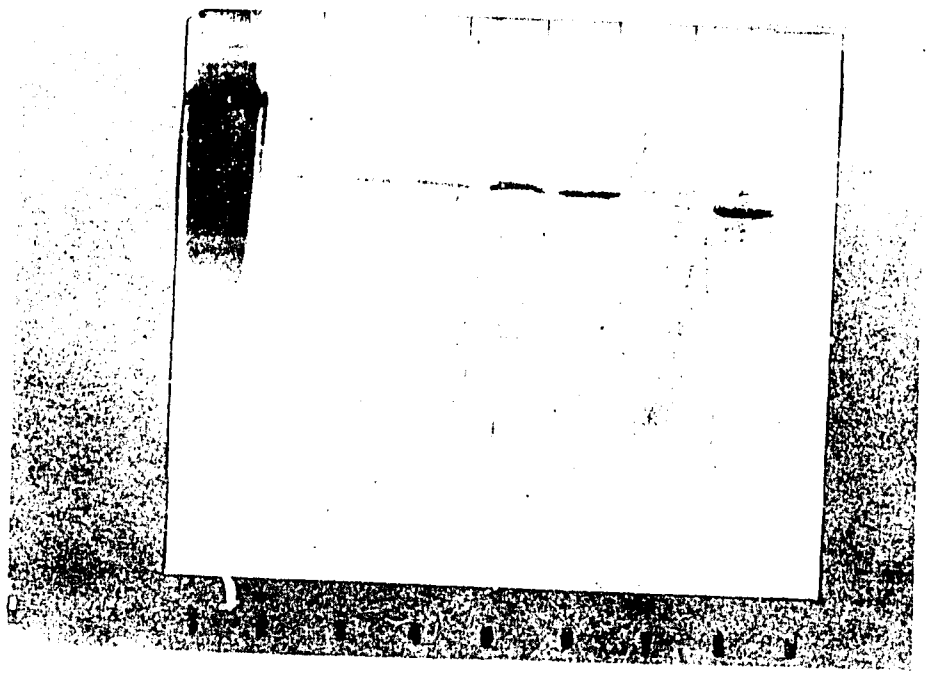

Figure 4 D

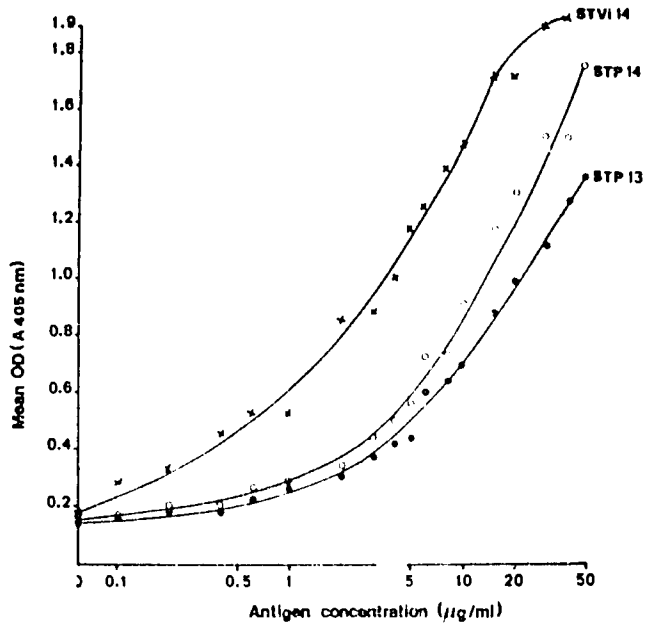

Figure 5 Detection of Antigen of S. typhi by STP13 (●) STP14 (o) and STVi14 (x) Using Modified Double Antibody Sandwich ELISA. y axis: various concentrations of protein antigens (for STP13 and STP14) or VBE antigen (for STVi14) of S. typhi in µg per ml. x axis: the mean of optical densities (OD) at 405 nm.

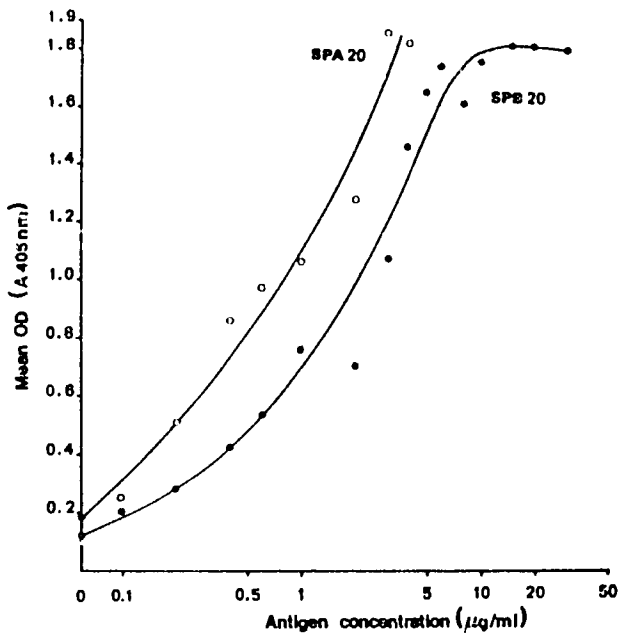

Figure 6 Detection of Bp Antigen of S. paratyphi A by SPA20 (o) and S. paratyphi B by SPB20 (●) Using Modified Double Antibody Sandwich ELISA. y axis: various concentrations of the Bp antigen of S. paratyphi A or S. paratyphi B in µg per ml. x axis: the mean of optical densities (OD) at 405 nm.

FY 1985

"RI-PLASMID ROOT CULTURE AS A POTENTIAL SUBSTRATE FOR IN VITRO PROPAGATION OF MYCORRHIZAL FUNGI" (5.244)

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ABSTRACT

VA mycorrhizal fungi are normally maintained and propagated in pot cultures with their host plants. Growth in artificial media was not possible while limited growth has been obtained with root cultures of tomato (Miller-Wideman and Watrud, 1984.) due to root thickening or lignification and the presence of few lateral roots.

This project attempted to use tumour-inducing bacteria to activate root formation to obtained and maintain active root culture system with large number of lateral roots in order to facilitate penetration and growth of mycorrhizal fungi. Ri-plasmid habouring bacteria, Agrobacterium rhizogenes, was found to be effective in inducing root formation in carrot, potato and certain legumes, particularly peanut. Root cultures could be maintained and propagated successfully with a high number of lateral roots. Infection of potato root cultures with Glomus fasciculatus was also obtained. Future research plan includes studies of effectiveness on mycorrhizal root inocula as well as induction of sporulation under the two-member (root-mycorrhizal fungus) culture system.

INTRODUCTION

VA mycorrhizal fungi, which form symbiotic association with plants, can not be propagated in artificial media but have been shown to infect root cultures of tomato (Miller-Wideman and Watrud, 1984). Infection was, however, constrained by root thickening and lignification as well as by limited formation of lateral roots. Furthermore, the cultivation of roots depended on plant hormones in the culture medium.

On the other hand, Agrobacterium is known as an oncogenic agent and can neoplastically transform cells of many kinds of plants into tumour cells. Agrobacterium-induced tumours can be removed and cultured in vitro where they can continue to grow indefinitely (Hooykaas and Schilperoort, 1986).

It is therefore, proposed to use Agrobacterium to induce and accelerate root formation in selected plants and to develop bacteria-free, fast-growing (tumour) root cell lines suitable for mycorrhizal associations with useful VA mycorrhizal fungi. The two-member root mycorrhizal fungus culture system could subsequently be used to propagate aseptically VA mycorrhizal fungi inocula.

MATERIALS AND METHODS

Bacterial Cultures

Agrobacterium tumifacicus ATCC 15955 and *Agrobacterium rhizogenes* ATCC 15834 were obtained from the American Type Culture Collection, U.S.A. through the Bangkok Microbiological Resource for SE Asia at Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The cultures were maintained in a nutrient agar medium under refrigeration.

VA Mycorrhizal Fungi

Glomus fasciculatus KUMD 8605 was isolated from soils in legume fields and shown to infect mungbean and potato in pot culture.

Plant Materials

Model plants used were carrot and potato. Other tested tumor-plants were five different leguminous plants, i.e. soybean, mungbean, peanut, winged bean and sugar pea.

Tumour Indication

Fresh carrots and potatoes were peeled and washed clean and surface-steriled according to the procedures of Lippincott (1969). They were then aseptically cut into disks 0.5 cm thick and 0.1 cm in diameter. Legume seeds were similarly treated and allowed to germinate. Wounds were implicated on the seedlings. Inoculation was performed by dropping two drops of 10^9 cell suspension to the cut tissue (disc) surface or the cut wound.

Nutrient Media for Root Cultures

The growth media for root cultures were modified from Murashige and Skoog (1962), without the addition of plant hormones.

RESULTS AND DISCUSSION

Phytogenicity of Agrobacterium

Both *A. tumifacicus* and *A. rhizogenes* caused tumour growth formation when tested with the potato model tissue system. *A. tumifacicus* produced unorganized compact clumps of tissue growth which were hard and brittle in texture while *A. rhizogenes* produced white tender lateral roots (Figure 1). *A. rhizogenes* was chosen for further studies with other test plants.

Susceptibility of Test Plant to *A. rhizogenes* Infection

Infection experiments revealed that soybean seedlings showed no response while winged bean seedlings showed slight abnormal fluffy cell

differentiation at the wound sites. Mung bean, peanut and sugar pea showed positive response with varying degrees of tumour root formation.

Transplants of tumour roots, after bacterial decontamination by successive cultivation on a carbenicillin-containing medium, was most successful with peanut (Figure2) and with the two model systems of potato and carrot. Root tumour cell lines of peanut, potato and carrot could be and are being maintained through periodic transplantation.

VA Mycorrhizal Infection of A. rhizogenes-induced Root Cultures

Potato tumour root cultures with extensive branching and root hair formation, were used, initially, to develop the two-member (root-VA mycorrhizal fungus) culture system. It was found that A. rhizogenes induced root were susceptible, as hosts, to mycorrhizal association with the test fungus, Glomus fasciculatus (Figure 3).

CONCLUSION

1. Bacteria-free hairy-root tumour cell lines of carrot, potato and peanut have been successfully developed and maintained.

2. Root cultures with extensive lateral root formation could be successively transplanted and propagated to obtain effective in vitro substrate for cultivation of mycorrhizal fungi based on the two-member culture system of potato tumour root and Glomus fasciculatus.

FUTURE RESEARCH PLAN:

1. Studies on the effectiveness of the mycorrhizal root inocula.

2. Induction of sporulation of mycorrhizal fungi.

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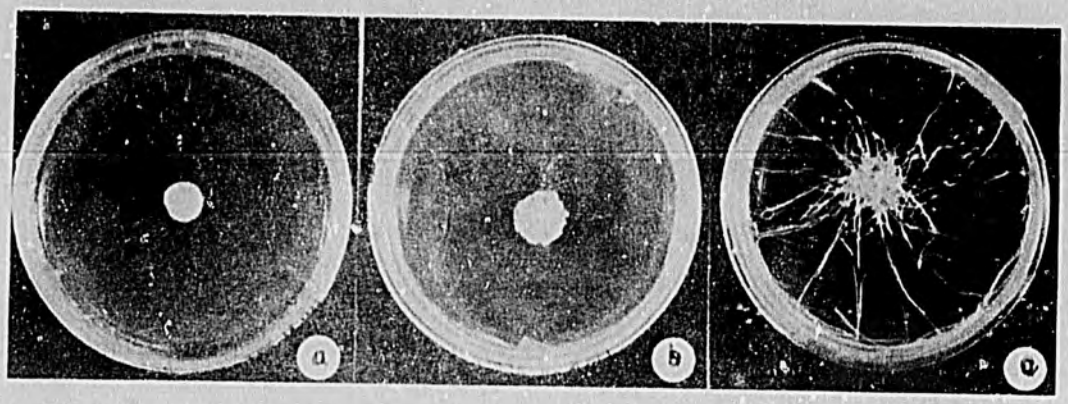

Figure 1 Tumour Formation on Potato Disc

(a) control (b) infected with A. tumifaciens

(c) with A. rhizogenes

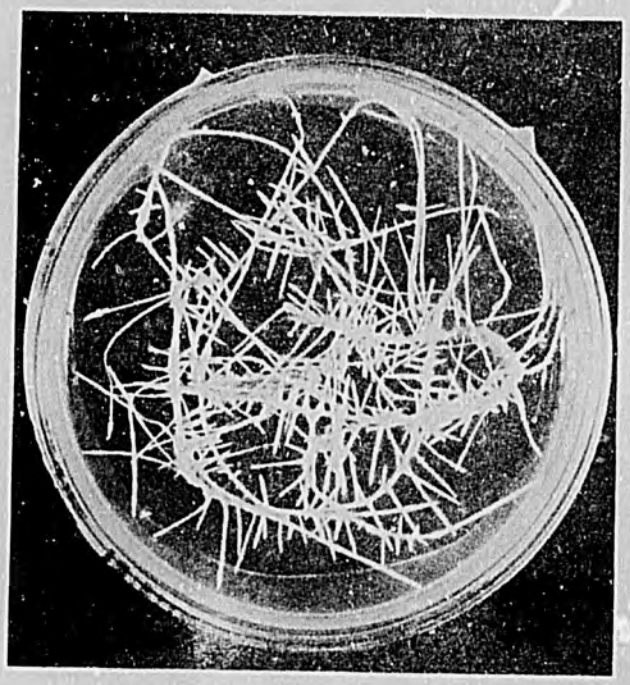

Figure 2 Bacteria-free Transplant of Tumour Root of Peanut Seedling

Figure 3 The Root- VA Mycorrhizal Fungus System of Potato and Glomus fasciculatus Showing Vesicle Formation (arrows).

FY 1985

ANTIGENIC DIVERSITY OF *Plasmodium vivax* (5.232)

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ABSTRACT

Monoclonal antibodies were raised against the blood stages of the malaria parasite, *Plasmodium vivax*. Based on IFA patterns, these monoclonal antibodies (MAB) could be classified into 5 groups. Group I MAB gave bright, generalized staining of all blood stages. Group II MAB reacted strongly with merozoites or their organelles. Group III MAB reacted intensely around the perimeter of individual merozoites with the intraschizont merozoites taking the form of a cluster of grapes. Group V MAB reacted strongly with the surface of trophozoites and schizonts. Group VII MAB reacted irregularly with parasites and internal components. By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with ^{125}I-antimouse immunoglobulin, these monoclonal antibodies to *P. vivax* reacted with proteins of several molecular weights comprising Mr. > 200,000 (PV > 200), 135,000 (PV 135), 115,000 (PV115), 100,000 (PV100), 95,000 (PV95), 85,000 (PV85), 39,000 (PV39), 36,000 (PV36), and 30,000 (PV30). The IFA patterns and the target antigens related to these monoclonal antibodies are now being investigated for further study of the antigenic diversity of *P. vivax*.

INTRODUCTION

Malaria remains prevalent throughout tropical and sub-tropical areas and almost one-third of the world's population is exposed to the risk of infection. The number of estimated malaria cases is 150 millions per year. The mortality and morbidity directly attributable to malaria constitute major constraints on economic progress in the tropical and subtropical areas of the world (1). Failure of global eradiacation is attributable primarily to the practical difficulties of implementing an appropriate programme in countries that have a relative lack of public health facilities. These practical problems have been compounded by the spread of insecticide resistance in mosquitoes, by alterations in vector behavior and especially by the widespread appearance of chloroquine resistant strains of malaria parasites (2).

Another factor which could contribute to the maintenance of malaria transmission in the endemic areas is the carrier. Such carriers can be found in both *P. falciparum* and *P. vivax* infection. *Vivax* primary attacks, if untreated, may last for three weeks to months or longer. As the attack wanes, paroxysms may become less severe and are irregular in periodicity. In perhaps half of all cases, relapses occur following a period of weeks, months or even years without symptoms causing a latent

infection and persisting low grade parasitaemia (3). This asymptomatic state may have following negative effects including maintenance of transmission in the endemic area, and development of recrudescence with the patients becomes debilitated, perhaps from intercurrent infection or immunosuppression. Besides, if at any time blood from such a patient is used for a transfusion, the recipient will unfortunately experience a typical attack of quartan malaria (3).

At present, little is known about the nature of the relapse. One theory is that malaria parasites occur as two populations, one developing in the liver to produce merozoites which invade the blood after the normal prepatent period of 7-8 days, and the remaining in the parenchymal cells for periods in a form of arrested development, the so called "hypnozoites", which ultimately complete their development and cause relapes (4). It is not clear whether these two forms are antigenically different, and if this is the case, it should be possible to distinguish between these strains by typing with a panel of MABs the strains causing initial parasitaemia and the strains causing recrudescense. The objectives of this study are: first, to produce and characterize these monoclonal antibodies with respect to the size of the antigenic molecules to which they react; secondly, to determine whether there is antigenic diversity among different strains of P. vivax using an indirect fluorescent antibody test and a panel of monoclonal antibodies and to investigate whether distribution of different antigenic types is related to geographical locations and thirdly, to demonstrate whether the strains recrudescent after anti-malarial drug therapy are the same or different from those causing initial clinical symptoms.

MATERIALS AND METHODS

Preparation of P. vivax Antigens

Enrichment of P. vivax infected RBCs was achieved by using the SEC-G-25-Percoll method described recently (5). Blood was collected in heparinized tubes (15 IU heparin/ml) from 86 vivax malaria patients (PV 001-PV086) admitted to the Hospital for Tropical Diseases during January 1985 and February 1987. The specimens were either processed immediately or stored overnight at 4°C.

A one ml aliquot of heparinized whole blood was passed through a column containing an equal volume of sulfoethyl cellulose and Sephadex G-25 previously equilibrated with phosphate buffer pH 7.5. The column was then washed with approximately 40 ml of the buffer until the column was free from RBCs. The eluate was centrifuged. the supernate removed and the cell sediment restored to 50% hematocrit. The cell suspensions from several tubes were pooled and two ml each was layered on top of discontinuous gradients comprising successive layers of two ml each of 75%, 60%, 50% and 40% of Percoll in the same buffer. After centrifugation at 2,600 rpm for 10 min at 20°C, two and sometime three bands were obtained. The first and second bands were located in the layers of 50% and 60% Percoll and the third in the interface of 60% and 75% Percoll. The cells in the first and second bands were pooled and called "upper band",

whereas the third band was called "lower band". Smears were made from the blood prior to passage through the column and after Percoll gradient centrifugation, stained with Giemsa and the results examined by light microscopy. Cells in the upper and lower bands consisted of both trophozoites and ring stages with more mature trophozoites and a relatively higher percentage of trophozoites in the upper band.

Production of MAB:

Three-eight week old BALB/c mice were immunized at different time periods each with five to six intraperitoneal injections of washed $5x10^7$ - $1x10^8$ parasites consisting mainly of rings and trophozoites of P. vivax emulsified in an equal volume of Freund's complete adjuvant initially and an incomplete adjuvant subsequently at an interval of 1-2 weeks. At a time when high anti-blood-stage antibodies were detectable in their sera, the mice were immunized intravenously for one week with $1x10^8$ washed parasites in physiological saline. Spleen cells taken from the mice three days after the last immunizing dose were fused with Sp2/0 myeloma cells (6) according to the techniques of Galfre and Milstein (7) with minor modifications. MABs produced were screened by the indirect fluorescent antibody test.

Indirect Fluorescent Antibody Test (IFAT)

Blood smears were made from washed blood from patients with vivax malaria in both ring and trophozoites stages. The IFAT assay was carried out using acetone-fixed blood stages of appropriate isolates as antigens (8). After 2 hours of reactions with the MABs, the washed slides were stained with fluorescein isothiocyanate-conjugate goat anti-mouse Ig (GAM) for one hour and the results were examined.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot analysis

Electrophoresis was carried out in a vertical slab gel apparatus (BioRad, U.S.A.) using the Laemmli and Favre method (9). After electrophoresis, the gels were cut and used for protein staining or electroblotted onto a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, California, U.S.A.) according to the Towbin et al. method (10). After blotting, the unchanged sites on the membrane were blocked by soaking the strips in phosphate buffered saline containing 3% gelatin and treated with 2 ml MAB at 26°C for 2 hours. The strips were then washed 5 times with PBS containing 0.05% Tween 20 and then treated with ^{125}I labeled anti-mouse immunoglobulin (Amersham, England) at 26°C for 30 minutes. Nitrocellulose strips were washed as above, dried and autoradiographed on Kodak XOOmat RP films (Eastman Kodak Co., N.Y., U.S.A.) with a Kodak X-Omatic Casette at -70°C.

RESULTS AND DISCUSSION

Based on their immunofluorescent reactivities demonstrated with P. falciparum, the anti-P. vivax MABs were categorized in groups I, II, III, V and newly designated group VII. Group I MABs produced uniformly bright

generalized staining of all blood stages. Group II MABs reacted strongly to merozoites within schizonts as well as organelles. Group III MABs reacted to the surface membranes of merozoites with the appearance of a cluster of grapes. Group V MABs reacted strongly to the surface of schizonts and trophozoites. Group VII MABs reacted irregularly to parasites and internal components. The number of monoclones and their IFA patterns is given in Table 1.

Table 1 Monoclones and Their IFAT Staining Patterns

Group	Staining patterns	Number of monoclones
I	Bright generalized staining of all blood stages	67
II	Strong reactions to merozoites as well as organelles	21
III	Strong reactions to the surface membranes of merozoites with the appearance of a cluster of grapes	5
V	Strong reactions to the surface of schizonts and trophozoites	16
VII	Strong irregular staining of internal components of trophozoites	46

Some of these MABs with the same pattern of fluorescence have been further characterized by Western blot analysis (Table 2).

Table 2 Western Blot Analysis of the Molecules of *P. vivax* Antigens Reacting with Some Anti-*P. vivax* Monoclonal Antibodies

Molecules of *P. vivax* antigen in kilodalton to which monoclonal antibodies react	Number of monoclonal antibodies
PV30 K	4
PV100 K	2
PV > 200 K	3
PV135 K	3
PV115 K, PV95 K, PV85 K, PV39 K, PV36 K	14

CONCLUSION

This project has been successful in the producting 155 hybrids against P. vivax. These hybrids recognized at least 10 antigenic molecules. Further characterization of these MABs is in progress. We hope that some of these MABs recognized different epitopes of P. vivax antigens. A panel of these MABs would help in the demonstration of diversity of this plasmodial species.

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FY 1985

"DEVELOPMENT OF IMMUNODIAGNOSIS AND IMPROVEMENT OF IMMUNOTHERAPY OF SNAKE VENOM POISONING" (5.221)

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BACKGROUND

Snake venom poisoning remains an important health problem in Thailand and many other developing countries. Although specific horse antivenins against major poisonous snakes are available in most of these countries, a significant fraction of snake bite victims die and many who survive suffer some degree of permanent injury.

In the management of snake venom poisoning, at least two important problems are encountered and need to be overcome. First, the identification of the snake species is extremely important but difficult in the absence of the culprit snake. This is so because many snakes of the same family produce chemically and pharmacologically similar toxins leading to similar clinical signs and symptoms[1]. Immunochemically, however, these toxins are distinct and the specific antivenin must be used for neutralization. Polyvalent horse antivenin has not been produced as yet in Thailand and the currently used monovalent antivenins show insignificant cross-neutralization[2]. Thus, species diagnosis of the culprit snake is of utmost importance to the physician in charge of snake venom poisoned victims.

The second major problem involves the quality of the horse antivenin used in the treatment of snake bite victims. The antivenins available until recently have been crude horse "hyperimmune" sera. These sera are of low neutralizing potency and thus a large volume (30-600 ml) of these sera must be administered in an average poisoning case[3]. It is not surprising that a high incidence (30-50 %) of hypersensitivity reactions has been observed in patients receiving the horse sera[4,5].

This project involves several research activities which, directly or indirectly, will help solve or alleviate the problems mentioned above.

A. Purification and Characterization of Elapid Postsynaptic Neurotoxins

Elapid postsynaptic neurotoxins are highly potent toxins of snakes in the Elapidae family; they bind specifically and strongly with acetylcholine receptors at the neuromuscular junction[6]. This binding leads to inhibition of neuromuscular transmission and muscular paralysis and death by respiratory failure. These toxins are therefore the most important components in these venoms and thus, are the targets in many aspects of this research. The pure toxins are needed for several studies

e.g., ELISA of anti-toxin antibody assays, specific chemical modifications to increase their immunogenicity, studies on the mechanism of toxin neutralization using monoclonal antibodies etc.

For these studies, the principle postsynaptic neurotoxins of N. siamensis, B. fasciatus and O. hannah have been purified. In the case of O. hannah, two toxins have been purified and are currently being characterized with respect to their amino acid compositions and their amino and carboxyl terminal residues. A previous attempt[7] of such characterization has been reported but the results appear equivocal. Monoclonal antibodies against the single major neurotoxin of N. siamensis have been produced and we are beginning to produce similar reagents against the other elapid neurotoxins. These will greatly assist in the characterization and in neutralization studies.

B. Immunochemical Studies of the Venoms of Six Poisonous Snakes of Thailand

By means of SDS-polyacrylamide gel electrophoresis and immunoblotting, the venoms of the six poisonous snakes of Thailand have been characterized in terms of the molecular sizes, abundancies, immunogenicity and immunological cross reactivities of the protein constituents in each venom. The data obtained not only provide useful information on the venom components of the Thai snakes but also give direct evidence accounting for some puzzling previous observations. For example, the frequently observed difficulty in producing high potency antivenins against the elapids can now be rationalized in terms of the extremely low immunogenicity of the elapid postsynaptic neurotoxins. The data also demonstrated a lack of immunochemical cross-reaction of rabbit heterologous antisera towards elapid postsynaptic neurotoxins despite considerable cross reactions of others in consequential venom proteins, which has been inferred based upon clinically observed absences of cross neutralization of horse antivenins against closely related snake species.

The identification of venom proteins which cross-react with heterologous antisera will be useful in the development of an immunodiagnostic test for snake venoms. These cross-reacting venom proteins represent the greatest source of ambiguity in any immunological species diagnosis. Thus, should such complications arise in the development of immunodiagnostic tests, attempts will be made to specifically remove these cross reacting-proteins, enabling a monospecific antisera which do not cross react to be prepared

C. Development of Immunodiagnostic Tests for Snake Venoms

Various immunodiagnostic tests for snake venoms have previously been developed[8]. Most of these tests are ELISA-based which is useful but not without problems[9]. It is anticipated, however, that for a test to be useful in developing countries it must incorporate other properties in addition to the required speed, sensitivity and specificity. For example, it must be simple and require no special equipment or expertise; it must be cost-effective relative to the local per capita income; it should be stable in the climate, and under storage and transportation

conditions which may be far from optimal. For these reasons the immunodiagnostic tests being investigated under this project are based on passive hemagglutination (PHA) and reverse latex agglutination (RLA).

Using the Protein A purified rabbit polyclonal antibody, a PHA test and a RLA test for the six snake venoms have been developed. The sensitivity of the PHA test is about 0.4-8 μg venom per test while that of the RLA test is about 100 times more sensitive. This sensitivity should be adequate since considerably higher concentrations of venom have been found in patients. Cross reaction was observed only in 500-1000 fold higher concentrations of the heterologous venoms. Between the PHA and RLA tests, the sensitized latex particle showed additional advantage over the sensitized SRBC in that they are stable in cycles of freezing, thawing and lyophilyzation. The anticipated problem of nonspecific interference resulting from heterophile antibodies particularly prevalent in sera of rural people has largely been solved. The total test time of the RLA test is 10 minutes.

The RLA test developed will be field tested using serum or wound aspirates of victims of snake bites and the accuracy of the test determined.

D. Development of an *In Vitro* Assay of Antivenom Potency and the Improvement of the Quality of Antivenins

As briefly mentioned above, low potency antivenin in the form of crude horse serum is currently being used in Thailand for the treatment of snake venom poisoning. Some attempts have been made by the Government Pharmaceutical Organization of Thailand to produce pepsin-digested, ammonium sulfate precipitated "refined" globulin[10] with approximately 50% recovery of antibody activity. Because of the severe shortage of the crude horse antiserum, the overall benefit of this refinement becomes questionable. Thus methods should be found for the preparation of more highly potent antivenin by using better immunogens, better adjuvants and/or immunization schedules. In addition, optimization of the pepsin digestion and fractionation of the resulting $F(ab)'_2$ should be made so that the purity and recovery of the antibodies could be the highest possible. Experiments leading to such improvements become almost impossible without a simple, accurate, rapid and economical assay for the potency of the resulting antivenin. The currently used *in vivo* neutralization assay using mice is extremely laborious and expensive and limits the number of conditions that can be investigated. An *in vitro* assay of antivenom potency based on ELISA is therefore under study in our laboratory.

For the use of cobra crude venom, partially purified neurotoxin or purified postsynaptic neurotoxin, an ELISA for the quantitation of horse antibody against the snake venom/toxin has been set up. A dozen batches of horse antivenin against cobra venom are being assayed using both the ELISA described above and the *in vivo* neutralization assay to estimate the correlation of these two assays. The results obtained so far are quite promising. Thus, this ELISA may be a useful replacement for the *in vivo* neutralization assay.

Anticipating the validity of the ELISA for the quantitation of horse antitoxin antibody, various immunogenic forms of the cobra post-synaptic neurotoxin are being prepared. These immunogens will be tried in groups of rats using different adjuvants and immunization schedules. The best 2-3 immunogens will be used for further study in larger animals. In addition, procedures for the fractionation of horse antibody before and after pepsin digestion are being explored. Although high purity and recovery of the antibody are desirable, only procedures that are economical e.g., reusable, and adaptable to large scale(more than 50 litres of serum) are being explored.

ACKNOWLEDGEMENT

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FY 1985

"HYBRIDIZATION OF HALOTOLERANT YEAST FOR ALCOHOL FERMENTATION" (5.238)

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ABSTRACT

A total of 413 strains of halotolerant yeasts were isolated from 285 samples of various products. And 261 strains were chosen from 690 strains in stock culture. All halotolerant strains obtained were screened for potent strains to be used for hybridization with high ethanol producing strains of Saccharomyces cerevisiae at two levels of sodium chloride, 5 and 17 %. Strain 251, S013, S037, S065 and S090 were selected at 5 % NaCl. And at 17 % NaCl, strain 356, 370, 126 and 130 were selected. From nine strains of high ethanol producing S. cerevisiae, strain M30, AM1 and Sc90 were selected since they produced high ethanol at 40°C.

Successful protoplast fusion was obtained from the crosses of strain 251 and AM12, S037 and AM12, S090 and AM12 and S037 and Sc90. A total of 15 hybrid fusants were isolated from selective regeneration media. Ethanol production by these fusants in a salted molasses medium at 40°C is being investigated.

INTRODUCTION

From our own investigation and personal communication with local alcohol distilleries in northeastern and southern areas of Thailand where fresh water is scarce and brackish water is used in the mashing process, fermentation proceeds slowly and efficiency is reduced by about one-half. This is due to many complications, one of which is intolerance of the yeast strain employed to the high salt concentration of the fermentation mash. To solve this problem, the high ethanol-producing yeast strains employed in the mashing process must be modified to have halotoleration. Protoplast fusion is a novel means of hybridizing yeast that are either impaired in mating and spore formation or lacking a sexual phase altogether(5). Industrial strains of yeast normally fall into this categories. Although the concepts and techniques are well known, the hybridization of yeasts for the two distinct characteristics of halotolerance and high ethanol production has not yet been reported. Therefore, this work aims to construct a new hybrid from two different foundation strains, namely halotolerant and industrial ethanol producing strains through protoplast fusion techniques.

MATERIALS AND METHODS

Isolation and Collection of Halotolerant Yeasts

Halotolerant yeasts were isolated by enrichment techniques using salted molasses media (molasses of 10 or 5 % sucrose-equivalalent sugar concentration, 5 or 17 % sodium chloride corresponding to sugar concentration and 0.05 % ammonium sulfate) at 37 and 40°C. Various yeast strains in stock culture were collected and selected for the halotolerant strains based on the ability of growth in molasses medium containing 5 % NaCl, at 40°C.

Screening of Potent Halotolerant Yeast

Screening was carried out at 5 and 17 % NaCl and at 40°C. At 5 % NaCl, the composition of medium used was molasses of 20 % sucrose-equivalent sugar concentration, 5 % NaCl and 0.05 % ammonium sulfate. At 17 % NaCl, medium consisted of 5 % sucrose equivalent sugar concentration, 17 % NaCl and 0.05 % ammonium sulfate was used.

For the standing tube culture, the incubation period was 48 or 96 h. In the standing and shaking flask cultivation, 200 ml of medium in 500 Erlenmeyer flask were used. The incubation of the shaking flask cultivation was performed on rotary shakers at 70-80 rpm. Cultivation in fermenters was carried out in 5-liters fermenters using 3 liters working volume. Initial pH at 4.5 and 200 rpm agitation were used.

Collection and Screening of High Ethanol Producing Strains of *S. cerevisiae*

High ethanol producing strains being used in this work are shown in Table 1.

For primary screening, ethanol production in molasses media (molasses of 20 % sucrose equivalent sugar concentration and 0.05 % ammonium sulfate) was carried out using slow shaking flask cultivation, at 35°C. For final screening, ethanol production was carried out in fermenter at 40°C using the same media.

Isolation of Auxotrophic Mutant

Cells were mutagenized by treatment with ethyl methane sulfonate (EMS) following the Fink method (2) with some modifications. The treatment of spores by EMS was done following the Oshima and Takano method (3) with some modifications.

Isolation of Respiratory Deficient Mutant

Cells were treated with ethidium bromide following the method described by Seki et al (4).

Protoplast Fusion

Protoplast were prepared using the enzyme zymolyase (5,000 or 20,000) following the Arima and Takano method (1). Protoplast fusion was performed in polyethylene glycol (PEG) and calcium ion by the method described by Seki et al (4).

Screening of Hybrid Fusant

Screening for high ethanol producing fusants was carried out using salted molasses medium at 40°C.

Analysis

Ethanol concentration was determined by Gas-liquid chromatography (Shimadzu GC-9A) using propanol as an internal standard. Cell concentration was measured as optical density of cell suspension at 660 nm after washing with 0.1 N HCl by Spectrophotometer (Shimadzu UV-240). Sugar concentration was determined by phenol sulfuric acid method and high performance liquid chromatograph (HPLC).

RESULTS AND DISCUSSION

Isolation and Collection of Halotolerant Yeasts

A total of 413 strains of halotolerant yeasts were isolated from 285 samples of various products. From 413 strains, 302 strains were isolated using molasses media containing 5 % NaCl at 37°C and 60 strains were obtained at 40°C. Another 43 strains were isolated using molasses media containing 17 % NaCl at 37°C and 9 strains at 40°C.

Six hundred and ninety yeast strains collected from stock cultures were tested for the ability to grow in molasses media containing 5 % NaCl at 40°C. The results showed that only 261 strains could grow in salted molasses media and be considered as halotolerant yeast strains.

Screening for Potent Halotolerant Yeasts To Be Used as Parental Strains

A total of 674 strains of halotolerant yeast obtained by isolation and collection were screened at two levels of sodium chloride, 5 and 17 %. At each sodium chloride concentration, the screening was divided into 3 steps.

At the 5 % NaCl level, good ethanol producing strains were expected to be obtained. Standing tube cultivation was used for primary screening. In order to obtain the strain which produced ethanol at the highest rates 48 hours of incubation was chosen for the tests. Fifty-two strains which produced ethanol more than 2.5 % by weight and did not show pellicle forming characteristic were selected. These 52 strains were further screened by standing flask cultivation at 40°C. Seven strains, i.e. 016, 251, S019, S037, S013, S065 and S090, which produced ethanol 2.0-3.4 % by weight after 24-48 hours were selected for final screening.

The final screening was carried out in the fermenter and the result of ethanol production by the selected strains is shown in Fig.1. The results revealed that strains, S013, S037 and 251 produced almost the same amount of maximum ethanol concentration but at different incubation periods. Strain S0131 produced 3.1 % at 60 hours while strain S037 and 251 produced 3.1 % at 52 hours 3.0 % at 72 hours, repectively. Strain S065, S090, S019 and 016 produced ethanol 2.9 % at 67 hours, 2.7 % at 72 hours., 2.2 % at 72 hours. and 1.4 % at 60 hours., respectively. From this result, the strains S013,S037, 251, S065 and S090 were selected to be used as the halotolerant parents.

To obtain halotolerant yeast which could grow at high concentrations of sodium chloride, all isolated and selected halotolerant strains were tested for their ability to grow in molasses media containing 17 % NaCl at 40°C. Primary screening was performed using standing tube cultivation. Incubation was prolonged to 96 hours since cultivation conditions were stressed due to high salt concentration and high temperature. Fifty-one strains whose growth gave cell concentration which were determined as optical density rates at 660 nm after 96 hrs were selected. In secondary screening shaking flask was used for cultivation and incubation time was reduced to 72 hours in order to obtain fast growing halotolerant strains. In this step, 11 strains whose optical densities were increased to 3.03-6.22 after 72 hours were selected. They were strain 126, 062, 092, 356, 394, 366, 090, 246, 130, 229 and 370. And because strain 346 could produce 1.72 % ethanol by weight, it was also selected for final screening. The time courses of growth and ethanol production by these selected strains were investigated in molasses media containing 17 % NaCl by shaking flask cultivation, at 40°C. The results of growth by these 12 strains are shown in Fig.2a. The results demonstrated that, strain 370 gave the highest cell concentration. Observed OD at 660nm was 4.8 after 72 hours. Optical density of cells obtained by strain 356 and 126 were 4.61 and 3.54 respectively. The other 9 strains did not show much difference in optical density. Based on only one aspect of cell growth, the selection of strains was difficult. Therefore, the amounts of ethanol produced by these 12 strains were compared. We found that only 4 strains produced ethanol in amounts more than 1 % by weight. Those were strain 356, 370, 126 and 130 which produced 1.6, 1.6, 1.5 and 1.4 % ethanol by weight, respectively (Fig.2b). These 4 strains were selected for hybridization with high ethanol producing strains.

Screening of High Ethanol Producing Strains of *S. cerevisiae* To be Used as Another Parental Strain

To obtain high ethanol-producing strains for hybridization with halotolerant strains, ethanol production by nine collected strains was investigated. The results indicated that *S. cerevisiae* Sc90 which is currently used in most factories produced the highest ethanol concentration, 8.8 % by weight after 48 h. Strain B120, AM12, M30, 281 and 191 produced 8.6, 8.3, 8.2, 7.7 and 7.7 % ethanol by weight, respectively. The other strains N1, TJ3 and TJ1 produced 7.2, 6.7 and 4.2 % ethanols by weight, respectively. Considering on cell concentrations, most strains produced maximum cell concentration at 24h. Corresponding to the results of the ethanol production tests, six strains which produced more than

7.5 % ethanol by weight were selected for final screening in the fermenter, at 40°C. The results of ethanol production by 6 selected strains are shown in Fig.3. The maximum amount of 7.5% ethanol by weight was produced by S. cerevisiae M30, while AM12 and Sc90 produced 7.3 and 5.9 % respectively. Strains 191, B120 and 281 could not grow and produce ethanol under such conditions. Therefore, S. cerevisiae M30, AM12 and Sc90 were selected to be used as the high ethanol producing parents.

Protoplast Fusion

Each of selected halotolerant strains and selected high ethanol producing strains of S. cerevisiae was hybridized using protoplast fusion technique.

To facilitate the selection of fusants, auxotrophic mutant was used as one parental strain and a respiratory deficient mutant was used as another parental strain.

Auxotrophic mutants of selected halotolerant strains were isolated by EMS. We found that only the strains screened at 5 % NaCl gave auxotrophic mutants after treatment with EMS. Therefore the strains selected at 17 % NaCl were mutagenized by NTG.

Respiratory deficient mutant of high ethanol producing strains, M30, AM12 and Sc90 were successfully isolated.

Protoplast fusion techniques were successful in the crosses of strain 251 and AM12, S037 and AM12, S090 and AM12 and S037 and Sc90. From the cross of strain 251 and AM12 four fusants were selected in minimal media containing glycerol as the sole source of carbon. From the crosses of S037 and AM12, S090 and AM12 and S037 and Sc90, three, five and three fusants were selected, respectively.

The fusants obtained are being screened for high ethanol production in salted molasses media at 40°C.

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Table 1 High ethanol producing strains of *Saccharomyces cerevisiae* being used in this work.

Strain	Background
S. cerevisiae Sc90	Ayudthaya Alcohol Factory, Thailand, stock culture of KU.
S. cerevisiae M30	Surat Thani Alcohol Factory, Thailand, stock culture of KU.
S. cerevisiae 191	Isolated from mold bran of vinegar factory in China, stock culture of KU.
S. cerevisiae 281	Uniditified source, stock culture of Institute of Food Research and Product Development, KU.
S. cerevisiae TJ1	Isolated from molasses, stock culture of KU.
S. cerevisiae TJ3	Isolated from molasses mash, stock culture of KU.
S. cerevisiae N1	Isolated from mash of Awamori brewing (Okinawa spirit),stock culture of Department of Fermentation Technology, Osaka University, Japan.(4)
S. cerevisiae AM12	A hybrid obtained by protoplast fusion between TJ1 and N1.(4)
S. cerevisiae B120	Unidentified source, stock culture of KU.

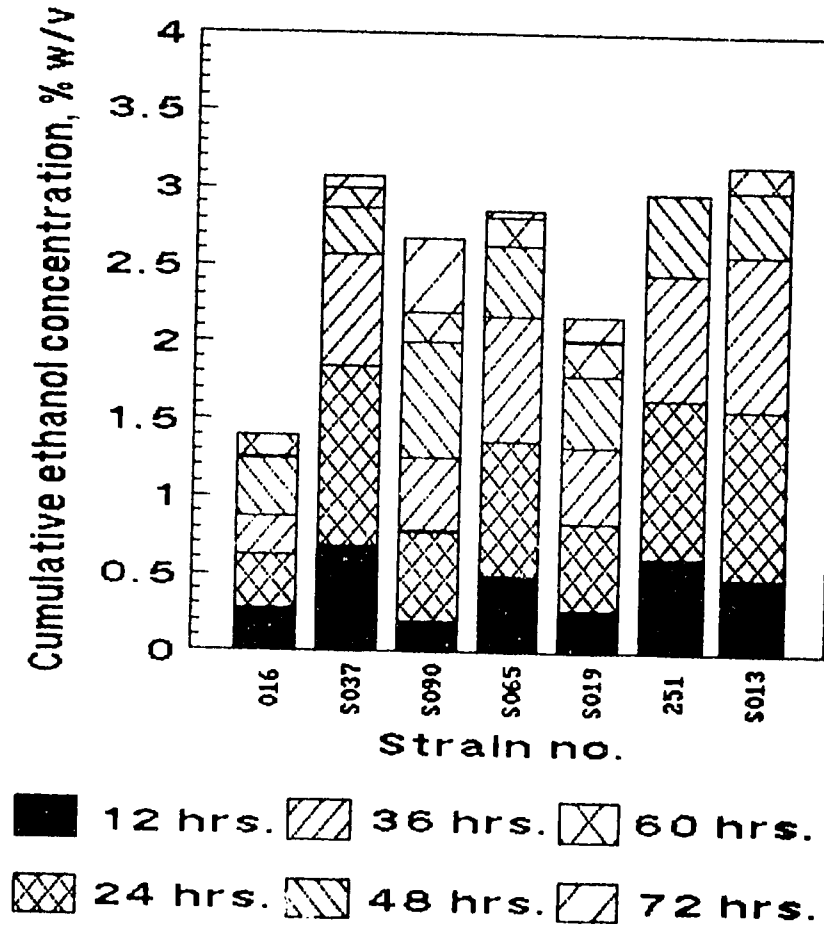

Figure 1 Ethanol Production by Selected Halotolerant Strains in Molasses Medium Containing 5% NaCl at 40°C.

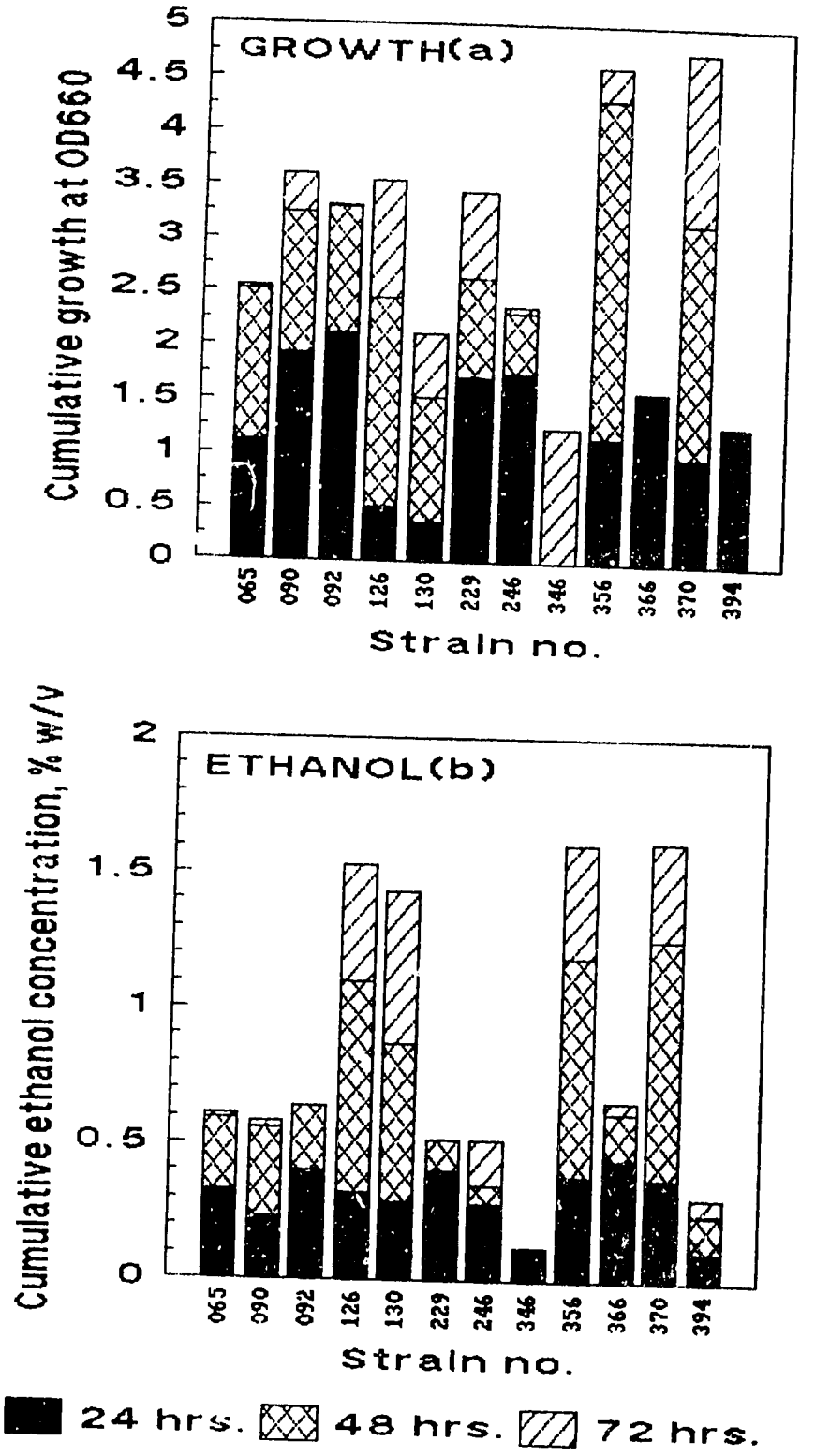

Figure 2 Growth (a) and Ethanol Production (b) by Selected Haloto-Strains in Molasses Medium Containing 17% NaCl at 40°C.

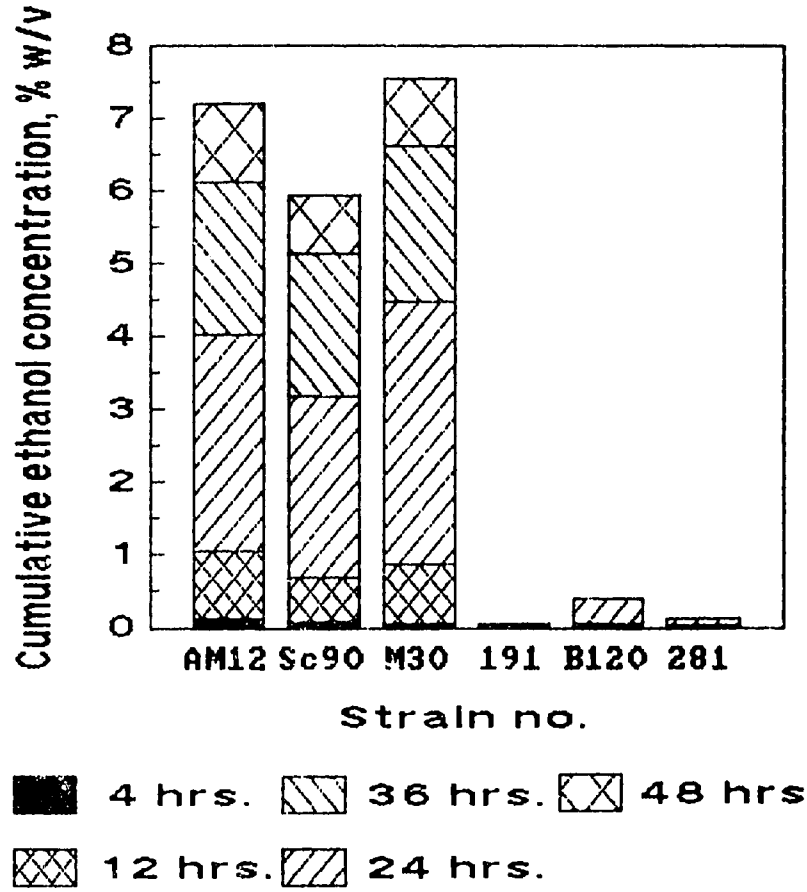

Figure 3 Ethanol Production by Selected High Ethanol Producing Strains in Molasses Medium at 40°C.

FY 1986

"IMMUNOPATHOGENESIS OF DENGUE HEMORRHAGIC FEVER/DENGUE SHOCK SYNDROME" (6.391)

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ABSTRACT

1. Kinetic studies of the peripheral blood leukocytes in patients with DHF/DSS revealed that neutrophils and monocytes were predominant in the first few days of the disease. Later on, both total leukocytes and neutrophils as well as monocytes declined to their lowest values 1-2 days before the subsidence of fever. Subsequently, the atypical lymphocytes increased to a peak at the time of shock or defeverescence and then decreased to normal levels 3-4 days afterwards. The eosinophils gradually increased during the convalescent phase of the disease.

2. The complement system which is actively involved in DHG/DSS coincided with the onset of shock and leakage. Measurement of the complement fragments C3a and C5a revealed that C3a markedly increased during the shock phase and rapidly declined to normal levels in the convalescent stage. However, the fact that the level of C5a did not significantly increase suggested that C3a may be the major mediator responsible for the development of shock in DHF/DSS.

3. The circulating immune complexes as detected by C1q binding and conglutinin binding assays could not be detected in large number of patients and, when detected, they were present in rather small amounts.

INTRODUCTION

Despite many active investigations in dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in all aspects of the disease during the past decade, DHF/DSS still remains one of the major health problems not only in Southeast Asia but also in the Pacifics and lately in the Carribean areas. DHF/DSS is a severe febrile disease of children and adolescents characterized by sudden onset of fever, nausea, vomiting, abdominal pain, hepatomegaly, petechial hemorrhage, epistaxis, hematemesis, melena and shock on the fifth to seventh day of illness with significant mortality. The major pathophysiologic hallmarks in DHF/DSS are shock and excessive hemorrhage which are the leading causes of death. Previous studies suggested that although immunological processes are involved in the pathogenesis of this disease, the exact mechanisms are largely unknown. Further investigation is needed to understand the whole immunopathophysiologic process, especially factors leading to the development of shock,

which will lead to better treatment and ultimate prevention of this disease by vaccination. This report describes the results of the first part of the research project during the first year of study which consisted of the study of the kinetics of peripheral blood leukocytes, and the determination of complement activation and immune complexes during the course of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).

MATERIALS AND METHODS

1. Patient Population Studies

Children diagnosed as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) victims who were admitted to the Department of Pediatrics, Siriraj Hospital and Children's Hospital, Bangkok, Thailand were included in this study. Approximately 100 serologic-proven cases of either primary or secondary dengue hemorrhagic fever were studied in detail during the course of illness. The criteria for the clinical diagnosis of DHF/DSS and grading of disease severity were used as described previously.[1]

2. Kinetics of Peripheral Blood Leukocytes in Different Stages of Disease

The peripheral blood leukocytes were obtained every day beginning from the first day of admission to approximately 5-7 days of hospitalization, from 52 confirmed cases of DHF/DSS. The pattern and nature of various kinds of leukocytes were determined sequentially by Wright's stain using light microscopy, with particular attention paid on the accurate enumeration of monocytes, mature lymphocytes and atypical or transformed lymphocytes. The absolute number of these peripheral white blood cells was then calculated from the total white cell count.

3. Complement Assays

Assay for complement components, mainly C_3, C_4, factor B, C_7 and C_9 were performed using radial immunodiffusion techniques with monospecific antisera. Functional assays for the classical and the alternative pathways were determined by methods previously described.[2, 3] The complement activities in vivo and in vitro were evaluated by various assays to detect different degree of complement activities as follows:

a. Assay for the presence of C3a des-arg and C5a des-arg by radioimmunoassay. [4]

b. Detection of C3d by rocket immunodiffusion assay. [5]

c. Detection of the soluble terminal complement complex (SC 5b-9) by a modified ELISA method. [6]

4. Immune Complexes

Assays for the presence of immune complexes in EDTA plasma were employed by 2 different methods:

a. Clq binding test. [7]

b. Solid phase conglutinin binding.[8]

Heat aggregated human IgG was used as the standard control.

RESULT AND DISCUSSION

1. Kinetics of Peripheral Blood Leukocytes in Different Stages of Disease

The studied patients included 52 serologically proven secondary dengue infection cases, aged 9 months to 12 years, in whom 4 were classified by their disease severity as grade 1, 24 patients as grade 2, 20 patients as grade 3, and 4 patients as grade 4. The peripheral blood leukocytes were studied daily in the acute stages and once in the convalescent stages of the disease. The study revealed that the total number of leukocytes was augmented on the first day of disease (approximately 5 days before subsidence of fever) due to the substantial increase in neutrophils (Figure 1). Thereafter the total number of leukocytes suddenly decreased to a nadir on the second day before the subsidence of the fever. It is believed that this reduction could be attributed to both the decrease in production by the bone marrow and the increase in destruction of leukocytes by the reticuloendothelial system. Then the total number of leukocytes gradually rose to its normal value on the first and second days after defeverescence. The subsequent rise of total leukocytes was mainly due to the increasing number of the lymphocytes during the stages of shock or defeverescence. This rapidly increased number of total lymphocytes was mostly caused by the rise of atypical lymphocytes which reached their highest values on the day of shock or subsidence of fever (Figure 2). The number of atypical lymphocytes gradually decreased to normal value 4 days after shock or defeverescence. Evidently, the increase in atypical lymphocytes was due to an immunological response to secondary dengue infection resulting in the increased number of B lymphocytes. The role of cell-mediated immunity, especially T-lymphocyte subpopulations, remains to be studied in the next part of this project. Regarding to the number of monocytes, it was found that the number of monocytes increased in the early days of disease and then declined to a minimum at 3 days before shock or defeverescence (Figure 2). This finding supported the hypothesis that monocytes or macrophage may play central roles in the pathogenesis of this disease by acting as the primary site of dengue virus replication prior to being destroyed by T-lymphocytes or natural killer (NK) cells. [9] Eosinophils and basophil cells were also found in this research to be greater in number in later stages of the disease, which is believed to be a reaction following the inflammations of the acute stage.

2. Complement Activation

It has been suggested that some of the complement breakdown products, especially C3a and C5a, might be potential candidates causing rapid extravasation of plasma, leading to shock. Using the technique described by Chenoweth and Hugli[4], EDTA plasma was collected at different phases of the disease from 50 patients with DHF/DSS seen at the Department of Pediatrics, Siriraj Hospital, and examined for the presences of C3a and C5a. Thirty five and thirteen patients were classified as grade 2 and grade 3 respectively. The remaining two patients were classified as grade 4 and 1. The preliminary results are summarized in Figure 3 and Figure 4. In Fig. 3, C3a was significantly high at the time of subsidence of fever or coincident with the time of shock in grade 3 and 4. The levels returned to normal within 24 to 48 hours. No significant rise of C5a was seen (Fig. 4). Our data demonstrated a brief burst of C3a levels in patients with grade 2, 3 and 4 but did not demonstrate any differences among the groups. The peak of C3a coincided with the peak of disease during the shock phase, indicating that C3a probably participates in the pathogenesis of the DHF/DSS. The fact that C5a was not raised in DHF/DSS did not exclude its participation in the pathogenesis of shock. The C5a was shown to be a very potent spasmogen and a strong chemotactic agent. It also caused prolonged vascular leakage in the presence of polymorphonuclear leukocytes, an action not found in C3a.[10] In theory at least, C5a seems to be a better candidate causing severe vascular leakage in DSS. It is probable that C5a acts locally (possibly in the liver and lung) and is sequestrated at the site of activation. Webster et al have shown that C5a accumulated in the lung when injected intravenously.[11]

3. Immune Complexes

Figure 5 summarizes data of circulating immune complexes (CICO as detected by C1 binding (C1qBA) and conglutinin binding (KBA) assays performed in our laboratory during the 1986 season. Only few DHF/DSS patients showed raised levels of either C1qBA or KBA; and even if the levels were abnormal they were marginally raised above the standard normal controls, i.e. slightly more than 40 ugm of heat aggregated immunoglobulin G per ml of serum. In our hands at least, circulating immune complexes, as defined by the two assays, could not be detected in large number of patients and, when detected, they were present in rather small amounts. When the two assays were used to analyse sera from patients with post streptococcal glomerulonephritis and SLE, the assays could detect large amount of immune complexes. The actual role of immune complexes in DHF/DSS needs to be reinvestigated. Immune complexes might not be major factors in the activation of the complement as has previously been postulated.

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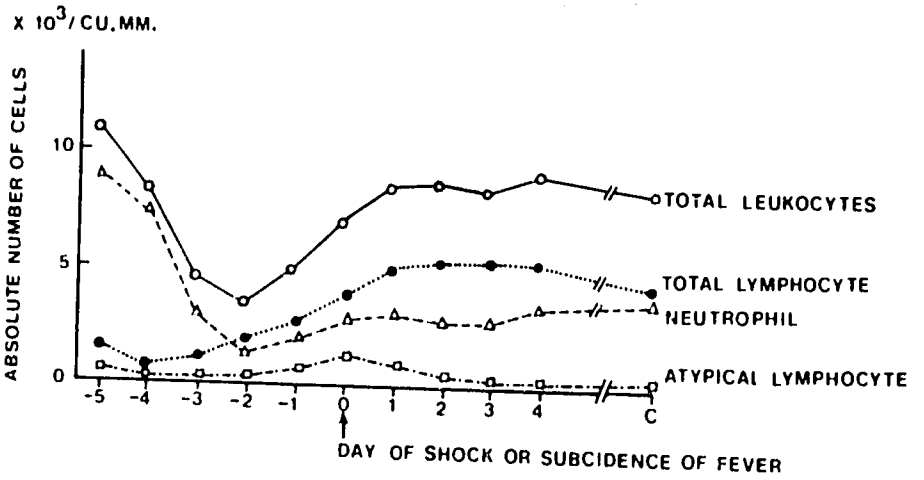

Figure 1 Serial Determination of Periopheral Blood Leukocytes in Different Stages of DHF/DSS

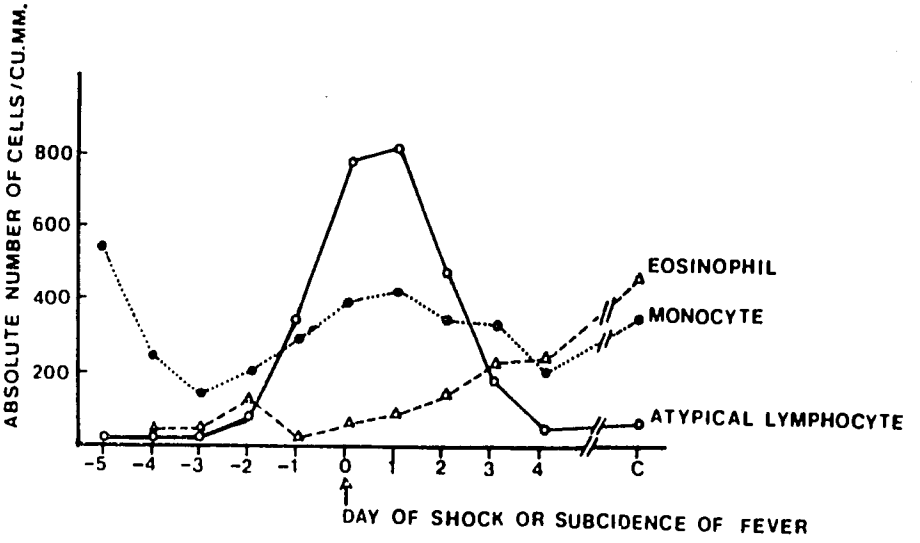

Figure 2 Serial Determination of Atypical Lymphocytes, Monocytes and Eosinophils in Different Stages of DHF/DSS

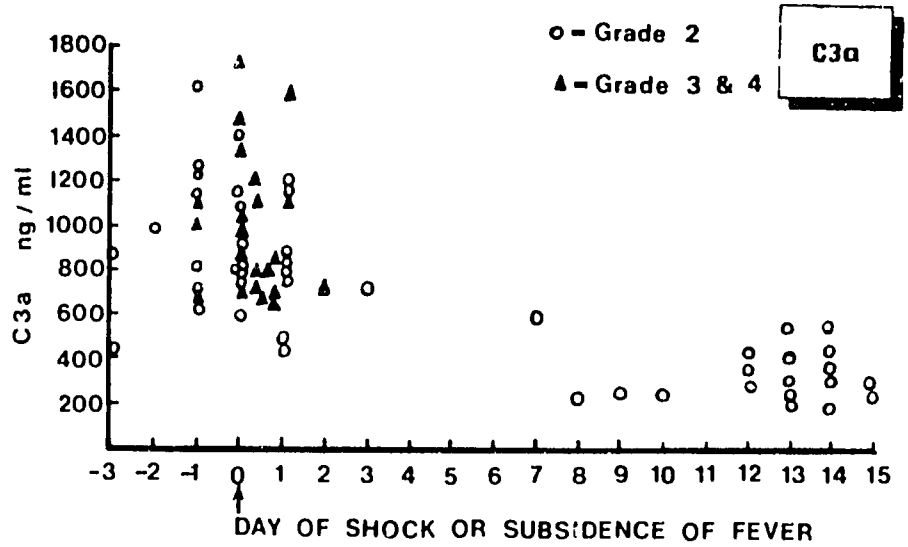

Figure 3 C3a in Plasma of Dengue Patients Taken at Different Periods Relative to the Day of Shock or Subsidence of Fever.

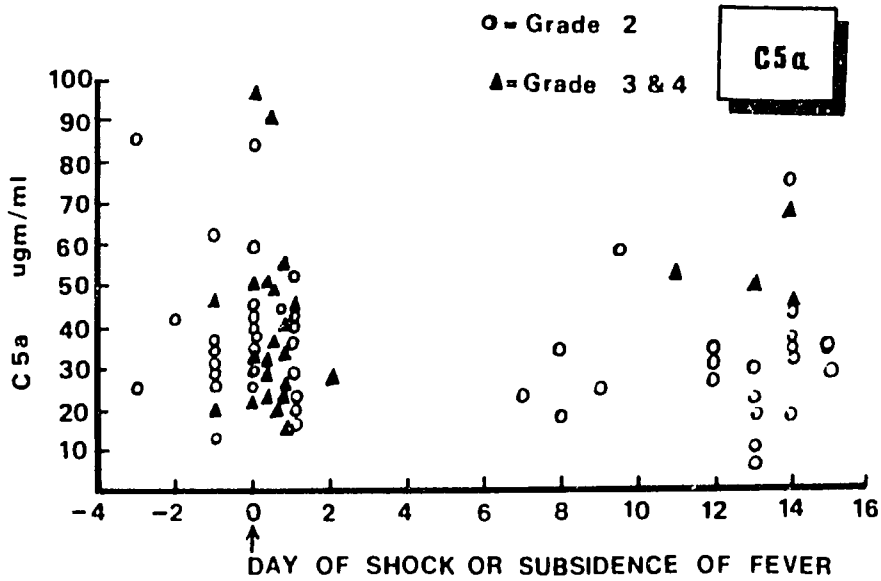

Figure 4 C5a in Plasma of Disease Patients Taken at Different Periods of the Disease.

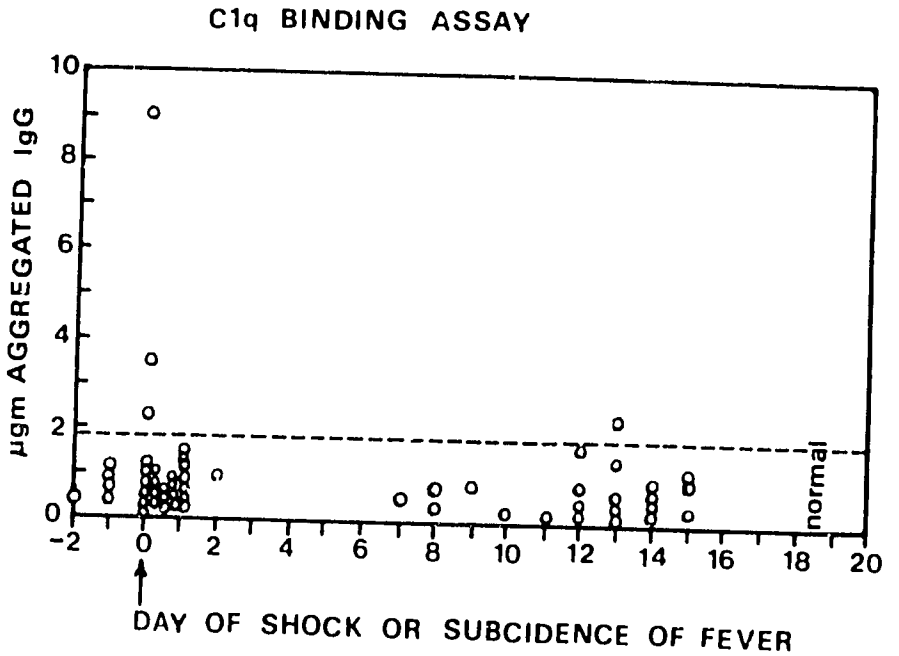

Figure 5A

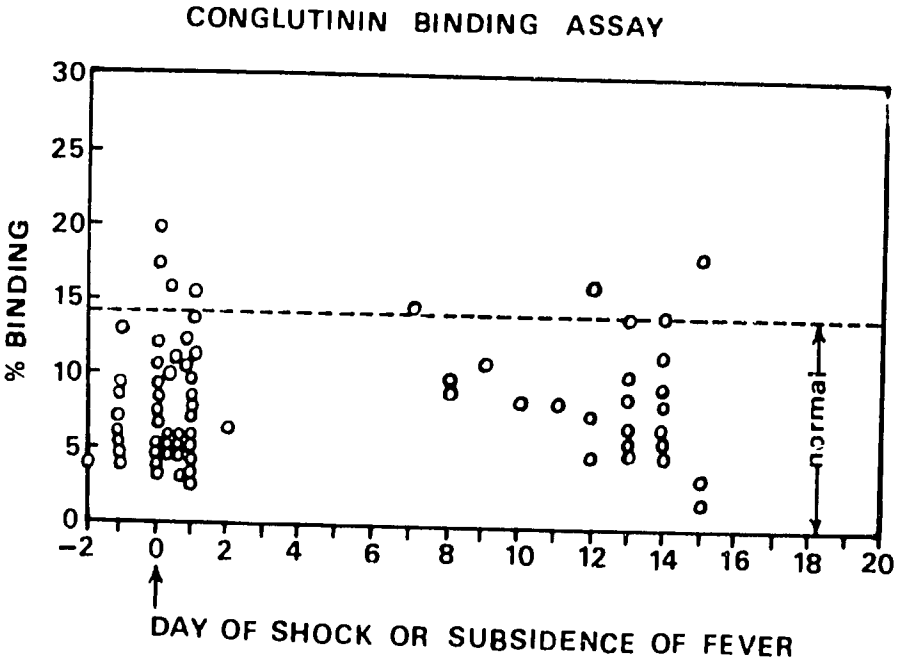

Figure 5B Levels of Immune Complexes as Detected by Two Assays in DHF/DSS at Different Periods Relative to the Day of Shock or Subsidence of Fever (A) Clq Binding Assay (B) Conglutinin Binding Assay.

FY 1986

"COLLECTION AND UTILIZATION OF TAMARIND GERMPLASM" (5.243)

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INTRODUCTION

Tamarind (Tamarindus indica L.) trees are long living and may reach an age of 100 years or more. They are found throughout the tropics either as individually planted trees or, more often, in random stands which developed from discarded seeds. Although India produces over 250,000 tons of tamarind fruit pulp annually, most of their pulp comes from untended trees, with only a few small plantations or orchards. In most countries the fresh fruit pulp is consumed as a delicacy or used as the basis of beverages. The fruit may be eaten fresh or it may be preserved for later consumption.

Tamarind trees grow well on poor, infertile soils, are drought resistant, need little care after planting, and produce dense wood. Tamarind has an excellent potential for use as a plantation crop, not only as a source of fruit pulp, but also as a source of biomass. The fact that tamarind also has a capacity for nitrogen - fixation means that it could be used to improve soil fertility, particularly the fertility of the poor soils which are the kind usually available for growing plantation crops. However, in order to successfully grow tamarind as a plantation crop, a germplasm collection must be established and evaluated. Optimizing the choice of clones for propagation is a necessary first step in profitably utilizing the potential of the species. Through screening and selection of the germplasm collected for biomass production, new fast growing tamarind types which produce large amount of foliage (for human and animal consumption) and fuelwood, will become available. Tamarind has potential use as a source of biomass for many developing countries of the tropics. It is planned to share the germplasm collections with other developing countries, for further testing and utilization, and to share the results of this research by making available seeds and clones required by tamarind growers. Recommendations for efficient plantation practices are being collected and are also available to other growers. Similarly, concurrent research on fast - growing sweet and sour tamarind types which bear early and annually, and yield large amount of fruit and fruit pulp of improved quality has been undertaken. As more results become available, further interest in the cultivation of tamarind as a cash crop is anticipated. Tamarind would be a suitable cash crop for many developing countries. The establishment of demonstration plantations would be an appropriate intermediate step, which should follow the conclusion of this project. The proposed research aims at using tamarind as a multipurpose tree, i.e., stems for use in furniture making, branches for use as fuelwoods, young leaves for human and animal consumption, falling leaves for use as compost, fruit pulp for use as food and beverages and seeds for

use in glue and animal feed products, etc. Through the prospect of such multipurpose potential, growers of tamarind may find a considerable incentive. Thus, this project is expected to have impact on economic and social development of many developing countries, including Thailand.

OBJECTIVE

The objectives of this project are to obtain, establish and evaluate tamarind germplasm distributed in the tropical regions. Pod yield and quality as well as rate of biomass production is to be assessed. The accessions are asexually propagated and further evaluated. Attempt is being made to develop dwarf rootstock through genetic and cultural means. Some nitrogen - fixing species have been identified and will be assessed as possible sources of more productive rootstock. Scope for increasing nitrogen - fixing character of these trees through employment of alternate Rhizobium strains will be investigated.

TECHNICAL WORK PLAN

The objectives of this project will be achieved through the following phases of works :

a. Germplasm Collection
b. Germplasm Testing
c. Rootstock Study
d. Rhizobium Study
e. Demonstration Plot Established and Cultivation Practices Determined

These steps are described next:

Germplasm Collection

1. Procurement of specimens

Tamarind pod collection has been carried out through the provincial agricultural extension officers by means of pod competition and specimen collection.

2. Adoption of selection criteria

2.1 Biomass Production
1) Fast growth rate and number of stomata.
2) Plant growth habits including angle of branching.

2.2 Fruit Production
1) Primary selection : based on RV-formula
(Real value of pulp = Pulp Wt. x %Pulp Wt. / 100)
2) Plant growth habits : based on shape of canopy.
3) Regularity or bearing.
4) Uniformity of fruit ripening.
5) Pod characteristics : based on pod size and pulp yield.

Testing of Germplasm

1. Biomass production

   1.1 The growth of plants in pots
   To determining the dry matter of root, stem, branch and leaf beside these the nutritive values of leaf, both young and aging, is analyzed for protein, fat, carbohydrate, fiber, carotenoid, vitamin A, ascorbic acid, etc.

   1.2 The growth of plants in plots
   The individual accession will be grown in plots at spacing of 2 x 2 meters with 6 replications, each consisting of 4 plants.

2. Fruit production

   2.1 Observation of mother plants
   The aspects will be observed in fruiting behavior, fruit setting characters and uniformity of ripening pods.

   2.2 Field test of selected grafted plants
   The individual accession will be grown in plots at spacing of 6 x 6 meters with 6 replications, each consisting of 4 plants. The observations will be made on the following aspects.

   - growth (eg. girth, length of stem, height, shape and volume of canopy)
   - flowering (eg. earliness)
   - fruit setting (eg. number of fruit set)
   - marketing quality of pod

Rootstock Study

1. The Search for Dwarf Rootstock

The seeds collected from the untended dwarf phenotypic expressions will be sown to determine the number of stomata from the young seedlings. The test will indirectly identify whether the plants are those of dwarf types, expressed as fewer stomata per unit area than the normal one.

2. The Search for Appropriate Rootstock Plants other than Tamarind

An attempt will be made to develop the dwarf rootstocks by using other plant species than tamarind such as Sudan senna (*Cassia acutifolia*), Indian senna (*C. angustifolia*), Manila tamarind (*Pithecellobium dulce*) ete.

3. Chemical Treatments for the Production of Dwarf Rootstock

Dwarf rootstocks may be induced by mean of chemical treatments e.g. SADH, MH, CCC and Paclobutrazol.

4. Use of Nodulated Leguminous Plants for Rootstocks Rootstocks could possibly provide adequate nitrogen fixation by the nodule bacteria, Rain tree (Samanea saman Merr.) and Sesban (Sesbania grandiflora Desv.) may be tried for this purpose.

Rhizobium Studies

The studies of Rhizobium symbiosis phenomena will be investigated by the following steps.

- Rhizobium Isolation
- Isolation of Rhizobium from Tamarind
- Screening for the potent Rhizobium Strain.
- Inoculation of the Experiment Plots with Rhizobium.

Cultivation Practices and Demonstration Plots

Cultural practices will be carried out in order to determine the optimum technology for maximum production.

Highlights of Works

1. A primary selection criterion has been formulated, that is Real value of pulp = Pulp Wt. x % Pulp Wt. / 100

2. A total of 51 accessions of sour tamarind type have been selected from 1,811 specimens throughout the country.

3. The superior accessions of sour tamarind have been found with various characteristics viz. jumbo-size pod type (13-15 ripe pod/kilogram), very high pulp percentage (79.4%), highest Real Value of pulp (RV. = 21.3894), gold brown color of pulp which is very rare and attractive to the consumer, very loose pulp and for sweet tamarind tastiness.

4. Most of accessions are found in the provinces along the Mae Khong River close to the Laos border.

5. Some accessions of sour and sweet tamarind types are established in a demonstration plot at Khon Kaen University Campus.

FY 1986

DEVELOPMENT OF METHODS FOR IMMUNODIAGNOSIS OF LIVER FLUKE INFECTION (6.392) - APPROACHES TO DEVELOP IMMUNODIAGNOSTIC METHODS FOR LIVER FLUKE INFECTION CAUSED BY *Opisthorchis viverrini*

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Liver fluke infection is still an important endemic disease in many parts of the world. among the three important flukes that cause the disease in humans, *Opisthorchis viverrini* (*Ov*) is the fluke found in Thailand and parts of Laos, Cambodia and Vietnam. Opisthorchiasis is among the diseases of greatest public health importance in Thailand, as it has been estimated that at the present time there are at least 7 million Thais infected by this parasite, representing approximately 15% of the total population. Moreover, the prevalence of *Ov* infection among the population in northeastern Thailand has increased from 3.5 million cases in 1965 to 5.4 million cases in 1981[1]. In some villages in the endemic areas, a prevalence of more than 90% has been reported. All age groups including infants are known to be infected[2].

In the past, the liver fluke infections caused by *O. viverrini*, *O. felineus* and *Clonorchis sinensis* have been reported sporadically from non-endemic areas including North America and Western Europe, but at the present time increasing numbers of cases have been reported. One main reason is due to the ever increasing number of immigrants and refugees from less-developed countries within the last few years. Another possibility includes the consumption of fish imported from endemic areas.

Current methods for diagnosis of human liver fluke infection are based on the demonstration of fluke eggs in stools, and on some occasions also in duodenal fluid or bile (for review, see Sirisinha[3]). Although such an examination is reliable and permits the identification of flukes, it is unfortunate that such a technique is useful only when the intensity of infection is high and is reliable only in the hands of experienced laboratory personnel. It is relatively simple to detect cases with heavy infection when several thousand eggs per g of feces are present. However, the diagnosis of cases with light infection is uncertain, can be easily missed and the results may vary from one sample to another. Moreover, a decision is even more difficult to make in individuals having mixed intestinal parasitic infections. The latter situation is not unusual for people residing in the *Ov* endemic areas. On the other hand, when the infection becomes chronic and so severe as to result in biliary obstruction, eggs will not be found in the fecal samples and in order to demonstrate the presence of fluke eggs one has to use bile secretion for such an examination. However, obtaining bile is neither simple nor practical and is not without danger. Therefore, if a more simple and objective diagnostic method is available, the problem would be alleviated. Moreover, when it becomes available, such a method would facilitate epidemiological study as well as the monitoring of the efficacy of individual and mass anthelmintic treatment and reinfection.

The development of sensitive and specific serodiagnostic assays requires a thorough understanding of the host-parasite relationship which, in turn, depends on information regarding the parasite antigens and host immune response to these antigenic stimuli. Therefore, our initial study is focused on characterization of Ov antigens and the immune response in patients with opisthorchiasis and in animals experimentally infected with this parasite. Once sufficient information regarding these matters becomes available, then we will be ready to develop appropriate assays for this important parasitic disease.

Characterization of Ov Antigens

Only limited information is currently available on O. viverrini antigens. It was reported by Janechaiwat and her associates several years ago that sera from patients with opisthorchiasis and hamsters experimentally infected with Ov metacercariae could precipitate at least 5-7 parasite components as analyzed by immunoelectrophoresis[4]. However, some of these components also reacted with sera from individuals infected with unrelated parasites. Thus, some of these components were not Ov specific. More recently, Ruppel et al.[5] showed that protein components of adult Ov were highly complex which is not surprising. However, the interesting discovery was that almost half of the total protein in the extract was a peptide having a molecular weight of 18,000-19,000 daltons. In our preliminary investigation, we analyzed Ov antigens by radioimmunoprecipitation, SDS-PAGE and autoradiography and found many parasite-specific proteins, some of which were present in more than one developmental stage[6].

More recently, we found that a protein in adult crude extract having a molecular weight around 89,000 daltons appeared to be Ov specific[7]. Using radioimmunoprecipitation techniques, this component failed to give significant reactions with sera from patients with other parasitic infections including paragonimiasis, schistosomiasis, filariasis, gnathostomiasis, toxoplasmosis and malaria. Moreover, it failed to react with sera from animals experimentally infected with G. spinigerum, A. cantonensis, S. mansoni, S. japonicum, S. makongi, S. hematobium, P. heterotremus and filaria worms. The 89-kD component also represents a major protein found in the culture fluid obtained by maintaining the parasites in vitro in a protein-free culture medium. Rabbit antiserum prepared by immunizing with such metabolic products reacted almost entirely with this 89-kD protein by radioimmunoprecipitation. Our preliminary observation showed that the antisera gave positive immunofluorescence to Ov eggs and reproductive fluid. Other lines of evidence indicate that Ov eggs are antigenic in humans and animals[3-10]. It should be mentioned that the 89-kD protein was also present to some extent in the metacercarial stage, and therefore, appeared not to be stage-specific. Kinetic studies of the humoral immune response in hamsters experimentally infected with Ov showed this polypeptide to be highly immunogenic since antibody reactive with it could be detected within 15 days of infection[11].

In addition to the 89-kD component, components with M.W. of 116, 78, and 20 kD also appeared to be specifically associated with adult flukes. Unlike these components which appeared to be highly immunogenic, the 16-17 kD duplex, which represented the most abandant protein in the crude Ov extract, was a rather weak immunogen and it reacted only weakly with human and animal sera used in this study. Our limited data on detergent extracts of adult Ov suggested that the latter components was largely associated with the surface tegument of the parasite

Immune Response in Opisthorchiasis

Generally both humoral and cell-mediated immune components are stimulated following appropriate antigenic exposure. Although several groups of investigators have reported an antibody response in human and animal opisthorchiasis, there is practically no definitive evidence for a specific cellular response against the Ov antigens. Indirect evidence from pathological studies suggests that cell-mediated immune mechanisms may be involved in the pathogenesis of the disease[12,13]. We were not able to demonstrate specific cellular responses to the parasite antigens until some time after treatment of the infected hamsters with praziquantel[14]. In fact, we recently reported an immunodepressive state with regard to unrelated antigens in hamsters experimentally infected with Ov[15]. These animals again exhibited normal responses following praziquantel treatment.

Antibodies to various Ov antigens have been demonstrated in the serum of patients with opisthorchiasis and of animals experimentally infected with O. viverrini[8-10,16,17]. Boonpucknavig and her associates[8] showed by immunofluorescence that these antibodies reacted to both eggs and surface teguments. In addition to these components, we have also observed immunofluorescence staining of the reproductive and gastrointestinal tracts and the developing miracidium inside the egg (unpublished observations).

We have shown recently that antibodies reacting to parasites antigens are present in both the serum and bile of humans and animals infected with O. viverrini[18]. Although in both specimens, IgG, IgA, IgE and, in a few cases IgM, antibody isotypes could be detected, such responses were more vigorous in the serum. While in the serum, the IgG response predominated, in the bile it was the IgA. Evidence available suggested that the biliary IgA antibody was selectively transported and/or locally produced by the hepatobiliary system of these patients.

Although both systemic and local immune responses can be detected, their protective values and diagnostic usefulness are still poorly understood. We previously showed that the parasites were still alive after a period of exposure to antibodies[19]. Moreover, the addition of peritoneal exudative cells from either infected or uninfected animals to the system did not alter the outcome (unpublished observations). On the other hand, the parasites could be killed by the complement alone[19]. It was clearly demonstrated that the parasites could

activate the complement via an alternative pathway. A scanning electron-microscopic study showed that the parasite tegument was extensively damaged[19]. However, some parasites could recover and it is possible that tegument shedding is an evasive mechanism of O. viverrini against attack by the host. There is no evidence regarding other host factors that adversely influence the survival of parasites, either in vivo or in vitro. The ability of O. viverrini to survive in immune hosts has been demonstrated previously by our group[20,21].

Development of Methods for Immunodiagnosis of Opisthorchiasis

Several possible approaches can be undertaken to develop appropriate immunodiagnostic methods for opisthorchiasis. The popular current approach is to detect antibodies in the serum of suspected individuals. The immunoelectrophoresis method used by Janechaiwat et al.[4] was not sensitive enough for this purpose. The semi-quantitative immunofluorescence method recently reported by Boonpucknavig et al.[8] also lacks sensitivity and is not practical for routine diagnostic work. The enzyme-linked immunosorbent assay employed by Srivatanakul et al.[16] and Wongratanacheewin et al.[18] has sufficient sensitivity and specificity provided that a suitable antigen is available. Currently both groups use crude somatic extract prepared from adult O. viverrini. However, such a preparation is highly complex and therefore it is expected to obtain cross reactivity. A systematic analysis of Ov antigens is a necessary first step in identifying a specific parasite (Ov) antigen(s). We have recently characterized and identified several specific Ov antigens[18]. Among them, the 89-kD protein appeared to be the most promising component that should be investigated further. However, it is present only in trace quantities in the crude somatic extract and it is technically impossible to purify it for general use by classical physicochemical approaches. Furthermore, although it is present as a major parasite component in the in vitro culture fluid, it is rather tedious to produce in large quantities. Therefore, to achieve this, it is likely that a genetic engineering approach will be undertaken. Such an approach is now being carried out in our laboratory. Although our initial attempts thus far have been negative, we feel that it is not at all impossible once a system functions at its maximum potential.

In addition to the detection of specific antibodies, it is also possible to develop an immunodiagnostic technique for the detection of Ov antigens in those suspected of being infected with the parasite. These antigens can be eggs or other soluble parasite antigens. Again, this approach requires highly specific reagents and here monoclonal antibodies could play a significant role. We are now currently attempting to produce monoclonal antibodies for such a purpose.

Hopefully one or several of these various approaches will be successful. If this is the case, it should be possible for other investigators to systemically employ a similar approach for infections caused by other parasites, particularly those involving mucosal surfaces.

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FY 1986

"CHANGES OF CYANOGENIC GLYCOSIDES AND RELATED ENZYMES IN CASSAVA ROOTS DURING STORAGE AND PROCESSING" (6.404) : CYANOGENIC GLYCOSIDES IN CASSAVA (MANIHOT ESCULENTA CRANTZ)

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ABSTRACT

Chronic cyanide toxicity is a health threat to the poor, who survive on cassava diets. The toxicity is attributed to the cyanogenic glucosides, linamarin and lotaustralin. In this project, the biotransformation of the glucosides was studied with the aim to lessen the toxicity. Linamarase, the linamarin-hydrolyzing enzyme, was found in abundance in the cassava petiole, stem and root cortex. The enzyme was purified and characterized. Glucosyl transferase, the last enzyme in linamarin biosynthesis, was present in young leaves. It is being purified and characterized. During storage, an apparent synthesis of linamarin in cassava roots was confirmed. A procedure was established to isolate linamarin and lotaustralin from cassava root cortex for use in research and as a standard for determining cyanide content in tapioca products.

INTRODUCTION

Stored roots of cassava (Manihot esculenta Crantz), also called tapioca or manoic, provide an important source of carbohydrates for some 500 million people in 30 tropical countries. After rice, maize and sugar cane, cassava is the fourth most important source of calories. In 1986, the world cassava production was 143 million tons. Thailand and Brazil were the top producers, each producing about 20 million tons. Thailand has been the world's largest exporter of tapioca products. In 1986, the nation exported about 6.5 million tons, earning approximately 19,000 million baht which was about equal to the earnings from rice export in the same year. A survey last year showed that the area used for cassava cultivation in Thailand was nearly 8 million rai (equivalent to 12,800 Km or 2.5% of total land of the country). World-wide, about two-thirds of cassava is consumed as human food and one-third as animal feed. For human consumption, half is eaten as cooked fresh roots and the remainder as processed flours or meals.

Hidden in this agro-economic profile is a health problem. Cassava is one of the 200 plant species which possess cyanogenic glucosides . In cassava, the cyanogenic glucosides known today are linamarin (or phaseolunatin; 2-hydroxy-isobutyronitrile-D-glucopyranoside) and lotaustralin (or methyl linamarin; 2-hydroxy-2-methylbutyronitrile-D-glucopyranoside), with the former being the major poison. The average content of cyanogenic glucosides in cassava roots is about 100 mg/kg. Thus, the world

production of these natural products in cassava amounted to 14,000 tons in 1986. The same glucosides are also present in lima bean, linen flax and white clover. The cyanogenic glucosides are toxic because of the free cyanide liberated from their hydrolysis. Thus, use of cassava as a staple diet can cause chronic cyanide toxicity, which can lead to such health problems as goiter, cretinism, tropical toxic neuropathy and tropical diabetes. In short, long-term consumption of cassava by man or animals obviously does not cause death but health can be impaired.

To minimize the health threat, three approaches have been taken. (a) A limit of 100 mg HCN/kg dry weight of tapioca products has been set up as a trade standard. (b) Several post-harvest processing methods have been devised to reduce the cyanide content. (c) Selective breeding and cultivation of low-cyanide species are encouraged. Science and technology have contributed much to (b) and (c). This project is an attempt to understand the biotransformation of the cyanogenic glucosides (mainly linamarin) in cassava, using molecular and cellular approaches. Linamarin metabolism is better-known in white clover and flax than in cassava even though more men and animals are exposed to it in the latter. It is anticipated that an understanding of linamarin metabolism in cassava will lead to (a) a rational approach to prevent post-harvest deterioration of cassava roots; and (b) a logical design for cyanide-free species. Both are desirable but not yet possible at present.

Studies in white clover and flax have shown that valine is converted by plant microsome to acetone cyanohydrin. The latter is then conjugated to glucose by reactions with UDP-glucose to form linamarin. This reaction is catalyzed by glucosyl transferase. Linamarin hydrolysis to yield acetone cyanohydrin is catalyzed by linamarase. The unstable acetone cyanohydrin then breaks down spontaneously or enzymically into acetone and cyanide.

This report covers the work done in the first year. It contains studies on linamarase, glucosyl transferase and linamarin in cassava.

MATERIALS AND METHODS

Cassava roots, measuring 30-35 cm in length and 6-8 cm in diameter, stems, petioles and leaves were purchased from a Bangkok market.

Linamarin was either bought from Calbiochem or prepared from cassava root cortex. Two methods were used to prepare crude linamarin from the cortex. One involved the use of ice-cold 0.1 M phosphoric acid according to Kojima et al. The other employed 80% ethanol at 65-70°C for extraction, according to Nambisan and Sundaresan. Linamarin was assayed as HCN released after treating with an excess amount of linamarase.

To isolate pure linamarin and lotaustralin, the crude ethanol extract was concentrated under reduced pressure to give a dark brown residue. The residue was chromatographed in a silica gel column and eluted with a mixture of chloroform:methanol:water in the ratio 20:3:1 to yield lotaustralin and 6:4:1 to yield linamarin. The products were checked by TLC and NMR.

Linamarase was extracted from cassava tissues in 0.1 M acetate buffer, pH 5.5, according to the Cooke et al method. It was further purified by ammonium sulfate precipitation at 65% saturation, followed by gel filtration using a column of Sepharose 6B and chroma to focusing in pH range of 7 to 4 or 5 to 1.

Linamarase activity was assayed using linamarin as substrate by a modified Locas and Sotelo procedure. Alternatively, an artificial substrate, p-nitrophenyl-D-glucopyranoside, was used in the assay according to the method of Evans.

Glucosyl transferase was extracted from fresh young leaves in 0.15 M phosphate buffer, pH 7.6, containing 5% polyvinyl polypyrolidone and 1 mM DTT. After centrifugation at 100,000 x g for 1 hr, the enzyme was collected by ammonium sulfate precipitate at 30-70% saturation.

RESULTS AND DISCUSSION

Four major studies are described below:

1. Linamarase

The enzyme was purified from cassava root parenchyma by Cooke et al. Among various tissue extracts, it was shown that cassava petiole, stem and root cortex possessed much more linamarase than the root parenchyma (Table 1). The enzyme was separately purified from petiole, stem and root cortex as described elsewhere. The enzyme from each source was found by SDS-PAGE to have a subunit molecular weight of 63,000. The native structure was oligomeric having a molecular weight of approx. 600,000. The purified enzyme was separated by chromatofocusing into three isozymes with pI of 4.2, 3.5 and 2.8. With linamarin as the substrate, the enzyme exhibited a pH optimum of 7 and Km of 1-2 mM. With p-nitrophenyl-D-glucopyranoside, the enzyme showed a pH optimum of 8 and Km of 13-18 mM. The linamarase activity was inhibited by -gluconolactone and isopropyl-D-thioglucopyranoside. Thus, purified linamarase can be easily obtained from cassava petiole, stem and root cortex. The enzyme will be useful not only in research but also in cyanide determination in the tapioca trade.

Table 1 Distribution of Linamarase in Cassava Tissues (mean SD)

Ammonium sulfate fraction from	Preparation (n)	Protein/fresh weight (mg/ml)		Specific activity (mol/min/mg prot.)	
Leaf	4	0.32	0.23	0	
Petiole	5	0.92	0.65	2.85	2.02
Stem	4	1.25	0.27	1.76	1.10
Root cortex	3	0.28	0.14	1.56	1.53
Root parenchyma	3	0.39	0.01	0.05	0.04

2. Glucosyl Transferase

The enzyme was found to have a pH optimum of 8. It catalyzed the transfer of the glucosyl moiety from UDP-glucose to an alcohol acceptor. Acetone cyanohydrin was the best acceptor while ethanol, glycerol, isopropanol, butanol and phenol were not. The molecular weight of the native structure was estimated to be 46,000.

3. Root Deterioration

Harvested cassava roots were allowed to deteriorate at room temperature. After 3 days, the roots became soft. By 6 days, fungal growth on the surface was noted. When the roots were cut open, browning of the tissue was observed after 3 days of storage and became quite marked after 6 days. Linamarin and linamarase were mostly found in the root cortex. After 3 days of storage, the root cortex linamarase, free cyanide and protein content dropped while linamarin level rose (Table 2). The finding confirmed that of Kojima et al which suggested that linamarin synthesis is possible in cassava root during storage and deterioration. Future studies will search for ways to prevent root deterioration. If this is achieved, post-harvest storage of cassava roots will be possible and beneficial to the farmers.

Table 2 Changes in Linamarin, Free cyanide, Linamarase and Protein in the Cassava Root Cortex during Storage at Room Temperature

Parameter (n = 8)	Section	Day 0		Day 3	
Linamarin (mol/g wet wt.)	Head	1.13	0.82	1.40	1.01
	Middle	0.82	0.92	1.22	0.79
	Tip	1.40	1.05	1.82	0.68
Free cyanide (mol/g wet wt.)	Head	0.37	0.36	0.21	0.16
	Middle	1.12	0.73	0.79	0.77
	Tip	0.82	0.65	0.36	0.50
Linamarase (mol/min/mg)	Head	0.95	1.65	0.31	0.66
	Middle	0.70	1.19	0.23	0.60
	Tip	0.27	0.49	0.15	0.39
Protein (mg/g wet wt.)	Head	0.07	0.02	0.09	0.03
	Middle	0.05	0.04	0.07	0.03
	Tip	0.05	0.01	0.08	0.02

4. Linamarin Isolation

A procedure was worked out to isolate linamarin and lotaustralin from the crude ethanol-extract of the cassava root cortex. In a preparation, 62 gm of the crude extract yielded 1.23 gm of linamarin and 74 mg of lotaustralin. Efforts were being made to improve isolation yield and to characterize certain unknown fractions containing sugar and cyanide. Although linamarin is available commercially, it is expensive. In the case of lotaustralin, there is no commercial source at present. Both will be useful in our project and in cyanide determination in tapioca trade.

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FY 1986

APPLICATION OF MONOCLONAL ANTIBODIES AGAINST Entamoeba Histolytica IN TROPICAL MEDICINE RESEARCH (6.419)

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ABSTRACT

Thirteen positive hybrids secreting antibodies against the HM-1: IMSS strain of E. histolytica have been successfully produced by using a hybridoma technology. Based on their immunofluorescent reactivities, these antibodies were classified into 3 groups. The first group, consisting of nine hybrids, reacted to all components of amoebic trophozoites comprising the cytoplasmic granule (G), the membrane (M), the cytosol (C) and the released products (RP). The second group, comprising only one hybrid, reacted only to the cytoplasmic granules (G) and the third group, consisting of three hybrids, reacted only to the cytosol (C). Four hybrids of group I (F1W6, F1W16, F1W19 and F1W35) and one hybrid of group III (F1W23) were cloned by a limiting dilution technique. Intense IFA staining (3+) was observed with MAB from 2 F1W6 subclones, 6 F1W19 subclones, and all 5 F1W35 subclones. Isoenzyme typing of E. histolytica was also studied. Two axenically cultivated strains (HM-1:IMSS and HK-9) and five other multixenically cultivated E. histolytica isolates from Thai patients belonged to zymodeme II whereas the Laredo strain belonged to a zymodeme pattern different from that of E. histolytica.

INTRODUCTION

Amoebiasis, a disease caused by E. histolytica with or without clinical symptoms occurs throughout the world especially in tropical and subtropical climates, and in places with poor sanitary conditions and immigration of symptomatic and asymptomatic amoebiasis patients. It has been estimated that 480 million people are infected with E. histolytica and some 48 million people suffer from invasive amoebiasis[1]. The majority of amoebic infections are asymptomatic. In Thailand, the point prevalence varies from 1.4 to 4.2% depending on the region[2]. The high percentage of asymptomatic cases suggests that there could be differences in invasiveness and/or pathogenicity among different isolates of amoebae and this hypothesis was subsequently proved to be the case. The pathogenic E. histolytica have been shown to have several properties different from non-pathogenic amoebae. The pathogenic amoebae belong to certain zymodemes[3], have more cytotoxin[4], exert efficient contact mediated cytolysis of target cells[5], have high collagenase activity[6], and are able to phagocytize red blood cells. Among the properties associated with pathogenic E. histolytica, none except the erythrophagocytosis is easily established. We hypothesize that the pathogenic amoebae have certain

antigens not present in the non-pathogenic amoebae. Demonstration of such antigens should be possible using monoclonal antibodies (MAB)s specific for those antigens using an indirect immunofluorescent antibody (IFA) technique. Such MAB should react only to the pathogenic and not to the non-pathogenic E. histolytica or other E. histolytica-like amoebae. The objective of this study is to produce mouse MABs capable of differentiating between pathogenic and non-pathogenic amoebae by the IFA technique and, if possible, to apply these antibodies to detect soluble amoebic antigens in clinical specimens.

MATERIALS AND METHODS

Production of Mouse MAB for Typing of the Pathogenic E. histolytica

Amoebae: Two axenically E. histolytica trophozoites strains comprising HK-9 and HM-1:IMSS kindly provided respectively by Drs. LS Diamond (NIH, Bethesda, Md.) and GB Bailey (Morehouse School of Medicine, Atlanta, GA) were used. A strain of Laredo was purchased from ATCC. They were cultured mostly in TPS-1 media and occasionally in TYI-S-33 according to the techniques of Diamond[7] and Diamond et al[8].

Immunization, Cell Fusion, Screening and Selection of Hybrids: Three eight week-old BALB/c mice received four intraperitoneal injections of ultrasonicate trophozoites of the HM-1:IMSs strain incorporated in Freund's complete adjuvant initially and in the incomplete adjuvant subsequently with dosages of $2x10^5$, $5x10^5$, 10^6 and $2x10^6$ trophozoites respectively. A week after the last immunizing dose, the IFA titers in each of the three mice were 1:2560, 1:2560 and 1:1280. Two mice with high antibody titers were immunized intravenously three days before fusion with the soluble extract of ultrasonicated amoebae (2 x 10^6 trophozoites). The spleen cells were fused with $Sp_2/0$ myeloma cells at a ratio of 10:1 according to the Galfre and Milstein[9] technique using 4,000 MW polyethylene glycol (Sigma Chemical, St. Louis) as a fusant. The cells were suspended in a selective medium comprising RPMI-1640, 5 x 10^{-2} mM hypoxanthine (Sigma), 1 x 10^{-2} MM azaserine (Sigma), 15% fetal bovine serum (Flow Laboratory), 0.1% kanamycin to give a concentration of 1 x 10^6 per ml. A volume of 220 ul was dispensed in a 96 well plate (Costar, Mass of Nunc Kamstrup, Denmark) and incubated at 37°C in 5% CO_2 in air. After 7-10 days, the supernatant from the growing hybrids was removed and tested for anti-amoebic antibodies by the indirect IFA test against whole ethanol-fixed amoebic trophozoites of the HM-1:IMSS, HK-9 and Laredo strains and by ELISA against the saline extracts of these three strains.

Cloning of Hybrids Secreting Anti-E. histolytica Antibodies: This was done by limiting dilution techniques.

Isoenzyme Typing of E. histolytica

The amoebic lysates of eight strains of E. histolytica comprising three axenically cultivated HK-9, HM-1:IMSS and Laredo and five multixenically cultivated amoebae (HTH-4:MUTM, HTH-7:MUTM, HTH-8:MUTM, HTH-17:MUTM AND HTH-18:MUTM) in TYSGM-9[10] or Robinson's media[11] were

subjected to electrophoresis and isoenzyme typing for hexokinase (HK), malic enzyme (ME) glucose phosphate isomerase (GPI) and phosphoglucomutase (PGM) according to the Sargeaunt et al.[3] technique.

RESULTS AND DISCUSSION

Production of MAB for Typing of Pathogenic E. histolytica

In one successful fusion, hybrids were present in 95 of 600 wells, of which 13 wells secreted anti-E. histolytica antibodies (Table 1). Based on IFA staining patterns, these oligoclonal antibodies were arbitrarily divided into three groups. The first group comprising nine hybrids reacted to all components of amoebae including the granules (G), the membranes (M), the cytosol (C) and the released products (RP) from amoebae. All of them reacted variably to the HM-1:IMSS and the HK-9 strains of E. histolytica and did not react to the Laredo strain. With ELISA, the supernatant from all hybrids reacted to the HM-1:IMSS antigens more intensely than those of the HK-9, and none reacted to the Laredo strain. The second group, comprising only one hybrid, reacted to cytoplasmic granules only. The their group, comprising three hybrids, reacted only to the cytosol. With IFA, it was shown that all MAB produced reacted to the HM-1:IMSS, HK-9 but not the Laredo strains. With ELISA, it was shown that two of these MAB (F1W65, F1W52) also reacted against the Laredo strain. This result suggests that IFA reactivity is more specific than ELISA.

Table 1 Indirect Fluorescent Antibody (IFA) and ELISA Reactivities of Oligoclonal Antibodies from Hybrid Cells Against Two Strains of E. histolytica and a Laredo Strain.

Group	Staining pattern	Designation	IFA			ELISA		
			HM-1	HK-9	Laredo	HM-1	HK-9	Laredo
I	Generalized staining of amoeba including the granules, cell sap plasmalemma, internal membrane, and released products	F1W6	2+	2+	-	0.23	0.09	0
		F1W15	2+	2+	-	0.51	0.05	0
		F1W16	3+	3+	-	1.24	1.02	0
		F1W19	2+	2+	-	0.16	0	0
		F1W20	1+	1+	-	0.18	0.12	0
		F1W35	2+	2+	-	1.26	0.36	0
		F1W61	3+	3+	-	0.12	0	0
		F1W72	3+	3+	-	0.49	0.07	0
		F1W95	1+	-	-	0.28	0.22	0
II	Cytoplasmic granules	F1W65	1+	1+	-	0.33	0.44	0.24
III	Cytosol	F1W23	1+	-	-	0.01	0.06	0
		F1W30	1+	1+	-	0.16	0.14	0
		F1W52	1+	-	-	1.25	1.24	1.24

Hybrids from five positive wells comprising F1W6, F1W16, F1W19 and F1W35 from group I and F1W23 from group III were cloned by limiting dilution techniques. Ten monoclones from F1W6, F1W19 and F1W23 and five monoclones from F1W16 and F1W19 were subjected to further study by IFA and ELISA. Intense IFA staining (3+) was observed with MAB from 2 F1W6 subclones, 6 F1W19 subclones, all 5 F1W35 subclones but not with the products from the F1W16 and F1W23 subclones.

Isoenzyme Typing

It was shown that two strains of axenically cultivate amoebae (HK-9 and Hm-1:IMSS) as well as five other multixenically grown amoebae (HTH-4:MUTM, HTH-7:MUTM, HTH-8:MUTM, HTH-17:MUTM and HTH-18:MUTM) belonged to zymodeme II. The axenically grown Laredo strain conformed to group of E. histolytica-like amoeba.

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FY 1986

TEAK (Tectona Grandis) TISSUE CULTURE (6.427)

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ABSTRACT

Research on teak tissue culture in Thailand is a joint study between the Royal Forest Department and the Chiang Mai University. The main purpose is to develop techniques of teak tissue culture for plantation establishment and improvement programmes. There are four components in this study, i.e. in vitro cultures of seed, shoot-tips/buds of mature trees, callus and anthers. The in vitro culture of seed is aimed at propagating progenies of the selected families for the advanced breeding programme. The in vitro culture of shoot-tips/buds of mature trees is aimed at mass propagation of selected clones for the plantation establishment programme. The purpose of the in vitro cultures of callus and anther is to create new clones and/or varieties for the breeding programme. Preliminary results and the modified culture techniques used in this study are briefly presented in this paper.

INTRODUCTION

At present, the remaining teak natural forest area and the total teak planting area in Thailand are 25,000 sq.km and 133,000 ha, respectively (Kaosa-ard, 1986). To accelerate the planting programme, the Teak Improvement Centre (TIC) was set up by the Royal Forest Department (RFD) in 1965. The objectives are to operate the breeding programme, to produce genetically improved seed for the planting programme and to conduct research supporting the improvement and planting programmes of this species.

Due to low production of improved seed in the seed orchards in conjunction with poor germination behaviour of the seed, the supply of seed and planting stumps for the large scale planting programme is insufficient. Various propagation techniques, e.g. cuttings and tissue culture, of this species have been explored.

A series of studies on teak tissue culture in Thailand was first conducted in 1983-1985 by a research team from the RFD and Chiang Mai University (CMU) (Kaosa-ard, Apavatjrut and Sombun, 1985). It was established that there is a high potential for developing techniques of tissue culture of this species for mass propagation. The scope of the project has been enlarged with the support of the USAID/PSTC programme. The research team of this extended project consists of researchers from the TIC, the Faculty of Agriculture, CMU and the Faculty of Science, CMU. The term of support is May 1986 - April 1989.

OBJECTIVES

1. To develop tissue culture techniques of teak for commercial mass propagation.
2. To develop techniques of callus and anther cultures for the improvement programme.
3. To observe field performance of plants derived from the developed culture techniques.
4. To estimate the comparative cost of production of planting stumps produced by the developed methods and the conventional methods when the improved materials (seed and clones) are used.

MATERIALS AND METHODS

1. Materials

1.1 Plant Materials : Teak fruit, shoot-tips/buds, and flower buds, used in this research project were genetically improved materials from the TIC, Ngao, Lampang.

1.2 Laboratories : Laboratory studies were conducted at the Faculty of Agriculture and the Faculty of Science (CMU).

1.3 Nursery : Nursery operations for plant production and field testing were conducted at the TIC.

2. Methods

There are four main components in this series of studies. These components include:

2.1 In Vitro Culture of Seed

Teak fruit from TIC seed orchards were cut. The fruit's white seeds were removed. The seeds were sterilized by shaking in 10% (v/v) chlorox solution for 10 minutes and rinsing 3 times with distilled water. The treated seeds were placed individually into test tubes containing White (1963) culture medium (without hormone) for germination.

After germination, the sterile seedlines (3-5 cm in height) were removed, cut into nodal segments and transferred to cultures in flasks containing various tested culture media for promoting auxillary shoot regeneration.

2.2 In Vitro Culture of Shoot-tips/Buds of Mature Trees

Shoot-tips and buds (about 5 cm in length) of mature trees (22-year-old) were taken from the TIC clone bank at monthly intervals. They were placed in ice boxes and transported (about 150 km) to the laboratory.

In the laboratory, the shoot-tips/buds were washed, shaped into small (1 cm) pieces and sterilized by shaking in 10-20 % (v/v) chlorox solution for 10 minutes and rinsing 3 times. They were then trimmed into 0.5 cm pieces and transferred singly to cultures in test tubes containing culture media for promoting shoot growth and development. After

shooting, the shootlets (3-5 cm in height) were removed, cut into nodal segments and cultured for shoot multiplication in the freshly prepared culture media.

2.3 In Vitro Culture of Callus

Basal callus from the cultured explants in 2.1 and 2.2 was trimmed and used as callus sources for the study. The prepared callus was sub-cultured for growth and development in various modified culture media under different environmental conditions, e.g. light and temperature combinations.

2.4 In Vitro Culture of Anther

Teak flower inflorescences were taken from the TIC breeding orchard and kept in plastic bags in ice boxes for transportation to CMU. The flower buds of different sizes or developmental stages were histologically studied for their anther development. The suitable buds for anther culture study were selected, shaken in 10% (v/v) chlorox solution for 10 minutes and rinsed 3 times. They were then dissected and their anthers were transferred to culture in the tested culture media to promote their growth and development.

RESULTS

1. In Vitro Culture of Seed:

Teak seeds can be sterilized safely by using the technique described in 2.1. After culturing, the seeds started germinating within 2 weeks.

After germination (about 45 days), seedlings of size 3-5 cm in height or with 3-4 pairs of leaves appeared to be the most suitable size for nodal segment cultures.

The culture medium which was most suitable for auxillary shoot regeneration and multiplication of teak nodal segments culture was the T17 medium. The composition of the developed T17 culture medium was as follows:

Composition of the T17 Culture Medium

1/2 SH	Macronutrients (Schenk and Hildebrandt, 1972)		
MS	Micronutrients (Murashige and Skoog 1962)		
	Organic Components		
	Glycine	2	mg/l
	Nicotinic Acid	0.25	"
	Pyridoxine HCI	0.25	"
	Thiamine HCL	0.25	"
	Hormones		
	IBA	0.3	"
	BAP	1.0	"
	Sucrose	30.0	g/l
	Agar	10.0	"
	pH	5.7-5.8	

Under favourable growth conditions, the multiplication rate and cycle was about 2-4 shootlets/cultured explant/ 45 days. The regenerated shootlets could be removed for remultiplication or for rooting about 45 days after culturing.

To remultiplicate, the removed shootlets were cut into nodal segments and transferred to culture in the freshly prepared T17 culture medium.

To produce plantlets, the shootlets of size > 3.0 cm in length, which are the most suitable size for rooting were selected. Culture medium was thoroughly removed from shootlets through rinsing. The were line-planted in plastic containers covered with lids. Sterile fine sand was used as the rooting medium. During the rooting period (2-3 weeks), the moisture and humidity inside the containers were kept at near saturation point. The light intensity was low or under heavy shading conditions. The temperature was 25-30°C. The planted shootlets were ventilated for a short time (3-5 minutes) 3 times a day. Under these conditions the rooting percentage was as high as 90-100%. After rooting, the containers were transferred to warm and humid conditions (in glasshouse) for 2 weeks for further growth and development of plantlets before they were transplanted in soil.

2. **In Vitro Culture of Shoot-tips/Buds:**

The results obtained in this series study can be summarized as follows:

Sterilization Techniques : There was a marked seasonal variation in contamination rate of cultured explants using 10% (v/v) chlorox solution for 10 minutes, the lowest (10-30%) contamination rate was in the shoot/bud sprouting season (April - June) whereas the highest (80-100%) was in the shoot/bud dormant season (November-March). During the dormant season, the contamination rate could be slightly reduced by sterilizing explants with 20% (v/v) chlorox solution for 10-20 minutes or with 0.1% (w/v) mercuric chloride solution for 10-20 minutes. The high contamination rate during this period was mainly due to the embeded microorganisms in the shoot/bud tissue.

Culture Medium for Shoot Growth : The culture medium suitable for shoot growth and development of cultured shoot-tips/buds from mature trees was also the T17 medium as mentioned in Section 1 (above).

Shoot Growth and Development : There were marked seasonal and clonal variations in growth and development of cultured explants (shoot-tips/buds). Explants excised during the sprouting season (April-June) had a higher shooting potential than those excised during the dormant season (November-March). In contrast, the basal callus of the cultured dormant explants was much larger than that of the cultured sprouting explants. Within the culture season, the rate of shoot and basal callus development in cultured explants also varied from clone to clone. More studies are required to clarify the degree of clonal variation.

Multiplication : The shoots (about 5 cm in height) were removed, cut into nodal segments and transferred to culture for auxillary shoot multiplication in the freshly prepared T17 culture medium. The culture procedures for shootlet production were similar to those mentioned in Section 1 (above).

Plantlet Production : About 45 days after subculturing, the shootlets were removed, individually separated from their cultured explants and rooted under the conditions reported in Section 1 (above).

Nursery Lining : The plantlets (> 3.0 cm in height) could be line-transplanted in nursery beds safely for future planting stump production. Under observed conditions in May-June (1987) it was found that the survival rate of the transplanted plantlets was as high as 80% (i.e. transplanting during the raining period and the beds were shaded with cloth and/or split bamboo. Growth and development of the transplanted plantlets are being observed.

3. *In Vitro* Culture of Callus:

Basal callus derived from cultured explants (See Sections 1 and 2 above). were multiplied in the modified T17 culture medium (IBA deleted and BAP added up to 6 mg/l) and placed under continuous supplied light. The multiplied callus was being cultured and treated for morpho/embryogeneses.

4. *In Vitro* Culture of Anther:

Flower Bud Selection : The result of histological study indicated that the suitable flower buds, of which yellow/jelly anthers contain uninucleate microspores, were the 1.5-2.0 mm-diameter buds. The smaller buds usually contained green/jelly anthers in which the sporocytes were in the meiosis stage, whereas the anthers of the bigger buds were usually yellow/dense and contain binucleate pollen grains. The flower buds could be stored safely for 3-5 days in the refrigerator before being used as sources of explants for anther culture.

Sterilization : The selected flower buds could be sterilized safely (> 65% with 10% (v/v) chlorox solution for 10 minutes and rinsed for 3 times with distilled water.

Culture Medium : There are four types of modified media which were found to be suitable for promoting pollen growth and callus formation of the cultured anthers. These culture media are as follows:

Composition of culture media suitable for teak anther culture.

(1) MS basal medium (Murashige and Skoog, 1962) + 0.1 K + 2.0 pCPA (mg/l)

(2) MS basal medium (Murashige and skoog, 1962) + 0.1 K + 0.5 2,4-D (mg/l)

(3) B5 basal medium (Gamborg and Wetter, 1968) + 0.1 K + 2.0 pCPA (mg/l)

(4) SH basal medium (Schenk and Hildebrandt, 1972) + 0.1 BAP + 2.0 pCPA (mg/l)

Cytological Study : Under suitable growth conditions, the division of uninucleate microspores in the cultured anthers could be observed. The chromosome number (n = 16) of the growing callus derived from the cultures was very similar to that of the sporocytes in the anthers of selected flower buds. The regenerated callus was the haploid callus.

Treatments for Morpho/Embryogeneses : The derived callus is being cultured and treated for its morpho/embryogeneses. Different concentrations and types of particularly hormones and vitamins in combination with environmental treatments are being tested for inducing morpho/embryogeneses in the cultured callus.

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SHOOT BUD FORMATION FROM COWPEA TISSUE CULTURE

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ABSTRACT

Attempts have been made to induce shoot bud formation from cultures of cowpea immature embryos. The immature embryos were cultured on the modified Murashige and Skoog (MS) medium which consisted of MS inorganic salts and B5 vitamins. The medium was supplemented with various combinations of benzyladenine (BA) and napthaleneacetic acid (NAA) concentrations and with various proline concentrations. In a separate experiment, the effect of relative concentration of MS minor elements were investigated. It was found that only a concentration of BA in the medium had a pronounced effect on shoot buds formation. The optimum concentration of BA appeared to be at 10 uM. The effects of NAA on the rooting ability of cowpea shootlets grown in vitro were also investigated. The result demonstrated that 0.1 uM NAA was the optimum concentration for root induction and root growth.

INTRODUCTION

Cowpea (Vigna unguiculata), a grain legume species, is an extremely important source of plant protein for Africa. The plants can be grown under marginal moisture and poor soil conditions. It is an agronomically and nutritionally useful alternative for cassava. Thus, it is also a promising leguminous plant for the northeast region of Thailand. Considerable efforts have been made to study the cowpea as a possible source for food products which would relieve the protein malnutrition of Northeast Thailand[6].

Although cowpea is becoming a promising leguminous plant for the northeast, the average yield of local varieties is low, ranging from 200 to 300 kg/ha, due to plant susceptibility to a number of diseases and insect pests. Therefore, various attempts have been made to improve cowpea varieties. It is well known that the developing technology of plant tissue[7] culture can be used to produce plant variation which may include new and valuable agronomic traits. This has led to the use of this technology in cowpea improvement programs.

For this approach to be useful in the cowpea improvement program, it must be demonstrated that regeneration of cowpea plants from cells or tissues which have been in culture for several months is possible. Previous efforts of in vitro culture of cowpea have been limited to embryo culture and anther culture[4] which are sought as solutions to hybridization problems, and to shoot-tip culture[3,7] which has yielded whole plant regeneration. To the best of our knowledge, full cowpea

plant regeneration through callus or cell suspension cultures has never been reported. Therefore, research on the development of methodologies for cowpea tissue culture has been undertaken in the hope that this new technology for developing new plant varieties can be of use to the cowpea breeding program.

The experiment reported herein is part of the main project supported by USAID. It presents evidence of adventitious bud formation on cotyledons lacking a growing point from cultured immature cowpea embryos.

MATERIALS AND METHODS

Immature embryos were selected as explant sources because of their juvenile nature and high regenerative potential. Cowpea plants (Vigna unguiculata c.v. IT82E-889) were field grown at Khon Kaen University. Pods were harvested and washed with soap. After a 20 sec soaking in 70% ethyl alcohol, the pods were dissected and the immature seeds removed and placed in sterile distilled water. These seeds were rinsed and transferred for further disinfection to a solution containing 5% (v/v) Clorox (commercial bleach possessing 5.25% sodium hypochlorite) plus a few drops of Tween 80 for 15 min. The seeds were subsequently rinsed three times in sterile distilled water for at least 5 min each time. The embryos ranging in size from 1.5-2.0 mm, were aseptically removed from the immature seeds and placed in culture media. All media consisted of MS mineral elements[5] and B_5 vitamins[2]. Different growth regulators such as BA(10-30 µM), NAA (0.06-0.60 µM) and an amino acid, proline, (0-30 mM) were incorporated into the media. In a separate experiment, the effects of relative concentration of MS minor elements and MS FeEDTA were investigated. All media were adjusted to pH 5.8 and solidified with 0.7% Difco agar. The cultures were incubated at 25-28°c in darkness. Observations were taken at the end of 8 weeks.

RESULTS AND DISCUSSION

Immature cowpea embryos were cultured in darkness due to the precocious germination effect of light. The results of a factorial experiment on the effect of BA, NAA, and proline are shown in Tables 1 and 2. When no proline was added, it was clearly shown that the highest shoots occurred in the medium which contained BA at 10 µM and NAA at 0.2 µM. High concentration of both BA (30 µM) and NAA (0.60 µM) tend to inhibit shoot formation. However, when the percentage of embryos which formed buds (data not shown) were compared, it was clear that only BA exerted an effect on shoot bud formation. The total mean concentration of NAA in every trial was essentially the same (44 %, 46%, 45 %), while the average mean concentration of BA in every trial increased from 29 % at BA 3 uM to 52 - 54 % at BA 10,30 µM respectively. The addition of proline did not have any effect on the number of shoots formed (Table 1). However, the frequency of shoot bud formation was slightly enhanced by the addition of proline at 10 mM (50%) while proline at 20 mM caused a slight reduction in this frequency (38%) (Table 2). Addition of proline resulted in a considerable reduction on the degree of browning of callus tissues. It was also found that there was no interaction between the growth regulator effect and the proline effect. The effect of BA and NAA on the frequency

of shoot bud regeneration and on the average number of shoot buds/culture were again confirmed in the treatments supplemented with proline by comparisons of the overall mean of BA and NAA (Table 1, 2). Therefore, it can be concluded that BA is necessary for shoot bud formation in cultured immature cowpea embryos.

The increasing of relative concentrations of MS minor elements did not have any effect on the number of shoot buds formed (Table 3). This result was different from that of Barwale et. al.[1] who reported that the percentage of organogenesis in callus cultures from immature soybean embryos was increased with the increase of MS minor elements levels. However, their result did not show the number of shoots produced.

In a separate experiment, the effect of increasing concentrations of MS FeEDTA were also studied. Although there was no effect on the formation of bud (data not shown), it has been observed that shoot growth increased up to the level 4 and decreased at the level 6 (Table 3).

The effects of NAA on rooting ability of cowpea shootlets grown in vitro were investigated. The results demonstrated that 0.1 uM NAA was the optimum concentration for root formation and root growth (Table 4).

The study demonstrated that cultured cowpea immature embryos can differentiate and form adventitious buds. Future study, involving the maintenance of regenerative capacity and exploring cell culture, is needed in order to determine that cowpea tissue culture can be useful for cowpea improvement programs.

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Table 1 Effect of Various Concentrations of BA, NAA, and Proline on the Average Number of Shoot Buds per Explant from Cultured Immature Cowpea Embryos. Observations were taken eight weeks after the cultures were initiated, using CV. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins.

NAA (uM)	Proline (0mM)				Proline (10mM)				Proline (20mM)				Over-all X of NAA
	BA (uM)				BA (uM)				BA (uM)				
	3	10	30	$\bar{X}$	3	10	30	$\bar{X}$	3	10	30	$\bar{X}$	
0 06	2.0±0.0	3.2±1.3	1.1±0.1	2.1	1.7±0.5	2.9±0.1	1.6±0.5	2.1	2.0±0.5	2.6±0.5	1.9±0.3	2.2	2.1
0.20	2.1±0.2	4.6±0.9	1.4±0.5	2.7	2.4±0.4	4.9±0.9	1.6±0.7	3.0	1.8±0.2	3.3±0.7	1.8±0.5	2.3	2.7
0.60	2.1±0.1	3.1±1.1	1.4±0.9	2.2	2.1±0.9	3.2±0.6	1.7±0.5	2.3	2.6±0.5	3.2±0.6	2.2±2.3	2.7	2.4
X	2.1	3.6	1.3	2.3	2.1	3.7	1.6	2.5	2.1	3.0	2.0	2.4	

Overall X of BA; BA 3 uM = 2.1, BA 10 uM = 3.4, BA 30 uM = 1.6

Table 2 Effect of Various Concentrations of BA, NAA, and Proline on the Percentage of Embryo which Formed Buds from Cultured Immature Cowpea Embryos. Observations were taken eight weeks after the cultures were initiated, using CV. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins.

NAA (µM)	Proline (0mM)				Proline (10mM)				Proline (20mM)				Over-all X of NAA
	BA (µM)				BA (µM)				BA (µM)				
	3	10	30	$\bar{X}$	3	10	30	$\bar{X}$	3	10	30	$\bar{X}$	
0.06	27±5.8	49±13.7	57±15.0	44	29±1.0	62±0.0	56±9.4	49	20±14.1	45±8.9	50±10.0	38	44
0.20	31±1.7	54±18.9	53±20.8	46	37±5.8	60±7.8	58±2.7	52	20±14.1	52±9.0	52±9.6	41	46
0.60	20±10.0	53±7.3	52±17.7	45	31±10.5	57±15.0	56±14.5	48	14±8.9	44±11.0	43±20.8	34	42
X	29	52	54	45	32	60	57	50	18	47	48	38	

Overall X of BA; BA 3 µM = 26, BA 10 µM = 53, BA 30 µM = 53

Table 3 Effect of Higher Concentrations of MS Minor Elements on the Average Number of Buds in Cultured Immature Cowpea Embryos. Observations were taken eight weeks after the cultures were initiated, using CV. IT82E-889 with 40 embryos per treatment on MS medium with B_5 vitamins supplemented with BA 10 uM, NAA 0.2 uM. The minor elements are H_3BO_3, $MnSO_4$, $ZnSO_4$, KI, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$.

Relative concentration of minor elements	number of shoot buds[a]
1	1.96±0.60
2	1.96±0.74
4	1.92±0.47
6	1.92±0.58

[a] Mean ± SD

Table 4 Effect of Higher Concentrations of MS FeEDTA on Shoot Growth in Callus Cultures from Immature Cowpea Embryos. Observations were taken eight weeks after the cultures were initiated, using CV. IT82E-889 with 40 embryos per treatment on MS medium with 13 uM BA, and 0.2 uM NAA.

Relative concentration of FeEDTA	Embryo size (mm)	
	1.0	2.0
1	1.97 ± 0.18[a]	2.10 ± 0.77
2	2.29 ± 0.41	2.41 ± 0.27
4	1.92 ± 0.73	2.44 ± 0.14
6	1.00 ± 0.00	1.25 ± 0.50

[a] Mean of shoot length ± SD expressed as a relative unit: 1 = 1-5 mm, 2 = 6-10 mm, 3 = > 10 mm.

Table 5 Effect of NAA Concentrations on Root Formation and Root Growth in Shootlets Obtained from Cultured Immature Cowpea Embryos on MS FeEDTA Medium. Observations were taken four weeks after the treatments, using CV. IT82E-889 with 20 shootlets per treatment.

NAA (uM)	Root number	Root length
0.1	2.15 ± 0.77[a]	2.00 ± 0.59[b]
0.3	1.72 ± 0.48	1.74 ± 0.45
0.5	1.40 ± 0.54	1.28 ± 0.38

[a] Mean of root number ± SD expressed as a relative unit: 1 = 1-3, 2 = 4-6, 3 = > 6.

[b] Mean of root length ± SD expressed as a relative unit: 1 = 1-10 mm, 2 = 10-20 mm, 3 = > 20 mm.

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IMPROVED PRODUCER GAS CLEANING SYSTEM (5.245)

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ABSTRACT

A rice husk down-draft gasifier has been designed and constructed, and its performance investigated. This gasifier supplies producer gas, which will, in the next stage of experimentation, be utilized to study the collection efficiency of the improved cleaning system. The producer gas generated from the gasifier was found to have a low content of hydrogen (0.06 -0.16% by volume) and a comparable content of carbon monoxide (6-12% by volume). The proximate analysis of rice husk and char indicates a high amount of ash (20.32% wt in rice husk and 43.6% wt in char). As a result of proximate analysis, about 50% of rice husk remains as char in the gasification.

INTRODUCTION

Gasification is a thermochemical process which converts coal and biomass to combustible gas, normally known as "producer gas". Producer gas can then be used in an internal combustion engine. Biomass gasification - different from coal gasification - produces gas containing high content of tar and dust. It has been found that tar content can vary between 5.7 and 27 gm/cm^3 of hot producer gas[1]. Tar laden gases can quickly gum the engine valves and necessitate a stoppage. The entire engine, in some cases, has to be pulled apart and cleaned[2]. Additionally, dust or gas-borne particles in the producer gas can easily damage the engine. As a consequence, cleaning the producer gas or eliminating tar and dust from the gas is an unavoidable step before the gas is supplied to the internal-combustion engine.

In normal practice, a producer gas cleaning system consists of a cyclone, a filter, and sometimes a scrubber. Kaupp and Goss[3] have investigated the following systems: a dry packed-bed filter, dry cyclone, wet cyclone, and sieve plate scrubber in combination with dry packed-bed filter of rice husk char. They found that the fourth cleaning system is a highly efficient combination. However, the pressure drop across the packed-bed filter is high.

Apart from the cyclone, which can efficiently remove particles with sizes greater than 5 microns[4,5], many investigators have been studying other cleaning devices that will then be connected to the cyclone. These include a packed bed of rice husk[6,7], wet scrubber[6], and fabric filter[7]. These devices - particularly the fabric filter - can effectively remove particles, but high pressure drop can be encountered[5,7].

Recently, the authors have introduced a novel producer gas cleaning system[8]. The system consists of a cyclone, three wet impingers, and

standard filter paper. We found that the three impingers have a total efficiency of 97% in removing tar and dust from the gas generated from the gasification of eucalyptus wood, and the pressure drop across each impinger is only 1.5 cm of water. The result are encouraging. At present, the above cleaning system is being further investigated for its collection efficiency when connected to a rice husk gasifier.

MATERIALS AND METHODS

Instruments

A schematic arrangement of the cleaning system is illustrated in Figure 1. A down-draft gasifier was designed and constructed as shown in Figure 2. This gasifier was scaled down ten times from the commercial size, designed by Mr. C. Vongvanrungruang. The material used in the construction was stainless steel. The gasifier had a height of 150 cm, and a diameter of 97 cm; it sat on a stand, 100 cm high. Inside the gasifier, there was a stirrer run automatically at the speed of 4 rpm. Rice husk was conveyed at a maximum rate of 500 kg/hr from a hopper sitting on the floor. The hopper could hold 40 kg of rice husk and the conveyor rotated at the speed of 200 rpm. At the bottom of the gasifier was a water reservoir which could collect ash falling from the gasifier during gasification. The gasifier was also jacketed.

Next to the gasifier were a wet scrubber, a 2-hp blower, a cyclone, an impinger and a demister. The wet scrubber had a height of 150 cm and cross-section area of 40 x 40 cm; there were 5 inclined plates inside. The cyclone was designed according to the standard practice; its upper cylindrical part had a diameter of 30 cm and a height of 6 cm, the lower part tapered to a diameter of 7.5 cm. The wet impinger was a cylinder, 28 cm in diameter and 100 cm long. (Figure 3). The producer gas flowed via a central duct, through a nozzle with a diameter of 1.75 cm. This inlet duct could be moved vertically. The demister was employed to remove water from the gas. It had a diameter of 50 cm. and was filled with rice husk, about 70 cm high (Figure 4).

Analysis of Gas Composition

The producer gas was collected in a U-tube before it entered the wet impinger. It was then analyzed for the content of hydrogen, nitrogen, oxygen, and carbon monoxide in a MS5A column, and the content of air and carbon dioxide in a porapak N column. Analyse were carried out by means of gas chromatography.

Proximate Analysis of Rice Husk and Char

Char, in this work, was the residue of rice husk after gasification. Both char and rice husk were analysed, by the proximate method for moisture, ash, volatile matters, and fixed carbon. A sample was heated in an oven at 110 C for 1 hour, and the decrease in weight was recorded. This decrease represented the amount of moisture in the sample. The sample was further heated at 750°C until the weight no longer changed; the decrease in weight at this condition was the ash content. Finally, the

sample was heated at 950°C, and the drop in weight of the sample then noted; the value obtained represented the amount of volatile matter. The residue of the sample was normally taken as fixed carbon.

RESULTS AND DISCUSSION

The producer gas leaving the cyclone was found to have a temperature of about 30°C; its typical composition, analysed in the gas chromatograph, is illustrated in Figure 5. The gas contained a very low amount of hydrogen, varying from about 0.06% by volume to 0.16% by volume (Figure 5a). Compared with the hydrogen content in the producer gas from eucalyptus wood (2-5% by volume)[8], the obtained value indicated that rice husk may not be a suitable material for hyrogen production. Carbon monoxide content, as shown in Figure 5b, altered between 6% by volume and 12% by volume; the result was comparable with that obtained in wood gasification. Since hydrogen and carbon monoxide are the major combustible constituents in producer gas, the heating value of the gas greatly depended on the contents of these two compounds. With the heat of combustion of hydrogen at 57.8 kcal/gmol[4] (water in vapour state) and that of carbon monoxide at 67.6 kcal/gmol, the heating values of the producer gases obtained from wood and rice husk gasification were approximately 8.49 and 6.82 kcal/gmol, respectively. Thus, at the same energy requirements, the rice husk gasifier must be able to generate gas about 20-30% more than the wood gasifier. However, the conclusion should not be taken as a sole decisive factor in preference of a wood gasifier to a rice husk gasifier. Other factors such as the plant location, and the cost of biomass should be included in the consideration before any decision is made.

Figures 5c and 5d illustrate the variations of oxygen and carbon monoxide contents in the producer gas. Oxygen varied from 4% by volume to 10% by volume, and carbon monoxide content lay between 14% by volume and 20% by volume. Both results were higher than those in producer gas from wood gasification (4.5-5.5% for oxygen and 11-13% for carbon dioxide)[8]. This seems to indicate that rice husk is more difficult gasify to than wood.

The proximate analysis of rice husk and char is summarized in Table 1. The rice husk was found to be composed of 6.70% moisture, 20.32% ash, 61.05% volatile matter, and 11.93% fixed carbon. The ash content of the rice husk was rather high when compared with ash content of 1.69% in ponderosa pine charcoal[9] and 0.2-2.0% in wood[10,11]. The high value of ash content is due to the high content of silica in the rice husk.

Table 1 Proximate Analysis of Rice Husk and Char.

Content (% wt)	Rice husk	Char
Moisture	6.70	3.15
Ash	20.32	43.6
Volatile Matter	61.05	16.36
Fixed Carbon	11.93	36.89

After gasification, the char was composed of 3.15% moisture, 43.6% ash, 16.36% volatile matter, and 36.89% fixed carbon. By assuming that all the ash in the rice husk remained in the char, a simple calculation revealed that every 100 gm of rice husk gasified would yield 46.6 gm of char. In other words, about 50% of rice husk remained as char in gasification. If the above method were applied to the pyrolysis of saw dust[12], it would be found that about 8% of saw dust remained as char. This roughly indicated the poor convertibility of rice husk.

CONCLUSION

Rice husk, as shown in this study, looks as if it is not a suitable material for gasification. However, due to its availability and very low cost, rice husk retains its popularity among the investigators in the field of biomass gasification. The present work concerns itself mainly with the operation of the down-draft gasifier. In the next stage of investigation, the performance of the impinger will be studied; a mathematical relation between the collection efficiency of impinger and its configuration will be derived, using dimensional analysis methods. The obtained relation will be applied in scaling up the impinger.

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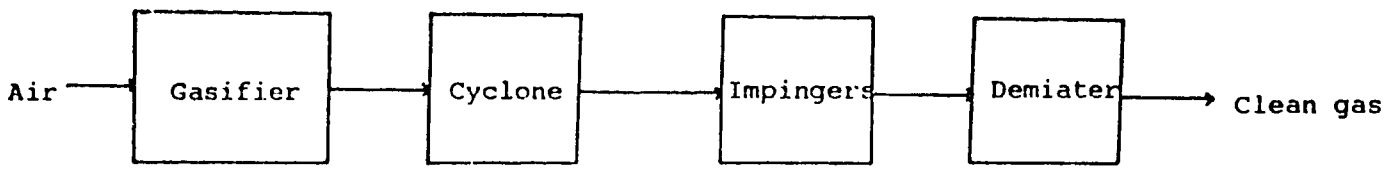

Figure 1 Schematic Arrangement of the Cleaning System

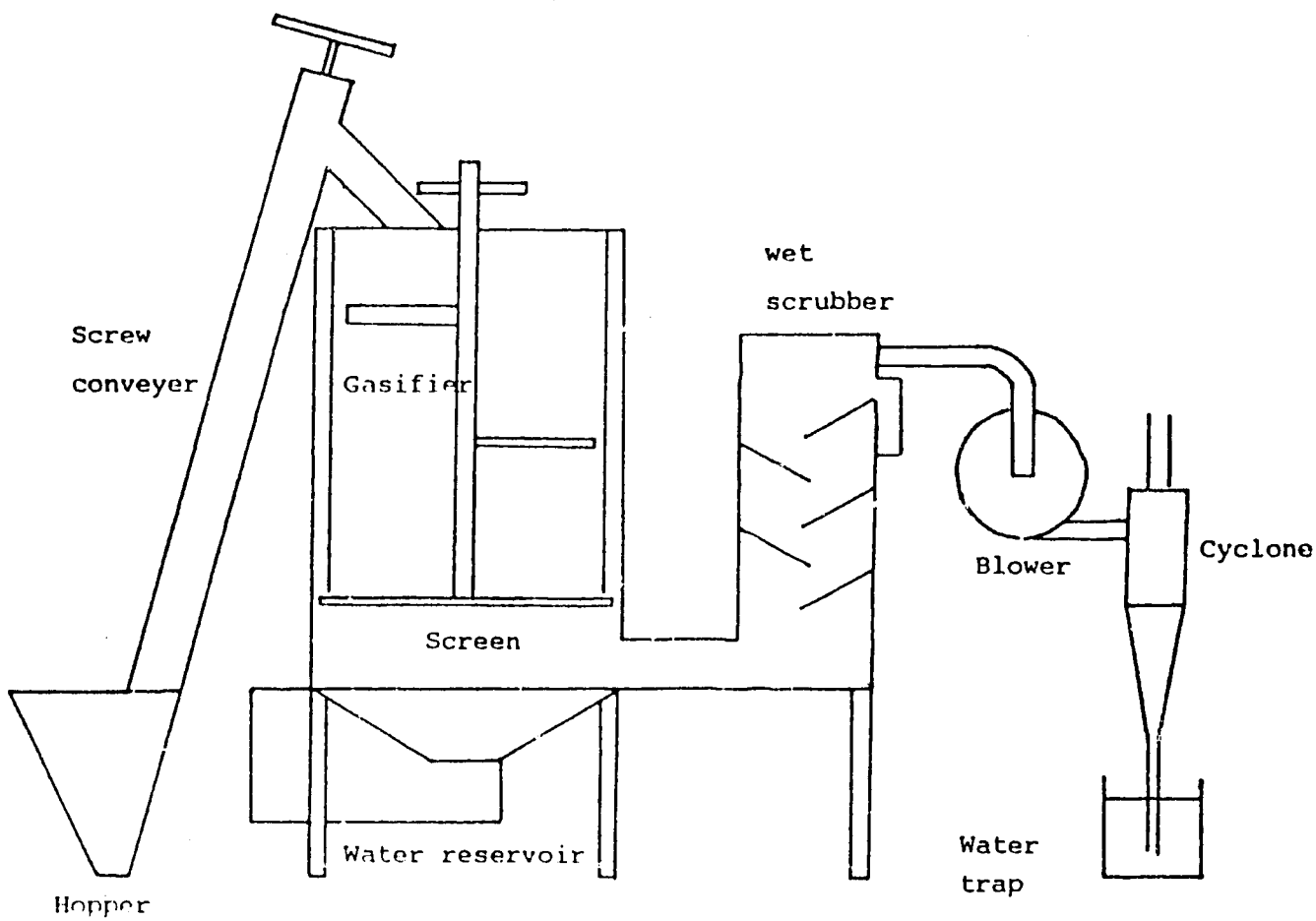

Figure 2 Configuration of Gasifier and Cyclone

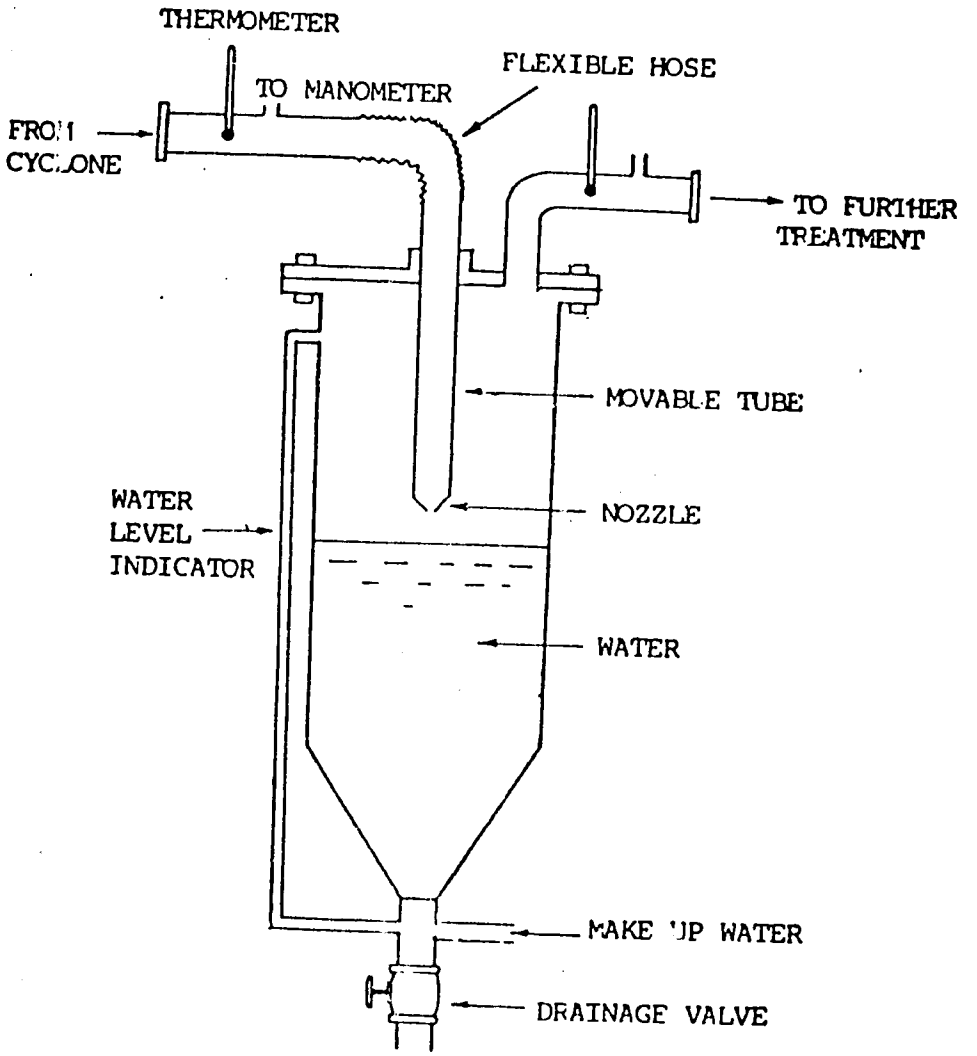

Figure 3 Details of a Wet Impinger

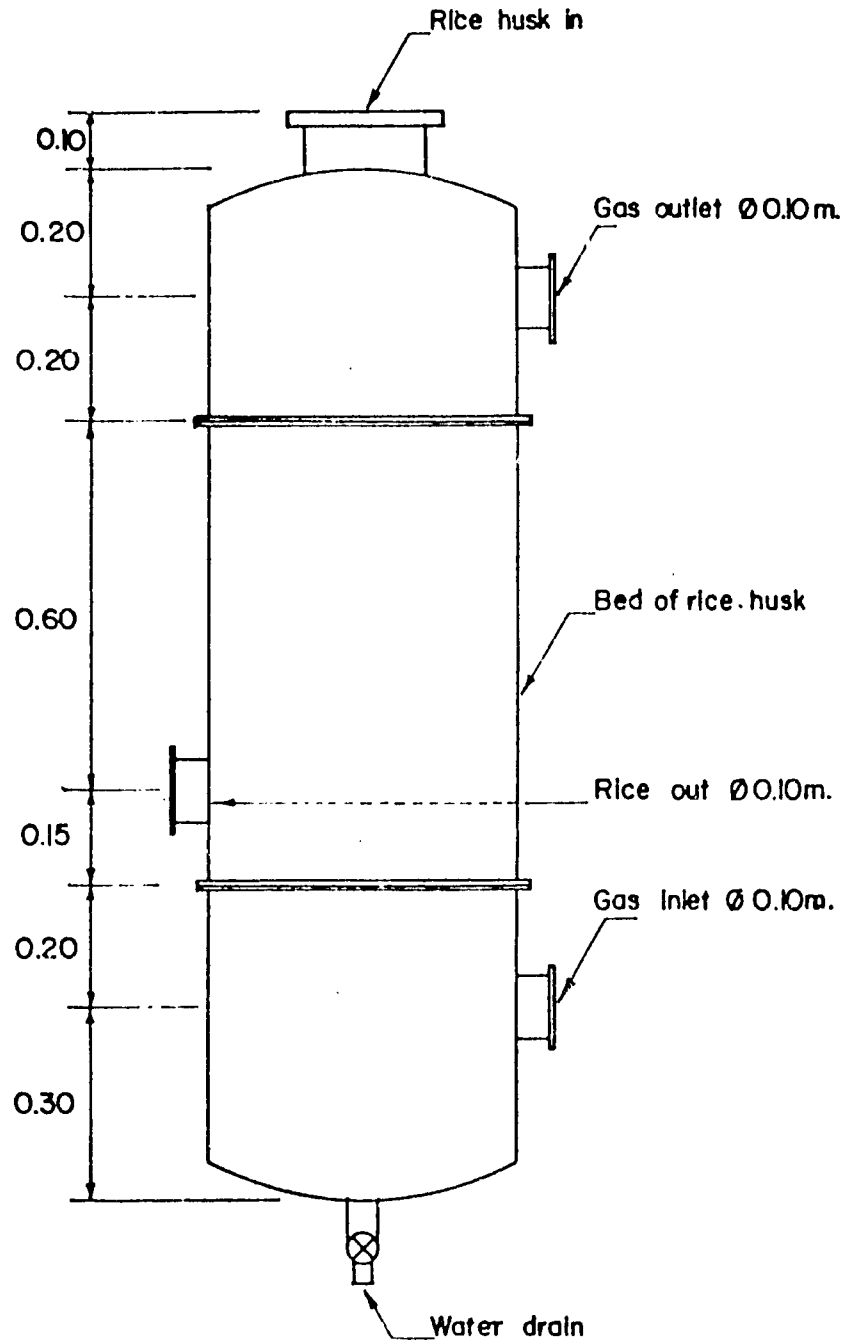

Figure 4 Details of a Water Vapor Demister

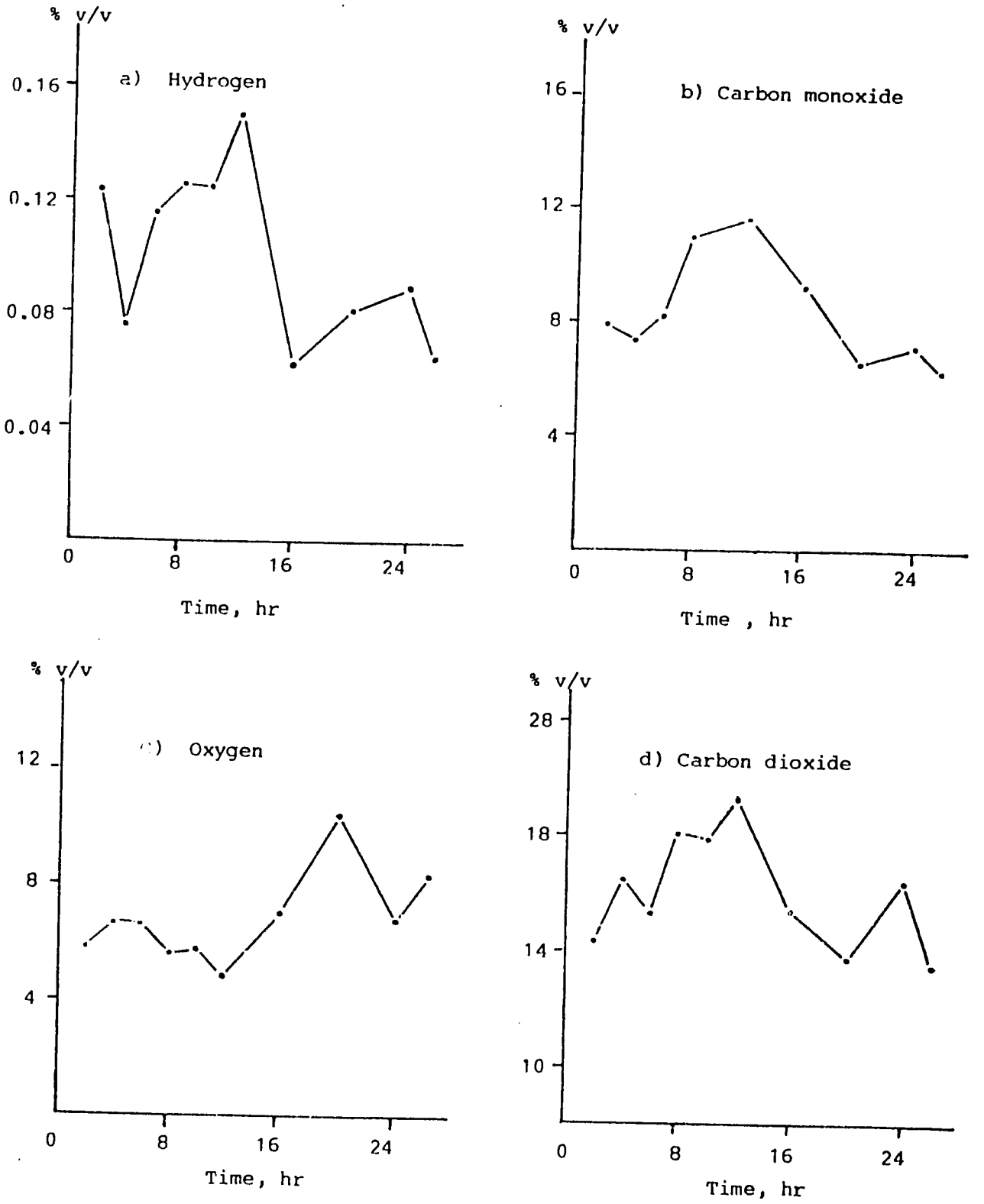

Figure 5 Composition of Producer Gas

FY 1986

BIOTECHNOLOGY APPLICATION FOR CHARACTERIZATION AND SELECTION OF BETTER YIELDING RUBBER CLONES (6.400) : 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE FROM LATEX OF Hevea brasiliensis

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ABSTRACT

Hevea 3-hydroxy-3-methylglutaryl coenzyme A reductase (Mevalonate : NADP Oxidoreductase; EC 1.1.1.34) was partially purified from fresh latex of clone RRIM 600. The latex was fractionated by centrifugation at 49,000 g for 40 min. Bottom fractions were pooled and used for enzyme purification. The enzyme was solubilized from the bottom fraction (lutoid) by freeze-thawing in the solubilization buffer containing 1% Brij 35 and 20% glycerol. Affinity chromatography (agarose-hexane-HMG-CoA) was used in the final step of purification. SDS-PAGE of the enzyme showed a molecular weight of 43,000 daltons as the enzyme subunit.

Different activity levels of HMG-CoA reductase in latex from various known yield-ranking clones were observed. The effects of the yield stimulant Ethrel on the level of HMG-CoA reductase activity were studied. The stimulant showed no effects on the enzyme in latex obtained from Ethrel treated trees as compared to the untreated controls.

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes reduction of HMG-CoA to mevalonic acid, an important step in isoprenoid biosynthesis. The enzyme has been extensively studied in both animals (1,2) and microorganisms (3). In higher plants the reaction catalyzed by HMG-CoA reductase has been proposed to be the first committed reaction step unique to the biosynthesis of a wide range of isoprenoid compounds (i.e. carotenoids, sterol, isoprenoidquinones, abscisic acid, gibberellic acid and some naturally occuring cytokinins) as well as natural rubber (4,5). The presence of HMG-CoA reductase has been shown to occur in all plant species tested such as sweet potato roots (6), radish seedlings (7), tobacco seedlings (8); sygamore tissue culture (9), rubber latex and leaf tissue (4,10,11) etc.

HMG-CoA reductase in the latex of Hevea brasiliensis has been shown to be membrane bound (12), similar to that found in animals. The enzymatic activity of HMG-CoA reductase in the latex was first demonstrated and characterized by F. Lynen (13). Our investigation showed a temporal variation of the enzyme activities during a 24 hour cycle (4). The level of HMG-CoA reductase activity observed closely correlated to that of the

rubber content in latex (4), suggesting the importance of this enzyme in the regulation of rubber biosynthesis. In order to assess the significant regularly role of HMG-CoA reductase in rubber biosynthesis, purification of the enzyme is required for further characterization and refined studies.

In this study we presented methodology developed for solubilization and purification of Hevea HMG-CoA reductase. Activity levels of latex HMG-CoA reductase obtained from various rubber clones as well as the rubber yield were investigated. The effects of Ethrel treatment on the latex enzymic activity were studied and also reported in this study.

MATERIALS AND METHODS

Chemicals : Commercial sources of chemicals used were as follows: DL-3-^{14}C-HMG-CoA (New England Nuclear), DL-HMG-CoA, DTT, NADPH, polyoxyethylene either W-1 (Brix W-1) and mevalonolactone (Sigma); Affi-Gel-Blue (Bio-Rad); agarose-hexane-HMG-CoA type 5 (P-L Biochemicals) and DEAE-Sephadex A-50 (Pharmacia). All other reagents and biochemicals were analytical reagent grade.

Collection and Fractionation of Latex : The latex was obtained from several regularly tapped (S.2/D.2) Hevea brasiliensis trees of various clones as specified in the text (each approximately 17 years old). After tapping, the latex was collected for 15 min in an ice-chilled beaker. The pooled latex was fractionated by centrifugation at 49,000 g for 40 min. The pellets at the bottom of centrifuge tube, "bottom fraction", were separated and pooled. The bottom fractions were used for the assay of HMG-CoA reductase activities and enzyme purification. The bottom fraction was quite stable when stored frozen at -10°C.

Assay of HMG-CoA Reductase Activity : The HMG-CoA reductase activity of the bottom or the active protein fraction (0.1-1.8 mg) was assayed on 0.05 ml of buffer x (0.1 M KCl, 10 mM DTT, 5 mM EDTA, 0.1 M K_xPO_4, pH 7.0) containing BSA 0.1 mg, NADPH 0.4 umoles and DL-3-^{14}C HMG-CoA 120 nmoles (1000 dpm/nmole). The mixture was incubated at 37°C for 20 or 30 min as indicated. The reaction was stopped by the addition of 6 ul of 10 N HCl. Unlabelled mevalonolactone was then added, and the mixture was allowed to incubate for another 30 min for complete lactonization of the reaction product (mevalonic acid). The precipitated protein was removed by centrifugation. The product of the enzyme reaction, mevalonolactone, was analyzed by TLC using benzene/acetone (1:1, v/v) as a solvent and developed under iodine vapor according to the Iijima et al (14) method. The zone of the mevalonolactone spots was scraped into a scintillation vial containing 10 ml of dioxane flour. Samples were allowed to stabilize overnight before being counted in a Packard liquid scintillation counter.

Enzyme Solubilization and Fractionation : Frozen samples of bottom fraction were thawed at room temperature and homogenized in an equal

volume of solubilization buffer A (0.1 M K_xPO_4, pH 7.0, 10 mM DTT 0.1 M KCl, 5 mM EDTA) which contained 1% Brij W-1 and 20% glycerol. The detergent Brij W-1 was used in the solubilization of the enzyme according to the methods described by Bach et al. (15) A glass Potter Elevehjem homogenizer with a tight-fitting glass pestle was used in homogenization. After 10 downward passes of the hand-driven pestle, the suspension was incubated at 37°C for 30 min. The mixture was then rehomogenized and centrifuged at 190,000 g for 60 min at 4°C. The resulting supernatant which contained the solubilized enzyme was isolated. This was then fractionated by the slow addition of saturated ammonium sulfate solution to a final concentration of 40%. After incubation at 40°C temperature for 3-6 hours, the mixture was centrifuged at 25,000 g for 30 min. The yellowish uppermost protein layer was dissolved in buffer B containing 6% Brij W-1 and dialyzed against buffer B (50 mM K_xPO_4, 5 mM EDTA, 0.1% Brij W-1, 10 mM 2-Mecaptoethanol) before subjected to further purification by chromatography.

Chromatographic Procedures : The dialyzed sample in buffer B was slowly loaded onto a column (40 x 2.5 cm) of DEAE Sephadex A-50, previously equilibrated with buffer B. The column was washed with buffer B with a flow rate 0.5 ml/min. The enzyme protein was eluted by adding KCl into buffer B at a stepwise concentration of 50, 500 and 1000 mM respectively. The enzyme fractions were pooled and concentrated by ammonium sulfate precipitation (90% saturation). The protein pellets were collected by centrifugation at 25,000 xg for 30 min and dialyzed against buffer B. This active fraction was then loaded in a Affi-Gel-Blue (100-200 mesh; 75-150 u) column (1.5x5 cm), previously equilibrated with buffer B. The column was washed with buffer B before eluted with the same buffer by the addition of 500 mM and 1000 mM KCl respectively. Active fractions were pooled and loaded onto a 1 ml HMG-CoA-hexane agarose column, previously equilibrated with buffer B. The enzyme activity was eluted by adding 100 mM KCl, 100 mM KCl and 5 mM NADPH mixtures, 750 mM KCl and 1000 mM KCl, respectively.

Gel Electrophoresis : Polyacrylamide gel electrophoresis (PAGE) was run either in the absence or presence of sodium dodecyl sulfate (SDS) using the Weber et al. (16) method. The Wray et al. (17) method was used for silver staining.

Protein Determination : The protein concentration was determined by the Lowry et al. (18) method.

RESULTS AND DISCUSSION

Preparation of Soluble HMG-CoA Reductase : Various attempts to solubilize HMG-CoA reductase from the bottom fraction of fresh latex were carried out using different detergent compounds. Efforts to solubilize the enzyme with Triton x-100, Brij 35 and Tween 80 were unsatisfactory. However, we found that maximum activity can be obtained in soluble forms by using polyethylene ether W-1, similar to those reported for etiolated radish seedlings by Bach et al. (15). After the solubilization process, all enzyme activity was present in the soluble or supernatant fractions. The enzyme activity remained stable in the solubilization medium.

Purification of Solubilized Reductase Enzyme : The concentration and purification of HMG-CoA reductase was achieved by ammonium sulfate fractionation and affinity chromatography. Affi-Gel-Blue, having a bio-specific affinity for nucleotide-requiring enzymes, and agarose-hexane-HMG-CoA were used in the final affinity chromatography steps. Data for typical preparation and purification are presented in Table I. The specific activity of the reductase enzyme solubilized from a freshly prepared bottom fraction ranges between 0.4 to 0.7 nmole $min^{-1}mg^{-1}$. Solubilization leads to a 1.78 fold purification so that the specific activity of the enzyme in the soluble supernatant after centrifugation is around 0.7 nmole $min^{-1}mg^{-1}$. Further purification was achieved by fractionating the supernatant with the addition of saturated ammonium sulfate solution to achieve a final concentration of 40%. The uppermost yellowish enzyme protein layer was collected by centrifugation at 25,000g for 20 min. The ammonium sulfate fractionated enzyme resulted in about a five-fold purification. This was further purified by chromatography in a DEAE-Sephadex A-50 Column. The elution profile obtained is shown in Fig. 1. The reductase activity was eluted from column with buffer B containing 0.5 M KCl. The pooled fraction contained approximately 85% of added activity and gave a 22-fold increase in specific activity. Further purification on Affi-Gel Blue (Fig. 2.) and HMG-CoA-hexane agarose columns (Fig. 3) led to purification and 77- and 257- folds respectively. Polyacrylamide gel electrophoresis (in the presence of SDS) of 10 ug of protein from the purified enzyme was carried out. The enzyme with a specific activity of 105 nmol $min^{-1}mg^{-1}$ applied to the gel exhibited one single major protein band (Fig. 4). The calibrate subunit molecular weight or Hevea HMG-CoA reductase was approximately 43,000 daltons (Fig. 5). The subunit molecular weight of HMG-CoA reductase from Hevea was similar to that reported for radish seedlings (15) and considerably higher than that of rat livers (19,20). The purified enzyme showed a single prominent band with one faint minor band in the nondenaturing gel electrophoresis as shown by the silver staining procedure. Investigation of further experiments for HMG-CoA reductase molecular weight determination and enzyme kinetics are in progress.

Comparison Studies on Activity Levels of Latex HMG-CoA Reductase from Various Clonal Types : It was earlier shown that HMG-CoA reductase activity varies diurnally and its activity was closely associated with dry rubber content in the latex (4). Our comparison studies on the extent of variations in reductase enzyme activity among various rubber clones is shown in Fig. 6. It is interesting to note that different clonal types gave different specific activities. High specific activity levels were obtained from the clones with higher rubber yield (i.e. KRS 208, KRS 209 and RRIM 600 etc.). The high correspondent correlation was shown to be statistically significant.

Effect of Yield Stimulant Ethrel on the Level of HMG-CoA Reduc- tase Activity : Ethrel is known to prolong the flow time of latex with- out increasing the dry rubber content. Our results reported in Table II show that there was apparently no increase on HMG-CoA reductase activity in latex obtained from rubber trees tapped after a week of Ethrel application as compared to the untreated control. This evidence suggests that

the HMG-CoA reductase activity level was not affected by ethylene released from Ethrel. Higher total rubber yield on Ethrel treated trees was probably due to the lengthening of flow time by expansion of the rubber drainage area below the tapping cuts and not due to an increased rate of rubber biosynthesis. Further investigations on molecular actions of the yield stimulant on rubber biosynthesis enzymes are in progress.

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Table 1 Purification Protocol of *Hevea* HMG-CoA Reductase

Fraction/step	Total volume (ml)	Total protein (mg)	Total activity (nmoles MVA formed /min)	Specific activity (nmoles MVA formed /min.mg)	yield (%)	purification (fold)
Bottom fraction in Buffer B	80	848	347.7	0.41	100	1
+ Brij w-1 (1.1)	100	866.7	449.5	0.52	129.3	1.3
+ Glycerol (20%)	95	570	404.7	0.71	116.4	1.78
190,000 x g supernatant	20.5	136.4	267.4	1.96	76.9	4.8
0-40% $(NH_4)_2SO_4$	109	24.8	227.4	9.19	65.4	22.4
DEAE-Sephadex	6	7.3	88.6	12.13	25.5	29.6
0.90% $(NH_4)_2SO_4$	10	1.5	47.3	31.95	13.6	77.9
Affi-Gel Blue	4	0.37	39.1	105.65	11.3	257.7
HMG-CoA-hexane-agarose						

Table II Effects of the Yield Stimulant Ethrel on the Level of Latex HMG-CoA Reductase*

Clonal types	Ethrel treatment	HMG-CoA reductase activity nmoles MVA formed/min.mg
RRIM 600	-	7.53
RRIM 600	+	5.41
GT 1	-	1.20
GT 1	+	1.12

* Bottom fraction protein used for each incubation ranged from 0.4-0.6 mg.

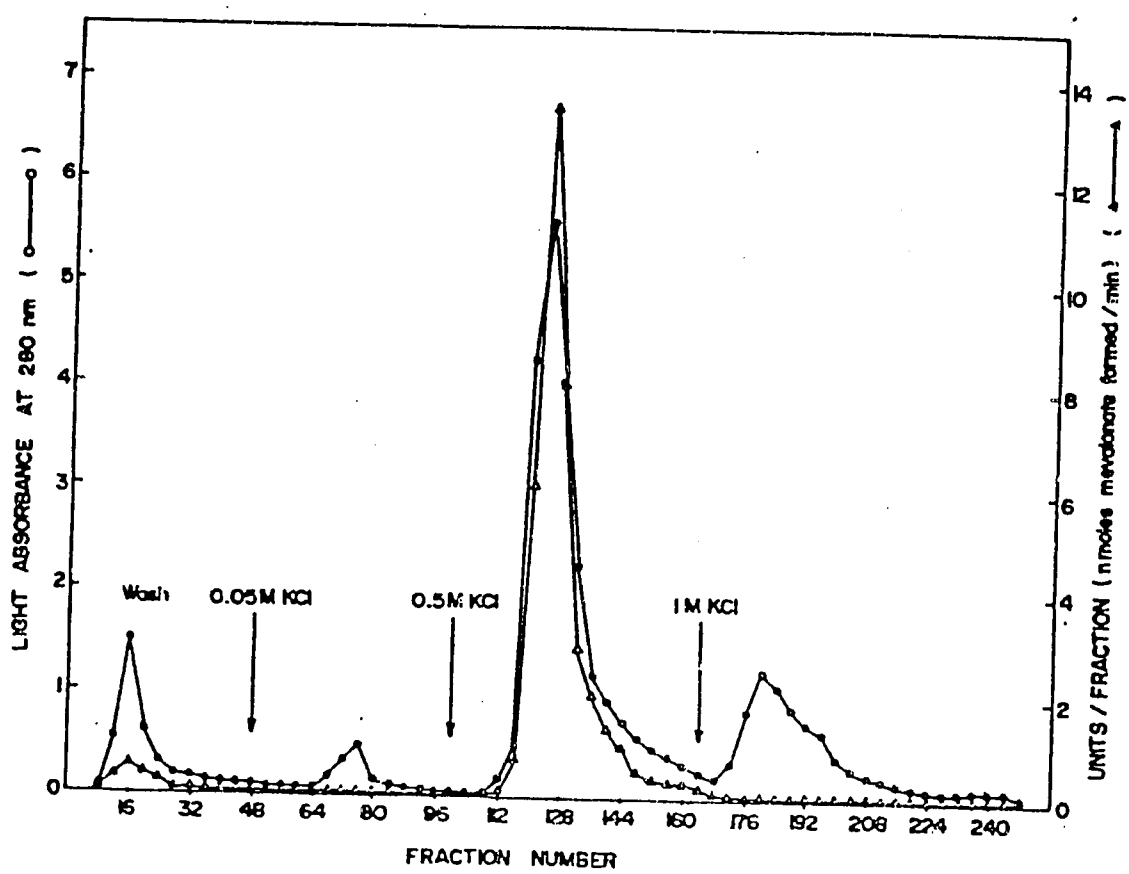

Figure 1 Purification of Solubilized *Hevea* HMG-CoA reductase from DEAE-Sephadex A 50. The position of arrows indicates the changes of salt concentrations.

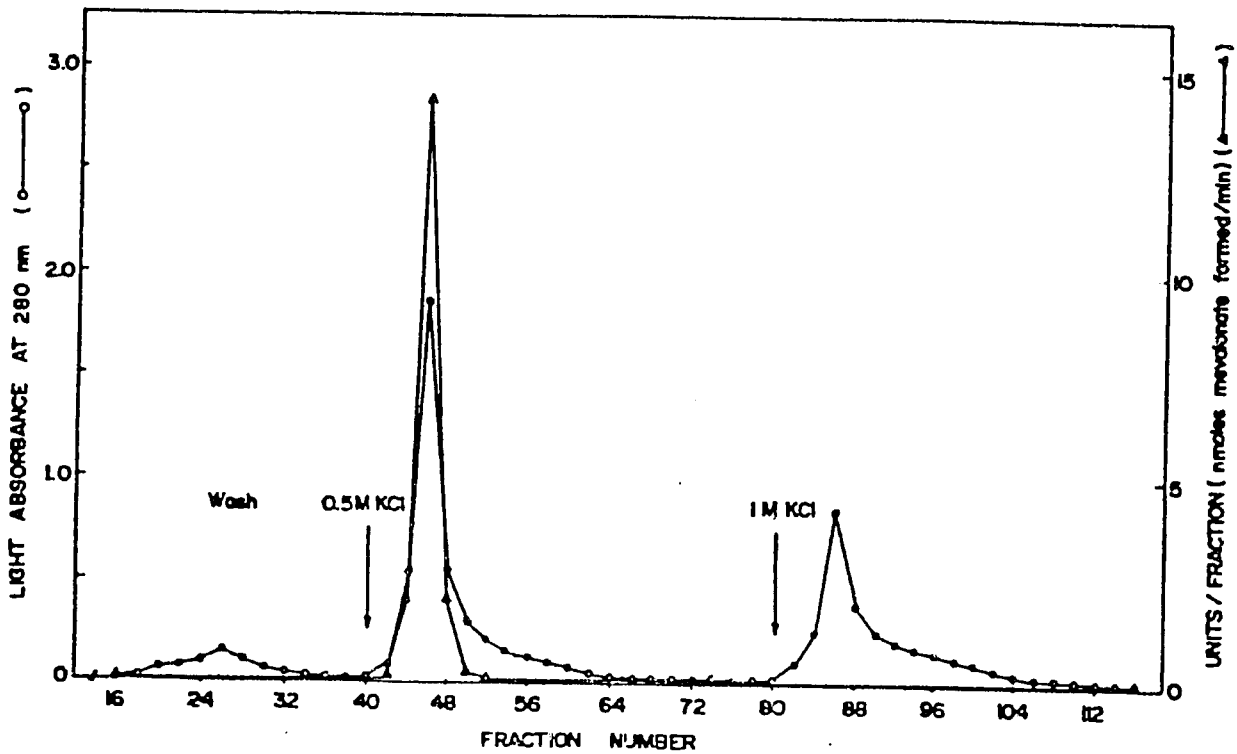

Figure 2 Purification of Active Fractions Obtained from the DEAE-Sephadex Column on Affi-Gel Blue Column. The position of arrows indicates the changes of salt concentrations.

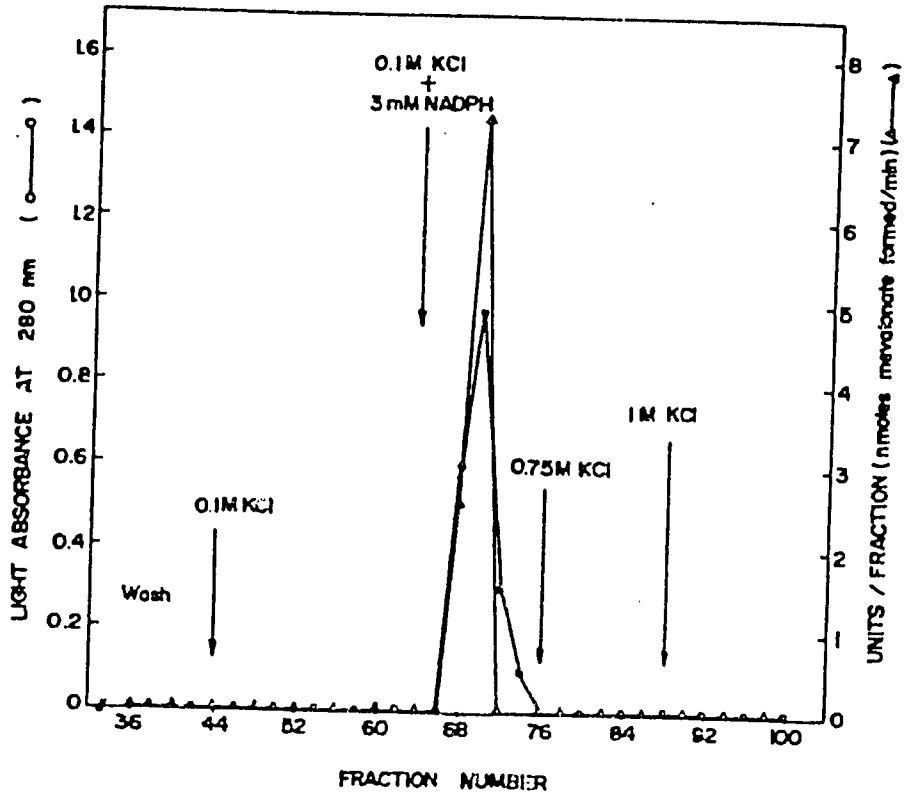

Figure 3 Purification of *Hevea* HMG-CoA Reductase on Affinity Chromatography Column Using HMG-CoA-Hexane-Agarose. Arrows indicate changes of salt concentrations with or without NADPH addition.

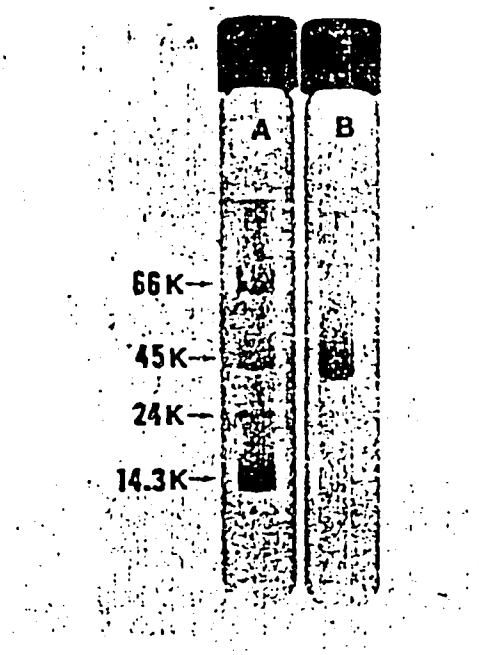

Figure 4 SDS-PAGE of Purified *L. pea* HMG-CoA Reductase (gel B). The relative positions of protein standards are indicated on the gel at left (gel A).

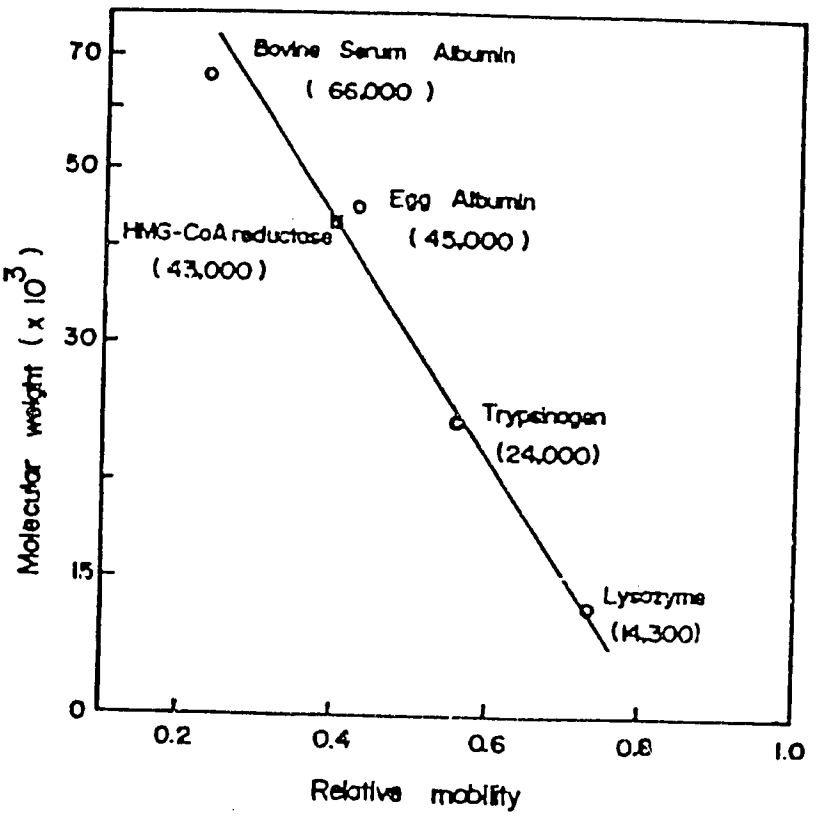

Figure 5 Molecular Weight Calibration of *Hevea* HMG-CoA Reductase Monomeric Polypeptide. Both purified enzyme and standard proteins were investigated on separate gels under 8% SDS-PAGE.

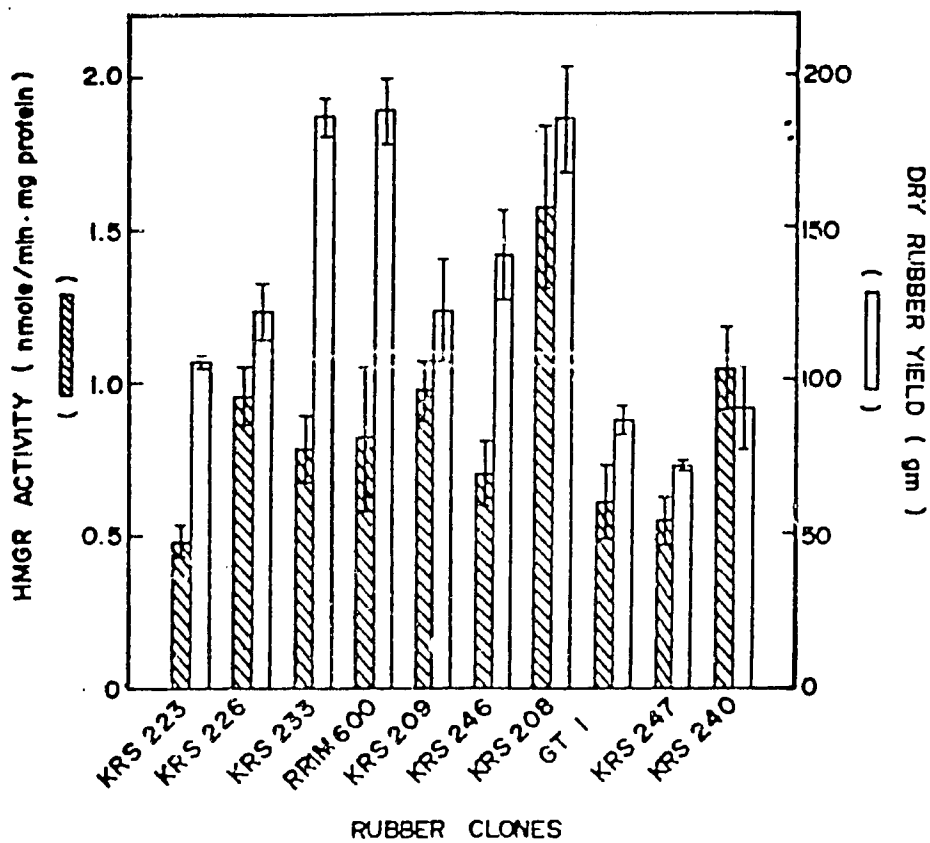

Figure 6 Correlation of Latex HMG-CoA Reductase-Specific Activity (▨) and Dry Rubber Yield (▭) Among TEn Rubber Clones. Each value is the mean I S.E. of results obtained from 3 experiments. Three trees were used per *Hevea* clonal type.

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IDENTIFICATION OF SURFACE ANTIGENS AND PRODUCTION OF MONOCLONAL ANTIBODIES IN *Schistosoma japonicum* (CHINESE & PHILIPPINE) (6.390)

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ABSTRACT

The patterns of ^{125}I-labelled surface proteins and immunoprecipitations by mouse antisera to *S. japonicum* (Chinese) (ISCH, to *S. japonicum* (Philippine) (ISPH), to *S. mansoni* (ISMA) and human immune serum to *S. japonicum* (Philippine) (IHPH) are identical for adults of both strains of *S. japonicum*. The ^{125}I-labelled surface proteins consist of 18 bands, with the heaviest labelled at bands 180,000; 158,000; 128,000 and 15,000. Immunoprecipitations by ISCH, ISPH, AND IHPH result in similar patterns. The most intense band that stands out in contrast to others is 128,000, which is not precipitated by ISMA, a mouse antiserum against *S. mekongi* (ISME), or mouse antiserum against *S. hematobium* (ISHE); thus it is the most unique and specific antigen detected only in *S. japonicum*. Other bands with moderate precipitation against ISCH, ISPH and IHPH are 205,000; 180,000; 158,000; 45,000; 38,000; 34,000; 30,000; 26,000 and 23,000. It should be noted that mouse and human antisera produce identical patterns and that these bands show slight to moderate cross-reactions with ISMA. Bangkok 150,000 is probably non-specifically precipitated, since it is always intense against all antisera as well as non-immune serum. In addition, it was found that band 86,000 is specially and heavily precipitated against ISME and ISHE. The rest show as similar the light cross-reactions to ISME and ISHE as to ISMA. In conclusion, adults of *S. japonicum* (Chinese & Philippine) show a major and non-cross reacting surface antigen to ISMA, ISME and ISHE at 128,000 and 158,000, and a specifically crossreacting antigen to ISME and ISHE at 86,000. The rest of the surface proteins show light to moderate cross-reactions to ISMA, ISME and ISHE.

INTRODUCTION

Human schistosomiasis is still a serious public health problem in China, the Philippines, Indonesia and Indo-Chinese states that include parts of Laos, Cambodia and Vietnam[1-4]. A few sporadic cases of human schistosomiasis were reported in the northern, north-eastern parts of Thailand bordering Laos and Cambodia and in the southern part of Thailand[4], and Pahang and Perak states of Malaysia[5-7]. In Indonesia, schistosomiasis is endemic in the region around Lake Lindoe in central Sulawesi[8-9]. Two predominant species of schistosomes were found to be responsible for schistosomiasis in the Orient, namely, *Schistosoma japonicum* and *Schistosoma mekongi*[3-4]. The former consists of various geographic strains; of which the Chinese and Philippine strains are

medically the most important because of their widespread occurrences. On the other hand, S. mekongi is responsible for schistosomiasis in the Lower Mekong Basin area that encompasses parts of Laos, Cambodia and north-eastern Thailand.

One of the main strategies for control of schistosomiasis is laboratory study that will ultimately lead to the production of vaccines, that can be conveniently administered to the population at risk. The rationale for the development of vaccines is based on the findings that when human hosts receive primary infection, both humoral and cell-mediated immunities are induced. These concomitant immunities protect hosts against reinfecting schistosomulae, though adult worms from the primary infection evolve an evasion mechanism that makes them resistant to the hosts' immunities[10]. It has now been established both in vivo and in vitro that young schistosomulae are only susceptible to immune effector mechanism within the first few days of their transformation from cercariae[11,12]. The primary goal of using vaccines is, therefore, to induce immune mechanism in hosts that can kill the schistosomulae at this stage. It is amply demonstrated that the immune attack occurs on the surface of young parasites; and reciprocally, older parasites can evade host immune attacks through the modification of their outer membrane. Hence, biochemical and immunochemical characterization of parasite tegument, especially that of the outer membrane, is the key to understanding the interactions between the host immune system and the parasite, and the latter's evasion mechanism. In addition, the identification and characterization of major surface antigens that can elicit concomitant immunity in hosts is essential if some of them are to be selected for use in the new immunodiagnostic methods as well as for an eventual production of vaccine. Therefore, in this project our major objectives are as follows:

1. To identify and characterize antigens on the surface of the teguments of S. japonicum (Chinese & Philippine), and to study cross-reactions with surface antigens of other schistosome species in order to define major antigens that can illicit concomittant immunity in hosts;

2. To produce monoclonal antibodies (MAB)s against these surface antigens, so that MABs can be used to detect major surface antigens that confer protective immunity. Consequently, these antigens and MABs can be used for improvement of immunodiagnostic methods and possibly future production of vaccines.

In this report the first part of the results concerning the identification and characterization of major surface antigens of adult S. japonicum (Chinese & Philippine) are presented.

MATERIALS AND METHODS

Collection of adult schistosomes. S. japonicum (Chinese & Philippine) cycles were maintained in Oncomelania hupensis hupensis and Oncomelania hupensis quadrasi under laboratory conditions. Adult worms were obtained from Swiss albino mice, infected 8-week previously with

cercariae, by portal perfusion with 0.15 M sodium chloride and 0.05 M sodium citrate. The worms were washed 5 times with Minimum Essential Medium (MEM) (GIBCO) before being used.

Collection of antisera. Infected mouse antisera were obtained from Swiss albino mice, each infected with 30 cercariae for 8 weeks, by heart puncture. Animals were bled and the blood was allowed to clot at 25°C for 3 hours. The clots were removed and the antisera were centrifuged at 1000 x g for 5 minutes to obtain clear supernatants. Normal mouse serum was obtained in a similar manner from uninfected mice, and the normal human serum was obtained from volunteers. In addition, human antiserum against S. japonicum (Philippine) was kindly provided by Professor E.G. Garcia of the Institute of Public Health, The University of Philippine, Manila, The Philippines.

Surface antigen labelling with ^{125}I. Adult male S. japonicum were radiolabelled by ^{125}I using the Iodogen method of Fraker & Speck, (1987)[13] by using 100 uCi $Na^{125}I$, 100 ug Iodogen (Piere Chemical Company) per 100 adult male parasites. The labelling time was 5 minutes and this was stopped and chased with 5 mM KI. Labelled parasites were rapidly frozen and thawed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA and 1 mM PMSF (TNEP buffer) in cycles using dry-ice 10 times.

Immunoprecipitation of ^{125}I surface antigens by immune sera. Immunoprecipitations were set up using labelled surface antigens from freeze-thawed fractions of tegument at 100 ul (10^6 ct/min) per aliquot, and these were clarified by incubation with 10 ul of normal serum. After 10 minutes the complexes were precipitated by the addition of 100 ul of 10% protein-A Sepharose and centrifuged at 2000 x g for 10 minutes at 4°C. Supernatant were incubated with species-specific immune sera as well as immune sera against other schistosoma for 30 minutes at 37°C, and kept further overnight (18 hrs at 4°C). The antigen-antibody complexes were then precipitated with protein-A sepharose. The immunoprecipitates were centrifuged at 2000 x g for 10 minutes at 4°C to obtain a pellet and then washed 3 times in phosphate buffer saline pH 7.4. Each of the pellets were then treated with 50 ul of sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS: 5% B-Mercaptoethanol and 10% Glycerol) and boiled for 2 minutes to complete the dissolution.

Electrophoresis and autoradiography. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli (1971)[14] method. Electrophoresis was performed on 12.5% gels. The voltage was set at 100 volts and gel run for 4.5 hrs. After drying, the gels were exposed to X-ray film (Kodak XO mat RP film) using intensifying screens. Autoradiographs were developed in Kodak GBX using developing time of 2 minutes.

EM autoradiography (EMARG) of labelled worms. In order to monitor the sites of ^{125}I labelling EMARG was performed. The worms were washed after ^{125}I labelling, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 18 hrs. at 4°C. After rinsing 3 times in cacodylate buffer, the worms were postfixed in 1% OsO_4 in cacodylate buffer for

2 hrs. and washed 3 times with a cacodylate buffer. Following washings, worms were dehydrated and embeded in Araldite 502. About 300-500 A thin sections were cut with Proter-Plum MT-2 ultramicrotome and coated with Ilford L-4 emulsion maintained at 45°C and diluted 1:1 (V/V) with H_2O. Autoradiographs were exposed, developed in undiluted Dektol developer (Eastman Kodak Company) for 2 mins., fixed for 10 mins., washed in water, dried and stained with lead citrate and uranyl acetate.

RESULTS AND DISCUSSION

Identification and Comparison of Immunogenic Surface Proteins and Glycoproteins of Adult S. mansoni, S. japonicum (Chinese & Philippine) by Immunoprecipitation (Tables 1 to 3; Plates 1-3)

Immunoprecipitation of surface antigens from S. mansoni (Table 1; Plate 2 A, B)

For comparison with oriental schistosomes, we have watched adult S. mansoni under similar conditions. Our results indicate that the ^{125}I-labelled surface proteins of adult S. mansoni consisted of 15 bands, with the most intense bands of M.W. 26,500; 29,500 and 15,000, and moderately intense ones 64,000; 58,000 and 42,500. Fourteen bands were precipitated by mouse immune serum against S. mansoni (ISMA), with the heaviest precipitation at 15,000, 26,500 and 29,500. Mouse immune sera against S. japonicum (Chinese & Philippine - ISCH & ISPH), against S. mekongi (ISME), and against S. haematobium (ISHE) all showed cross-reactions with S. mansoni antigens (MA), with heaviest precipitations at 15,000, 26,500 and 29,500, respectively. However, we believe that band 15,000 is non-specific, since it was precipitated by all immune sera, as well as non-immune serum. This also occurs in other parasites, and it is possible that this band represented small molecular weight, fast moving lipoproteins or glycolipids of the surface membrane.

The surface antigens of S. mansoni have been characterized previously by many groups of investigators, with slightly varied results. By using radio-labelling with ^{125}I or ^{3}H and one-dimension SDS gel electrophoresis, differences in the number and exact molecular weights have been reported; however, most investigators generally agree that there are about 7 to 15 bands of surface proteins and glycoproteins, ranging in molecular weights from 12,000 to 200,000 daltons. Most labelled proteins and glycoproteins could be immunoprecipitated with antisera from patients and infected animals; the reported molecular weights of major precipitated protein and glycoprotein antigens vary considerably[15-25]. Taylor et al.[19], utilizing iodosulphanilic acid to label schistosomulae, could precipitate polypeptides with MW 185,00; 68,000 and 24,000 daltons with infected human sera. Dissous et al.[20], on the other hand, could immunoprecipitate polypeptides with MW 100,000; 40,000; 37,000 and 32,000 and 20,000 - 25,000 with immuned rat and human sera, following the ^{125}I labeling of schistosomula surface antigens by the lactoperoxidase method. Using similar techniques, Simpson et al.[24], showed that there are seven major proteins with MW 94,000, 68,000, 45,000, 40,000 to 32,000, 22,000 and 16,000 on the surface of

3-hour schistosomulae. On the other hand, Samuelson & Caulfield[23] using galactose oxidase/Na B^3H_4 revealed 12 bands of major surface glycoproteins and 7 glycolipids on newly transformed schistosomulae. By contrast, Strand et al.[22] could immunoprecipitate up to 15 bands of glycoproteins (MW 200,000 to 12,000) isolated from the surfaces of cercariae and adult parasites by ConA-affinity chromatography. Indeed, the number of immunoprecipitable surface proteins and glycoproteins in schistosomulae can be as high as 35 bands if resolved by two-demensional gel electrophoresis 22. In general, it was agreed that, despite the difference in morphology of various developmental stages from cercaria to adult, it was found that the surface proteins of cercariae and various stages schistosomulae and even adults also showed marked similarity[15,23,26,29].

Furthermore, despite the variations in the number of bands and their molecular weights, recent studies revealed that schistosomulae, lung-stage and adult worms share some common surface antigens which may become "hidden" in the latter[28,29]. Dissous & Capron (1983)[28] demonstrated a common antigenic determinant shared by surface molecules with MW 38,000 daltons on newly transformed schistosomulae and 115,000 daltons on adult parasites. Payares et al. (1985)[29] also showed that schistosomulae, lung stage and adult worms bear prominent common surface antigens with MW 32,000 to 38,000 and 20,000 daltons, whereas an antigen with MW 25,000 daltons was found only in lung stage and adult worms. Furthermore, it was shown in these studies that antisera against the purified membrane of adult parasites and monoclonal antibodies raised against adult antigens can readily kill schistosomulae *in vitro*. This evidence strongly supported the concept of "concomitant immunity", and demonstrated the possibility that certain surface antigens may be selected as potential candidates for the ultimate production of vaccines.

Immunoprecipitation of surface antigens from adult *S. japonicum* (Chinese & Philippine) (Table 2 & 3; Plates 1,1 C, D & 3 A, B).

The results obtained from the EMARG indicate that over 95% of positive silver grains occurs on the membrane covering ridges or pits of the parasites' tegument (Pl. 1); therefore, it is safe to assume that the labelling by ^{125}I occurs, primarily on the proteins associated with the surface membrane of the parasites' tegument. The patterns of ^{125}Ilabelled surface proteins and immunoprecipitations by ISCH, ISPH, ISMA and human immune serum against *S. japonicum* (Philippine) (IHPH) are identical for both strains. The ^{125}I-labelled surface proteins consist of 18 bands, with the heaviest labelling at bands 180,000; 158,000; 128,000 and 15,000. Immunoprecipitations by ISCH, ISPH, IHPH result in similar patterns. The most intense band that stands out in contrast to others is 128,000 which is not precipitated by ISMA, ISME, or ISHE: thus it is the most unique and specific antigen detected only in *S. japonicum*. Other bands with moderate precipitation to ISCH, ISPH and IHPH are 205,000; 180,000; 158,000; 45,000; 38,000; 34,000; 30,000; 26,000 and 23,000. It should be noted that mouse and human antisera produce identical patterns and that these bands show slight to moderate cross-reactions with ISMA. Like *S. mansoni* band 15,000 is probably non-specifically precipitated, since it is always intense to all antisera as well

as non-immune serum. In addition, it was found that band 86,000 is specifically and heavily precipitated to ISME and ISHE. The rest show similar and light cross reactions to ISME and ISHE as to ISMA.

In conclusion, adults of S. japonicum (Chinese & Philippine) show major and non-cross reacting surface antigens to ISME, ISME and ISHE at 128,000 and 158,000, and specifically cross-reacting antigen against ISME and ISHE at 86,000. The rest of the surface proteins show light to moderate cross-reactions to ISMA, ISME and ISHE.

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Table 1 Immunoprecipitations of ^{125}I-labelled Surface Proteins of Adult Male *S. mansoni* by Mouse Antisera to *S. mansoni* (ISMA), *S. japonicum* (Chinese & Philippine -ISCH & ISPH), *S. mekongi* (ISME) and *S. hematobium* (ISHE), and Comparisons to the Resulted on Adult *S. mansoni* as Reported by Shah and Ramasamy (1982)[15] and Hayunga (1979 a, b)[16,17].

(Refer to Plate 2 A & B)

Immunoprecipitations of adult *S. mansoni* ^{125}I-labelled Surface proteins as reported by		^{125}I labelled surface proteins of *S. mansoni*	Immunoprecipitations of adult male *S. mansoni* ^{125}I-labelled surface proteins by				
Shah & Ramasamy (1982)* (MW X 10^{-3})	Hayunga et al 1979 (ab)** (MW X 10^{-3})	(MW X 10^{-3})	ISMA	ISCH	ISPH	ISME	ISHE
			(ME X 10^{-3})				
-	-	> 205 (+)	++	+	+	+	+
> 150	-	160 (+)	++	+	+	+	+
102	100	105 (+)	++	+	+	+	+
78	78	80 (+)	+	-	-	-	-
-	68	64 (++)	++	+	+	+	+
59	60	58 (++)	++	-	-	-	-
-	-	50 (+)	-	-	-	-	-
45	43	42.5(++)	++	+	+	+	+
-	36	37 (+)	+	-	-	-	-
35	-	34 (+)	+	+	+	+	+
-	30	29.5(++)	++	++	++	++	++
-	26	26.5(+++)	+++	++	++	++	++
22	20	22.5	+	+	+	+	+
-	-	19 (++)	++	+	+	+	+
-	12	< 15 (+++)	+++	+++	+++	+++	+++
		(15 bands)					

"+" sign indicates relative intensity of the protein bands; "+" = light; "++" = moderate; "+++" = intense

* Shah, J. & Ramasamy, K. (1982) Int. J. Parasitol. 12, 451-461. Hayunga, E.G., Murrell, K.D., Taylor, D.W. & Vannier, W.E. (1979 a) J. Parasitol., 65, 488-496.

** Hayunga, E.G., Murrell, K.D., Taylor, D,W. & Vannier, W.E. (1979 b) J. Parasitol., 65, 497-506.

Table 2 Immunoprecipitations of ^{125}I-labelled Surface Proteins of Adult Male *S. japonicum* (Chinese by Mouse Antisera to *S. japonicum* (Chinese & Philippine - ISCH & ISPH), Human Antisera to *S. japonicum* (Philippine - IHPH)*, and Mouse Antisera to *S. mekongi* (ISME), *S. mansoni* (ISMA) and *S. hematobium* (ISHE). All samples were pre-absorbed with normal mouse or normal human sera.

(Refer to Plate 2 C & D)

^{125}I Labelled Surface Proteins	Immunoprecipitated by ISCH,ISPH,IHPH	Immunoprecipitated by ISME, ISHE	Immunoprecipitated by ISMA
(MW X 10^{-3})	(MW X 10^{-3})	(MW X 10^{-3})	(MW X 10^{-3})
205 (+)	++	+	+
180 (++)	++	+	+
158 (++)	++	-	-
128 (++)	+++	-	-
114 (+)	+	+	+
86 (+)	+	+	-
68 (+)	+	+	+
62 (+)	+	+	+
56 (+)	-	-	-
48 (+)	-	-	-
45 (+)	++	+	+
38 (+)	++	+	+
34 (+)	++	+	+
30 (+)	++	+	+
26 (+)	+	+	+
23 (+)	+	+	+
19 (+)	+	+	+
15 (+++)	+++	+++	+++
(18 bands)			

"+" sign indicates relative intensity; "+" = light, "+" = moderate; "+++" = intense.

* IHPH - kindly supplied by Prof. E. Garcia, Institute of Public Health, University of the Philippine System, Manila.

Table 3 Immunoprecipitations of ^{125}I-labelled Surface Proteins of Adult Male *S. japonicum* (Philippine) by Mouse Antisera to *S. japonicum* (Chinese & Philippine - ISCH & ISPH), Human Antisera to *S. japonicum* (Philippine - IHPH), and Mouse Antisera to *S. mansoni* (ISMA). All samples were pre-absorbed with normal mouse or normal human sera.

(Refer to Plate 3 B)

^{125}I Labelled Surface Proteins (MW X 10^{-3})	Immunoprecipitated by ISCH,ISPH,IHPH (MW X 10^{-3})	Immunoprecipitated by ISMA (MW X 10^{-3})
205 (++)	++	++
180 (++)	++	++
158 (++)	+	-
128 (++)	+++	-
114 (+)	+	+
86 (+)	+	+
68 (+)	-	-
62 (+)	+	+
56 (+)	-	-
48 (+)	-	-
45 (+)	++	++
38 (+)	++	++
34 (+)	++	+
30 (+)	++	+
26 (+)	++	+
23 (+)	++	+
19 (+)	-	-
15 (+++)	+++	+++
(18 bands)		

Plate 1 Electron microscopic autoradiography (EM-ARG) to detect the ^{125}I-labelling sites on the tegument of S. japonicum (Chinese & Philippine) following iodination by the iodogen method.

A & B) EM-ARG of the tegument of an adult male S. japoncium (Chinese).

Notice that most silver grains (arrow heads) lie either just outside or on the membrane of pits (Pt) and ridges (Ri). X 20,000.

Plate 2 Immunoprecipitation patterns of ^{125}I-labelled surface proteins from adult male S. mansoni (MA), S. japonicum (Chinese - CH) by mouse immune sera against S. mansoni (ISMA), S. japonicum (Chinese & Philippine - ISCH & ISPH), S. hematobium (ISHE), S. mekongi (ISME), and human immune sera against S. japonicum (Philippine - IHPH). The gels used in all cases were 12.5% SDS-PAGE, and all samples were preabsorbed with non-immune mouse serum (NMS).

A & B) duplicate experiments on S. mansoni

C & D) duplicate experiments on S. japonicum (Chinese), in "D" different lots of mouse antisera (1, 2, 3, 4) were used.

Plate 3 Immunoprecipitations of ^{125}I-labelled surface proteins from adult male S. japonicum (Philippine) (PH) and S. mekongi (ME) by ISMA, ISCH, ISPH, ISME, ISHE and IHPH. All samples were preabsorbed with normal mouse serum (NMS), and the gels used were 12.5% SDS-PAGE.

A) S. japonicum (Chinese) - for comparison

B) S. japonicum (Philippine)

C) S. mekongi - for comparison

Plate 1

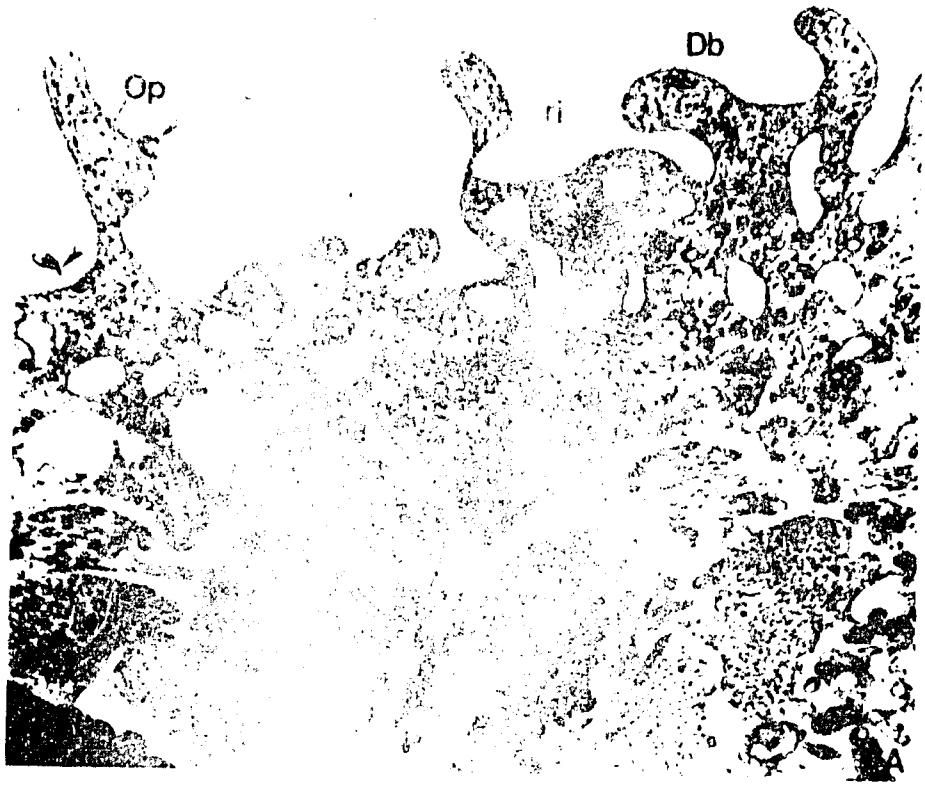
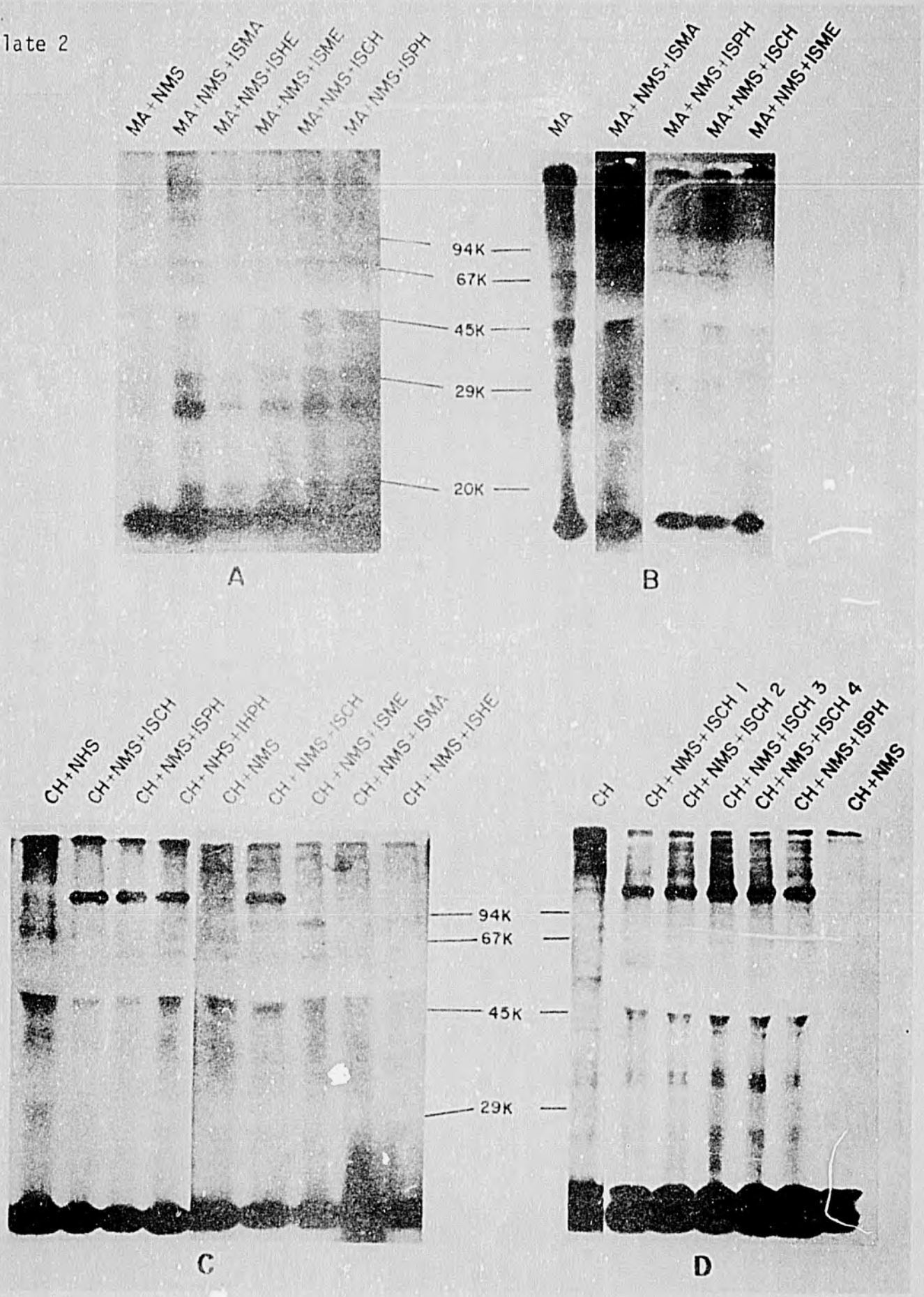


Plate 2


MA+NMS
MA+NMS+ISMA
MA+NMS+ISHE
MA+NMS+ISME
MA+NMS+ISCH
MA+NMS+ISPH
94K
67K
45K
29K
20K
A
MA
MA+NMS+ISMA
MA+NMS+ISPH
MA+NMS+ISCH
MA+NMS+ISME
B
CH+NHS
CH+NMS+ISCH
CH+NMS+ISPH
CH+NHS+IHPH
CH+NMS
CH+NMS+ISCH
CH+NMS+ISME
CH+NMS+ISMA
CH+NMS+ISHE
94K
67K
45K
29K
C
CH
CH+NMS+ISCH 1
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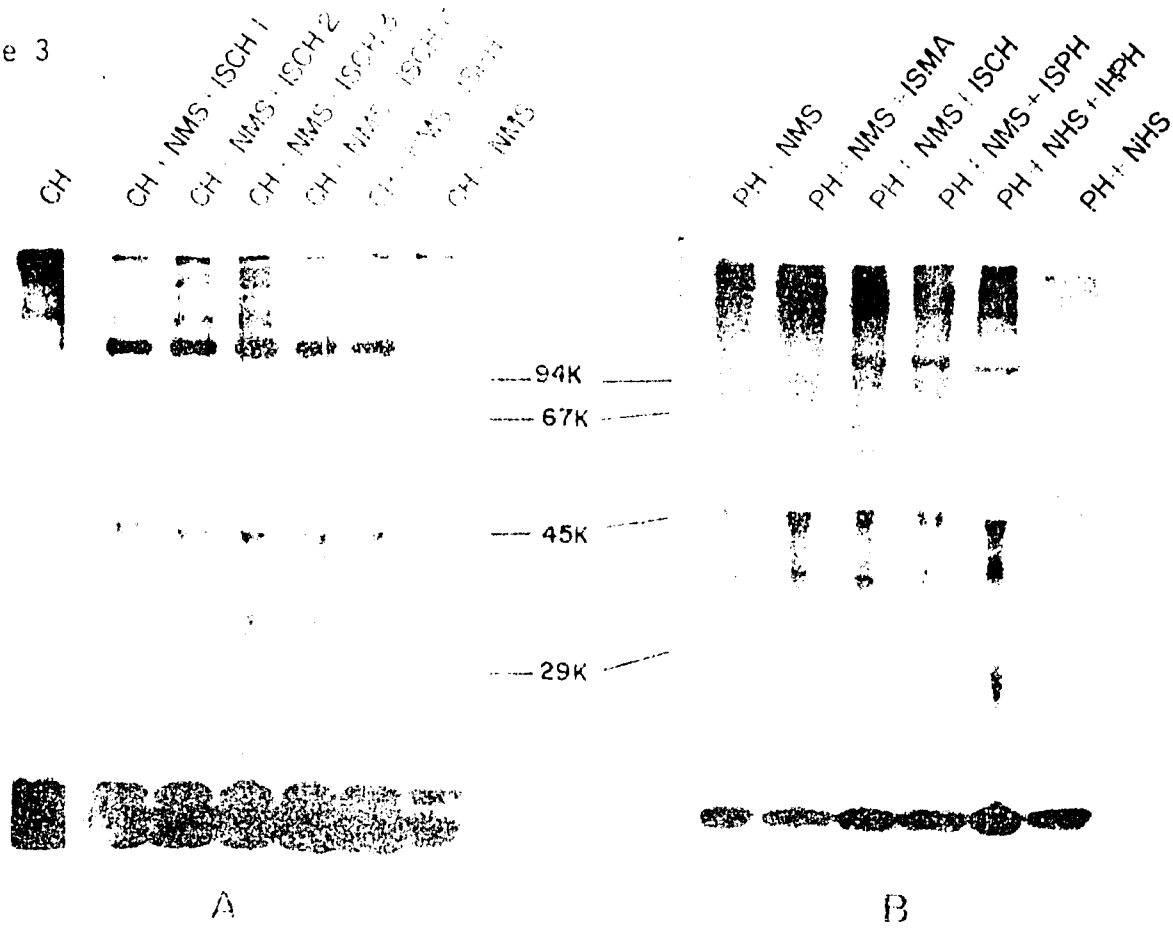
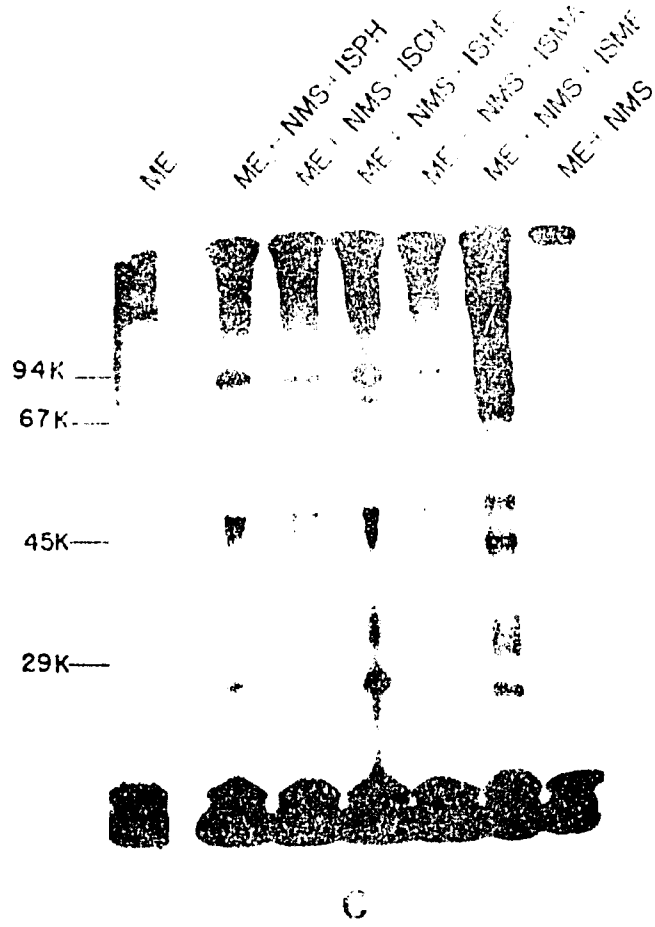
CH+NMS+ISPH
CH+NMS
D

Plate 3


CH
CH + NMS + ISCH 1
CH + NMS + ISCH 2
CH + NMS
PH + NMS
PH + NMS + ISMA
PH + NMS + ISCH
PH + NMS + ISPH
PH + NHS + IHPH
PH + NHS
94K
67K
45K
29K
A
B


ME
ME + NMS + ISPH
ME + NMS + ISCH
ME + NMS
94K
67K
45K
29K
C

PART II

U.S.-ISRAEL COOPERATIVE DEVELOPMENT RESEARCH PROGRAM

(CDR)

250a

FY 1985

ENHANCING GENETICS VARIABILITY AND BREEDING IMPROVED SESAME (S. INDICUM) CULTIVARS (C5-147)

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ABSTRACT

Thailand - Israel cooperative research project on sesame breeding for a high yield and desirable characteristics has been conducted since 1985. A collection of sesame lines and cultivars were studied and crosses were made from selected parents in both Thailand and Israel. Combining determinate mutants and non-shattering characteristics to the adapted high yielding types, the selection was made from segregating populations by the plant to row method. F4 progenies are being grown now at U-bol Field Crop Research Center in 1987 for further selection. Cultivars with high yield, determinate and non-shattering characteristics are desirable.

INTRODUCTION

Sesame is one of the world's most important oil crops. It is grown mostly in developing countries and by small farmers. Sesame seed is rich in oil content (50%) of excellent quality and protein (25%) of sulfur-containing amino acids. Sesame meal has been used for animal feed and defatted flour can be used for human consumption.

Sesame has important agricultural advantages: it produces set seeds and large yields under relatively high temperatures, and it can store soil moisture without rain or irrigation. It can be rotated with other main crops in areas and the seasons when other crops cannot be grown.

However, very little research in the past has been devoted to sesame. Limited research has been done in less developed countries where funds and scientists are scarce. No International Research Center has sesame as its mandated crop. Furthermore, sesame research in developing countries can not benefit from research done in developed countries, as has been the case in many other crops, because sesame is a very minor crop or is not grown in developed countries. Therefore, mean yields of sesame are very low although potentially they could be much higher. Various factors are responsible for the low yield, such as erratic rainfall, low input levels, uneven ripening of the capsules, dehiscence and seed shattering, diseases and insects.

Yield components of sesame consist of many characteristics of the sesame crop itself and its related functions that contribute to seed yield. All of these characteristics must be improved by plant breeding

either singly or in combinations. The plant characteristics are as follows:

- Height
- numbers of days to first flowering
- numbers of days to 50% flowering
- number of branches per plant
- length of branches bearing capsules
- number of capsules per node
- number of capsules per branch and per plant
- number of seeds per capsules
- seed size (weight per 1000 seeds)
- seed weight or seed yield per plant
- number of plants per unit area

Due to the several components and their variations, this sesame breeding will concentrate on only a few characteristics that mainly contribute to yield and economical value. They are as follows:

- Seed size and seed color
- Non-shattering or semi-shattering
- Determinate
- Upright growth, no lodging
- Early ripening
- High oil and protein content
- Disease resistance
- Drought resistance

Although sesame has been grown in Thailand for a very long time, planted areas and production of sesame have not been increased during the last ten years. Thai sesame farmers are small holders who farm in rainfed and drought-prone land with low-fertility soil. Annual planted area is about 35,000 to 46,000 ha while production ranges from about 21,000 to 30,000 tons. The average yield per hectare is relatively low, about 600-700 kg/ha.

Sesame is a versatile crop which can be grown in many parts of the country. Sesame production areas in Thailand are in the North, Northeast and the Central Regions.

Objectives of this project are to create widely based segregating populations of sesame for selection and breeding of improved cultivars for Thailand and Israel, which will be determinate and nonshattering. The determinate type of sesame will mature within a short time span with non-shattering capsules, thus facilitating harvest with minimum seed losses and shorter growing season.

MATERIALS AND METHOD

At the present, all sesame cultivars are indeterminate, i.e. the plants produce leaves flowers and capsules continuously as long as there is moisture available in the soil. At harvest time, the capsules on the harvested branches will be over mature, mature and immature. This makes

seed losses unavoidable. A determinate genotype, discovered by Dr. Ashri, will reduce seed losses and together with non-shattering or semi-shattering seeds hence it will minimize seed losses to acceptable levels.

Most of effort in this project is concentrated on breeding determi- nate cultivars adapted to various growing conditions in Thailand and Israel using the determine mutant induced by Dr. Ashri (1, 2) and the local sesame cultivars of desirable types.

Materials in this project are as follows.

1. Collection of cultivars from land varieties and introduced cultivars and lines from foreign countries including those determinate mutants and non-shattering lines.

2. Early generation crosses made from hybridization of early and high yielding genotypes with semi-shattering genotypes from collection and single seed descent and plant to row method of selection were used in order to select genotypes which are early, high-yielding, and non-shattering.

3. Early generation crosses made from diallele and reciprocal crosses of seven selected large seed and high yielding cultivars, (Roi-et 1, MKS-1-81111, Local white Chaibadan, Local Black Nakorn Sawan, Terras 77, Shang Cai Purple Flowers, and ICB-30-P4-(M3)). Again single seed descent and plant to row method were used to select large seed high yielding cultivars.

4. 57 second generation of segregating lines were produced from crosses of high yielding cultivars and determinate mutants in Israel. Single seed descent was used in F3 and plant to row selection was employed in F4.

This cooperative breeding project made it possible for Thai sesame breeders to obtain seeds of cultivars of segregated populations and advanced selected lines of genotypes adapted to Thai conditions and needs. The project also enabled us to speed up the program by shifting and shar- ing the breeding materials between the two countries.

The project was planned for 3 years of work in both Israel and Thai- land (June 1985 - June 1988).

RESULT AND DISCUSSION

Project in Israel started later than planned because Dr. Ashri, the Israeli principal investigator, was on a one year sabbatical leave. How- ever, the materials he developed earlier were available to start the pro- ject in Thailand without delay.

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At Ubol Field Crops Research Center, 243 lines collected locally and from foreign sources were planted in September 1985 and

harvested in December 1985. The number of days to first flowering, days to 50% flowering, and days to harvest, seed yield and seed weight were recorded. Twenty lines of high seed yield and other desirable characteristics were selected for further study. Some lines in the study were non-shattering but seed yields were extremely low.

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1. Approximately half of the whole collections of sesame lines (318 lines) were planted in the early and late rainy seasons in order to study in detail all 33 characteristics according to the IBPGR description for both seasons and the plant's interaction to seasonal variations.

In the early rainy season of 1986, some results were as follows.

- maturity date 83 - 124 days
  83 - 90 days - 113 lines

- Seed yield 1.6 - 253.0 kg/rai
  101 - 140 kg/rai - 19 lines
  more than 141 kg/rai - 2 lines.

- Seed weight 1.360 - 4.763 gm/1000 seeds
  3.5 gm/1000 seed - 19 lines

In the late rainy season, little change was observed in the maturity date. Seed yield, seed weight and number of capsules per plant were significantly lower than the early crops. All lines in both seasons were indeterminate and shattering. They were non-branched, basal branched
and top branched.

2. Hybridization Program.

2.1 Twenty-three crosses of several high yielding large seeded varieties were made in the early seasons of 1986. F1 seed were planted in the late season at Ubol Field Crops Research Center and F3 seed were planted at Supanburi Field Crops Research Center in the dry season with irrigation. F4 seeds harvested from F3 were supposed to be planted at Ubol Field Crops Research Center in June. However, it was postponed to late season due to lack of rain in early season 1987.

2.2 The hybridization program was conducted for early and high yielding varieties (Roi-et 1 and MKS-1-8111) with five lines of semishattering types in order to incorporate the non-shattering characters into standard and new varieties. F1 seeds from 16 crosses were planted at Ubol Field Crops Research Center in 1986. F2 and subsequent F3 were planted at Supanburi Field Crops Research Center with irrigation in the dry season and F4 was to be planted at Ubol Field Crops Research Center in the rainy season of 1987 with plant-to-row method of selection.

3. Selection in the segregation population. Fifty seven families of F2 seed made from crosses of determinate, non-shattering and high yielding types in Israel were sent and planted at the Supanburi Field Crops Research Center in the 1986 dry season with irrigation. Plant to row selection in F4 in the rainy season at Ubol Field Crops Research Center will be employed in the rainy season of 1987.

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All F4 lines were planted at Ubol Field Crops Research Center as planned in the early rainy season. At the present, plants are in the late vegetative stage. Early rainy season collection has had to be postponed due to the lack of early season rain this year.

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Table 1 Average Grain Yield, Plant Height and Number of Days to Maturity of Different Grain Types of Amaranth Growth Under Three Different Altitudes During 1983 to 1986 in Chiang Mai, Thailand.

Genotype	Grain type	Lowland rainfed (300 MSL)			Upland (800 MSL)			Highland (1000 MSL)		
		Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)	Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)	Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)
1. 83S-27	African	163 ab*	117 b*	460 a*	157 ab*	135 d*	3228.3 a*	166 d*	148 bc*	2605 a*
2. 79S-1040	African	123 bc	96 cd	387 b	222 bc	140 d	2886.2 b	107 f	106 c	650 ef
3. R-158	Mexican	153 ab	114 bc	450 a	154 ab	133 de	2983.1 b	149 de	148 bc	1860 bc
4. 82S-1041	Mexican	162 ab	117 b	390 b	164 a	127 de	2391.0 c	160 de	120 de	2033 b
5. 82S-SP130	Nepal	145 b	111 bc	80 d	117 bc	133 de	1154.0 f	165 d	144 c	1583 c
6. 82S-1008	Nepal	130 bc	111 bc	70 d	108 bc	106 f	1078.8 f	69 g	114 de	247 fg
7. 82S-SP153	Spike	127 bc	83 d	200 c	95 bc	111 f	1180.0 [illegible]	87 fg	113 de	1330 cd
8. 82S-674	Spike	112 c	99 c	130 c	95 bc	109 f	1293.6 ef	72 fg	118 de	360 f
9. 82S-1023	Mercado	128 bc	118 b	340 b	107 bc	123 e	1989.5 d	137 e	121 de	841 de
10. 81S-1044	Mercado	140 b	99 c	237 c	156 ab	130 de	2368.1 c	152 de	128 cd	1150 d
11. 81S-1004	Prima	105 c	83 d	60 d	91 c	105 f	1053.3 f	60 g	121 de	437 ef
12. A-982	South American	-	-	-	128 b	178 b	51.0 h	203 bc	170 ab	233 g
13. A-713	South American	-	-	-	123 bc	202 a	34.0 h	198 c	190 a	342 fg
14. A-718	Aztec	-	-	-	163 a	171 bc	619.5 g	252 a	170 ab	758 e
15. A-723	Aztec	205 a	185 a	58 d	183 a	164 c	1524.7 e	225 b	166 b	1537 c
	Average	141.08	111.08	242.66	130.8	137.8	1586.0	146.5	138.5	1064.0

* Values within a column followed by the same letter are not significantly different at 5% level of probability using New's Duncan Multiple Range Test (DMRT).

FY 1985

CASHEW NUT COMPACT SIZED SHELLER (C5-301) : MODEL AE(KKU)^2

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ABSTRACT

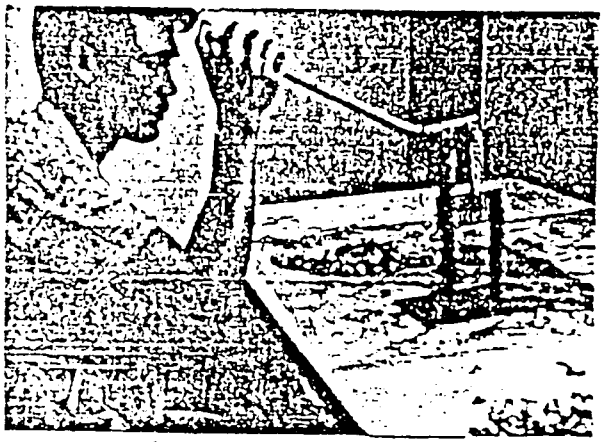
The Model AE (KKU)^2 cashew nut sheller was designed and developed to give high shelling performance when compared with other existing manually-operated shellers in Thailand. When used to shell cashew nut whose density was approximately 170 nuts/kg, with medium-skilled operator and without pretreatment of the nuts, the obtained shelling rate was 0.68 kg (kernels)/h or 5.44 kg (kernels)day (8h), with whole-kernel recovery of 85%. The sheller had a compact size of 10 cm base width, 13 cm base length and 35 cm overall height, with a total weight of 1.5 kg. The Model AE(KKU)^2 sheller had a proposed cost of 200 - 300 baht and was expected to help cashew nut farmers and small manufacturers in Thailand sharply boost their incomes.

INTRODUCTION

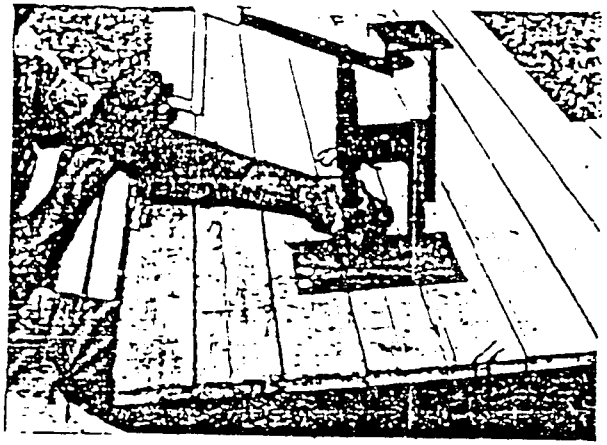
The cashew nut is a rapidly emerging cash crop in Thailand. Shelled cashew nuts offer far greater local income and export potential than the unshelled variety. The local price of unshelled nuts is 10 - 25 baht/kg, compared with 120 - 250 baht/kg for shelled nuts. It takes about 3.5 kg. of unshelled nuts to make 1 kg of kernels and therefore, cashew nut farmer who shells his own nuts before sale can increase his income from the crop by 200 - 300%. A survey carried out on cashew nut farmers and small manufacturers indicated that they needed a compact sized sheller which was inexpensive and could be manually operated, and another with higher shelling capacity and power driven. The research project therefore, concentrated first on the design and development of a manually operated sheller with emphasis on compactness, high performance and economy.

MATERIALS AND METHODS

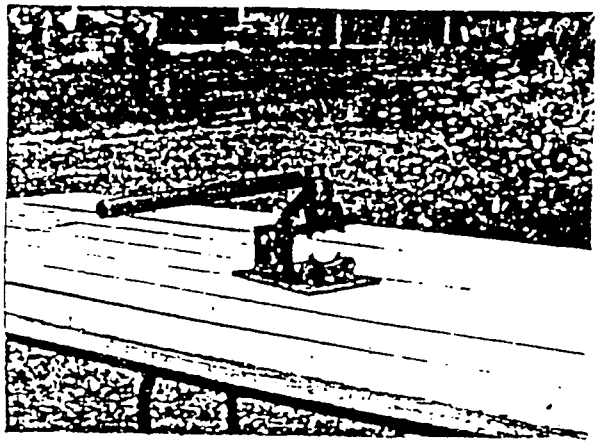
Two versions of the hand-operated sheller were designed and constructed at Khon Kaen University, both with shelling mechanisms for i) cutting the cashew nut shell and ii) twisting the halved shells open. The two prototypes, namely the Model AE(KKU)1 and the Model AE(KKU)2, were tested and evaluated, both for their performance and economy in comparison with 4 other versions of manually operated shellers, all of which were procured in Thailand. The testing and evaluation were done in order to determine the most promising sheller(s) to be introduced to cashew nut farmers and small manufacturers. The 6 prototypes of the manually operated shellers tested and evaluated were shown in Figure 1.



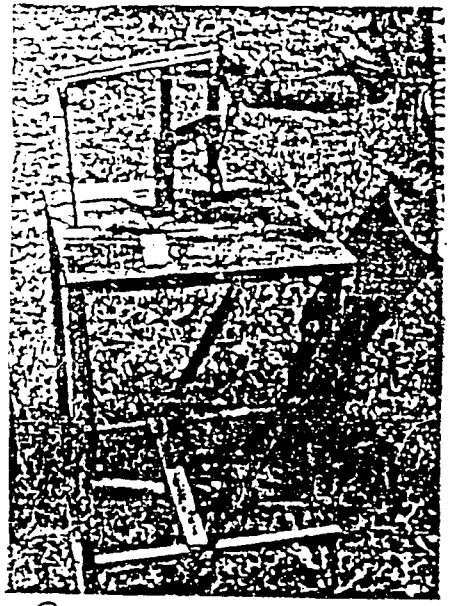
① The hand operated 'press-blade type' sheller.



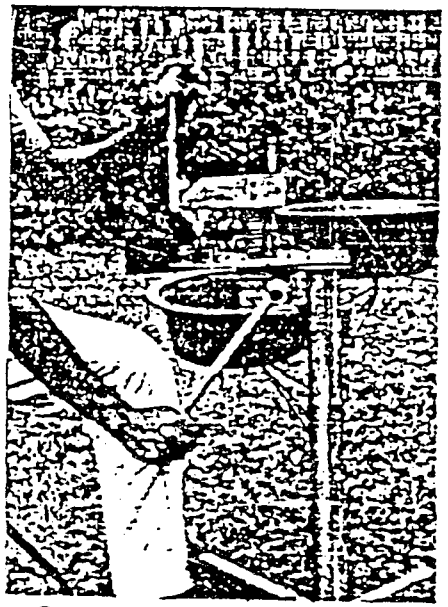
② The hand operated 'press/twist-blade type' sheller. (Model AE(KKU)1)



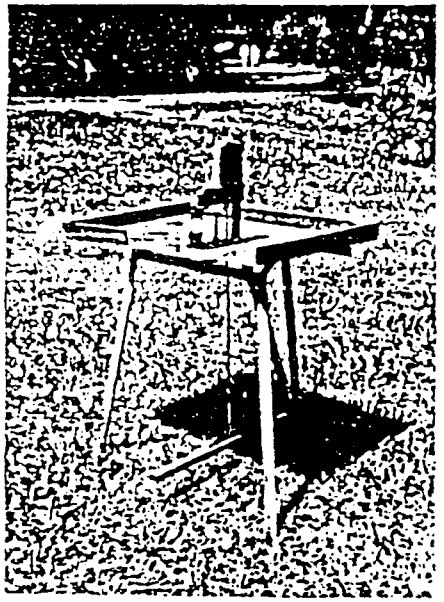
③ The hand operated 'press/twist-blade type' sheller. (Model AE(KKU)2)



④ The hand/foot operated 'press/separation-blade type' sheller.



⑤ The two hand operated 'press/separation-blade type' sheller.



⑥ The foot operated 'press-blade type' sheller.

Figure 1 Prototypes of Manually Operated Cashew Nut Shellers.

Factorial design was used and analysis of variance was employed for the interpretation of the test results. The performance of each sheller was evaluated by using the following indicators:

Shelling rate (kg/h) = weight of cashew kernels obtained per hour

$$\text{Whole-kernel recovery (\%)} = \frac{\text{wt. of whole kernels}}{\text{wt. of whole + broken kernels}} \times 100$$

The economy of each sheller was evaluated by considering the following:

- Amount of nuts to be shelled (kg/yr) to break even
- Payback period (yr)
- Net profit (baht/yr)

RESULTS AND DISCUSSION

Testing and evaluation of the 6 versions of the manually operated shellers indicated that the Model AE(KKU)2 sheller showed best performance and offered the lowest amount of nuts needed to be shelled to break even, and best payback period and net profit (1). The Model AE(KKU)2 sheller was then given a long term performance test covering a period of two months. The results indicated that the size and material of certain components had to be changed and that an additional component was necessary to ensure the machine's durability. The improved version of the sheller, its simple method of operation and its overall components are shown in Figures 2, 3 and 4 respectively. The refinements done on the sheller were as follows:

1. The size of the fulcrum brackets was increased to 4 x 30 x 30 mm and the diameter of the pin was increased to 16 mm.

2. The lower blade holder, originally made from a 4 mm thick steel strip, was changed to an angled steel piece of 4 x 25 x 25 mm plus 3 mm thick steel strip.

3. The lower and upper blades, originally made from a 3 mm thick steel bar, were changed to 1.5 mm thick flat spring steel.

4. The size of the spring was increased to 1) a 20 mm coil diameter and ii) a 2.8 mm wire diameter, and the spring was wound from one whole wire.

5. The ring, originally not existing, was added in order to minimize the breakdown and the bending of the screw end of the press/twist lever.

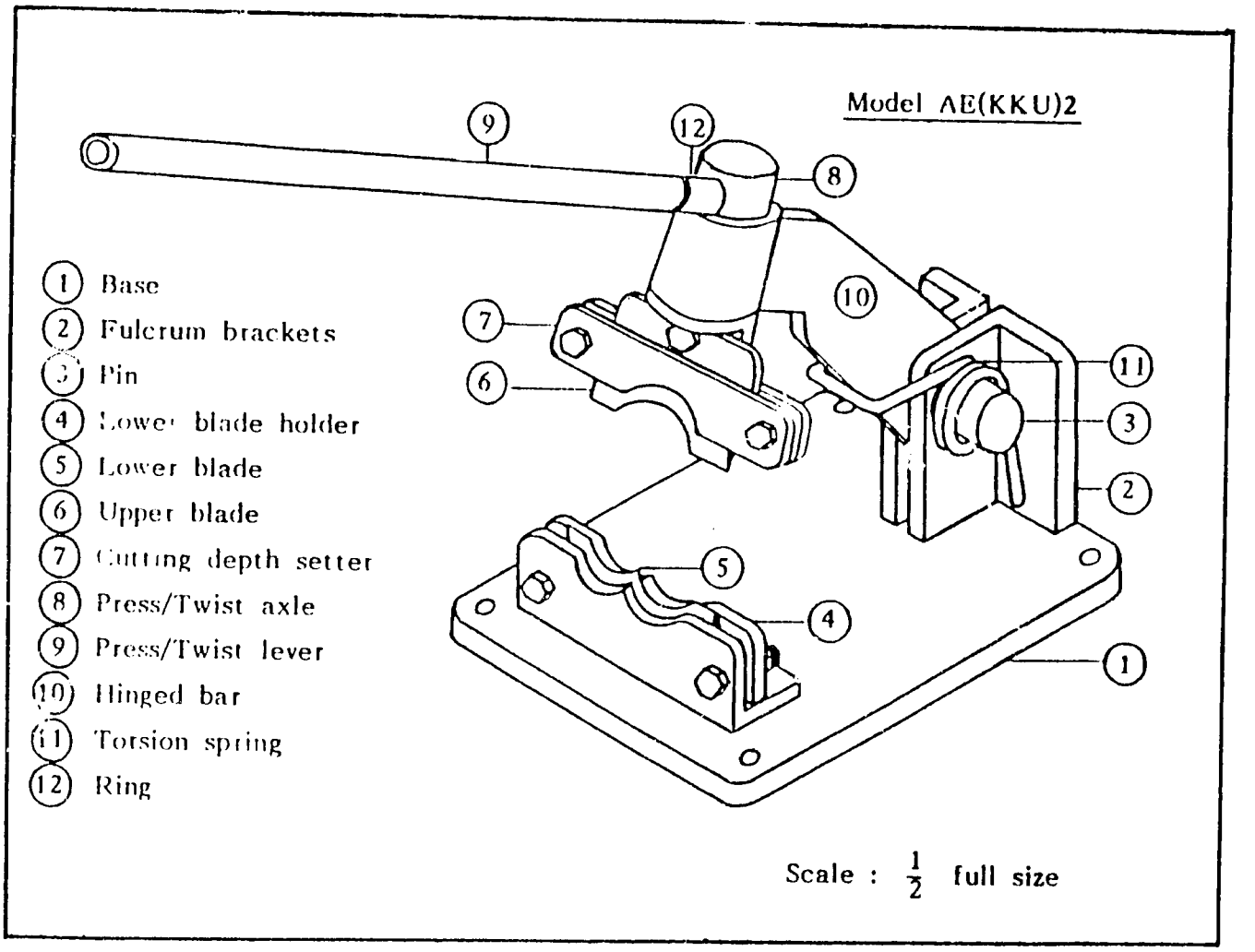
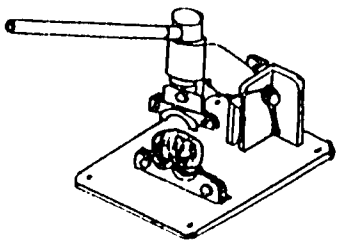
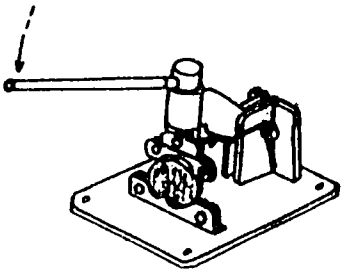


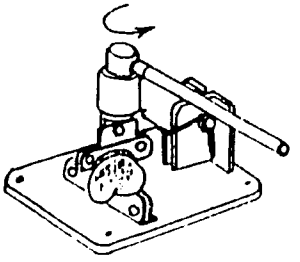
Figure 2 The Hand Operated 'Press/Twist-Blad Type' Sheller. (Model AE (KKU)2)



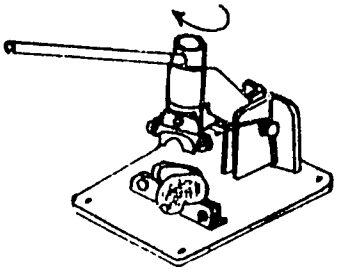
(a) Position the nut onto the lower blade.



(b) Press the lever down so that both the upper and lower blades cut into the shell.



(c) Twist the lever to the right or left to open up the halved shells.



(d) Release the lever and take out the shelled nut.

Figure 3 Operating Procedures for the Model AE(KKU)2 Sheller.

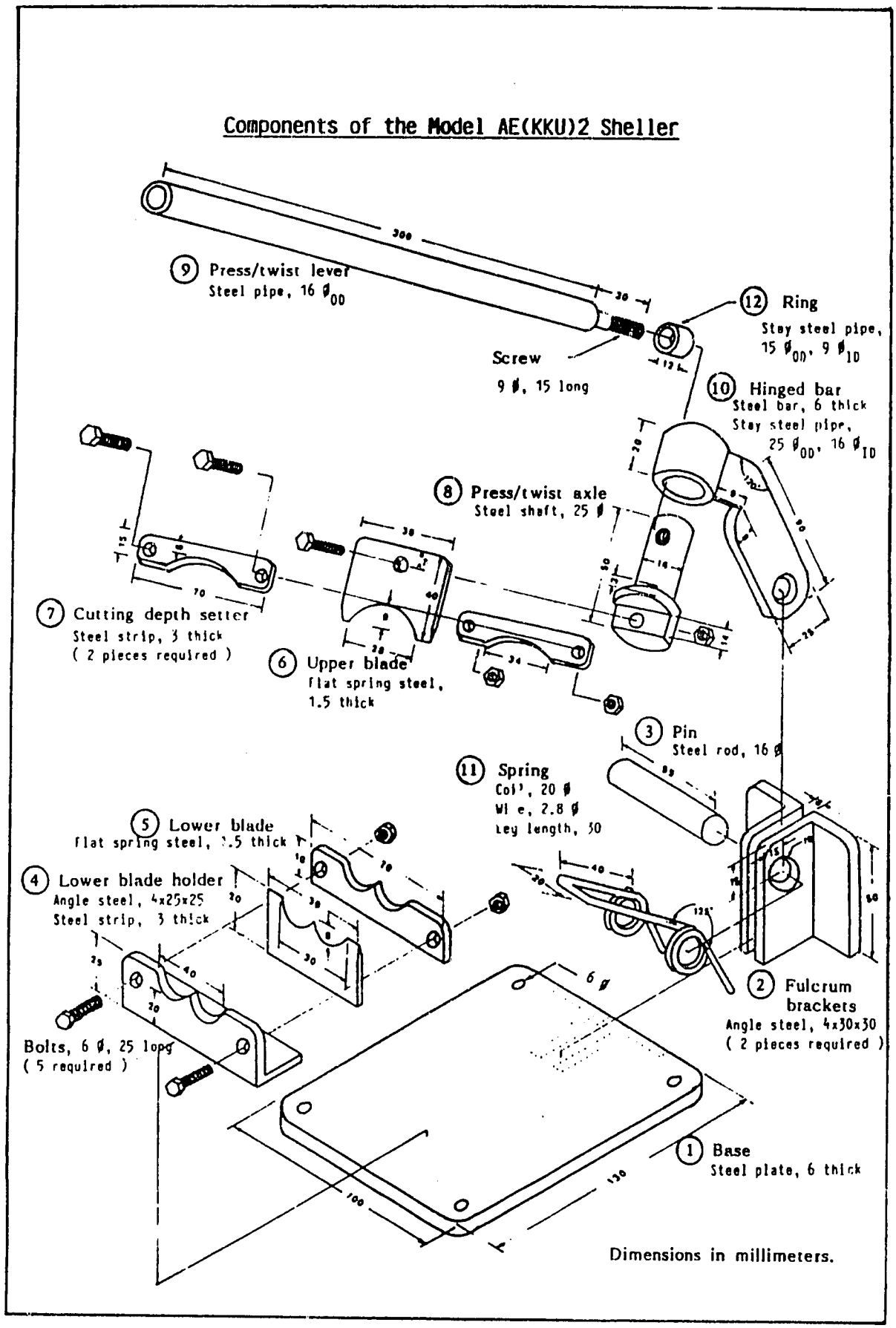


Figure 4 The Disassembled View of the Model AE(KKU)2 Sheller.

The Model AE(KKU)2 sheller was again tested and the latest results obtained were as follows:

Shelling rate	0.68 kg (kernels)/h
	(or 5.44 kg (kernels)/day(8h))
Whole-kernel recovery	85%

(The test was carried out in June 1987 using raw nuts whose densities were approximately 170 nuts/kg, with a somewhat skilled operator and without pretreatment of the nuts.)

For long term performance considerations, testing of the sheller during May - June 1987 indicated that it had the necessary durability because all the components functioned satisfactorily during the operation and did not show signs of fatigue at the end of the two-month test.

The economic evaluation of the sheller is as shown in Figures 5, 6 and 7. The results show that the minimum amount to be shelled and the payback period have practically low values, thus ascertaining the cost-effectiveness of the machine. Figure 7 shows that the more nuts that are shelled, the better the net profit is, therefore to obtain the highest possible profit the farmer or small manufacturer should make sure that he uses the sheller to shell the maximum amount of cashew nuts.

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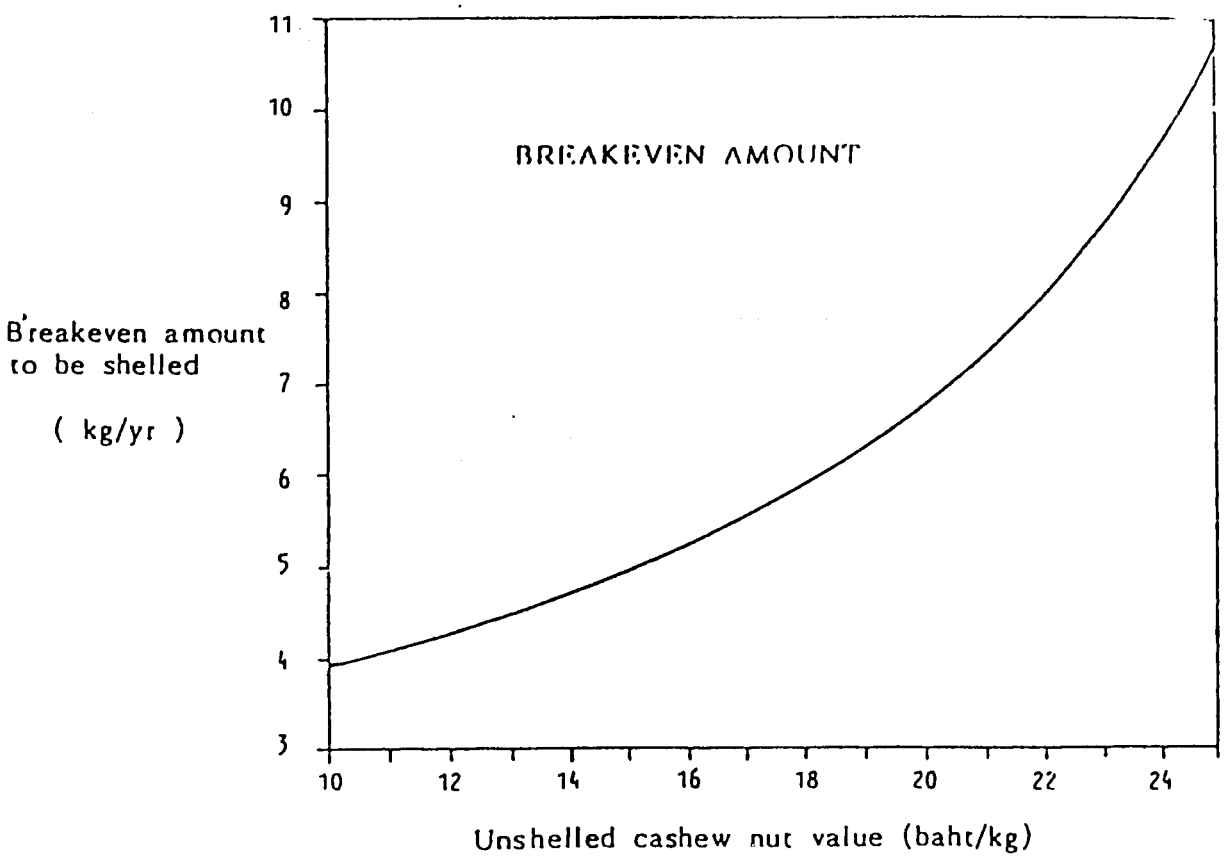

Figure 5 Relations Between the Minimum Amounts to be Shelled and the Unshelled Cashew Nut Value.

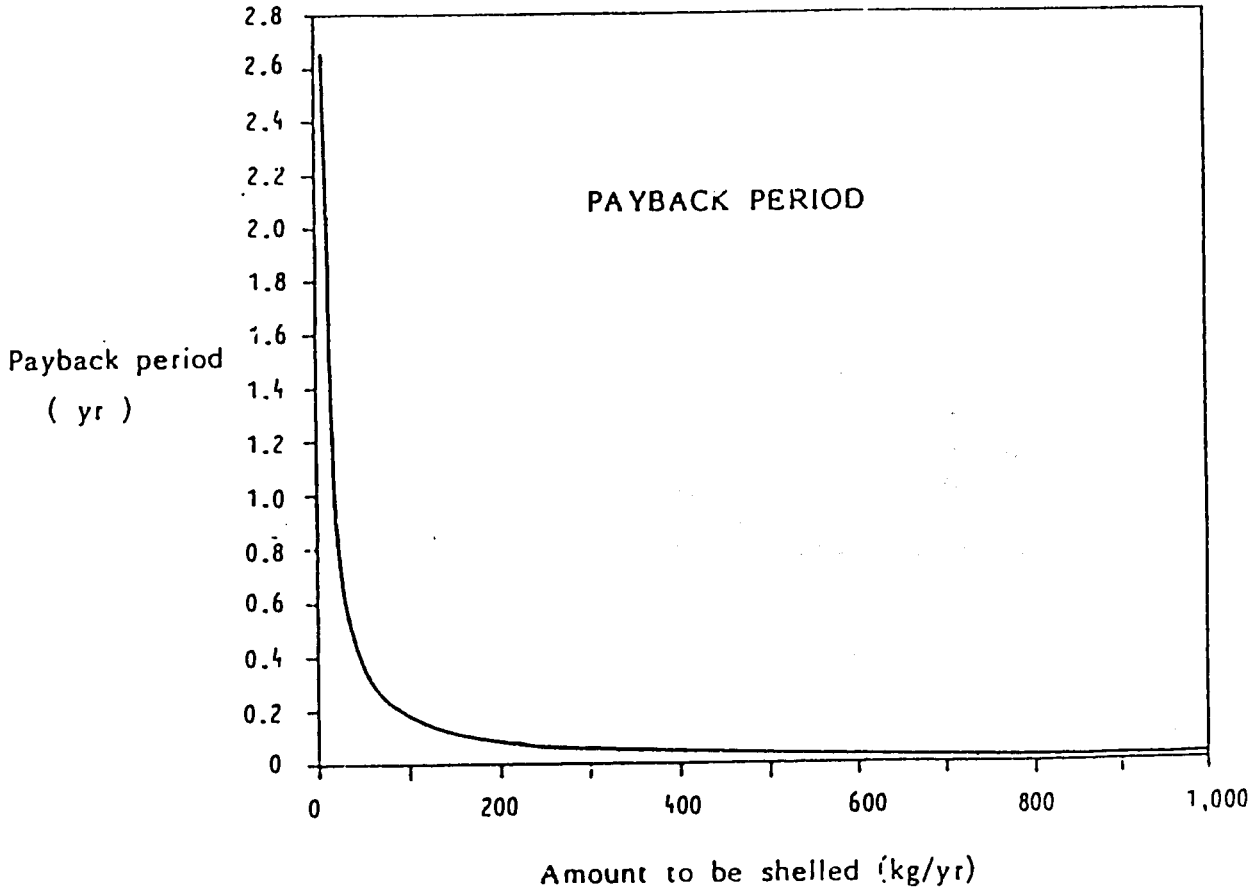

Figure 6 Relations Between the Payback Period and the Amount of Cashew Nuts to be Shelled.

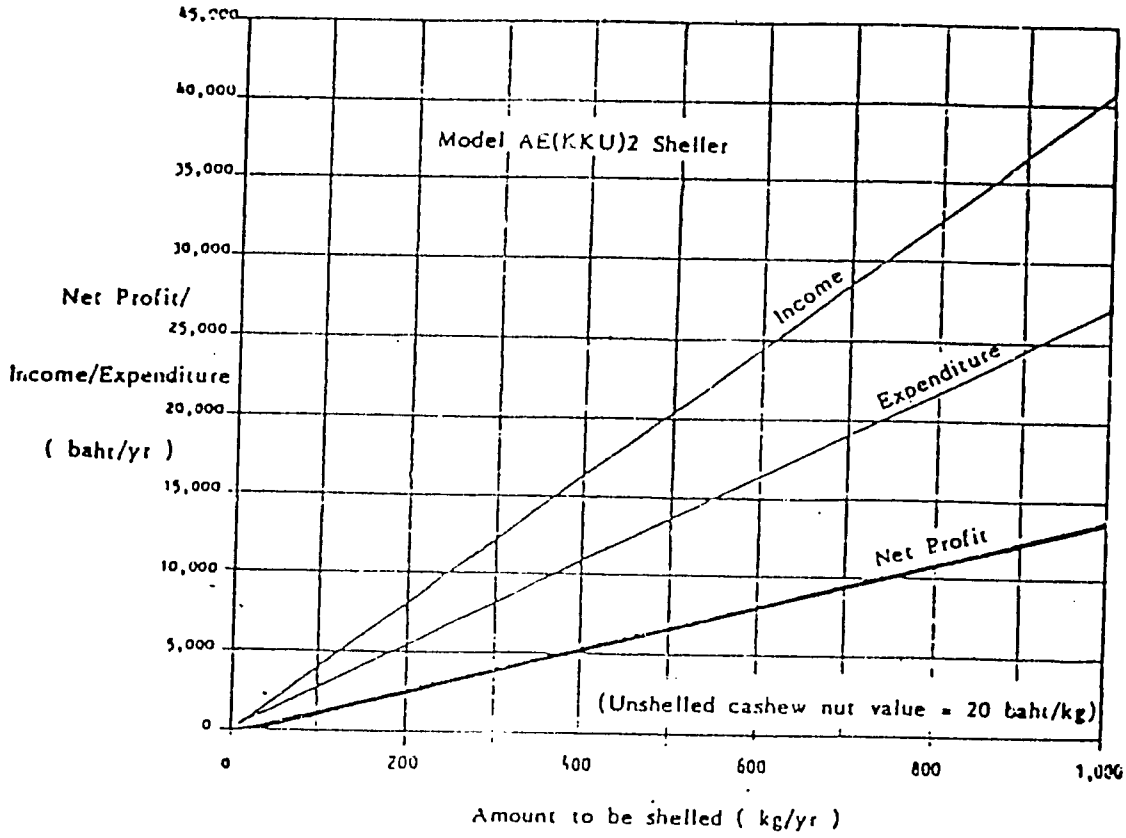

Figure 7 Relations Between the Net Profit and the Amount of Cashew Nuts to be Shelled.

Note : The fixed and variable values used in the economic analysis were as follows :

1. Machine cost	250	฿
2. Machine life	5	yr (approx.)
3. Maintenance cost	10	% of machine cost
4. Shelling rate	0.68	kg/h
5. Whole-kernel recovery	85	%
6. Wages for shelling	15	฿/kg (kernels)
	(10	฿/h (approx.))
7. Wages for inner skin peeling	12	฿/kg (kernels)
8. Conversion ratio between wt. of unshelled nuts and wt. of kernels	3.5 : 1	
9. Unshelled cashew nut value	20	฿/kg
10. Whole-kernel price	150	฿/kg
11. Halved-kernel price	110	฿/kg

FY 1986

STRAIN SELECTION OF *Spirulina algae* SUITABLE FOR MASS PRODUCTION

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ABSTRACT

The project contract between NIFI and Ben Gurion University was signed in October 1986. Two trips to North Eastern regions of Thailand for strain collection have been made; one in January and one in April 1987. Isolation of strains is in progress. Monoculture of four strains has been established. Laboratory determination of nutrient requirements, environmental sensitivities, production output and chemical composition of each isolated strain will be made. Further experiments under both moderately high and low technology operational systems will be used in final evaluations and strain selection for mass production.

INTRODUCTION

Spirulina sp. are blue green algae which probably have a highest protein content (60%-70%) among algae. Recent research concerning this algae has captured a great deal of attention around the world. Nowadays, mass culture of these algae is performed in many countries such as Japan, the United States of America, Israel, Taiwan, India, Thailand, Italy, Mexico and some African countries. Besides the high protein content which can be used as a protein food source for both human and animal feed, the *Spirulina* also contain a remarkable level of B-carotene and Vitamin B complex. These qualities have made *Spirulina* a very popular health foods in both Western and Eastern nations. Its high carotene can provide a great source of color in aquarium fish and deepen the color of poultry egg yolk to satisfy consumer preference.

Reports from Thailand (Boonsom 1970) also show that micro algae *Spirulina* species were found in natural waters of the northeastern part of the country. This is evidence that spirulina exists in Thailand and hence can be cultured. Therefore, close examination and strain selection of these indigenous species will provide a species to be introduced to country villagers of NE salty soil areas where other agricultural crops can not yield an acceptable income and a new source of food which has high value chemicals for aquaculture.

PLANS AND METHODOLOGY

1. Collection and Isolation

1.1 Collection of Samples

Samples of *Spirulina* strains were collected from their natural habitats in Northeastern Thailand. The area was divided

geographically into 3 sections, Upper NE, Lower NE and Eastern NE. At least two trips were made to each section; one at the end of the dry season when soil salinity was expected reach maximum, and others at the end of the rainy season when the salinity was expected to be lower than that during the first trip.

Measurements of DO, pH and temperature were made on the site. Other water qualities were examined in the laboratory by using standard methods. Phytoplankton collecting procedure were used to collect samples.

1.2 Isolation of Spirulina Strains

Samples that were collected from the field trips were brought back to the NIFI laboratory in glass bottles carefully carried in baskets which provided some sunlight. Isolations were conducted by using micro manipulation techniques. Standard Zarrouk's medium and standard BG11 medium were used in this stage. General morphological characteristics were also recorded.

2. Testing for Requirements and Sensitivities

2.1 Determination of the requirements

After obtaining the mono alga culture of the isolated strains, each strain will be tested for optimal temperatures and main nutrient requirements by using a turbidostat and spectrophotometer.

2.2 Testing for sensitivity and adaptability to the expected outdoor environment

The outdoor environment, i.e. high solar radiation, ion concentration and temperature range, and variations according to the area and the season will have effects on production of the algae. Therefore, those factors will be tested. Measurements of oxygen evolution and dark respiration will reveal the sensitivity or adaptability of the algae cultures after exposing them to those environments for a certain period of time.

2.3 Testing for adaptability to increased salinity

One of the objectives in this strain selection project is to obtain the most salinity-tolerant strain to be introduced into areas where soil salinity problems exist. Therefore, the strain which able to survive in wide range of salinity will be selected. Measuring photosynthesis by using oxygen evolution and dark respiration will identify the most effective strain.

3. Testing for Performance of the Better Strains Under Laboratory Conditions in an Outdoor Experimental Pond

3.1 Testing for pond operational parameters

Operational parameters i.e. depth of culture, rate of mixing, cell concentration, and harvesting frequency will be tested by

varying these parameters. The amount of chlorophyll, optical density, and dry weight will be measured and used as indicators.

3.2 Maximum growth rate under optimum condition

Maximum growth rate will be determined by using daily diluted culture of turbidostate methods. Daily recordings of the chlorophyll and protein contents, optical density and dry weight will be used as evaluation parameters.

3.3 Floating capability

The floating capabilities of the strains will help in pond mixing (less energy is required) and harvesting. This parameter will be determined by using an Inhoff cone.

3.4 Chemical composition of the biomass

Chemical analysis of the nutritional value and other economical chemical content i.e. Gamma Linolenic Acid (GLA), phycocyanin, vitamins, lipid carbohydrate, protein and antibiotic properties will be conducted.

4. Performance of the Selected Strain in Polyethylene-Lined Eastern Ponds in the NE

The tests will be carried out for two factors.

4.1 Daily output rate of the strain.

4.2 The ability to survive as a monoalga culture under the local low technology input operational system and the moderate high technology operational system.

The parameters that are described in (2) and (3) will be retested for each strain and each operational system. The results of this performance will be the final decisive factors for the best suitable strain of the area.

RESULTS AND DISCUSSION

The National Inland Fisheries signed the contract to conduct this project with the Ben Gurion University in October 1986. Until now, two trips have been made. One was to the upper Northeastern region in January 1987. In this trip, more than 30 samples of different localities were collected and 13 samples contained Spirulina alga. So far four different strains of Spirulina have been isolated and mono alga culture has been obtained.

The second field trip was held in April 1987 to the eastern section of the NE. 53 samples of water were collected and 11 samples contained Spirulina algae. Isolation is still in progress in both Thai and Israeli laboratories. The NE Part of Thailand especially the upper and eastern sections seem to be the locations for Spirulina alga for Thailand. They

are found all over the area in scarce numbers. Only few samples i.e. TH-11 that contain high concentration of spirulina have been found. These areas will be carefully examined in the future.

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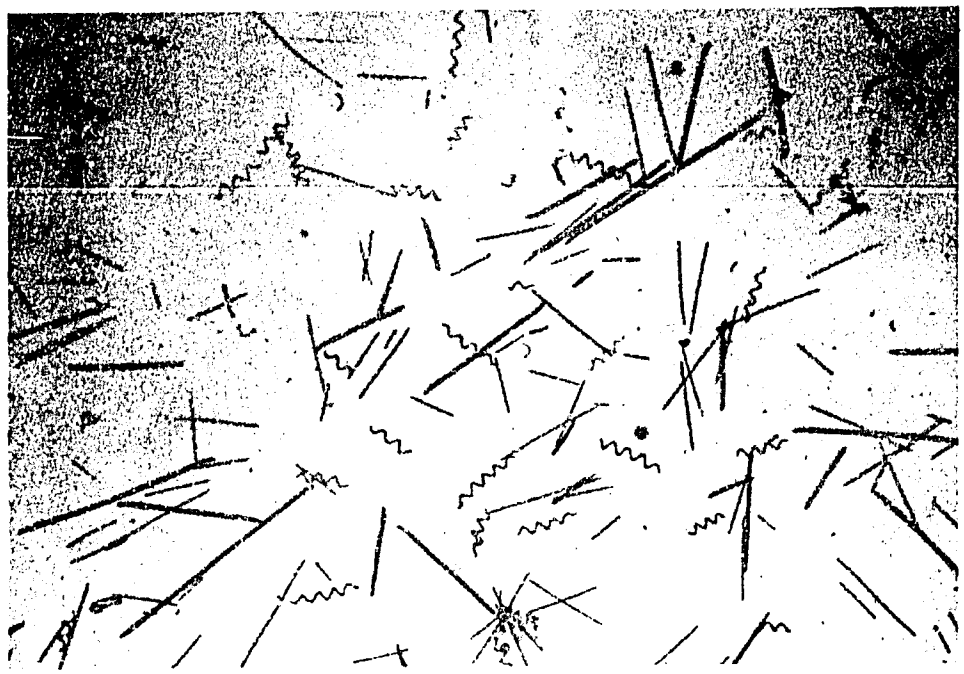
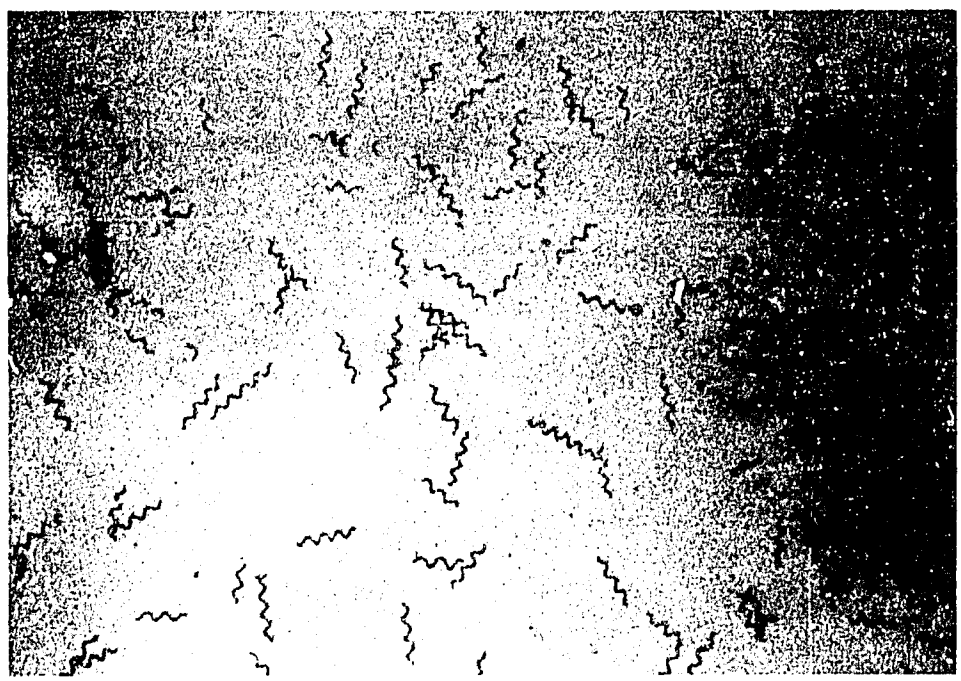


Spirulina sp. 16.5 x

Mahasarakham, January, 1987



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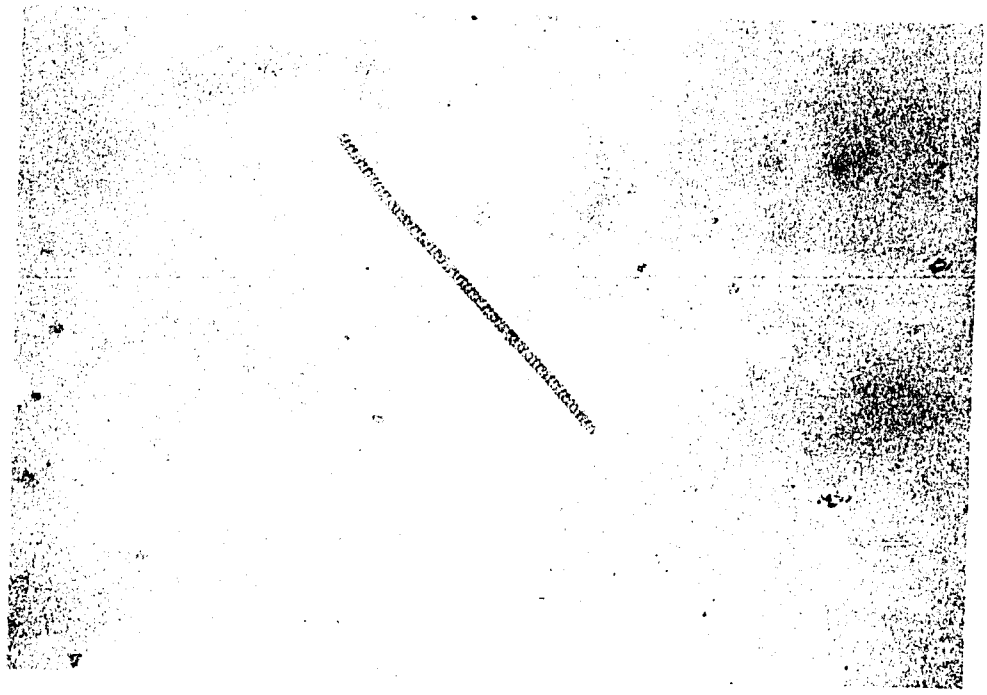
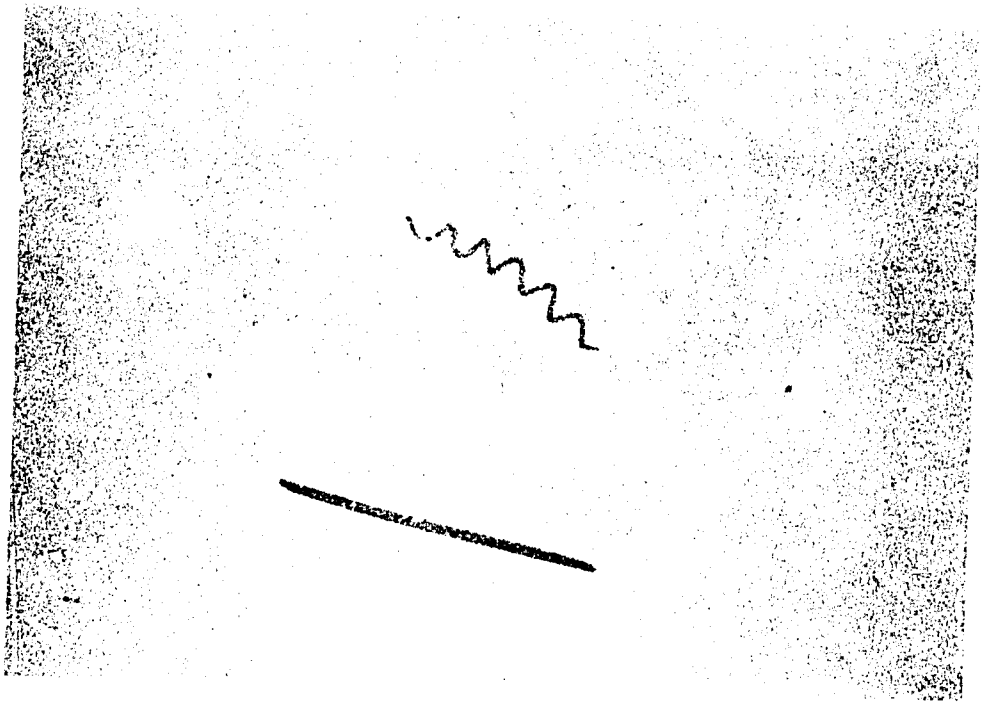
Khon Kaen, January 1987



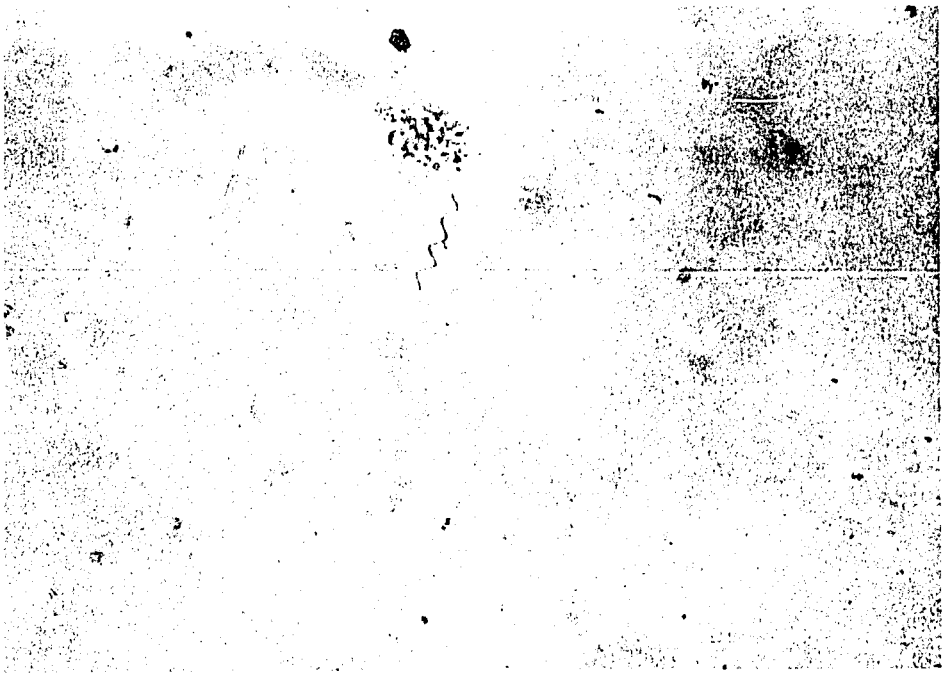
Spirulina sp. 82.5 x

Ubon Ratchatani, April 1987



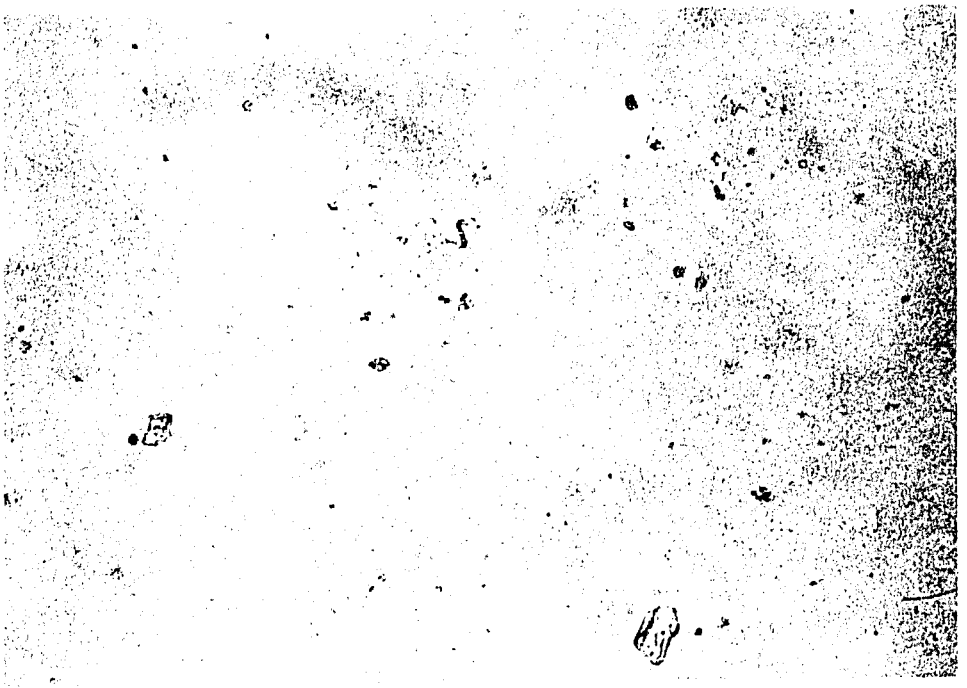
Spirulina sp. 41.25 x

Mahasarakham, January 1987



Spirulina sp. 82.5 x

Burirum, April 1987



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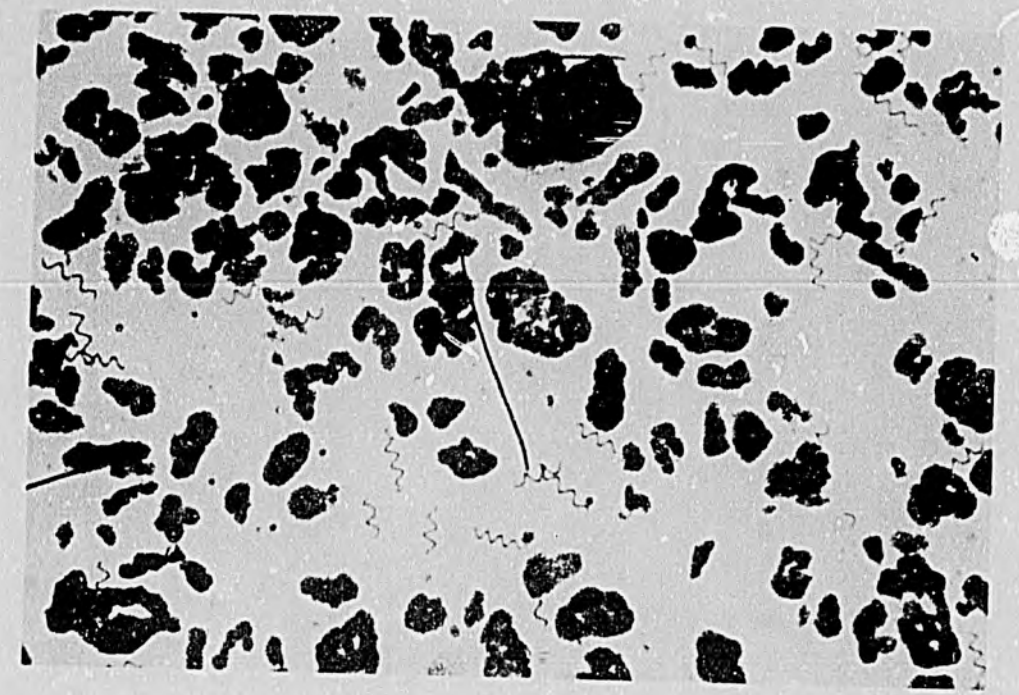
Burirum, April 1987



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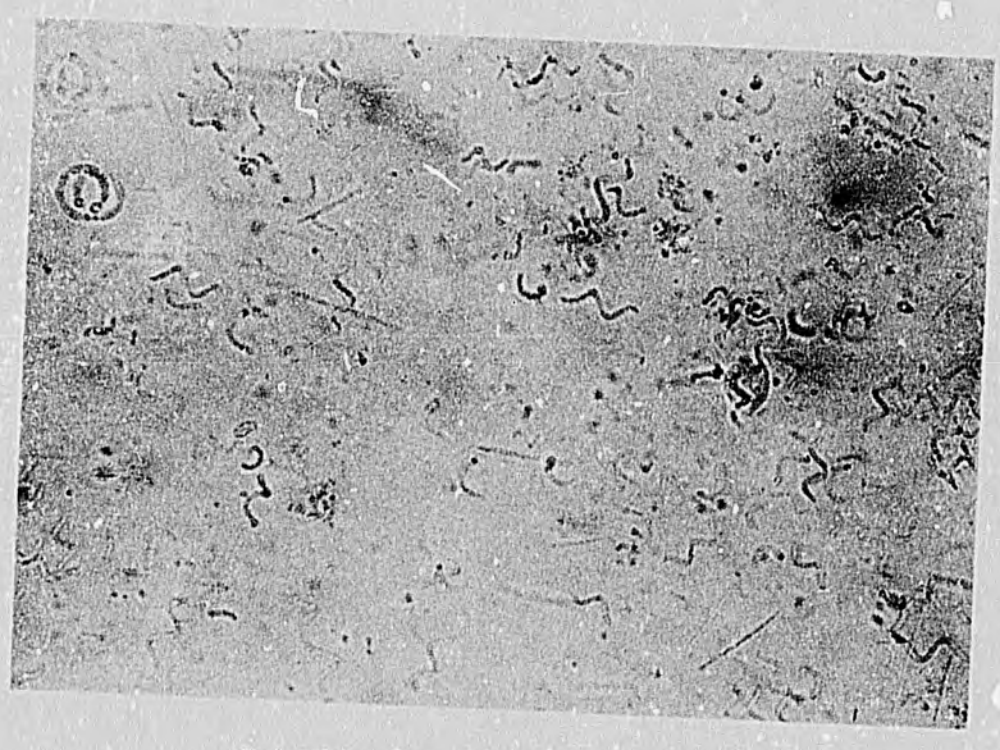

Spirulina sp. 82.5 x

Burirum, April 1987

FY 1986

SULFONAMIDE DRUGS AS A MALE ANTIFERTILITY AGENT. (C7-169) - I. EFFECTS OF SULFASALAZINE AND SOME SIMPLE SULFONAMIDES ON FERTILITY OF THE MALE RAT

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ABSTRACT

The possible antifertility effects of sulfasalazine and 6 other simple sulfonamides were studied in male Fischer rats. All drugs were suspended in corn oil and forced fed at a dose ranging 125-450 mg/kg daily for six weeks. The fertility of the treated males and controls (corn oil only) was tested by natural mating with untreated females at weeks 1, 3 and 5 during treatment and at weeks 1 and 3 after drug withdrawal. Four out of seven sulfa drugs tested showed a dose-related and reversible antifertility effect. The reduction of fertility occurred as rapidly as by one week after treatment. Fertility of the treated rats was fully recovered by one or three weeks after drug withdrawal. The potency of those showing the antifertility activity was in the following order : sulfapyridine > sulfanilamide > sulfasalazine > sulfacetamide; whereas sulfamerazine, sulfadimethoxine and sulfioxazole had virtually no effect at least at the dose of 450 mg/kg. The results suggest that the antifertility activity of sulfonamides does not require the presence of pyridine nor other heterocyclic rings at the N^1 position.

INTRODUCTION

Sulfasalazine, a compound formed by azo linkage between sulfapyridine and 5-amino salicylic acid, was introduced first in 1930 to treat rheumatoid arthritis (Goldman & Peppercorn, 1975). It has been later demonstrated to be useful in the therapy and prophylaxis of ulcerative colitis and regional entoritis (Baron et al., 1962; Misiewicz, 1965). Although these diseases predominantly affect men and women of reproductive age, the association between sulfasalazine treatment and male infertility was not recognized until in 1979 when both Toth and Levi and his colleagues independently reported reduced semen quality and infertility in sulfasalazine-treated men. The antifertility effects of sulfasalazine are completely reversible after drug withdrawal. These findings have since been confirmed both in man and animals by many investigators (for review see Giwercman & Skakkeback, 1986). However, the mode of action of sulfasalazine is largely unknown. Recent evidence indicates that the sulfapyridine moiety of the sulfasalazine molecule or its metabolites are responsible for the effects on male infertility (O'Morain et al., 1982a, b:

Cann & Holdsworth, 1984: McIntyre & Lennard-Jones, 1984; Shaffer et al., 1984). Whether other sulfonamides have similar effects on male fertility is still not known. Since sulfapyridine is considered to be relatively toxic in man other sulfa drugs which suppress male fertility and are less toxic should be found. In this report, the possible antifertility effects of some simple sulfonamides having the N^1 position substituted by various functional groups and having different physicochemical as well as pharmacokinetic properties were studied.

MATARIAL AND METHODS

Chemicals

All drugs were of reagent grade and were purchased from Sigma Co., St. Louis, USA. Corn oil (Mazola) was obtained from local supermarket.

Animals

Albino rats of the Fischer strain were obtained from and maintained at the Animal House, Faculty of Science, Mahidol University. The male rats aged between 90-120 days and the female aged between 40-50 days were housed separately in groups of 5-10 animals under a 12:12 h lighting regime, and with free access to pellet feed (Gold Coin. Singapore) and water.

Fertility testing

Every male rat was screened for fertility capability before grouping and drug treatments. Each male was housed with two virgin females in a hanging cage with food and water ad lib for one week. Mating was confirmed by the presence of spermatozoa in the vagina smears done every morning. The copulated females were separated and kept for 8-10 days at which time pregnancy was examined after sacrifice and the number of fetuses and corpola lutea counted. Fertility of the male was assessed in terms of per cent fertility, which was evaluated from the percentage of the eggs successfully fertilized and the embryos implanted (number of fertuses over corpola lutea x100) in the females that showed the evidence of copulation. When both females were impregnanted by the same male, the average value was used.

One hundred and seven males with high percent fertility (greater than 80%) were randomly assigned to a control (12 animals) and 13 drug-treatment groups (5-11 animals each). Each of the experimental groups was force fed with one of the sulfa drugs shown in Fig. 1. The drug was suspended in corn oil and was given at a low (125 or 150 mg/kg), a medium (250 or 300 mg/kg) or a high (450 mg/kg) dose daily for 6 weeks. At the end of weeks 1, 3, 5, 7 and 9 the treated males and controls (receiving corn oil only) were subjected to fertility testing as described above. Thus, fertilities of the males at weeks, 1, 3 and 5 during treatment and weeks 1 and 3 after drug withdrawal were determined.

RESULTS AND DISCUSSION

In this study, mean fertility of the male rats in all groups ranged from 86.5 to 98.6% before treatments. Force feeding with corn oil had virtually no effect on fertility of the animals. However, when sulfa drugs were given, 4 out of 7 compounds showed some antifertility effects (Table 1). Thus sulfasalazine, sulfapyridine and sulfanilamide caused a dose-dependent reduction in per cent fertility during treatments and within one week after drug withdrawal, whereas sulfacetamide (150 mg/kg) had only a transient effect at week 1 during treatment. The potency of those which exhibited antifertility activity in decreasing order is as follows: sulfapyridine > sulfanilamide > sulfasalazine > sulfacetamide. On the other hand, sulfamerazine, sulfadimethoxine and sulfioxazole did not have any antifertility effects in the male rats.

The finding that sulfapyridine was more potent than sulfasalazine supports the previous studies that sulfapyridine is the active moiety of sulfasalazine (O'Morain et al., 1982a, b: Cann & Holdsworth, 1984; McIntyre & Lennard-Jones, 1984; Shaffer et al., 1984). The results in the present study also indicate that the antifertility activity of this series of sulfa drugs is independent of the presence of pyridine group or other heterocyclic rings at the N^1 position of the sulfonamide molecule. Thus, sulfanilamide, the most simple sulfonamide drug, is almost as potent as sulfapyridine. In order to determine whether the antifertility efficacy of the sulfa drugs depends on their water or lipid solubilities, or their half-lifes in the blood, their relative solubilities and plasma half-lifes were compared (Table 2). It is apparent that the ability to suppress male fertility is not related to the water and lipid solubilities nor the plasma half-life (if it is assumed that their pharmacokinetics in man is also applicable in the rat).

Table 2. Comparison between the relative antifertility activity of some sulfonamides and some of their solubility and pharmacokinetic properties.

Property	Relative quantity in decreasing order	Reference
Antifertility activity	A>B>C>D>E>F>G	present study
Water solubility	B>D>E>A, G>F	Anand (1979)
Lipid solubility	G>B>F>A>E>D	Anand (1979)
Plasma half-life (in man)	G>F>A, B>D>E	Anand (1979)

A = Sulfapyridine; B = Sulfanilamide; C = Sulfasalazine;
D = Sulfacetamide; E = Sulfioxazole; F = Sulfamerazine;
G = Sulfadimethoxine

In conclusion, we have demonstrated that four out of seven sulfonamides used in this study showed some antifertility activity in the male rats. The ability to reduce fertility is independent of their water and lipid solubilities or plasma half-life. In addition, the presence of pyridine or other heterocyclic rings at the N^1 position is not essential for their activities. However, the minimally required structure of the sulfa drugs for the antifertility activity in the males remains unknown.

ACKNOWLEDGEMENTS

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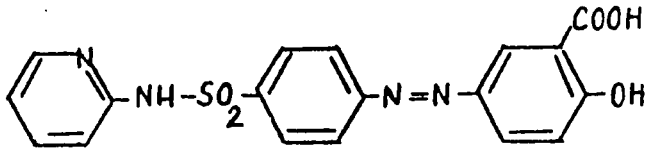
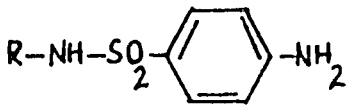


Sulfasalazine



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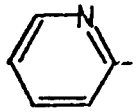
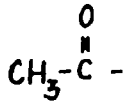
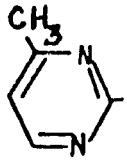
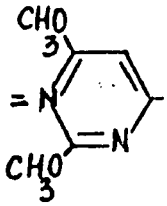
R =  Sulfapyridine

= H- Sulfanilamide

=  Sulfacetamide

=  Sulfamerazine

=  Sulfadimethoxine

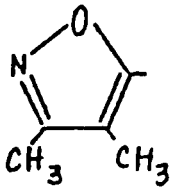
=  Sulfioxazole

Figure 1 Structures of Some Sulfonamides Used for Fertility Testing.

Table 1 Effects of Some S-lfonamide Drugs on Fertility of the Male Rats

Treatment group	Dose (mg/kg)	Fertility (%)					
		Before treatment	During Treatment Week-1	During Treatment Week-3	During Treatment Week-5	After Drug Withdrawal Week-1	After Drug Withdrawal Week-3
Control		94.3±2.4 (12)	96.5± 1.8 (10)	84.1± 2.8 (8)	90.6± 3.9 (11)	93.7± 2.4 (11)	92.8± 4.2 (4)
Sulfanilamide	150	92.0±3.0 (5)	91.8± 3.6 (4)	57.7± 9.5* (5)	44.5± 4.5*** (5)	74.6± 8.0** (5)	96.8± 1.9 (5)
	450	88.8±4.6 (6)	86.8± 6.6 (6)	56.3± 7.0* (5)	18.0± 9.0*** (4)	37.3±16.0 (5)	79.5± 9.9 (5)
Sulfacetamide	150	95.7±1.2 (12)	80.5± 7.0* (10)	70.0±10.0 (12)	85.9± 4.1 (9)	91.3± 2.5 (10)	-
	450	95.1±1.7 (11)	86.6± 2.8 (10)	70.6± 6.4 (9)	77.5± 7.3 (10)	81.5± 5.0 (10)	-
Sulfamerazine	150	93.5±2.4 (6)	90.8± 2.0 (6)	84.1± 1.8 (6)	73.4±10.1 (6)	91.3± 4.9 (6)	-
	450	96.5±1.8 (6)	85.6± 3.9 (5)	74.6± 9.3 (5)	83.4± 7.5 (5)	92.5± 4.3 (5)	-
Sulfadimethoxine	150	98.6±1.4 (7)	95.6± 2.9 (6)	82.5± 7.4 (4)	93.9± 1.9 (7)	90.2± 6.5 (6)	-
Sulfioxazole	150	93.5±4.0 (5)	95.5± 1.8 (4)	82.8± 4.3 (5)	98.3± 1.7 (3)	93.2± 5.5 (4)	93.3± 3.4 (3)
	450	89.2±4.1 (6)	97.0± 1.5 (6)	81.3± 7.3 (6)	82.1± 8.4 (6)	88.6± 5.6 (6)	87.4± 7.6 (6)
Sulfapyridine	125	94.6±2.3 (9)	76.2± 9.6* (5)	71.9±12.3 (4)	45.9± 4.8** (6)	-	-
	250	98.1±1.2 (8)	62.7±16.9* (7)	22.1±11.2*** (3)	25.1±11.5*** (4)	-	-
Sulfasalazine	300	86.5±4.2 (7)	87.3± 3.5 (5)	65.9±22.7 (4)	60.9±15.9 (3)	73.9±14.2 (6)	74.9±14.2 (7)
	450	94.5±2.9 (7)	92.3± 3.7 (6)	52.8±12.6* (6)	35.5±13.7** (6)	74.6± 8.5 (4)	94.4± 3.9 (4)

Values in paraentheses are number of animals.

*P<0.05; **P<0.01; ***P<0.001 when compared to the corresponding control group (F-test).

Table 2 Comparison Between the Relative Antifertility Activity of Some Sulfonamides and Some of Their Solubility and Pharmacokinetic Properties.

Property	Relative quantity in decreasing order	Reference
Antifertility activity	A > B > C > D > E > F > G	present study
Water solubility	B > D > E > A, G > F	Anand (1979)
Lipid solubility	G > B > F > A > E > D	Anand (1979)
Plasma half-life (in man)	G > F > A, B > D > E	Anand (1979)

A = Sulfapyridine; B = Sulfanilamide; C = Sulfasalazine; D = Sulfacetamide;

E = Sulfioxazole; F = Sulfamerazine; G = Sulfadimethoxine

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MODULATION OF MEMBRANE TRANSPORT: A BIOCHEMICAL APPROACH TO CHEMOTHERAPY OF MALARIA (C7-171)

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ABSTRACT

This paper reviews changes in transportation properties of the red cell membrane following malarial infection. Examples are given of transportation increases of small metabolite, anions and cations. In some cases, the transportation increases indicate the appearance of new transportation systems, which are likely to play an important role in intracellular parasite survival and development. Selective blockage of these new transport systems offer a potential approach to malarial chemotherapy. Alternatively, the new transport systems can be utilized to gain selective entrance of drugs or pro-drugs into infected cells. Proposed research will include investigation and modulation of transport of iron and folate compounds by malaria-infected red cells.

INTRODUCTION

Infection of the red blood cell by the malarial parasite is followed by wide-ranging changes of the host cell membrane[1,4]. Permeability changes form a prominent feature among these, and many of them are probably of crucial importance to parasite survival. For example, parasite dependence on glycolysis during the parasite is fast intracellular growth can only be satisfied by enhanced transport of monosaccharides, chiefly glucose[5-7]. Transportation changes are selective and not just a consequence of a general increase in membrane leakiness. While there is a marked increase of a glutamine influx into Plasmodium falciparum-infected red cells, the influxes of isoleucine, arginine and glycine are only slightly increased[8]. In addition to substrate specificity, the transportation changes are also specific for developmental stages of the parasite, occurring mostly at the stage of the mature trophozoite[8-11], and are also species-specific[12]. In some cases the selective changes may be due to modulation of existing host transportation systems by the parasite; in others, to the novel transport systems elaborated by the parasite. By whatever mechanisms, the specificities displayed by the transport changes point to new potential approaches in malaria chemotherapy, in which the specific changes may be either blocked, or utilized to bring appropriate drugs into the cells.

TRANSPORT OF SMALL METABOLITES AND ANIONS

By analyzing the kinetics of the entry of D-glucose and its non-metabolizable analog 3-O-methyl-D-glucose, into P. lophurae-infected duck red bloods cells, Sherman and Tanigoshi[5] showed that the enhanced entry

could be attributed to both carrier-mediated and simple diffusion components. We obtained a direct demonstration of the role of the carrier-mediated pathway, by showing that enhancement of the transport of D-glucose and 2-deoxy-D-glucose into P. berghei-infected mouse red cells was partly inhibited by cytochalasin B and phloretin[7]. Cytochalasin B is known to bind with high affinity to the glucose transported, while phloretin exerts a more general effect on transport systems. D-galactose and D-fructose also show a large transport increase, mostly insensitive to cytochalasin B. The specificity of the transport increase raises the possiblity of the presence of a new transport system on the infected red blood cell membrane.

This possibility was earlier raised by Ginsburg et al. who, by using a method of osmotic hemolysis by the transportable molecules, showed that the membrane of P. falciparum-infected human red blood cells became permeable to such substrates as sorbitol and glucose at near isotonic concentrations. The new permeability pathway was shown to be insensitive to cytochalasin B but sensitive to phloretin. Subsequent studies showed new permeability pathways to have pores of approximately a 0.7 nm equivalent radius, bearing a positive charge and facilitating movement of small anions and neutral molecules, but excluding cations. Substances the size of disaccharides or larger were excluded. Evidence was also presented[10] to indicate the need for parasite protein synthesis as an essential factor for the generation of the new permeability pathways. Phlorizin, a drug which inhibits P. falciparum growth in vitro, was shown to be a pore blocker, probably acting on the cytoplasmic side after gaining access to infected cells[13].

TRANSPORT OF CATIONS

Red blood cells have long been known to have a great influx of sodium and greater efflux of potassium during malarial infection[14]. With respect to calcium, a number of reports[15-17] have shown enhanced influx into a variety of infected cells. The enhanced influx is associated with changes in membrane properties, such as an increase in membrane fusion capacity[18]. Calcium has an important role in regulating the activity of many intracellular enzymes, and it is likely that the developing parasite has requirements for calcium in its metabolism. Its need to accumulate calcium within itself is probably due to the fact that, because of its intracellular location, the plasma source cannot be mobilized at a fast enough rate. At the schizont stage, the enhanced influx was accompanied by greatly enhanced lysis[17], raising the possibility that calcium may also have a role in the regulation of merozoite release.

The transportation status of another important cation, namely iron, is quite different. Although the intracellular parasite is surrounded by the hemoglobin in red blood cell cytoplasm, and when it feeds on this substance to leave the residual pigment hemozoin within its food vacuoles, the heme-bound iron is probably not utilized by the parasite due to its potential toxicity. The parasite probably elaborates a mechanism to obtain iron from the plasma instead. Recent evidence[19-21]

points to the appearance of a transferrin receptor on the infected cell, to sequester iron-bound plasma transferrin and deliver to the intracellular parasite. Our recent work (unpublished) has also raised the possibility of the existence of parasite-specific siderophores (small iron-binding molecules) in the infected red cells.

PROPOSED RESEARCH APPROACHES

A. General Outline

Our general approaches consist of studying the nature of new permeability pathways with implications for chemotherapy. The pathways which are used for the transport of metabolites, nutrients, and ions can be selectively blocked, thereby interfering with parasite metabolism. It is possible that some antimalarials act through such mechanisms. More intriguing perhaps is the possibility that some immune sera may block parasite development through interference with the new permeability pathways. It should also be possible in principle to induce blocking antibodies, in vivo or in vitro, if the antigens responsible for the new permeability pathways can be obtained and means of increasing immunogenicity can be increased. Screening for potential transport blockers can be made by either isotonic hemolysis method or uptake of radiolabelled substrates.

An alternative approach for chemotherapy is to utilize the new permeability pathways to gain selective entrance of drugs into infected cells. It was proposed that phlorizin acts through such a mechanism[13]. In a variation to this approach, selective entrance of pro-drugs is followed by parasite suicidal activation. An example is cleavage of transportable glucosides to yield toxic moieties within the parasite.

B. Iron Transport and Metobolism

We will place emphasis on the investigation of the possible presence of transferrin receptors on infected red blood cell membranes. An aim is to develop antibodies, probably monoclonal, against the parasite-directed transferrin receptor. The specific antibodies would block the binding of ferrotransferrin and hence the uptake of iron into the parasite. This would be lethal to the malaria parasite with no cytotoxic effect or interference on iron uptake and metabolism of the host cells.

Another approach is the possible utilization of possible parasite siderophore(s) (small iron chelator) in the cytoplasm of infected cells. The siderophore(s) would participate or facilitate in the iron uptake process. If this can be isolated and identified, the blockage or competition of the chelator function can be developed. This would lead to deprivation of iron specific to the malaria parasite which can be lethal to parasite development.

C. Transport of Folate Compounds and Antifolates

There are apparently two systems of transport for normal erythrocytes, one for 5-methyltetrahydrofolate and another for the parent

folate[22]. With our recent direct evidence of folate salvage by P. falciparum[23], it is desirable to investigate the possible presence of parasite-specific pathways for transport of folate compounds and antifolates. If these pathways are present, we will study their characteristics in detail, including their susceptibility to inhibitors, and possible relationship with other transport systems, eg, carbohydrates, anions. These studies should help in the design of new antimalarials based on the principles outlined above.

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HYDROLOGY OF RAINFED PADDY LAND

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OVERALL AIM OF THE RESEARCH

This research seeks to develop a mathematical model for a watershed containing predominantly paddy land area unique to Northeast Thailand. Such models for paddy land will be used for planning and management of water utilization in the agricultural watershed. The research itself constitutes a component in the overall research framework aiming at large scale implementation of small diversion weirs for supplementary irrigation of rainfed paddy land.

CONTRIBUTION

The research will provide answers relevant to many important development issues. These include: How much water is available in a stream as a result of a certain land use pattern? How much of this water can be used for supplementary irrigation of rainfed paddy land? What is the best scheduling for such supplementary irrigation? How many diversion weirs should be built for such irrigation? How can land erosion and sediment deposition at the weirs be minimized? The results of the research will also be applicable to developing countries in Southeast and South Asia of which the rural poor subsist on rainfed paddy cultivation. The research results and their policy implications will enable governments and international donors to help the rural poor in the area most needed.

INNOVATIVE APPROACH

The research formulation embraces the interactions of a natural process and the effect of human interferences. This natural process is important since it affects the livelihoods of hundreds of millions in many less developed countries. In the United States the hydrology of rangeland has been well established. This study could well be considered as a new parallel innovative discipline.

LINKAGES AND RELEVANCE TO REGIONAL DEVELOPMENT

A preliminary study of the hydrological process involved in watersheds containing paddy land was made by Khon Kaen University in 1978. At that time, an attempt was made to collect field data. The present research, being more comprehensive, is a vital component in the overall research framework conceived by the Water Resources and Environment Institute, Khon Kaen University. Together, these research components form a package of research activities the outcome of which can be used for large scale implementation of weir construction programs aiming at providing supplementary irrigation for paddy land. In order to see how

this research fits into the overall research framework and hence the likelyhood of its success, it is necessary to describe other research components. The following sections provide some elaborations. Large scale development of weirs for supplementary irrigation of rainfed paddy land and other domestic requirements in Northeast Thailand has been emphasized by the Royal Thai Government. Many problems have been encountered. The problems range from technical to social and economic problems. Initially, technical problems related to the appropriate design of the weir were prominent. At present it may be claimed that the technical problems related to weir design have been solved. Khon Kaen University, through its Faculty of Engineering and the Water Resources and Environment Institute (formerly Office of Water Resources Development) was the principal agency responsible for past research in weir design improvement. Since weirs can be built only at places on or close to streams, paddy land further away from them may not get any benefits. Therefore, only some portions of the rainfed paddy land will benefit from weirs. In order to assess the percentage of rainfed areas that can benefit from weirs, another research project entitled "Water Resources Spatial Zoning Study," is being conducted. This is depicted in the top box of Figure 1. The "Zoning Study" will provide potentials for the weir construction program. The information will be useful for two other development projects: the Small Scale Irrigation Project and the KKU-New Zealand Weir Program. This present research, "Hydrology of Rainfed Paddy Land," is viewed as a research project that will provide information and answers on physical aspects of watersheds. For example, it will be able to tell that weirs should not be built less than two kilometers apart in the same stream to prevent competition for water. This kind of information will be useful for setting strategies for implementation or even in preparation of rules relating to water utilization. Conversely, the two development projects will provide basic socio-economic information--pointing out important aspects in the process of model development in this research.

CONCEPTUAL FRAMEWORK

This research project consists of two major tasks: (i) field instrumentation and data collection (ii) model development, calibration and verification.

Field Instrumentation

Northeast Thailand was chosen as a target study area due to relevant development priority. In addition, the region contained several types of topography typical of Southeast and South Asian countries. For example, mountainous areas, undulating terrains, and alluvial plains were available. Five watersheds of different topography were selected as shown in Table 1.

Table 1 Watersheds Selected for Field Instrumentation.

Stream Name	Location District/ Province	Drainage Area km^2	%of Paddy Land	Topographic Type
1. Huai Yae	Chaturat/ Chaiyaphum	187	3.5	Foothill
2. Huai San Pan	Kudbak/ Sakhon Nakhon	50	49	Foothill
3. HuaiKhummum	Ubolrat/ Khon Kaen	244	26	Undulating terrain
4. Huai Som Yai	Nong Bua Lampu/ Udon Thani	76	61	Undulating terrain
5. HuaiBo	Pang Khon/ Sakhon Nakhon	82	80	Alluvial plain

Gaging stations will be set up for measuring important hydrologic data such as rainfall, streamflow, erosion and sedimentation data. Relevant meteorological data such as temperature, solar radiation, humidity and evaporation will be collected. Representative soil samples will be taken for laboratory testing to determine important properties such as soil classification, grain size distribution, infiltration characteristics and hydraulic conductivity. Instrumentation and data collection activities will be carried out extensively because there are very few existing data for watersheds of this size (about 100 to 200 km2). In addition to the five watersheds of Table 1, five more watersheds with similar characteristics will be selected for lesser degrees of field instrumentation. The collected data will be used for model verification. Collection of hydrologic and meteorological data will be carried out on a daily basis. Continuous data will be recorded for rainfall and streamflow during the rainy season to be used for hydrologic model calibration.

Model Development, Calibration and Verification

The development of models can be divided into two aspects. First, a water budget analysis of the watershed will be carried out. The analysis will include rainfall, evapotranspiration, streamflow and groundwater. Second, a more detailed rainfall-runoff model will be developed. A few popular models will be tested and refined to end up

with an appropriate model for paddy land watersheds Particular emphasis will be given to the initial abstraction and infiltration components. The model will be calibrated and parameters optimized using the collected data. Once calibrated, the model will be verified by applying it to watersheds other than those used in the model development and calibration. Activities to be carried out are scheduled as shown in Table 2. The project was started in May 1987 and is, at the time this manuscript was written, only in its third month. Current activities include procurement of equipment, field survey and instrumentation and collection of detail secondary data.

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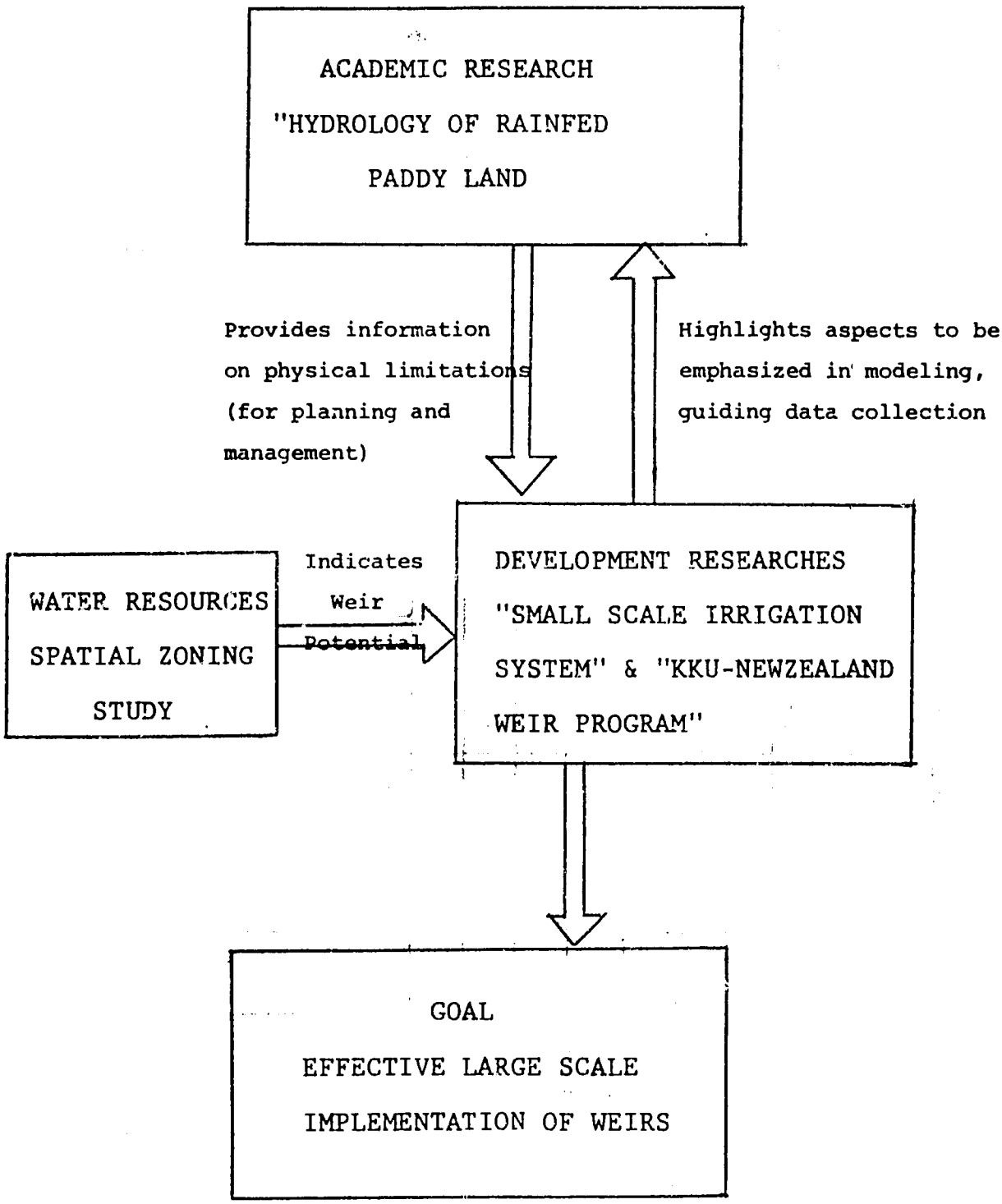
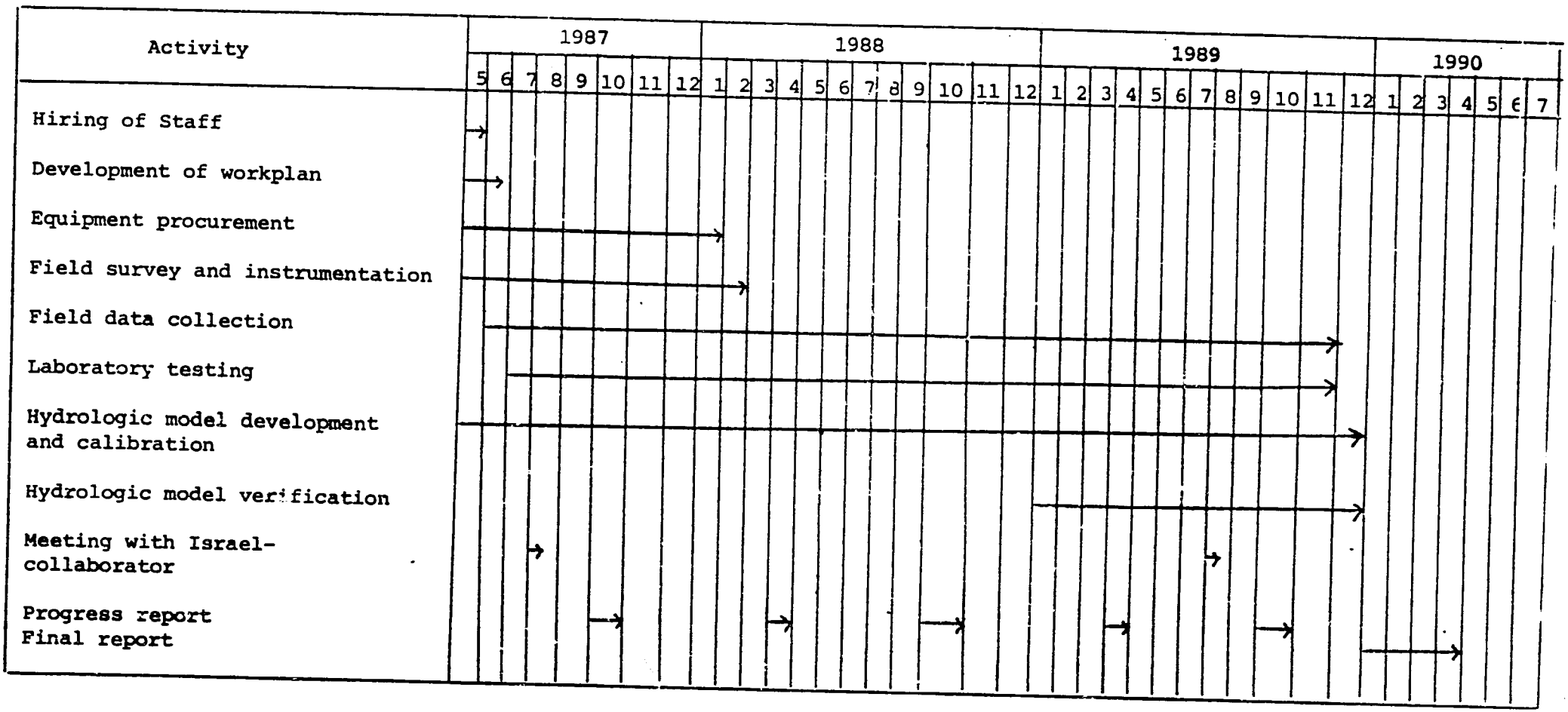


Figure 1 Research Framework for Agricultural Water

Table 2 Project Activity Plan (Hydrology of Rainfed Paddy Lands)



PART III

BOARD ON SCIENCE AND TECHNOLOGY FOR INTERNATIONAL DEVELOPMENT

(BOSTID)

7904

FY 1982

YIELD IMPROVEMENT. AGRONOMY, AND LOCAL USE OF AMARANTH (AMA-TH-2-83-9) - VARIETAL YIELD TRIAL IN GRAIN AMARANTH

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ABSTRACT

Grain amaranth (Amaranthus spp.), is an important food crop that is widely grown through out Central and South America and in some Asian countries for high quality protein food and animal feed. Studies on this newly introduced crop began in Chiang Mai, Thailand in 1983. Fifteen genotypes differing in morphological and grain types were evaluated for their adaptabilities and yield performances in these locations (lowland rainfed, upland and highland areas) with differences in altitude (300, 800 and 1,000 meters) above mean sea levels). Field studies were conducted on the farms (research stations) of the Faculty of Agriculture, Chiang Mai University, Thailand from 1983 to 1986. The results showed marked differences in high yielding stability and adaptation of various grain types of amaranth grown under different altitudes. African and Mexican grain types (A. cruentus) such as 83S-27, R-158 and 82S-1041 genotypes had higher yield and adapted better than the Mercado, Nepal, Spike and Aztec grain types (A. hypochon driacus). The South American grain type (A. caudatus) was less adaptable to lower altitude but performed well under highland locations. African grain type (83S-27) and Mexican grain type (R-158) gave the highest averages in grain yield of 3228 and 2938 kg/hectre, respectively, under upland conditions (800 MSL). Drought stress in the lowland rainfed conditions (300 MSL) was found to be the major constraint for grain amaranth production, while low temperature was the major limiting factor for the highland areas (|1000 MSL).

INTRODUCTION

In Thailand, vegetable amaranth has long been cultivated on small farms for edible leaves and animal feed. Amaranthus tricolor and A. dubius have been grown in semi-arid and tropical regions for more than 2,000 years (NAS, 1984). Vegetable amaranth is one of the cheapest dark green leafly crops in tropical markets and is described as a poor man's vegetable. Its leaves are a good source of carotene, iron, calcium, vitamin C, folic acid and other micronutrients. Grain amaranth is a new crop in Thailand, but it was one of the basic food crops of the Americas in pre-Columbians times. Three species of the genus Amaranthus produce large seed heads with edible seeds. A. hypochondriacus and A. cruentus are native to Mexico and Guatemala; A. caudatus is native to Peru and other Andean countries. Although grain amaranth is a food crop of the Americas, during the last century it was found to be a popular crop among hill tribes in India, Nepal, Pakistan and China. In the highlands of some Asian countries, it was found that grain amaranth was intensively

cultivated. Grain amaranth can be cultivated as a secondary crop in mixed croppings with maize, sorghum, millet, hot peppers or other food-crops (NAS, 1984, Joshi et al.,1983). With a protein content in the seeds of about 16 %, amaranth compares well with other crops such as wheat (13%), rice (7-10%) and corn (9-10%). Amarant protein, however, has nearly twice the lysine content of wheat protein, and in fact as much as is found in milk (NAS, 1984). Its protein can complement other cereals of low lysine content, while cereals complement amaranth in leucine. A diet which combines amaranth seed with other grain provides high quality protein (Grubben and van Sloen, 1981).

Amaranth seed can be used in breakfast cereals or as an ingredient in confections. It also can be parched or cooked into gruel or milled to produce a sweet, light colored flour suitable for biscuits, breads, cakes and other baked goods. When heated, the tiny amaranth grains pop and taste like a nutty flavored popcorn. The popped seeds are light and crisp and are eaten as a snack, or as a cold cereal with milk and honey (NAS, 1984). Grain amaranth can be grown in a wide range of environments with different yields in each location. It can be grown in dry areas with 600-800 mm of rain fall per year for tropical highlands up to an elevation of more than 3,000 meters. Grain amaranth is classified as a C_4 pathway crop. C_4 plants have higher efficiency for fixation of CO_2 and can convert with a higher ratio of atmospheric carbon to plant sugar per unit of water lost than C-3 pathway crops (NAS, 1984).

With an excellent nutritive value, the ability grow well in a wide range of climatic conditions, less susceptibility to diseases and insect damage, the ability to prevent soil erosion and to conserve soil moisture, grain amaranth would be a highly promising crop for the poor people that are lacking protein sources, especially the hill tribes peoples and pre-school children in Thailand. The significance of this research project is not only to serve the basic needs of the poorer people in the rural areas but also provide information about this crop for other developmental agencies and national development plans. Furthermore, the results obtained from this project will be a supportive basis for introducing a new crop in the multiple cropping system program for the upland and highland areas. If the yield of grain amaranth, which is a component crop in rice based systems, is improved, the income return of farmers will be increased.

The major objectives of the project studies at the Faculty of Agriculture, Chiang Mai University are, to explore the agronomic aspects of grain amaranth and to survey the potential for wider local production and use of amaranth by small farm holders. At pressent emphasis is on the varietal yield trial study. The objective of the trials is to evaluate the adaptabilities and yield performances of this newly introduced crop grown under three different altitudes in the Northern part of Thailand.

MATERIAL AND METHODS

The yield trials of collected genotypes of grain amaranth were conducted at three different locations which were classified as lowland

rainfed (300 MSL), upland (800 MSL) and highland (above 1,000 MSL), for four consecutive years from 1983 to 1986. During late May through June (rainy season), the land was plowed, rotovated and fertilized with 50-62.5-62.5 of $N-P_2O_5-K_2O$ kg per hectare and then rotovated again. One week after emergence, plants were thinned to 0.10 m. intra row spacing. Weeds were controlled by two hand weedings at 30 and 60 days after seeding. Carbofuran (Furadan 3G) was used for insect control and was applied at the rate of 2 gm per square meter. A randomized complete block design with four replications was used. A plot, measuring 3 m x 7 m, was assigned to each genotype. Data on some important physiological and agronomical characters such as epidemic of diseases and insects, flowering and maturity dates, lodging, shattering and plant height were recorded. Grain yield was harvested from an area of 15 m^2 from each plot. Seeds were dried at 70 C for 48 hours and all yields corrected to a standard of a 14 % moisture level. Dried seeds were weighed and expressed in term of kg per hectare. All the data was computed and analyzed.

RESULTS AND DISCUSSION

The results of the yield trial experiment are shown in Table 1. It was found that grain amaranth grown in the upland locations (800 MSL) produced the highest average of grain yield of 1,586 kg/hectare, whereas in the lowland rainfed (300 MSL), the lowest average in grain yield of 243 kg/ha was obtained. The African and Mexican grain types (A. cruentus) produced the highest grain yields and adapted better to diverse climatic conditions than the Nepal, Spike, Mercado, Prima and Aztec grain types. The South American grain type (A-713 and A-982) was less adaptable to lower altitudes (800 MSL) but performed well in highland areas (|1,000 MSL). It is evident that the South American grain type (A. caudatus) was more adaptable to cold conditions than the other grain types. In Nepal, this grain type can be grown in areas having up to an altitude of 3,000 meters (NAS, 1984). The averages of plant height were not significantly different among the three altitudes, while the maturity dates to these genotypes increased as altitude increased. It was observed that the Aztec and the South American grain types were not only late in flowering but also tall and late maturing. These two grain types took more than 6 months to mature for harvest (Senthong, 1984). It was found that drought stress in the lowland rainfed was the major limiting factor in grain amaranth production. The highest grain yield potential obtained from the 83S-27 (African grain type) genotype grown under lowland rainfed conditions (drought stress) was only 450 kg/ha, where as these genotypes produced a higher average grain yield of 3,228 and 2,605 kg/ha, under upland and highland conditions respectively. Senthong et al., (1987) reported that the average grain yield of 33 genotypes of grain amaranth grown under lowland rainfed conditions were very low (182 kg/ha) compared to the upland and highland areas whose average yields were 1,388 and 1,231 kg/ha respectively. This grain yield under the lowland rainfed conditions was therefore, limited by the variations in the amount and distribution of rainfall and by the biotic stress factors that discorage investment in technological inputs. Where irrigation has been possible, higer average in grain yields of 1,500 kg/ha of 20 genotypes of grain amaranth have been achieved in lowland irrigated areas (Senthong, 1984).

Dudal (1976) reported that 90 % of the global land area has significant agricultural problems. Of these, the most important is drought which is chronic. Drought stress influences various physiological processes associated with crop growth development and economic yield (Hsiao, 1973; Begg and Turner, 1976; Levitt, 1980). The average grain yield of 15 genotypes grown in highland areas (>1,000 MSL) was 33 % lower than those of upland location (800 MSL). This result was probably associated with lower temperature that might affect the yield performances of grain amaranth grown in this location.

CONCLUSIONS

1. Grain amaranth can grow and adapt well in the upland and highland areas in northern part of Thailand.

2. The African and Mexican grain types (A. cruentus) are more suitable to grow under a wide range of climatic conditions as compared to the other grain types.

3. Drought stress was found to be the major constraint for grain amaranth production at the lowland rainfed location, while low temperature is the problem in the highland locations.

4. More genetic variabilities should be generated for some specific purposes such as drought and cold resistance, by screening, introducing more germplasm and by crossing among parents which are diversed in origin and genetic bases.

5. All advanced promising genotypes or breeding lines should be further tested and selected continuously for 3 to 4 years until the most desirable traits are found.

ACKNOWLEDGEMENTS

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Table 1. Average Grain Yield, Plant Height and Days to Maturity of Different Grain Types of Amaranth Grown Under Three Different Altitudes During 1983 to 1986 at Chiang Mai, Thailand.

Genotype	Grain type	Lowland rainfed(300MSL)			Uplant (800 MSL)			Highland (> 100 MSL)		
		Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)	Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)	Plant Ht. (cm)	Day to Mat. (days)	Yield (kg/ha)
1. 83S-27	African	163 ab*	117 b*	460 a*	157 ab*	135 d*	3228.3 a*	166 d*	148 bc*	2605 a*
2. 79S-1040	African	123 bc	96 cd	387 b	122 bc	140 d	2886.2 b	107 f	106 c	650 ef
3. R-158	Mexican	153 ab	114 bc	450 a	154 ab	133 de	2983.1 b	149 de	148 bc	1860 bc
4. 82S-1041	Mexican	162 ab	117 b	390 b	164 a	127 de	2391.0 c	160 de	120 de	2033 b
5. 82S-SP130	Nepal	145 b	111 bc	80 d	117 bc	133 de	1154.0 f	165 d	144 c	1583 c
6. 82S-1008	Nepal	130 bc	111 bc	70 d	108 bc	106 f	1078.8 f	69 g	114 de	247 fg
7. 82S-SP153	Spike	127 bc	83 d	200 c	95 bc	111 f	1180.0 f	87 fg	113 de	1330 cd
8. 82S-674	Spike	112 c	99 c	180 c	95 bc	109 f	1293.6 ef	72 fg	118 de	360 f
9. 82S-1023	Mercado	128 bc	118 b	340 b	107 bc	123 e	1989.5 d	137 e	121 de	841 de
10. 81S-1044	Mercado	140 b	99 c	237 c	156 ab	130 de	2368.1 c	152 de	128 cd	1150 d
11. 81S-1004	Prima	105 c	83 d	60 d	91 c	105 f	1053.3 f	60 g	121 de	437 ef
12. A-982	South American	-	-	-	128 b	178 b	51.0 h	203 bc	170 ab	233 g
13. A-713	South American	-	-	-	123 bc	202 a	34.0 h	198 c	190 a	342 fg
14. A-718	Aztec	-	-	-	163 a	171 bc	619.5 g	252 a	170 ab	758 e
15. A-723	Aztec	205 a	185 a	58 d	183 a	164 c	1524.7 e	225 b	166 b	1537 c
	Average	141.08	111.08	242.66	130.8	137.8	1586.0	146.5	138.5	1064.0

Values within a column followed by the same letter are not significantly different at 5% level of probability using New's Duncan Multiple Range Test (DMRT).

FY 1982

INTERACTIONS IN LEGUME-NONLEGUME INTERCROPPING SYSTEMS (BNF-TH-3-83-16)

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ABSTRACT

A maize/ricebean intercrop was evaluated for its advantage over monocultures in terms of grain and nitrogen yield and the benefit from nitrogen fixation. Field experiments were carried out in Tropaqualf soils under rainfed conditions in Northern Thailand from 1983 to 1986. Intercrops and monocultures were compared at a constant overall density equivalent to 80,000 maize or 160,000 ricebean plants/ha, using de Wit's replacement series. Nitrogen fixation by the ricebean was estimated with the natural abundance 15N method, labelled 15N and ureide analysis.

Evidence of intercrop advantages was obtained from the analysis of relative yields and in terms of absolute yield of grain and nitrogen. Dry matter, grain and nitrogen yield of maize and ricebean in intercrops relative to their monoculture yields (relative yield, RY) were significantly greater than their respective share of the plant population. Relative yield totals (RYT) for grain, dry matter and nitrogen were always greater than 1.

Nitrogen fixation by the ricebean was found greatly enhanced by intercropping. Proportion of nitrogen derived from fixation increased 25% to 138% above that in monoculture. This enhancement of fixation was maintained long after the maize harvest. Despite their higher nitrogen yields, the intercrops took up the same amount of mineral nitrogen from the soil and fertilizer as monoculture maize. The intercrop surplus yield of nitrogen was created by fixation.

INTRODUCTION

The majority of Thailand's annual maize, which is grown on almost one million rai of land, is without the addition of fertilizer. An estimated 100,000 ha of this maize is grown in association with indeterminate traditional legumes, black seeded cowpea (*Vigna unguiculata*) and ricebean (*Vigna umbellata*). Ricebean, locally known as redbean or Loei redbean, is a common and traditional pulse of India and Southeast Asia. It is grown in association with major cereals, e.g. rice in Burma (Purseglove 1974) and maize in Thailand (Chulasai et al. 1985). In Thailand ricebean, mostly intercropped with maize, is grown on about 30,000 ha; 21,000 tons were exported in 1985 with a market value of 200 million baht (OAE 1986). Maize intercropped with ricebean is generally believed to be a more sustainable cropping system than monoculture maize and is presently being recommended for the remote highlands where yield declines rapidly after 2 - 3 years of maize monoculture (C. Suthi pers. comm.). This study sought to evaluate the advantage of maize/ricebean intercrop in terms of grain yield and nitrogen economy.

MATERIALS AND METHOD

The experiments were carried out from 1983 to 1986 under rainfed conditions in Chiang Mai, Northern Thailand. The soils were series of Tropaqualfs. The replacement series design (de Wit 1960) was used to compare intercrops with monocultures at a constant overall density of 80,000 maize 160,000 ricebean plants/ha. The treatments were factorial combinations of cropping systems and levels of combine nitrogen. Maize: ricebean ratios in the intercrop varied from 100:0, 75:25, 50:50, 25:75 and 0:100. Combined nitrogen ranged from 0 to 200 kgN/ha. Data collected include dry matter yield, nitrogen yield and grain yield. Nitrogen fixation was estimated from the natural abundance of 15N, labelled 15N and ureide analysis. Detail of the experiments have been presented elsewhere. (Rerkasem and Rerkasem submitted and Rerkasem et al. submitted).

RESULTS AND DISCUSSION

When both maize and ricebean yields are intercropped the dry weights of grain or nitrogen were almost always above the expected yield based on their proportions in the mixture, implying synergistic effects due to intercropping for both species (Figure 1, 2 and 3). Consequently, the RYT values were greater than 1, indicating a yield advantage of intercropping over monoculture. This intercrop advantage appeared unaffected by combined nitrogen. In all experiments ricebean consistently yielded the same amount of grain when intercropped, irrespective of maize: ricebean ratios, as in monoculture.

The RYT's for grain yields of the intercrop, weighted for the difference in crop duration, abbreviated as ATER (Hiebsch and McCollum 1987), for all four experiments ranged from 1.231 to 2.263, with a mean of 1.604±0.287.

Intercropped maize and ricebean yields, whether measured in terms of RYT's for dry matter and nitrogen yields for the period of associative growth or ATER's for grain yields, all indicated definite advantages of intercropping, compared with their monoculture yields. Similar advantages in legume/nonlegume intercrops have been observed by de Wit et al. (1986), Trenbath (1976) and Hiebsch and McCollum (1987). The fact that the yields of both maize and ricebean when intercropped were generally above the diagonals in the replacement diagrammes implies beneficial effects of intercropping for both species. In addition to the advantage in relative terms, there were also absolute advantages in terms of higher nitrogen yield and grain yields at a maize:ricebean ratio of 75:25. Since ricebean grain is 2.5 to 3.0 times the price of maize grain, on account of its higher nutritive value, a higher return can be expected from the intercrop than monoculture maize, even when the total weight of grain harvested in intercrop did not exceed that of the monoculture maize.

Nitrogen Fixation

The proportion of N_2 in ricebean that was derived from fixation varied somewhat between experiments and methods of measurement.

Nevertheless, the major finding that nitrogen fixation in ricebean was enhanced by intercropping was consistent with all sets of data (Table 1). The effect was similar to the effects of decreasing the level of combined N. The uptake of mineral nitrogen, soil and/or fertilizer, by ricebean was markedly reduced by intercropping, while that in maize was increased, relative to their respective shares of the population. This evidence suggests that mineral nitrogen was responsible for low contribution from fixation in monoculture ricebean. With intercropping, the mineral nitrogen was taken up by maize, thus lessening the depressing effects on ricebean N_2 fixation.

The amount of mineral nitrogen taken in the maize plus ricebean in intercrop never exceeded the amount taken by monoculture maize (Table 2). Estimated fixed N accounted for the nitrogen surplus yield of the inter-crop over that of the monoculture maize. This indicates that nitrogen fixation by the ricebean is the major advantage derived from maize/ricebean intercropping.

CONCLUSION

This study has shown that there is a clear advantage in intercropping maize with ricebean in terms of dry matter, seed and nitrogen yield. The intercrop advantage in nitrogen yield actually exceeded the best monoculture yield. This results from the efficient use of mineral nitrogen by the maize and enhancement of nitrogen fixation by the ricebean. The effects of intercropping in enhancing ricebean nitrogen fixation were maintained long after the maize harvest. The increased dependence on nitrogen fixation in intercropped ricebean has caused a similar amount of nitrogen to be fixed under intercrop and monoculture, despite a much higher nitrogen yield in the latter. The overall advantage of the maize/ricebean intercrop is therefore related to the efficient use of mineral nitrogen by the maize, and to fixation by the ricebean.

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Table 1 Contribution from Nitrogen Fixation in Ricebean Intercropped and Monoculture.

Year	kgN/ha	days from sowing	Maize : ricebean ratio 75:25	50:50	25:75	100:0	Method* of measurement
			Proportion of N from fixation				
1985	0	91	0.66	0.61	0.49	0.47	1
			0.86	0.84	0.63	0.36	2
		147	0.77	0.64	nd	0.48	1
1986	2[illegible]	100	0.90	-	0.76	0.72	3
	200	100	0.51	-	0.39	0.37	3

*Method of measurement: 1 = Ureide Analysis;
2 = Natural Abundance ^{15}N
3 = Labelled ^{15}N

Table 2 Sources of Nitrogen Taken Up by Maize and Ricebean in Intercrop and Monocultures.

KgN/ha	M:rb*	Maize N	Ricebean N		Cropping systems total N	
		S + F**	S + F	Fixed	S + F	Fixed
		.....kgN/ha.....				
1985						
91 days from sowing						
0	100:0	130.6	-	-	130.6	-
	75:25	122.0	16.5	31.8	138.5	31.8
	50:50	78.9	26.7	40.4	119.6	40.4
	25.75	54.2	70.9	58.3	125.1	58.3
	0:100	-	72.1	62.5	72.1	62.5
147 days from sowing						
0	100:0	-	-	-	-	-
	75:25	-	18.6	64.0	-	-
	50:50	-	28.3	50.3	-	-
	25:75	-	nd	nd	-	-
	0:100	-	74.4	69.0	-	-
1986						
20	100:0	74.34	-	-	74.34	-
	75:25	63.72	6.78	62.19	70.50	62.19
	25:75	42.51	28.65	97.90	71.16	97.90
	0:100	-	37.61	96.79	37.61	96.79
200	100:0	229.48	-	-	229.48	-
	75:25	182.73	27.87	31.78	210.6	31.78
	25:75	92.46	93.44	60.32	185.90	60.32
	0:100	-	122.15	55.45	122.15	55.45

* M:Rb = Maize:Ricebean

** Fertilizer Plus Soil Nitrogen

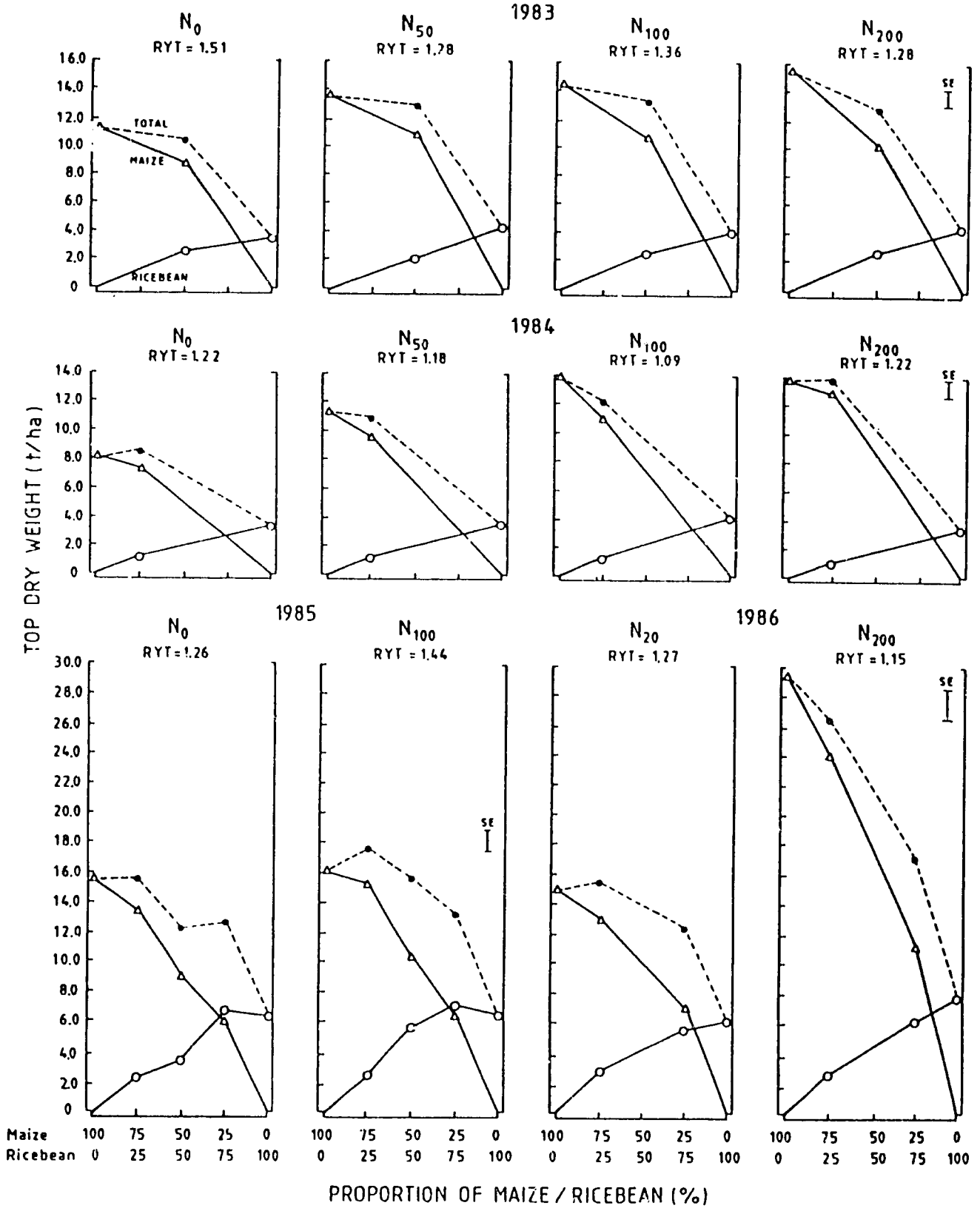

FIGURE 1. Effects of combined nitrogen and maize/ricebean proportion on top dry weight. Bars represent standard error (SE) of the total top dry weight.

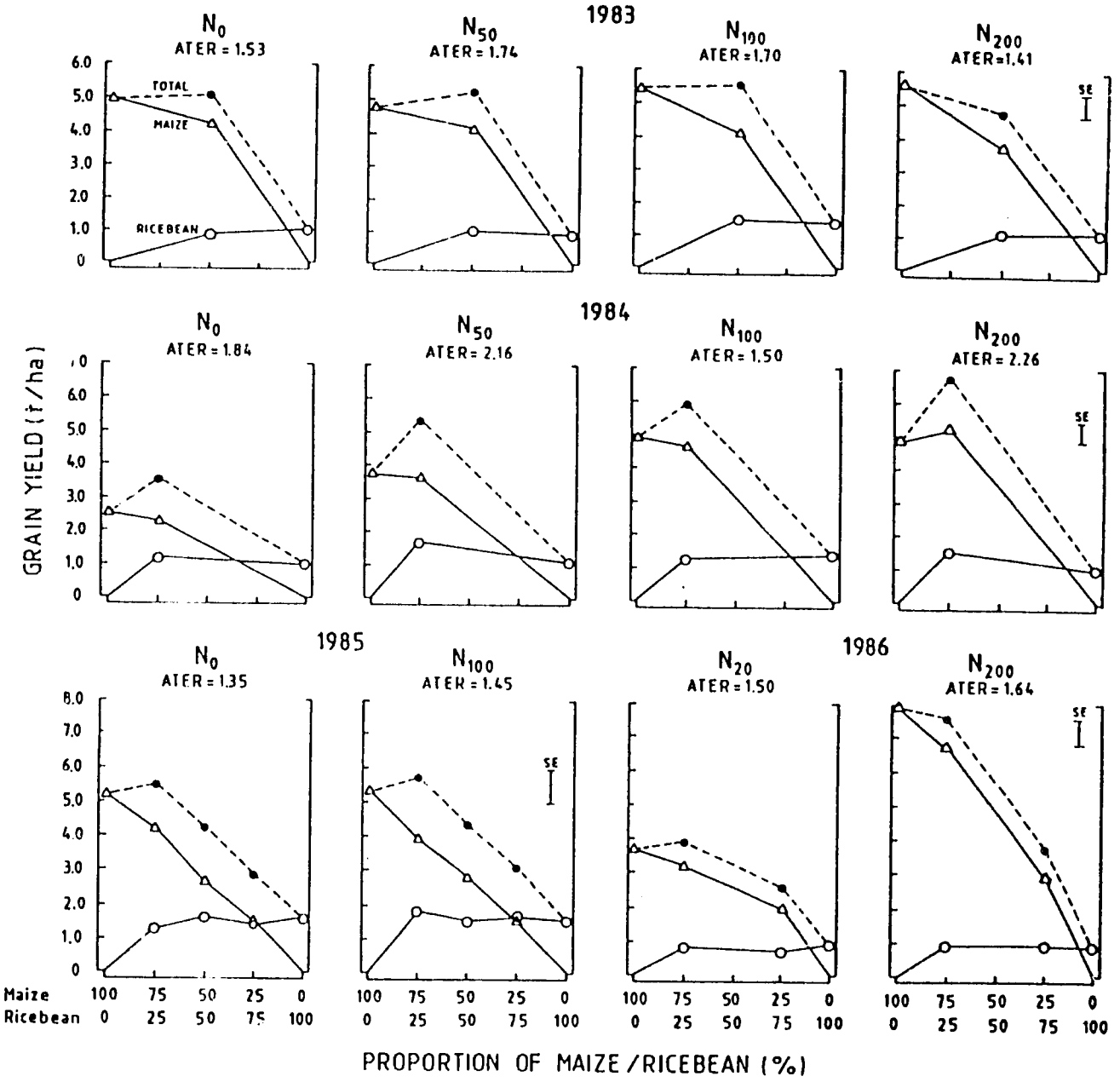

FIGURE 2. Effects of combined nitrogen and maize/ricebean proportion on grain yields. Bars represent standard error (SE) of the total grain yields.

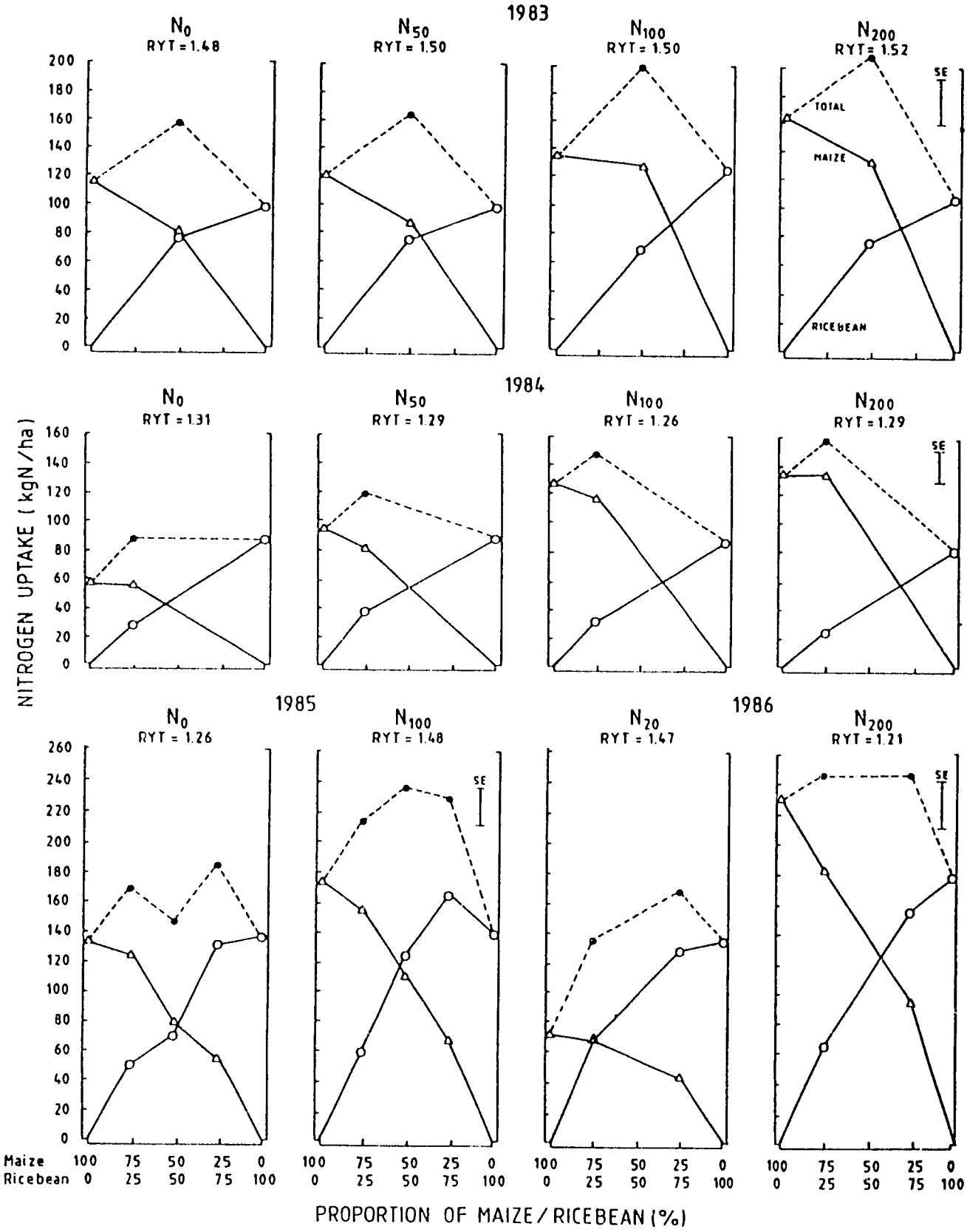

FIGURE 3. Effects of combined nitrogen on the uptake of total nitrogen (soil N and fixed) in the top of maize (▵—▵) and ricebean (o—o) in intercrop and monoculture at corn maturing stage; (•---•) indicates intercrop total.

FY 1983

SPECIES SELECTION AND POTENTIAL USES OF GRAIN AMARANTH AS AN INTRODUCED CROP (AMA-TH-4-83-18)

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INTRODUCTION

Grain amaranths were once extensively grown and used as staple foods nearly as important as corn and beans by the Incas and Aztes in the Americas. This plant went into oblivion after the collapse of the American Indian culture in the 15th century following the conquest of the Aztec Empire by Spanish conquistadors. The Spanish conquerors had strictly forbidden further planting and use of amaranth grains because of their use in pagan rituals and human sacrifice.

Revival of interest on grain amaranths began after the discovery of unusual protein quality in amaranth grains by an Australian plant physiologist, John Downton in 1972. Amaranth grains contain about 16% protein content, which is considerably higher than other grains. However, an exciting potential of grain amaranths lies in its high content of the amino acid lysine which is generally low in most cereals. The lysine content in amaranth grains is as much as is found in milk.

Identification of grain amaranths as an underexploited tropical plant with promising economic value by NAS in 1975 greatly stimulated public attention. Mixture of amaranth grain flour and conventional flour seems to provide an ideal staple with balanced amino acid composition for optimum health.

Protein deficiency is one of the common health problems in developing countries, especially in poor rural areas. Thailand is no exception; there is an urgent need for protein foods to alleviate protein deficiency problems in rural areas. Because of a decline in marine production, one of the most important protein food sources is decreasing. Therefore, it is necessary to look into other sources of protein food in order to meet the increasing demand.

Thailand Institute of Scientific and Technological Research (TISTR) received financial support from BOSTID-CRG in 1983 to undertake research on grain amaranth production and utilization in Thailand, especially in the northeast areas.

The objectives of the research program are to develop and select grain amaranth varieties for Northeast Thailand, to adapt grain amaranth to Thailand cropping practices, and to introduce this nutritious food grain to the public.

MATERIALS AND METHODS

This research program has been carried out in four main categories:

1. Grain amaranth germplasm, collection and evaluation.
2. Study of cultural practices
3. Study of food processing
4. Animal feeding trials.

Seventy-eight accessions of grain amaranth were introduced into Thailand at the beginning of the research program in early 1983. Nearly a half came from Rodale Research Center's Collection. The rest came from Brazil, India, and the Republic of China. Thirty accessions of grain amaranth were also collected in October 1986 from Nepal.

Classification of 78 accessions into various grain types was carried out during preliminary evaluation of monthly planting from April to August 1983 at TISTR's Chan Thuk research station. Plant accessions with poor adaptability were excluded from further evaluation at this stage. Varietal Trials of grain amaranth accessions were subsequently carried out within and between Mexican grain types, Mercado grain types, Nepal grain types and Aztec grain types. Selected accessions and plant types were then subjected to intensive studies in response to different cultural practice treatments of spacings, fertilizer rates, planting dates as well a responses to different methods of weed control.

Development of grain amaranth for Northeast Thailand was also carried out through plant breeding and selection. Evaluation of 10 F2 hybrids from Rodale Research Center was carried out to improve grain yield and adaptability to local conditions.

Study on food processing of amaranth grains was initially carried out in TISTR's Food Technology Lab but most of the works in the later period were carried out in the Institute of Food Research and Product Development, Kasetsart University. Grain amaranth food products were analysed for food composition, and acceptability tests were also carried out by 15 test panels.

Grain amaranth crop residues after removing of seed heads were analysed for animal feed value. A pig feeding trial was carried out with 15% and 30% replacement of conventional feed with amaranth crop residues. Replacements of 10% and 20% of roughage were also tested in dairy cows, while partial and total replacement of roughage were studied in beef cattle. Body weight gain and feed conversion ratio were evaluated in pig and beef cattle feeding trials. Milk production and compositions were evaluated in dairy cows.

RESULTS

1. Screening Trials

Early screening trials of 15 grain amaranths Mexican grain type showed that the number of growth parameters were significantly varied.

Grain yield varied from 775 kg/ha to 1,869 kg/ha but there was no significant difference in grain yield among 12 accessions which yielded more than 1,275 kg/ha. Therefore, other growth characters, especially lodging resistance and seed color, were taken into consideration in the selection of accession No. 1, 434, 1010, 1011 and 1015 to represent this group in future trials. Five grain amaranth accessions of Nepal grain types, IC 42254-14, 42255-5, 42290-17, 42293-15 and SP 130 which yielded 1,312 kg/ha to 1,575 kg/ha were also selected from the original 15 accessions.

Yield trials of four and five accessions of Aztec and Mercado grain types were also carried out in 1984 However, Aztec and Nepal plants produced the maximum grain yields of 144 and 850 kg/ha. Results from these trials indicate that the Aztec and Mercado plants in TISTR's collection have relatively low potential for grain production under local conditions.

2. Plant Date Trials

Studies on planting date trials of grain amaranths were carried out in 1984 and 1985. Grain amaranths were planted in the early and late rainy periods of the year in accordance with plantings of field crops in study areas. Results of this trial confirmed previous reports that grain amaranths are short-day plants. The plants had longer vegetative growth and took longer time to mature in early planting. Larger plants and higher grain yield were also recorded for early planting.

3. Spacing Trials

Evaluation on growth and grain production of three accessions of grain amaranth Mexican (1041, Mercado (1023) and Nepal grain type (IC 42290-17) in responses to 5, 15, 25 and 35 cm. plant spacings was carried out at Chan Thuk Research station in May 1984. Results of the study indicated that the maximum amaranth grain yield was produced under the closest spacing of 5 cm. and tended to decrease with increased plant spacing. Lodging was obviously a serious problem affecting Nepal and Mercado plants. The degree of lodging tended to increase with increased plant spacing, with the maximum values of 47.5% and 57.5% lodging recorded in Nepal and Mercado plots, respectively.

The second experiment was later planned and carried out in May and August 1986. Responses of the Mexican plant No. 1011 to four treatments of 1, 2, 3 and 4 plants/hill with plant spacings of 5, 10, 15 and 20 cm., respectively, were recorded and a broadcast treatment was conducted. All 5 treatments had equal plant populations of 166,667 plants/ha. Results of the study indicated that growth and grain yield of the Mexican plants were relatively unaffected by different distributions of plants within plots. Although plant competition tended to increase with an increasing number of plants/hill, wider plant spacing seemed to alleviate this problem. Hilling could not be carried out in broadcase plots and was likely responsible for low grain yield.

4. Fertilizer Trials

Responses of Mercado (1023), Mexican (1011) and Nepal (IC 17947) to four rates of 15-15-15 (NPK) fertilizer were carried out in farmer's plots, Muak Lek District and Thai-German Demonstration and Training Center in June and July 1985. Growth and grain production of grain amaranths were responsive to an increase in fertilizer. However, the Mexican plant showed a higher magnitude of responses indicating that Mexican plants would be better adapted to fertile conditions than Mercado and Nepal plants. And it was concluded that the rate of 312.5 kg/ha is adequate to sustain growth and grain production of grain amaranths within study areas.

5. Effects of Different Methods of Weed Control

Studies on the responses of grain amaranth Mexican plants No. 1011 were carried out at the Thai-German Demonstration and Training Center in May and August 1986. Results of the trial indicated that grain amaranth growth and grain production were relatively tolerant to weed problems. Hilling alone proved to be sufficient in sustaining grain amaranth productivity, with 7% reduction in grain yield compared to six fold increase from 210 kg/ha of weed in plots with conventional practices.

6. Food Processing Trials

Composite flour with 10-25% amaranth flour made from Mexican, Mercado and Nepal grains was baked into cakes, cookies, breads, local sweets and a snack called Khao Kriap. Results of proximate analysis revealed that addition of grain amaranth flours did not significantly improve protein content in these food products, except in Khao Kriap which is normally made from tapioca flour. Acceptability tests showed that food products with Mexican grain flour were the most acceptable.

7. Animal Feeding Trials

Substitutions of conventional feed and roughage with grain amaranth crop residues were evaluated over a period of 36 days in pigs and 60 days in beef cattle and dairy cattle.

Results of the pig feeding trials showed that up to 30% of ground amaranth crop residues can be fed and utilized by growing pigs. Up to 50% of amaranth straw replacement could be as good as grass forage for beef cattle. Dairy production was not affected by 20% replacement of roughage with amaranth straw especially protein content in milk. However, fat content in milk tended to increase when fed by amaranth straw.

CONCLUSION

Results of the trials carried out since 1983 provide understanding on the growth, development and productivity of grain amaranth under local conditions. The Mexican grain type was evidently well adapted to NE Thailand conditions. But poor distribution of rain, a common feature in the study areas could severely damage the plants in the early growth

period. Areas with high soil water retention will be necessary for successful amaranth grain production. Also, grain amaranth varieties with short growing periods have to be developed for NE Thailand to avoid drought problems which usually occur during planting season.

Substitution of conventional flours with grain amaranth flour seemed to have limited application. And the 20% to 25% replacement was far from the 50% needed for balancing the amino acid content. Pastries in Thailand are considered food commodities for middle or higher class families which have little protein deficiency problems. Thus, future work should mainly concentrate in developing processing procedures for supplementary baby foods accepted by people in rural areas.

Positive responses in animal feeding trials of grain amaranth crop residues have revealed potential uses of grain amaranth crops for animal feed. However, the use of whole amaranth plants which have much higher nutritional values will likely have more impact in this application.

FY 1983

STUDIES ON THE BIONOMICS OF *Anopheles maculatis* AND ITS ROLE IN MALARIA TRANSMISSION (MVR-TH-5-83-29)

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ABSTRACT

The biomomics of *Anopheles maculatus* complex and its role in malaria transmission were studied in the Sadao and Pakchong areas from January 1984 to July 1985. Morphological characteristics and polytene chromosomes were indentified simultaneously, with only 2.1% of individuals misidentified using the morphological key.

In Pakchong, *An. muculatus* species A was the most dominant species, followed by species B (form F), but species C was rare. The densities of species A and species B (form F) were high between July and November, with peaks in October. The female mosquitoes preferred to feed on cattle rather than on man, exhibiting a zoophilic habit. Biting activities of both species occurred throughout the night, with a major peak during the first quarter of the night on all seasons.

In Sadao, only *An. maculatus* species B (from E) was detected throughout the study period, with peak densities between February and June. Similar to *An. maculatus* species A and B (form F), the female mosquitoes species B (form E) preferred to feed on cattle rather than on man. Research on the contact between mosquitoes and man outdoors and indoors indicated that the mosquitoes in Sadao tended to associate with man more than the mosquitoes in Pakchong. Biting activity of this species demonstrated different biting peaks according to season.

Out of 4,430 guts of *An. maculatus* complex dissected, 10 (0.23%) were found infected with oocysts. No sporozoites were found in any of the 4,472 salivary glands dissected.

INTRODUCTION

Anopheles (Cellia *maculatus* Theobald occurs throughout the Oriental zoogeographic region. it is highly variable in both its morphology and ability to transmit malaria (Reid, 1968), and may represent another complex of species having little or no morphological differentiation (Reid, 1970). Studies on its biological difference were done by several authors (Christophers, 1931; Reid *et al.*, 1966). Cytogenetic evidence for a complex of species within the taxon of this species was reported by Green et al. (1985).

In Thailand, An. maculatus is a widespread species, particularly in the upper central, northern and northeastern parts of the country, and is found in forested and foothill areas. Evidently, it is one of the main malaria vectors in Malaysia, and has been incriminated only once in the Wang District of Narathiwas Province, in the southern part of Thailand, on the border with Malaysia. The breeding sites of this species are very similar to those of An. minimus which is prevalent throughout the year (Ismail et al., 1974, 1975 and 1978).

There are no recent studies on the bionomics and vector potential of the An. maculatus complex in Thailand prior to this investigation. Therefore, a series of careful investigations into the life history, bionomics, and role in transmission of this mosquito might not only be a means to settle its status vis-a-vis malaria, but also might provide biological and/or environmental indicators which could be employed to signal changes in receptivity levels. The information obtained could also be used for planning of effective control measures by the Thai Anti-Malaria Program.

The specific objectives of this investigation were described as follows:

1. to determine the role of Anopheles maculatus in malaria transmission,

2. to investigate the bionomics of An. maculatus complex.

MATERIALS AND METHODS

1. Selection of Study Sites

Two villages, in the Sadao and Pakchong districts, each with 130-212 houses and 590 to 600 inhabitants, were selected for this study. Sadao is located in village no. 9 (Ban Yang Ko), Prik Canton, Sadao District, Songkhla Province, southern Thailand. The houses are scattered in the form of small hamlets, and the area is covered with rubber plantations. Rubber tapping is the main occupation of the local people, facilitating malaria transmission in the area. Residual spraying with DDT with a dosage of 2 gm/m^2 was started in 1965, and this control measure is still being continued.

The Pakchong study area is located in village no. 5 (Ban Moh Mai Kaen), Mo Si Canton, Pakchong District, Nakorn Ratchasima Province, in the upper middle part of Thailand. The area is mountainous and is covered by forest. The main occupation of villagers is maize farming and forest reservation park laborers. This area has a long history of malaria since the beginning of the malaria eradication program. DDT residual indoor spraying has been used since 1960.

2. Entomological Techniques

Monthly entomological observations were conducted regularly to collect mosquitoes between January 1984 and June 1985. Collections of

mosquitoes were carried out on both human and animal bait, from 1800 hours until 0600 hours with a ten-minute rest each hour. Two humans indoors, two humans outdoors and one bovine were assigned to collect the mosquitoes on six consecutive nights each month studied.

The captured mosquitoes were first sorted into maculatus and other anophelines, and then kept in mosquito cups marked with the identification site and hour of collection, and held in coolers at ambient temperatures. All maculatus were blood fed until satiated at 24.00 and 06.00 hours per day to become half gravid by the following day. The collectors were supplied with appropriate prophylaxins against Plasmodium spp.

3. Identification and Dissection

The gravid maculatus were knocked out individually with ether, and then were identified using the morphological key provided by Mrs. Rampa Rattanarithikul (personal communication). The ovaries were dissected out and fixed in Carnoy's fixative for later ploytene chromosome identification. The guts and salivary glands were then examined for oocysts and sporozoites.

All other anophelines caught were processed in the following morning to be identified morphologically, and were dissected to examine the tracheoles of the ovaries (Detinova, 1962). In the parous specimens, the guts and glands were removed to examine for oocysts and sporozoites.

When the salivary glands were found to be positive, the slide was placed in an incubator at 30°C for 1 hour, and subsequently was reexamined using a specific IFA test to score the Plasmodium species.

4. Meteorological Records

Temperature and relative humidity were continuously recorded by a standard automatic thermohygrograph placed in a screen. Daily precipitation and hourly wind velocity were recorded during the highly bit collections.

RESULTS

1. Identification of the Genetic Species Within Anopheles maculatus

Morphological identification revealed 3 species of An. maculatus (Species, A, B (form F) and C) in the Pakchong area, and one species in the Sadao area (species B form E, identified by polytene chromosome). The results of testing the morphological key against the polytene chromosomal identification (Green et al., 1985) showed that 2.11% of the individuals were misidentified using the key.

2. Distribution

During the 18 month period of study (between January 1984 and June 1985), the density of An. maculatus complex found in Pakchong was

approximately 4.5 times greater than in Sadao with total numbers of 6090 and 1344, respectively. The numbers of An. maculatus species A, B (form F) and C in Pakchong were 4994 (82.0%), 1067 (17/5%), and 29 (0.5%), respectively. An. maculatus species a was the most dominant species in Pakchong, While An. maculatus species C was rare. An. maculatus species B (form F) was found in the Pakchong study area, while An. maculatus B (form E) was the only species found in the Sadao study area.

3. Seasonal Prevalence and Ulimatic Influence

3.1 Sadao

For the 18-month study period, monthly densities of female mosquitoes varied from zero to 34 mosquitoes per night. The densities were high (exceeding ten females per night) during March to June in both 1984 and 1985.

The monthly densities of An. maculatus species B (form E) showed a close relationship with monthly rainfall pattern. Moreover, heavy rains influenced by the northeastern monsoon washed away the larvae from their habitats and reduced drastically the number of adult female mosquitoes during September to December. The results also indicated that temperature and humidity exhibited positive correlation with mosquito densities. Rainfall and wind velocity were not significant factors.

3.2 Pakchong

Both An. maculatus species A and B (form F) were abundant during the rainy months, from May to October. The densities of both species rose in June to September, reached their peaks in October and decreased gradually until the next rainy season. Similarly the peak density of An. maculatus C occurred during the rainly season, even though only a few mosquitoes were caught.

The cool season in Pakchong is from mid October to mid February, and also is considered the dry season. During this time the density of adult mosquitoes was also low, which corresponded to the low number of mosquito larvae due to the dryness of breeding places.

4. Host Preference

In Sadao, the number of An. maculatus species B (form E) collected from the animal bait was 2.7 times greater than from the human bait (540 : 200), whereas in Pakchong, the numbers of An. maculatus species A, B (form F) and C collected from the animal bait were 168.2 (4877/29), 97.6 (1025/10.5), and 29.0 (29/0) times greater than from the human bait, respectively. The results indicated that An. maculatus species B (form E) associated with man more than An. maculatus species A, B (form F) and C.

5. Biting Cycle

5.1 Human Bait

The total number of An. maculatus species A caught in the summer, wet, and cool seasons were 10, 99 and 8, respectively. The biting peaks were at 1900 hours.

The total numbers of An. maculatus species B (form F) caught in the summer, wet, and cool seasons were 15, 17 and 5, respectively, and the biting peaks of both the summer and wet seasons were at 1900 hours. Only a few mosquitoes were caught during the cool season.

The total numbers of An. maculatus species B (form E) caught in the summer, wet and cool seasons were 523, 250, and 28, respectively, and the biting peaks of the mosquitoes in summer and wet seasons were similar, i.e. between 2000-2200 hours, and then decreased gradually until the morning. In the cool season, however, smaller numbers of mosquitoes were caught and most of them were caught between 2000-0200 hours.

5.2 Animal bait

The total numbers of An. maculatus species A caught in summer, wet and cool seasons were 337, 4165 and 375, respectively, and its biting peaks were similar for all three seasons, with major peaks during the first quarter of the night. The mosquitoes tended to bite throughout the night until 0600 hours.

The total numbers of An. maculatus species B (form F) caught in the summer, wet and cool seasons were 275, 671, and 81, respectively, and their biting pattern was similar to that of An. maculatus species A, except in the cool season, when its biting pattern fluctuated more than that of An. maculatus species a. This might be due to the smaller number of An. maculatus species B (form F) caught.

The total numbers of An. maculatus species B (form E) caught in the summer, wet and cool seasons were 415, 113 and 15, respectively. Its biting patterns for all three seasons were different. In the summer, the biting peak was sharp, between 2000-2200 hours, while in the wet season, the biting peak was at 2300 hours and its distribution was observed throughout the night. In the cool season, however, more biting was observed in the early morning.

6. Parasitology

In Sadao, out of 1,002 guts dissected, only a 0.6 oocyst rate (6%) was recorded, and out of 1,024 glands examined, there was no positive sporozoite rate detected.

In Pakchong, out of 3,428 guts dissected, only a 0.1 oocyst rate (4%) was recorded, and out of 3,448 glands examined, there was no positive sporozoite rate detected.

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FY 1984

DEVELOPMENT OF DNA PROBES TO DIFFERENTIATE SIBLING SPECIES OF *Anopheles dirus* AND *An. maculatus* (MVR-TH-6-84-25)

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ABSTRACT

Anopheles dirus, one of the major vectors of human malaria parasites in Thailand and Southeast Asia is a species complex of at least four distinct species identifiable by chromosome analysis of larva which is complicated, slow and impractical for epidemiological studies. This study develops DNA probes from species-specific DNA sequences for identification of the four isomorphic species. Species-specific DNA sequences of each of the four isomorphic species were constructed by either a "Direct cloning" or "Deletion enrichment" technique. Species D specific sequences were obtained by cloning Sau 3AI genomic DNA fragments into *E. coli* using pUC12 vector. From 7,000 recombinant clones 21 clones were species D specific. Three clones (pMU-D9, pMU-D10, pMU-D76) were selected based on their strong hybridization to the mosquito DNA. They could detect 5 ng of DNA, which represents one-hundredth of a single mosquito. pMU-D9, pMU-D10 and pMU-D76 contained DNA insert of 115, 289 and 124 nucleotides respectively and the DNA insert repeated approximately 18,000, 3,800 and 18,000 times per genome respectively. When used as a DNA probe, pMU-D76 could correctly identify individual mosquitoes including larvae, adult males and females.

INTRODUCTION

Anopheles dirus, a major vector of malaria parasites in Thailand and Southeast Asia belongs to a species complex. There are four informally designated species in the complex, species A, B, C and D, which have virtually isomorphic adult females. Identification of wild-caught females depends on rearing families from them and examination of larval chromosomes. This method is complicated, slow and impractical for epidemiological studies. As a consequence, the facts of whether the four isomorphic species are equally good vectors of malaria or some are relatively harmless are not clearly understood.

Other methods of species complex identification include analysis of ovarian polytene chromosome , enzyme electromorphs, and cuticular hydrocarbon analysis. However, none of the methods has succeeded in identification of *An. dirus* isomorphic species.

Recombinant DNA technology has been applied to obtain species-specific DNA fragments which could be used as DNA probes for identification of

insect vectors of onchocerciasis, strains and species of some human parasites and pathogens. Reported here is an attempt to apply recombinant DNA technology to search for species-specific DNA sequences and to use them as DNA probes for a practical means of identification of members of the An. dirus complex.

CONSTRUCTION OF SPECIES-SPECIFIC DNA SEQUENCES

An iso-female line of each species (species A, B, C or D) was used as a source of DNA. Females were used; males being excluded so as to avoid any Y-chromosome DNA which is male-limited. DNA was fragmented to completion with Sau3A1 and ligated to pUC12 at the BamH1 site. Recombinant E. coli clones were produced and screened by colony hybridization. From 7,000 recombinant clones of An. dirus D, twenty-one clones showed strong binding to P-DNA of the species D but weak or negative hybridization to that of species A, B and C. Plasmids were extracted from these clones which were then digested with EcoRI and analyzed by agarose gel electrophoresis (Fig.1). The majority of the plasmids had very small inserts ranging from 100-300 base pairs. Exceptions were clones 44, 47 and 67 which contained about 450, 750 and 1,800 base pairs respectively. These DNA fragments did not hybridize with the ^{32}P-labelled DNA from the species A, B and C. They hybridized with the ^{32}P-labelled DNA from the species D which indicated that these DNA fragments were species D specific. These DNA fragments were further tested if they would hybridize with the DNA from species D alone. The recombinant plasmids containing potential species D specific DNA fragments were purified in a CsCl gradient, labeled with P and then used as DNA probes from all four species which were dot-spotted onto nitrocellulose. The recombinant plasmids which hybridized specifically to the dot-spotted DNA of species D were clones 7,9,10,31,44,47,53,67,73 and 76 (See Fig. 1). A typical result is shown in Fig. 2 which demonstrates the specific binding of clones 9, 10 and 76 to species D. These plasmids could detect as little as 5 ng of DNA which represents approximately one hundredth of the total DNA extractable from a single mosquito.

SEQUENCE OF THE SPECIES D SPECIFIC FRAGMENTS

The species D specific nucleotide sequences in pMU-D9, pMU-D10 and pMU-D76 were identified by the chain termination method and shown in Fig. 3. The sequence in pMU-D9 was 115 base pairs long, that in pMU-D10 289 and in pMU-D76 124. pMU-D9 had an almost identical sequence to the first 115 bases in pMU-D10. The nucleotide sequence in pMU-D76 was unique.

COPY NUMBER OF SPECIES D SPECIFIC DNA FRAGMENTS

The number of copies of DNA fragments in clone 9 (pMU-D9), clone 10(pMU-D10) and clone 76(pMU-D76) was estimated by comparing the intensity of hybridization of the insert DNA sequence to itself and to total species D DNA as shown in Fig. 4. The DNA fragments in pMU-D9, pMU-D10 and pMU-D76 as deduced from DNA sequencing were 115, 289 and 124 respectively, assuming that genome size of An. dirus was 160,000 Kb . The copy numbers of insert DNA in pMU-D9,pMU-D10 and pMU-D76 were estimated as 1.8 x 10^4, 3.8 x 10^4 and 1.8 x 10^4 per genome respectively.

GENOMIC ORGANIZATION OF THE SPECIES D SPECIFIC DNA FRAGMENTS

DNA of all four species was digested with restriction endonucleases, subjected to agarose gel electrophoresis, Southern transfer onto nitrocellulose, and probed with ^{32}P-labelled pMU-D9, pMU-D10 or pMU-D76. As shown in Fig. 5, these DNA probes hybridized specifically to the species D DNA. All three probes gave the same pattern of a ladder with 400 base pair spacing when the genomic DNA was digested with HinfI. This suggests that the three probes adjacently sit on the genomic DNA. Furthermore, these three sequences should be on the satellite DNA as had been previously demonstrated in Drosophila genomes.

IDENTIFICATION OF SPECIES D AMONG THE ISOMORPHIC SPECIES

pMU-D76 was used to probe homogenates from individual insects that had been spotted onto nitrocellulose paper. Such individuals include larvae, adult males, and adult females. Some of the latter were specimens kept at room temperature for over two months. A typical result is shown in Fig. 6. In all cases, only specimens of species D showed a positive hybridization to the probes. There was no need to make a specific DNA extraction from these individuals. As little as one hundredth of the homogenate from a single mosquito could be successfully detected. This high sensitivity suggests that the radioisotope label may be replaced with a more practical, non-isotope label such as biotin/enzyme-linked streptavidin.

Other species of Anophelene mosquitoes including An. balabacencis, An. eucosphyrus, An. maculatus A, An. maculatus B, An. takasagoensis did not hybridize with the ^{32}P-labelled pMU-D76 probe. It appears that the sequence is highly specific to the An. dirus species D.

In the future, wild-caught mosquitoes can be directly processed for epidemiological parameters such as sporozoite rates and age grading and the residual of the individual used for species identification by probes such as pMU-D76. Abdomen and salivary glands from a single individual have been successfully used for species identification using pMU-D76. A combination of DNA probes for Plasmodium falciparum infection and for species identification is being persued.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1: Agarose gel electrophoretic (i) and autoradiographic (ii) patterns of 21 potential species-specific plasmids for species D of the An. dirus complex. The EcoRl cut plasmids from (i) were transferred onto nitrocellulose, probed with ^{32}P-labelled species D DNA and autoradiographed for five days (ii). No hybridization was observed when transfers of gels like (i) were probed with ^{32}P-labelled species A, B or C DNA. Lamda HindIII was used as a molecular-weight marker.

Figure 2: Dot-blot analysis of the plasmids pMU-D9, pMU-D10 and pMU-D76 that were specific to species D of the An. dirus complex. DNA from all four species were denatured and spotted in duplicate onto nitrocellulose paper (columns A, B, C and D) at the indicated concentrations. These dot-blots were probed with ^{32}P-labelled plasmid DNAs and autoradiographed for one day. Only species D showed the hybridization signal.

Figure 3: The nucleotide sequences of pMU-D9, pMU-D10 and pMU-D76.

Figure4: Comparison of autoradiographic signals between self-hybridization of species-specific DNA fragments and the hybridization of the DNA fragments to the total genomic DNA of An. dirus D. Varying amounts of genomic DNA from An. dirus D and of DNA inserts from pMU-D9, pMU-D10 and pMU-D76 were dot-spotted onto nitrocellulose which was subjected to hybridization with ^{32}P-labelled DNA inserts (specific activity 3 x 10 cpm/ug).

Figure 5: Southern hybridization pattern of the genomic DNA of species of the An. dirus complex cut with HinfI. The mosquito DNA was restricted, electrophoresed, transferred onto nitrocellulose and probed with ^{32}P-labelled pMU-D9, pMU-D10 and pMU-D76. The plasmid hybridized only to species D DNA. Lamda DNA was used as the molecular-weight marker.

Figure 6: Single individual mosquitoes, of known identity, from the four species of the An. dirus complex, probed with pMU-D76. Individuals were homogenized, treated with pronase; the supernatant directly spotted onto nitrocellulose and probed with ^{32}P-labelled pMU-D76. In all cases pMU-D76 bound only to species D individuals. The number 5, 8, 12, 16, 18, 19, 22, 34, 39 represents single individuals identified by metaphase chromosome analysis (Baimai et al 1984) as species A; 14 and 36 as species B; 6, 9, 10, 11, 13, 17, 21, 26, 27, 29, 31, 32, 35, 40 as species C; 1, 2, 3, 4, 15, 24, 25, 28, 33, 38 as species D and 7, 20, 23, 30, 37 as unidentified samples.

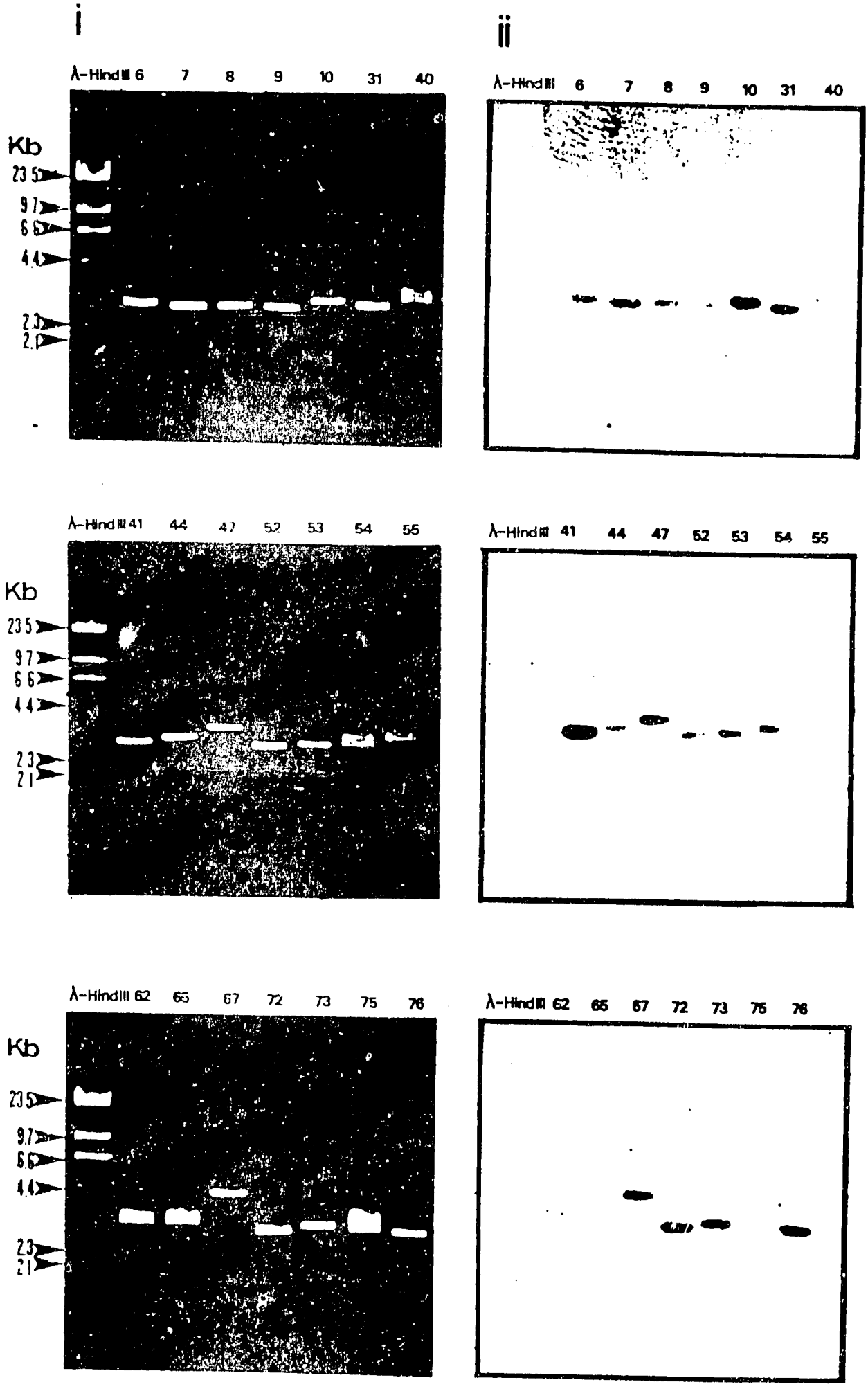
i
ii
λ-HindIII 6 7 8 9 10 31 40
Kb
23.5
9.7
6.6
4.4
2.3
2.1
λ-HindIII 41 44 47 52 53 54 55
λ-HindIII 62 65 67 72 73 75 76

Figure 1

324

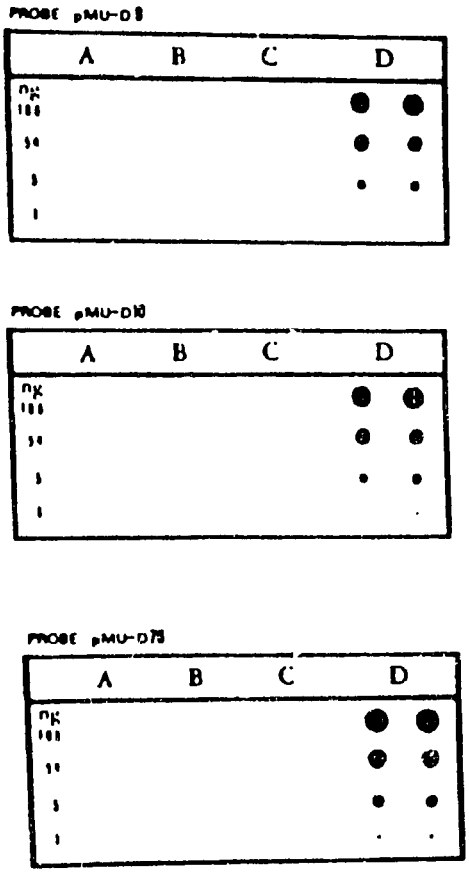

Figure 2

pMU-D9 SEQUENCE : 115bp ; 25A ; 31C ; 31G ; 28T

```
                10                  20                  30
5'  GATCCAATGG      AGGGCACAAA      AGTTATTAAC
3'  CTAGGTTACC      TCCCGTGTTT      TCAATAATTG
                40                  50                  60
    TTTTCCGATT      TCGGGTGCGC      GCAGTGACGC
    AAAAGGCTAA      AGCCCACGCG      CGTCACTGCG
                70                  80                  90
    GCGCGGCGAG      TGCGACGGAA      ACTTGCTCCG
    CGCGCCGCTC      ACGCTGCCTT      TGAACGAGGC
                100                 110                 115
    TGCACAACTT      TTCCGTTTCT      CAACC  3'
    ACGTGTTGAA      AAGGCAAAGA      GTTGG  5'
```

pMUD-10 SEQUENCE : 289 bp ; 70A ; 76C ; 59G ; 70T

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                10                  20                  30                  40
5'  GATCCAATGG      AGGGCACAAA      AGTTATTAAC      TTTTCCGATT
3'  CTAGGTTACC      TCCCGAGTTT      TCAATAATTG      AAAAGGCTAA
                50                  60                  70                  80
    TCGGGTGCGC      GCAGTGACGC      GCGCGGCGAG      TGCGACGGAA
    AGCCCACGCG      CGTCAGTGCG      CGCGCCGCTC      ACGGTGCCTT
                90                  100                 110                 120
    ACTTGCTCCG      TGCACAACTT      TTCCGTTTCT      CAACCGATCC
    TGAACGAGGC      ACGTGTTGAA      AAGGCAAAGA      GTTGGCTAGG
                130                 140                 150                 160
    TGCCCAAATT      TGCACCATAT      GCACATCTCT      TCGGGTGCTA
    ACGGGTTTAA      ACGTGGTATA      CGTGTAGAGA      AGCCCACGAT
                170                 180                 190                 200
    CAATATTCGC      TCTTCACCCG      ATCGGAAATC      GGAAAAAATA
    GTTATAAGCG      AGAAGTGGGC      TAGCCTTTAG      CCTTTTTTAT
                210                 220                 230                 240
    TTTTTTCCAA      CTTTTGGTAC      GTAAGGGGTA      CCCCTTTGGT
    AAAAAAGGTT      GAAAACCATG      CATTCCCCAT      GGGGAAACCA
                260                 260                 270                 280
    GTCGCCGAGC      AGATTCGAAA      ATCTTGAATA      ACTTTCTCAT
    CAGCGGCTCG      TCTAAGCTTT      TAGAACTTAT      TGAAAGAGTA
                289
    TTTCCAACC  3'
    AAAGGTTGG  5'
```

pMU-D76 SEQUENCE : 124 bp ; 32A ; 33C ; 34G ; 25T

```
                10                  20                  30
5'  GATCTGCACT      CGGCGTGAAT      TTGGTTACCA
3'  CTAGACGTGA      GCCGCACTTA      AACCAATGGT
                40                  50                  60
    TCGAATGTGC      GGAAAAAGTT      TTACCCCGTG
    AGCTTACACG      CCTTTTTCAA      AATGGGGCAC
                70                  80                  90
    CGCAGTGCGG      AACACGCCAG      ACTTGTTACA
    GCGTCACGCC      TTGTGCGGTC      TGAACAATGT
                100                 110                 120
    CACGGAAACA      GACCACGAAC      GTGTTACGCG
    GTGCCTTTGT      CTGGTGCTTG      CACAATGCGC
    124
    CACG  3'
    GTGC  5'
```

Figure 3

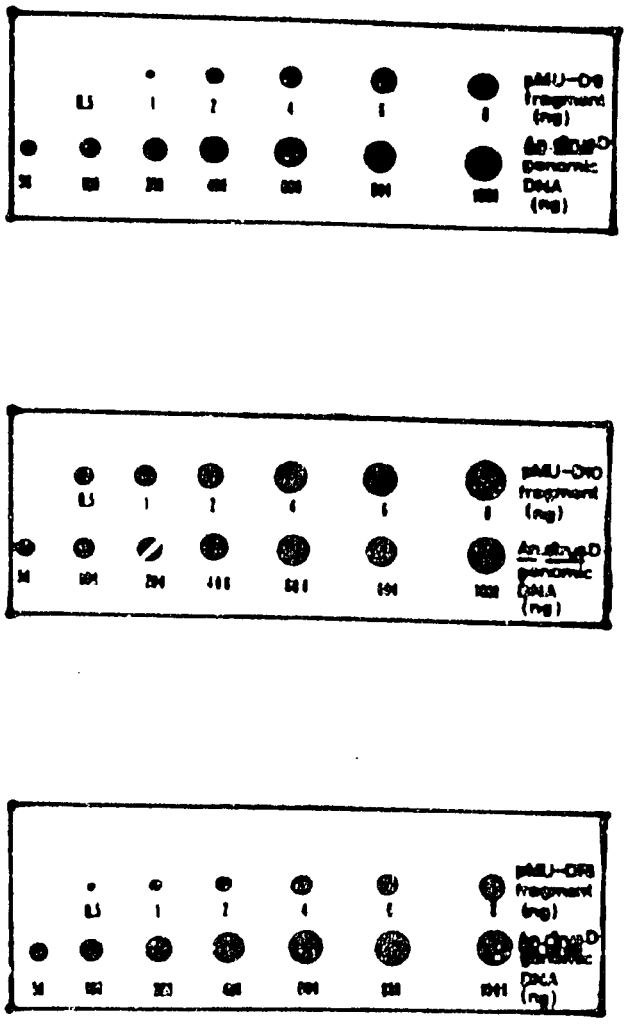
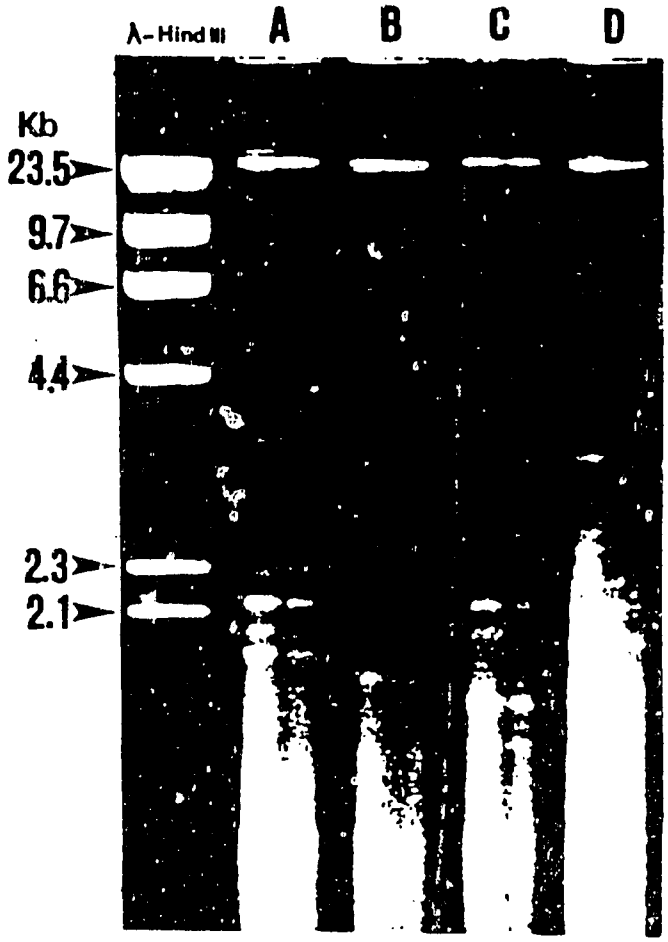
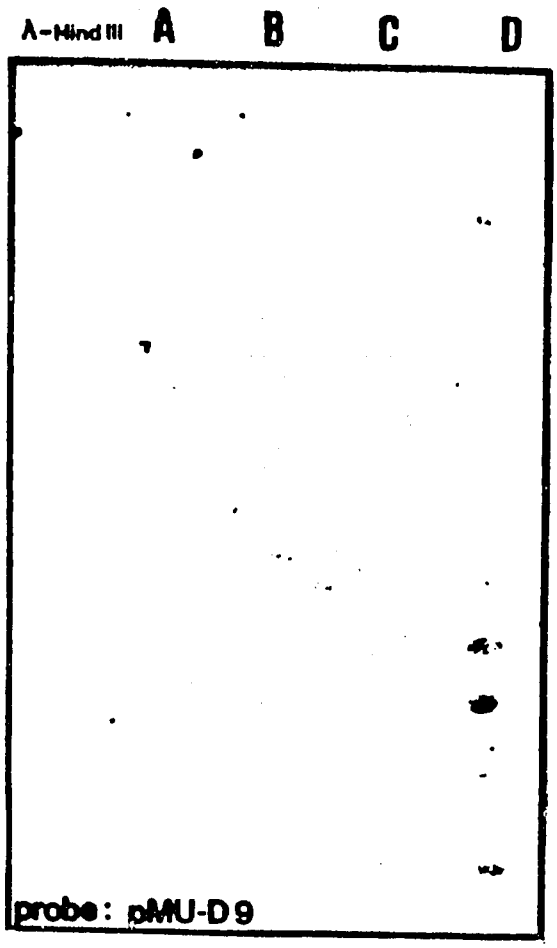
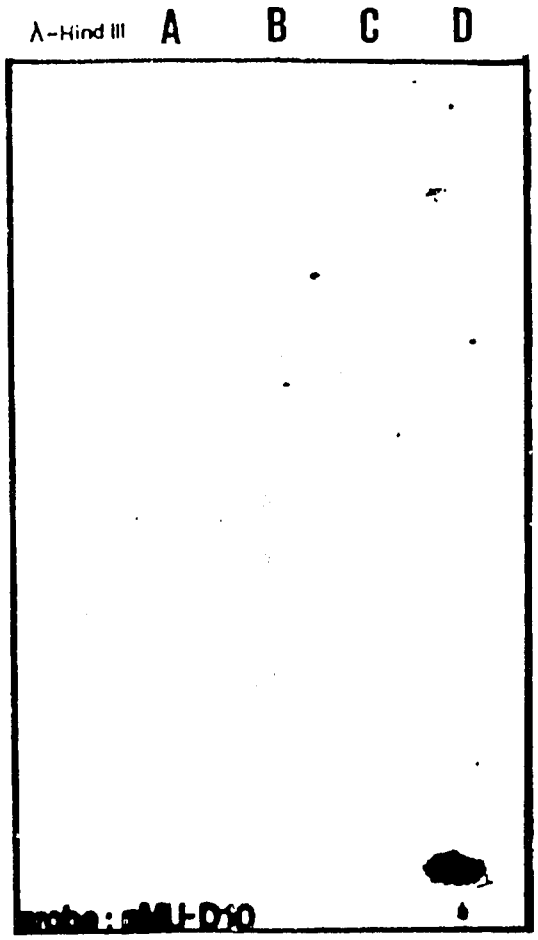


Figure 4


λ-Hind III
A
B
C
D
Kb
23.5
9.7
6.6
4.4
2.3
2.1


λ-Hind III
A
B
C
D
probe: pMU-D9


λ-Hind III
A
B
C
D

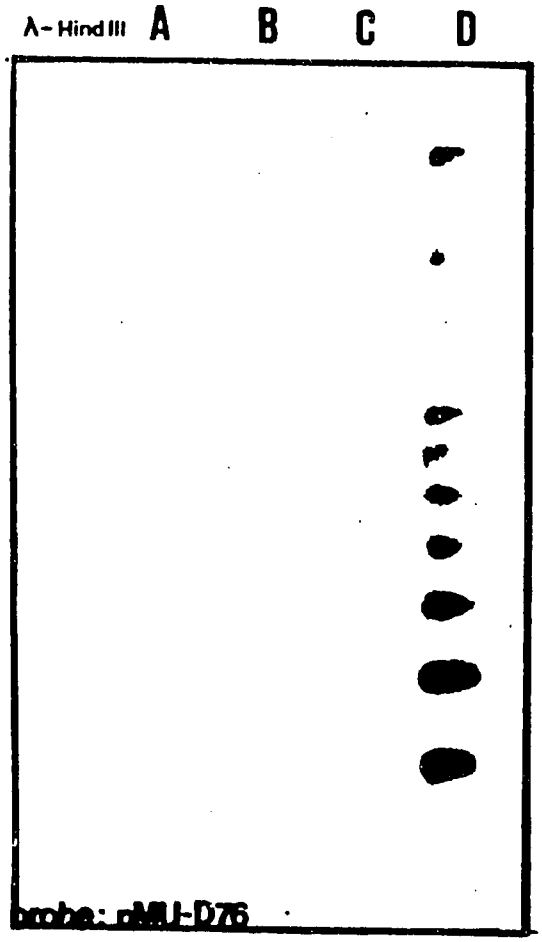

λ-Hind III
A
B
C
D

Figure 5

328

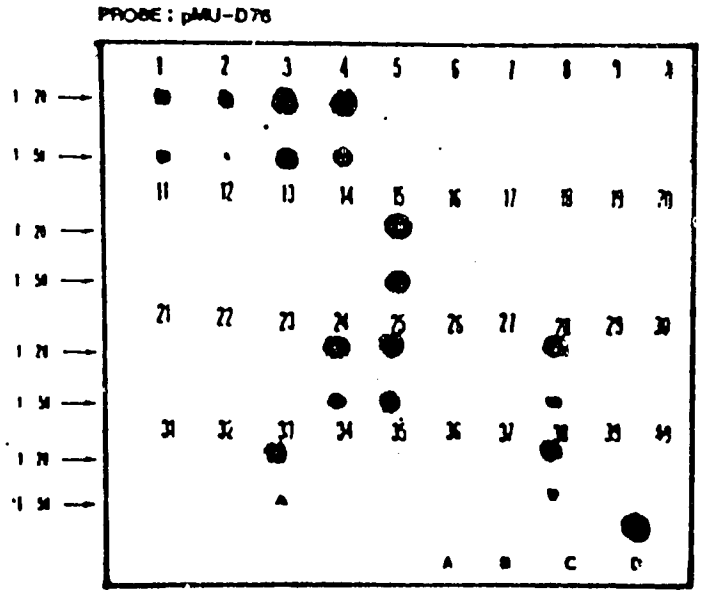


Figure 6

and Africa while A. filiculoides is indigenous to temperate regions. The cultivation of Azolla is a continuous process, however, requiring uninterrupted attention throughout the year. This is because at present only vegetative material can be used as innoculants. The use of sporocarps as planting materials has not been reported. Detailed studies of sporocarps of Azolla were reported by several investigators (Lucas and Duckett, 1980; Calvert et al, 1983; Campbell, 1983; Zhiyan, 1983; Konar and Kapoor, 1974; Fowler, 1975; Fowler and Stennett-Willson, 1978). It has been indicated that propagation of Azolla through sporocarps is possible (Konar and Kapoor, 1974; Peters personal communication). To the best of our knowledge, there have been no definitive reports concerning the use of Azolla spores as innoculants.

Soil fertility and structure can be improved when Azolla is continuously cultivated. Paddy fields in which Azolla have been cultivated required less energy for tillage and no nitrogen fertilizer when rice is planted. Unfortunately, the use of Azolla is still limited due to a number of major constraints. Among these problems, the greatest may be the lack of cultivation and propagation technology to maintain Azolla throughout the season.

The proposed research was to investigate the methods of propagation and maintenance of Azolla. The investigation comprises two major studies, vegetative propagation and spore propagation.

MATERIALS AND METHODS

Vegetative Propagation

Azolla materials

Nine Azolla accessions which ranked highly under greenhouse conditions were used for field studies (Table 1).

Glasshouse Study

Azolla species were cultured in a N-depleted nutrient solution in 8.5 cm diameter pots for a pH study and 12 cm diameter for insecticides and phosphorus concentrations studies. For the pH study, 6 levels of pH (4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) were used. In the insecticides studies, 4 concentrations of carbofuran; 0.375, 0.75, 1.5 and 3.0 kg/ha, and 3 concentrations of monocrotophos; 432, 864 and 1296 ppm, were used. For the phosphorus study, 8 concentrations; 0.05, 0.10, 0.37, 0.75, 1.50, 3.0, 5.0 and 15.0, were used. Treatments of all studies were arranged in randomized complete blocks with 3 replications.

Field Study

Experiments were conducted in three locations: Pathumthani Rice Research Center (Central), Prae Rice Research Center (North) and Khon Kaen Rice Experimental Station (Northeast). Two sets of experiments, strains selection and seasonal maintenance, were conducted at each location. For the strains selection experiment, Azolla accessions were

FY 1985

CULTURE, MAINTENANCE AND PROPAGATION OF Azolla UNDER TROPICAL CONDITIONS (BNF-TH-7-85-42)

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ABSTRACT

The aim of this study is to determine the methods for culturing Azolla throughout the season. Two methods are proposed; vegetative propagation and spore propagation. Vegetative propagation of suitable Azolla species for use at different locations is undertaken and a study of the factors governing the growth of Azolla; namely pH, phosphorus concentration, insecticide, toxicity and light intensities, is made. Studies are conducted in both glasshouse and field conditions. It is found that A. caroliniana, A. microphylla, A. pinnata C24,and several strains of A. pinnata are most suitable. The optimum pH for Azolla is between 4.5 to 6. The concentration of phosphorus could be from 0.37 up to 15 ppm. Two insecticides, carbofuran and monocrotophos, are recommended for controlling insects attacking Azolla and it has been found that the two insecticides are not harmful to Azolla, even used at a higher concentration than the recommended rate. Light intensities affect Azolla growth. Most Azolla could grow well under high light intensities of 100 % and 75 % of full sunlight in the low temperature months (rainy and cool seasons) and under 75 % in the hot season. Under 75 % light intensity appears to be most favorable for Azolla growth.

Propagation of Azolla through sporocarps was possible for some species. A. microphylla sporophytes were successfully propagated from sporocarps. A high percentage of spore germination was obtained from composting Azolla for two months while keeping the resulting spores moist.

INTRODUCTION

Azolla is a heterosporous aquatic fern which is widely distributed in tropical and temperate fresh water. It has a symbiotic relationship with a cyanobacterium, Anabaenaazollae (Moore, 1969). The Azolla-Anabaena symbioses have been widely used as biofertilizer for lowland rice production because the N-fixing cyanophyte can provide high total N to Azolla (Peters et al, 1981). It has been used traditionally in Vietnam and the Peoples' Republic of China as a fodder and as a source of nitrogen fertilizer for rice (Dao and Tran, 1979; Liu, 1979).

The genus is classified into seven species, namely A. caroliniana, A. filiculoides, A. mexicana, A. microphylla, A. ruba, A. nilotica and A. pinnata (Lumpkin and Plucknett, 1982). Two of the seven known species of Azolla, A. pinnata and A. filiculoides, have been used in cultivated systems. A. pinnata is indigenous to tropical South and Southeast Asia

cultured in 3x5 m clay plots. To each plot, 6 g of P_2O_5 and 4 g of K_2O were applied before culturing. Azolla was inoculated into each plot at the rate of 300 kg of fresh weight per hectare. Water depth of 10-15 cm was maintained in each plot until harvesting. Azolla were harvested within 1m area at 21 days after inoculation for determination of biomass and doubling time. Doubling time was calculated by the Mitchell's formula (1974).

The maintenance study was conducted under 4 light intensities, 100 %, 75 %, 50 %, and 25 % of full sunlight. Under each light intensity, three Azolla species which were selected for each location were cultured in two pot types (cement and clay construction). Two water regimes of 10 cm depth and saturatation were used. A factorial arrangement in randomized block designed was performed with three replications. The study was repeated every month until the completion of the season.

Spore Propagation

Design of Experiment

A factional arrangement was made in a randomized block within a split plot design with four replications. The main plots tested methods of collecting sporocarps. The sub plots were 3x4 factorial arrangements of types of media and concentrations of agar, respectively.

Methods of Collecting Spores

Azolla microphylla sporocarps were used. The three methods for collecting sporocarps were as follows; (a) "fresh spores" - sporocarps collected from fresh Azolla sporophytes, (b) "dry spores" - sporocarps collected from air dried Azolla sporophytes, (c) "composed spores" - sporocarps collected from fermented Azolla sporophytes. The procedure for separating sporocarps from sporophyte materials of the three methods was the same. Azolla materials were put into 2000 ml flasks and were shaken at 90 rpm for 2 hours. Azolla materials in the flask were put through a set of sieves containing sieving pans having pore sizes of 2830, 1000, 710, 500, 315 and 250 microns, respectively. Most Azolla sporocarps were retained on pans having pore sizes of 710 and 500 microns and they were further separated under a stereo microscope. The sporocarps were kept in a refrigerator until used.

Media

Three types of media were used: water; modified IRRI (Peter et al, 1980); and modified IRRI + 0.5 M NH_4Cl 5 ml/l. In addition to the liquid media, three concentrations (0.2, 0.5, and 1.0 %) of agar medium were prepared.

Culturing Method

Media were contained in 9.5 cm diameter glass petri plates. Fifty megasporocarps with massulae were placed in each plate. Plates

containing sporocarps were maintained at room temperature (25°C-30°C) under a light intensity of 40 uE/m^2/s with a 12 hr-12hr light-dark regime. Our previous study indicated that sporocarps of Azolla could not germinate under dark conditions, and that four uE/m^2/s induced an appreciable percentage of germination. Germination of megasporocarps (gametophyte developement) was examined after 10 days of culturing. The determination of germination was based on the opening of floats of a megaspore. Fertilization rates, as determined by sporophyte production from megasporocarp germination, were examined 15-20 days after culturing.

RESULTS AND DISCUSSION

Vegetative Propagation

Greenhouse Study

Effect of pH-on Azolla: Six Azolla accessions were used in this study and it was found that acidic condition was favored Azolla growth. The optimum pH range for Azolla was from 4.5 to 6 (Table 2).

Effect of insecticides on Azolla: Results showing in Table 3 and 4 indicated that high concentrations of carbofuran and monocrotophos were not harmful to Azolla. Azolla could withstand high concentrations of insecticides up to four times the recommended rate.

Effect of phosphorous on Azolla: The phosphorous requirement of Azolla is normally about 1.5 ppm. It was found in this study (Table 5) that Azolla could grow satisfactory in phosphorus concentrations between 0.37 to 15 ppm. This indicated that the application of phosphorus for Azolla could be flexible.

Field Study

Prathumthani: The first experiment was conducted in April and the second in May 1986. Performance of Azolla accessions was similar (Table 6). It was interesting that the exotic Azolla species, A. caroliniana and A. microphylla could adapt to tropical environments comparable to our indiginous species A. pinnata.

Khon Kaen: Two experiments were also conducted in April and May 1986. Both A. caroliniana and A. microphylla ranked higher in dry matter yield than the A. pinnata strains but the difference was not statistically significant. This might be due to high variations among treatments which were indicated by the high coefficient of variation. Significantly higher dry weight was obtained from A. caroliniana and A. pinnata KKN1 when the second experiment was conducted in May (Table 7).

Prae: In April experimental performance of A. microphylla was comparable to that of the A. pinnata stains and A. caroliniana had the lowest dry weight. The second experiment showed no significant differences between the dry weights of all Azolla accessions (Table 8).

Data from six experiments in three different locations showed that the two exotic species, A. caroliniana and A. microphylla, adapted to tropical conditions in Thailand. It was also observed that these two species were relatively less infected by fungal disease and insects as compared to the A. pinnata. This finding agreed with Lumpkin and Bartholomew (1986) who reported that A. microphylla was resistant to fungal attack and heat stress. Watanabe and Berja (1983) observed that A. caroliniana required lower temperature than A. pinnata. Poor growth was also observed in phosphorus limited conditions in the greeenhouse where temperature exceeded 35°C (Subudhi and Watanabe, 1981). Our field study in the hottest month of April showed that A. caroliniana performed equally to A. pinnata.

Seasonal maintenance of Azolla: Results of this study are summarized in Tables 9 and 10. Table 9 shows the effect of light intensities on Azolla growth. The data obtained from nine months (July to March) indicated that low light intensities between 25 % to 50 % of full sunlight were not suitable for culturing Azolla. Azolla could be cultured under light intensities of 100% and 75% from July until March in the central plain whereas in the North and Northeast it could be cultured only from July to December. The 75 % light intensities were in favor of Azolla growth during January and March in the North and Northeast. Seasonal performance of A. caroliniana 301 was better than the others in Pratumthani while A. microphylla 418 was better than the others in Prae and Khon Kaen (Table 10).

Spore Propagation

Germination data for megaspores collected from fresh Azolla sporophytes in different media are shown in Table 11. Modified IRRI medium with and without different levels of agar provided germinations significantly higher than the same medium with nitrogen added. On 0.2 % water agar, germination was comparable to that on the modified IRRI medium. The maximum germination of 49.2 % was observed on 0.2 % IRRI agar medium.

Fertilization followed the same patterns as germination, but was rather less. The lower fertilization might be due to the delay in antherozoid development.

Sporocarps collected from air dried Azolla sporophytes showed extremely poor germination (Table 12). The maximum germination was only 8 %. The poor germination might reflect that dormancy of the sporocarps had not been broken. Konar and Kapoor (1974) reported that Azolla pinnata remained dormant for 5 to 6 months before germination.

Germination and fertilization of sporocarps collected from fermented Azolla sporophytes were excellent (Table 13). The highest germination rate was 92.0 % whereas the lowest was 61.4 %. Adding 0.2 % agar in all media seemed to be satisfactory for good germination and fertilization. Fermentation of A. microphylla sporophyte for two months seemed to be adequate for breaking dormancy of spores.

A comparison of the fertilization of fresh, dry, and composed Azolla spores on three media is illustrated in Figure 1. A high percentage of fertilization was obtained from composed spores in all media. Even though the percentage of germination was similar for all media, water agar was favored for use because it remained relatively free of contaminants. Fungal and algal growth were usually observed in the IRRI medium, especially with added nitrogen, resulting in an interference with germination counting.

It has been reported by Haup (1985) that minerals at low concentrations, even as low as those leaching from a glass petri dish, could enhance spore germination of several fern species. In this study, however, there were no significant differences between the water agar medium and mineral medium (IRRI) in the germination of Azolla sporocarps. Thus, it can be recommended that water agar is adequate for germination of Azolla sporocarps and also that sporocarps should be fermented at least two months to obtain high fertilization. Sporophytes from sporocarp germination were successfully developed into mature sporophytes and they maintained healthy growth.

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Table 1. *Azolla* Species and Strains Used in This Study.

Azolla	Field site			Source
	Pathumthani	Prae	Khon Kaen	
A. caroliniana 301	+	+	+	IRRI
A. microphylla 418	+	+	+	IRRI
A. pinnata C 24	+	+	+	Central, Thailand
A. pinnata NE 14	+	+	+	Northeast, Thailand
A. pinnata BK 5	+	+	+	IRRI (Bangkok)
A. pinnata NE 17/19	+	+	+	Northeast, Thailand
A. pinnata SPT	-	+	-	North, Thailand
A. pinnata KKN 1	-	-	+	Northeast, Thailand
A. pinnata 701	+	-	-	IRRI

Table 2. Effect of pH on Dry Weight (g/pot) of *Azolla* Species

Azolla	pH						Average of azolla
	4.5	5.0	5.5	6.0	6.5	7.0	
A. caroliniana 301A	0.16	0.14	0.15	0.17	0.16	0.14	0.15b
A. microphylla 418	0.18	0.16	0.19	0.16	0.13	0.14	0.16b
A. filiculoides	0.13	0.13	0.15	0.13	0.15	0.10	0.13
A. pinnata NE 17/19	0.23	0.20	0.22	0.19	0.18	0.19	0.20a
A. pinnata NE 14	0.14	0.17	0.16	0.14	0.16	0.14	0.15b
A. pinnata BK 5	0.22	0.22	0.22	0.22	0.21	0.19	0.21a
Average of pH	0.17ab	0.17ab	0.18a	0.16bc	0.16bc	0.15c	

CV = 16.7 %

Table 3. Effect of Insecticide Carbofuran on *Azolla* Dry Weight (g/pot)

Azolla	Concentrations (kg/ha)					Average of azolla
	0	0.375	0.75	1.50	3.0	
A. caroliniana 301A	0.54	0.49	0.05	0.49	0.55	0.52 e
A. filiculoides	0.73	0.73	0.85	0.86	0.73	0.78 c
A. microphylla 418	0.92	0.95	0.96	0.94	0.99	0.95 a
A. pinnata NE 17/19	0.71	0.80	0.90	0.81	0.86	0.82 bc
A. pinnata NE 14	0.68	0.66	0.65	0.69	0.68	0.67 d
A. pinnata BK 5	0.81	0.91	0.87	0.86	0.82	0.85 b
Average of Insecticide conc.	0.73	0.76	0.80	0.78	0.77	

CV = 17.4 %

Table 4. Effect of Insecticide Monocrotophos on *Azolla* Dry Weight (g/pot)

Azolla	Concentrations (ppm) 0	432	864	1296	Average of azolla
A. caroliniana 301A	0.04	0.55	0.50	0.39	0.46 e
A. filiculoides	0.81	0.81	0.82	0.82	0.81 b
A. microphylla 418	1.01	1.11	0.96	0.96	1.0 a
A. pinnata NE 17/19	0.81	0.87	0.87	0.93	0.86 b
A. pinnata NE 14	0.63	0.60	0.59	0.48	0.57 d
A. pinnata BK 5	0.67	0.68	0.72	0.70	0.69 c
Average of Insecticide	0.72	0.77	0.74	0.71	

CV = 11.08 %

Table 5 Effect of Phosphorus Concentrations on *Azolla* Dry Weight

Azolla	0	0.05	0.10	0.37	0.75	1.5	3	5	15	Average
A. caroliniana 301A	0.18 p-r	0.16 p-r	0.25 m-r	0.54 f-j	0.70 c-f	0.86 ab	0.89 a	0.81 a-d	0.85 a-c	0.58
A. filiculoides	0.13 r	0.12 r	0.17 p-q	0.35 l-o	0.64 e-i	0.63 e-i	0.67 d-h	0.70 c-f	0.63 e-i	0.45
A. microphylla 418	0.14 qr	0.14 qr	0.15 qr	0.40 j-m	0.52 g-k	0.65 d-i	0.65 d-i	0.60 e-i	0.70 c-f	0.44
A. pinnata BK 5	0.14 qr	0.19 o-r	0.23 n-r	0.51 h-k	0.59 e-i	0.63 e-i	0.71 b-f	0.72 b-e	0.64 e-i	0.48
A. pinnata NK 14	0.26 m-r	0.22 n-r	0.30 m-q	0.58 e-i	0.60 f-i	0.66 d-i	0.70 c-f	0.63 e-i	0.69 d-g	0.52
A. pinnata NK 17/19	0.37 k-m	0.32 m-p	0.40 j-m	0.62 e-i	0.49 i-l	0.61 e-i	0.51 h-k	0.68 d-h	0.52 g-k	0.50
Average P	0.20	0.19	0.25	0.50	0.59	0.62	0.69	0.69	67	

CV = 17.55 %

Table 6. Performance of *Azolla* under Field Condition at Pathumthani Rice Research Center.

Azolla species	April		May	
	Dry wt. (g/m^2)	Doubling Time (days)	Dry wt. (g/m^2)	Doubling Time (days)
A. caroliniana 301 A	109.6	3.7	82.4	3.7
A. microphylla 418	111.2	3.7	53.6	3.9
A. pinnata 17/19	106.1	3.9	78.9	3.7
A. pinnata BK 5	104.4	3.9	68.3	3.9
A. pinnata C 24	97.6	3.9	92.7	3.7
A. pinnata NE 14	104.4	3.9	80.6	3.7
A. pinnata 701	115.2	3.6	72.2	3.7
	NS		NS	
CV (%)	8.14		20.21	

Table 7. Performance of *Azolla* under Field Condition at Khon Kaen Rice Experimental Station.

Azolla species	April		May	
	Dry wt. (g/m^2)	Doubling Time (days)	Dry wt. (g/m^2)	Doubling Time (days)
			1/	
A. caroliniana 301 A	103.5	3.7	108.3 a	3.6
A. microphylla 418	112.6	3.5	76.5 b	4.0
A. pinnata 17/19	77.5	4.1	42.7 c	4.8
A. pinnata BK 5	66.0	4.3	71.9 b	4.1
A. pinnata C 24	59.6	4.6	70.6 b	4.1
A. pinnata NE 14	64.7	4.3	80.7 b	4.0
A. pinnata KKN1	72.4	4.3	103.9 a	3.7
	NS			
CV (%)	33.9		20.8	

1/ Means in a colum followed by the same letter are not significantly different at the 5% level.

Table 8. Performance of *Azolla* under Field Condition at Prae Rice Research Center.

Azolla species	April		May	
	Dry wt. (g/m^2)	Doubling Time (days)	Dry wt. (g/m^2)	Doubling Time (days)
	1/			
A. caroliniana 301 A	41.6 c	4.8	59.8	4.1
A. microphylla 418	74.0 ab	3.9	83.5	3.7
A. pinnata 17/19	69.6 ab	4.2	76.8	3.8
A. pinnata BK 5	84.5 a	3.8	82.5	3.7
A. pinnata C 24	62.0 ab	4.1	78.5	3.8
A. pinnata NE 14	68.2 ab	4.2	82.2	3.7
A. pinnata SPT	56.5 bc	4.4	175.9	3.8
CV (%)	17.9		NS 17.1	

1/ Means in a colum followed by the same letter are not significantly different at the 5% level.

Table 9 Effects of Seasonal Light Intensities on *Azolla* Growth (g/m^2) at Different Locations.

Month	Prathumthani				Prae				Khon Kaen			
	100	75	50	25	100	75	50	25	100	75	50	25
July	105.8 a	95.1 b	55.1 b	-	78.0 b	83.0 a	27.5 c	7.1 d	68.1 a	63.2 ab	43.2 b	19.8 c
August	71.1 a	59.1 b	22.2 c	4.4 d	---------- Flooded ----------				52.7 ab	64.3 a	42.1 b	22.2 c
Septeaber	40.6 b	56.3 a	32.7 c	12.9 d	81.3 a	70.2 b	38.7 c	20.5 d	67.5 b	83.8 a	65.8 b	30.9 c
October	41.9 b	44.1 a	21.4 c	6.8 d	59.3 a	54.4 a	23.9 b	15.1 c	45.6 b	49.0 a	36.2 c	43.4 b
November	48.1 a	53.4 a	33.1 b	13.7 c	58.1 a	59.1 a	25.8 b	11.8 c	33.0 a	32.3 a	24.1 b	12.7 c
December	61.0 a	46.2 b	31.7 c	11.6 d	37.2 a	23.5 b	8.0 c	6.0 c	37.2 a	33.5 b	8.0 c	6.0 c
January	32.7 a	34.1 a	22.7 b	7.4 c	34.2 b	40.2 a	24.7 c	5.7 d	34.2 b	40.2 a	24.8 c	5.6 d
February	68.4 a	55.4 b	34.3 c	9.2 d	37.2 b	47.9 a	17.3 c	7.9 d	37.2 b	47.8 a	17.3 c	7.8 d
March	71.3 a	74.7 a	55.8 b	23.5 c	19.9 b	24.9 a	17.4 b	8.1 c	19.9 b	24.9 a	17.4 b	8.1 c
April												
May												
June												

Table 10 Seasonal Performances of *Azolla* Species of Different Locations (g/m^2)

Month	Prathumthani			Prae			Khon Kaen		
	M 418	C 301	PNB 14	M 418	PNB 14	PBK 5	M 418	C 301	PKKN 1
July	84.2 b	103.1 a	68.6 c	45.3 b	47.5 b	54.7 a	48.5 b	59.0 a	38.3 c
August	39.4 b	45.5 a	39.9 b	-------	Flooded	------	50.9 a	48.4 b	36.6 c
September	32.0 b	41.0 a	33.9 b	38.5 a	44.0 b	45.5 b	53.8 a	57.9 a	55.5 a
October	30.9 a	27.3 b	27.4 b	41.7 a	34.9 b	33.4 b	64.8 a	62.4 a	53.8 b
November	35.3 b	46.6 a	29.4 c	46.6 a	40.3 a	29.4 b	34.2 a	28.8 b	13.5 c
December	33.3 b	47.1 a	32.5 b	22.4 a	15.2 b	15.4 b	43.1 a	25.8 b	13.2 c
January	18.9 b	34.9 a	18.9 b	46.3 a	17.4 b	14.8 b	23.5 a	25.0 a	9.6 b
February	46.9 b	51.9 a	29.0 c	40.3 a	24.2 b	13.6 c	16.8 a	14.3 b	5.8 c
March	54.5 b	59.2 a	55.2 b	24.9 a	13.0 b	14.8 b	11.9 a	14.0 a	11.4 a
April									
May									
June									

Table 11. Germination and Fertilization of Fresh Spores in Different Media.

Media	Germination %	Fertilization %
	1/	
Water + 0 agar	12.6 def	8.0 de
Water + 0.2 % agar	39.2 ab	20.0 c
Water + 0.5 % agar	19.2 de	6.6 de
Water + 1.0 % agar	24.0 cd	9.4 de
IRRI + 0 agar	40.0 ab	37.4 a
IRRI + 0.2 % agar	49.2 a	30.6 b
IRRI + 0.5 % agar	36.0 abc	24.6 bc
IRRI + 1.0 % agar	37.2 abc	10.6 c
IRRI + N + 0 agar	15.2 def	2.6 de
IRRI + N + 0.2 % agar	6.0 df	0 e
IRRI + N + 0.5 % agar	1.2 f	0.6 e
IRRI + N + 1.0 % agar	5.2 ef	0.6 e
CV (%)	25.2	29.1

1/ Means in a column followed by the same letter are not significantly different at the 5 % level.

Table 12. Germination and Fertilization of Dry Spores in Different Media.

Media	Germination %	Fertilization %
Water + 0 agar	7.2	4.0
Water + 0.2 % agar	7.2	6.6
Water + 0.5 % agar	8.0	4.6
Water + 1.0 % agar	7.2	3.2
IRRI + 0 agar	6.0	5.2
IRRI + 0.2 % agar	7.2	6.0
IRRI + 0.5 % agar	5.2	4.6
IRRI + 1.0 % agar	4.0	3.2
IRRI + N + 0 agar	0.6	0
IRRI + N + 0.2 % agar	3.2	3.2
IRRI + N + 0.5 % agar	2.6	2.6
IRRI + N + 1.0 % agar	4.0	0.6
CV (%)	77.5	88.0

Table 13. Germination and Fertilization of Composted Spores in Different Media.

Media	Germination %	Fertilization %
	1/	
Water + 0 agar	78.0 bc	58.7 de
Water + 0.2 % agar	87.4 a	83.3 a
Water + 0.5 % agar	84.6 ab	82.7 a
Water + 1.0 % agar	77.4 c	72.7 c
IRRI + 0 agar	75.4 c	74.7 bc
IRRI + 0.2 % agar	84.6 ab	81.3 ab
IRRI + 0.5 % agar	86.6 a	83.3 a
IRRI + 1.0 % agar	90.0 a	86.7 a
IRRI + N + 0 agar	78.0 bc	71.3 c
IRRI + N + 0.2 % agar	92.0 a	85.3 a
IRRI + N + 0.5 % agar	72.6 c	58.0 d
IRRI + N + 1.0 % agar	61.4 d	34.7 e
CV (%)	9.87	11.10

1/ Means in a column followed by the same letter are not significantly different at the 5 % level.

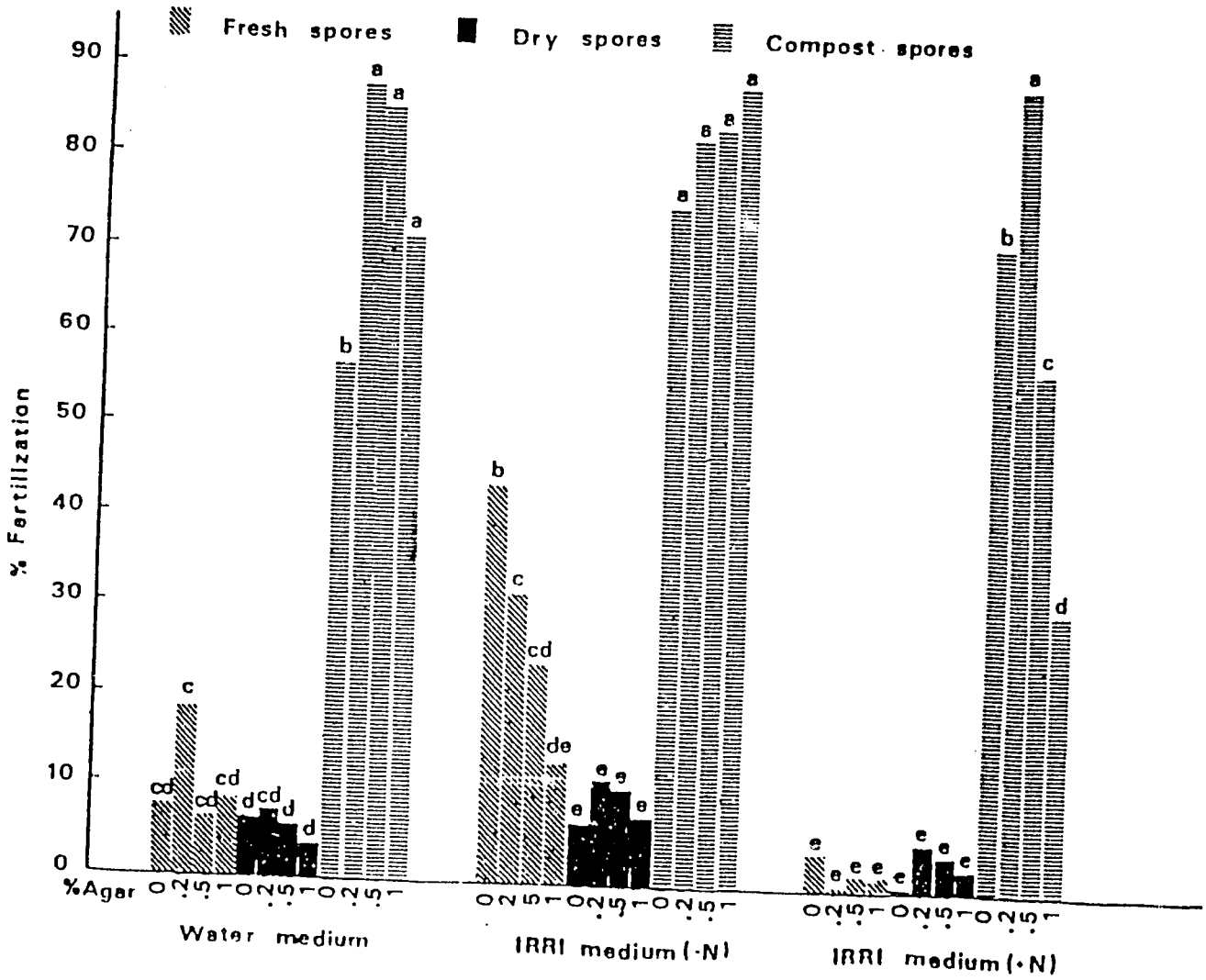

Figure 14 Fertilization of Azolla spores in Three Media.

FY 1985

ETIOLOGY AND DEVELOPMENT OF ACUTE RESPIRATORY INFECTION IN THAI CHILDREN IN URBAN COMMUNITY AND HOSPITAL (ARI-TH-8-85-51)

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ABSTRACT

A three year planned study on etiology and development of acute respiratory infections (ARI) in children under five years of age has been conducted simultaneously in Din Daeng community and Ramathibodi Hospital. The study was initiated in July 1985.

Etiologic studies included rapid antigen detection and isolation for bacterial, viral and chlamydial organisms. Serology for the above respiratory viruses and Mycoplasma pneumoniae were also performed.

Epidemiological profiles as well as incidence and prevalence of ARI were obtained.

Our preliminary results revealed a seasonal pattern of respiratory viruses causing ARI in young children. The respiratory syncytial virus accounted for more than 30 percent of all viruses involved and was the most common cause of bronchitis and pneumonia in this group of children.

The bacteriologic data obtained from children with ARI revealed a high percentage of recovery of S. pneumoniae and H. influenzae. The Percentage of strains resistant to antibiotics increased.

The knowledge obtained from this study will lead to future implementation of preventive and promotive measures for ARI as well as effective vaccine development.

INTRODUCTION

In Thailand, Acute Respiratory Infections (ARI) accounted for about 25% of deaths in children under one year of age. Forty to sixty percent of the children visiting general hospitals suffer from ARI and approximately 10% of them need hospitalization.

Only scattered information is available to clinically characterize or describe the disease, or identify the responsible pathogens. Information on the roles of certain respiratory viruses, Mycoplasma pneumoniae, Chlamydia trachomatis and other common bacterial agents of ARI namely Haemophilus influenzae, and S. pneumoniae is very limited. There is an immediate need for research on ARI to improve understanding and identify the most appropriate methods for addressing the problem.

Through the clinical research grant supported by BOSTID, the three year planned study on the etiology and development of acute respiratory infections in Thai children in an urban community and hospital has been carried on by the Mahidol University group in collaboration with Din Daeng Bangkok metropolitan health center.

OBJECTIVE

The objective of this study is to determine the morbidity and mortality, age and seasonal incidence, responsible pathogens, clinical correlation, risk factors, methods of rapid diagnosis, natural course of the

disease and establish guidelines for prevention and control of ARI in children under five years of age.

PATIENT SELECTION AND METHOD

The community under study is Din-Daeng, a congested area in the Bangkok metropolis with a low migration rate. There was a six-month preparation phase followed by a two-year cohort study, which included approximately 378 children under five years of age. This was conducted by the Faculty of Public Health and the Department of Pediatrics, Ramathibodi Hospital. Information on risk factors, natural history,etiologic agents and treatment was obtained.

Cross-sectional and case-control studies of moderate and severe ARI were conducted at the Department of Pediatrics, Ramathibodi Hospital in order to strengthen the information about the etiology, morbidity and mortality of moderate and severe ARI. This included approximately 500 patients per year. The criteria for diagnosis were based on clinical rather than laboratory findings. However,the correlation of the initial investigations such as radiological features, complete blood count and the clinical presentation was evaluated. The specimens obtained from the mild and moderate cases in the community were taken to the microbiology section, Department of Pathology, Ramathibodi Hospital for bacterial identification and to the virology section at Siriraj Hospital for identification of viral respiratory pathogens.

The specimens from moderate and severe cases of ARI seen at the hospital diagnosed as specific acute respiratory infections were sent to the above two places. In addition, identification of Mycoplasma pneumoniae and Chlamydia trachomatis were also performed.

For the hospital based study every child under five years of age with a history of ARI within a period of seven days who came to the hospital either from the Din Daeng community or other communities and was classified as having moderate or severe ARI according to our modified WHO criteria was studied (Table 1)

Complete histories and physical examinations were obtained from the above cases by our project nurse and responsible pediatricians in the project. The information gathered was recorded in a questionaire designed for the purpose.

Specimen collection for bacteriologic study included throat swab culture in cases and controls, blood cultures, urine and serum for bacterial antigen detection by the CIE technique was performed and taken to the bacteriology and immunology labs for processing. Nasopharyngeal aspiration was done by using an our modified aquarium suction pump and the nasopharyngeal aspirate was sent to the virology lab for rapid viral diagnosis and isolation of respiratory viruses i.e. Influenza A,B, Parainfluenza 1,3, Respiratory Syncytial Virus and adenoviruses.

Detection of Chlamydia trachomatis from nasopharyngeal aspirate by rapid antigen detection and isolation was done only in children below six months of age.

Acute and convalescent sera were obtained for serology of respiratory viruses. Blood was also drawn for a *Mycoplasma* titer.

The throat swab culture for the the community control group was started in January 1986 but for the hospital control group the culture was started in May 1986.

Table 1. Criteria for Grading of Severity of ARI Clinical Symptoms and Signs. (modified WHO criteria)

Symptom	Normal	Mild	Moderate (With symptoms of running nose, cough, fever)	Severe (With one or more of the symptoms in moderate and the followings)
Fever	None	< 38.5°C < 3 days	>38.3°c(axilla) > 3 days	
Masal discharge	None	Clear	Greenish or yellowish nasal discharge seen outside the nose.	
		Turbid, whitish with no fever	Whitish mucous nasal discharge seen with fever over 3 days	
Cough	None	Slight cough, infrequent no sputum	Vomiting after severe cough, Cough all day, productive cough.	
Breath-ing	Normal	Noisy breathing or stuffy nose	>50/min in children under 1 year or >40/min in children over 1 year	>60/min Snorring type of breathing and mouth breathing, flaring of alae nasi, chest wall retraction, cyanosis of nail beds
Others		Normal	Painful ear purulent discharge from ear canal	refused food and water convulsion severe lethargy

All cases with clinical diagnosis of croup, bronchitis, acute bronchiolitis, pneumonia and ten percent of the other conditions classified as moderate degree of ARI were randomly selected.

Table 2 ARI and Virus Identified in Moderated Cases at Ramathibodi Hospital
ARI - TH - 8 - 85 - 51 (Jan 1986 - Dec. 1986)

Clinical	Nasopharyngitis	Pharyngo tonsillitis	Croup	Bronchitis	Bronchiolitis	Pneumonia	Total
Organisms	(N=28)	(N=36)	(N=15)	(N=156)	(N=4)	(N=25)	(N=266)
Parainfluenza 1	1	2	3	5	-	1	11
Parainfluenza 3	4	2	3	13	-	1	23
Influenza A	-	4	1	4	-	1	10
Influenza B	1	-	-	-	-	-	1
RSV	1	4	-	27	1	8	41
Adenovirus	2	1	1	13	-	3	20
CMV	-	-	1	3	-	-	4
Others	1	-	-	3	-	-	4
Total (% of each syndrome)	10 (35.7)	13 (36.6)	9 (60)	68 (43.6)	1 (25)	13 (52)	114 (42.8)

Table 3 ARI and Virus Identified in Severe Cases at Ramathibodi Hospital
ARI - TH - 8 - 85 - 51 (Jan. 1986 - Dec. 1986)

Clinical Dx.	Croup	Bronchitis	Bronchiolitis	Pneumonia	Total
Organism	(N=30)	(N=5)	(N=8)	(N=101)	(N=144)
RSV	1	-	2	16	19
CMV	-	-	1	-	1
Parainfluenza	1	-	-	1	2
Parainfluenza 1	9	-	-	-	9
Parainfluenza 3	3	-	1	7	11
Influenza A	1	-	-	2	3
Influenza B	-	-	-	2	3
Adeno virus	-	-	-	8	8
Measles	1	-	-	2	6
Rhino virus	-	-	-	-	1
Others	-	-	-	2	2
Total positive (% of each syndrome)	15 50	0 (0)	4 (50)	41 (40.6)	65 (45.1)

Table 4. Comparison of Percentage of Potentially Pathogenic Bacteria Isolated from Throat Swab Culture of Patients and Controls in Hospital-Based and Community Population (Jan. 1986 - May 1987)

Organisms	Hospital patient (N=461)	Hospital control (N=240)	P-value	Community patient (N=681)	Community control (N=753)	P-value
H. influenzae	108(23.4%)	13(5.4%)	<0.0001	88(12.9%)	42(5.6%)	<0.0001
S. pneumoniae	69(14.9%)	12(5.0%)	<0.0001	86(12.6%)	85(11.2%)	0.4342
S. aureus	77(16.7%)	36(15.0%)	0.5607	48(7.0%)	42(5.6%)	0.2514

Table 5. Detection of *S. pneumoniae* and *H. influenzae* type B antigens by CIE in Cases of Childhood ARI (Jan - Dec. 1986)

	S. pneumoniae Urine No.	Urine %	Serum No.	Serum %	*H. influenzae* type b Urine No.	Urine %	Serum No.	Serum %
Total specimen tested	150		176		150		176	
CIE Negative	134	89.9	170	96.6	150	100	173	98.3
CIE Positive*	16	10/6	6	3.4	0	0	3	1.7

* Nine patients with positive *S. pneumoniae* antigen and two patients with positive *H. influenzae* type B antigens had the corresponding organism recovered from throat swab cultures.

RESULTS AND DISCUSSION

Preliminary data obtained from the hospital and community based population revealed seasonal distribution of respiratory viruses recovered during July 1985 through December 1986 from ARI children in the studied group (Fig 1.) RSV was isolated more frequently during July and August 1985 and during July through December 1986. Parainfluenza 1 and 3 were recovered more frequently during February through May 1986, Influenza A during May and June. Adenovirus was recovered all year but more during August through September.

Incidence and prevalence of ARI in community children during Jan 1986-June 1986 were shown in Fig. 2 and 3. The weekly incidence rate ranged from 11 to 31 episodes per 100 children-week and appeared to be highest during January through February. The weekly prevalence rate was between 22 and 37 episodes per 100 children-week.

Demographic information as well as information on risk factors, family background, personal health history were collected and being analysed.

Hospital based data obtained from moderate and severe cases of ARI revealed 42.8% and 45.1% respectively of respiratory viruses recovered. The respiratory syncytial virus (RSV) and parainfluenza viruses (PF) were the two most common viral agents found and associated with bronchitis and pneumonia. (Table 2 and 3). The percentage of RSV associated infections in this preliminary data was 31.3% of all viruses identified. Epidemiological profiles, clinical presentations of the RSV associated cases as well as pertinent laboratory findings are being carefully analysed and will be presented later.

Bacterial isolation from the throat swab cultures of ARI children and controls from both hospital and community based populations showed significant growth of the three bacterial pathogens known to cause ARI in children i.e. H. influenzae, S. pneumoniae and S. aureus respectively. (Table 4) There were also significant difference of H. influenzae and S. pneumoniae between the ARI cases and the controls ($P<0.0001$) but for the community group the significant difference was demonstrated only for H. influenzae ($P< 0.0001$). A high percentage of antibiotic resistant strains of the above two organisms was noted. Detection of S. pneumoniae and H. influenzae antigens by Counter Immuno-Electrophoresis (CIE) in severe cases of ARI revealed 10.6% positive with S. pneumoniae in urine, 3.4% in serum but only 1.7% positive for H. influenzae type B was identified in serum (Table 5) Confirmation and specific serotyping of the above two organisms are being done by the BOSTID reference laboratory. However, with the very low percentage of positive yield in our study, the specificity is still undetermined. More careful analysis of the clinical profiles obtained from each individual positive case may give clues to the significant value of the above test. There was only one case of Chlamydia trachomatis isolated from ARI case in our study. No cases of Mycoplasma pneumonia were found.

The above study revealed the great majority of children presented with acute repiratory infections had significant associations with respiratory viruses. A definite role for bacterial pathogens as primary causative agents was established only in very few cases.

Special emphasis on management of ARI in children should be stressed for supportive treatment as well as preventive and control measures.

The judgement for the appropriate use of antibiotics should be carefully considered as a high percentage of antibiotic resistant strains of H. influenzae and S. pneumoniae appeared to be increasing in the developing world and was noted in our study as well.

The future development of more effective bacterial and viral vaccines for prevention of ARI in all groups of children hopefully will reduce the morbidity and mortality of children due to ARI and lead to better health for children all over the world.

ACKNOWLEDGEMENTS

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Figure 1 Number of Positive Case of Respiratory Viruses Recovered from ARI Children Seen at Ramathibodi Hospital during July 1985-December 1986.

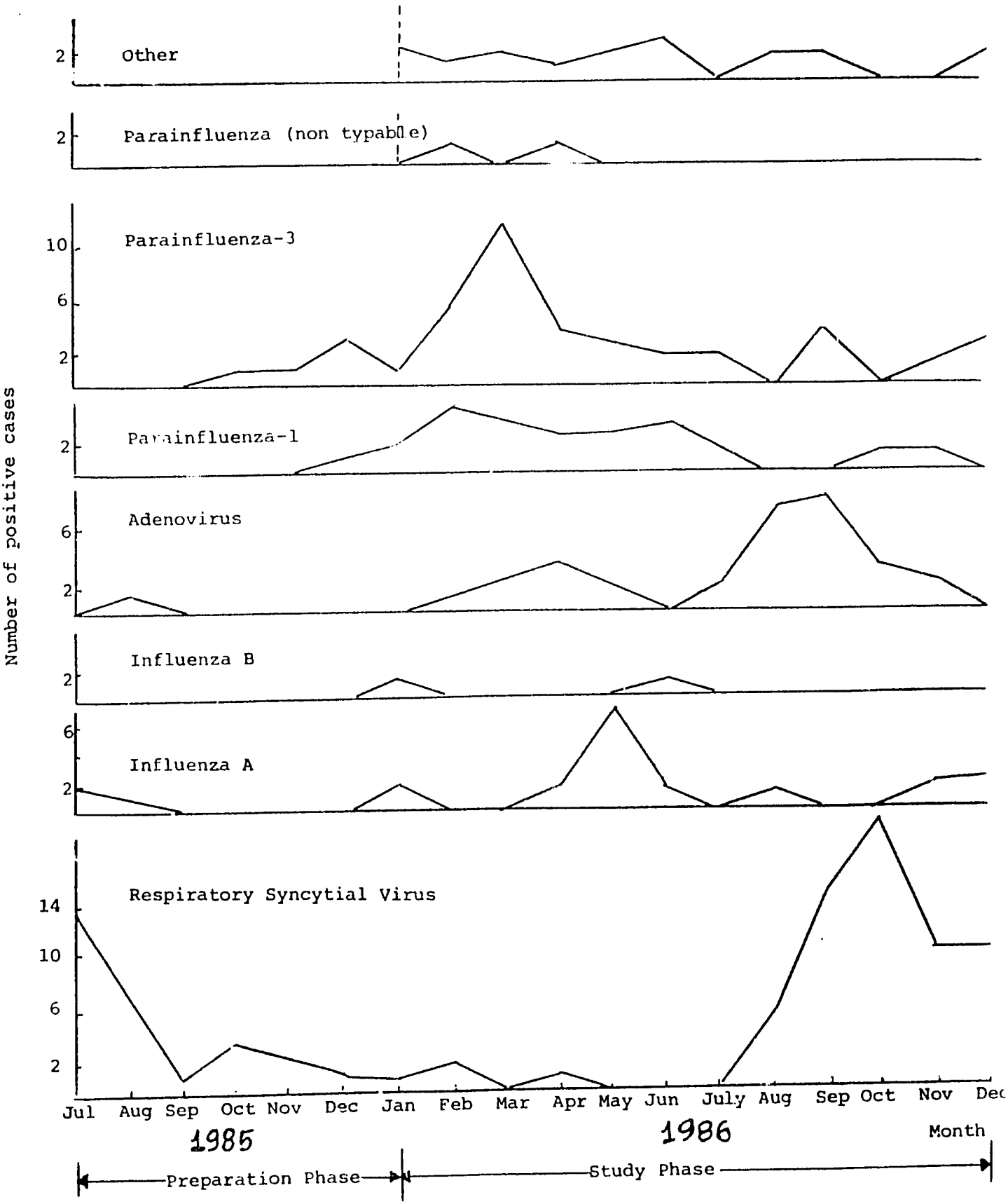


Figure 2

THE WEEKLY INCIDENCE RATE
(EPISODE) IN JANUARY - JUNE 1986)

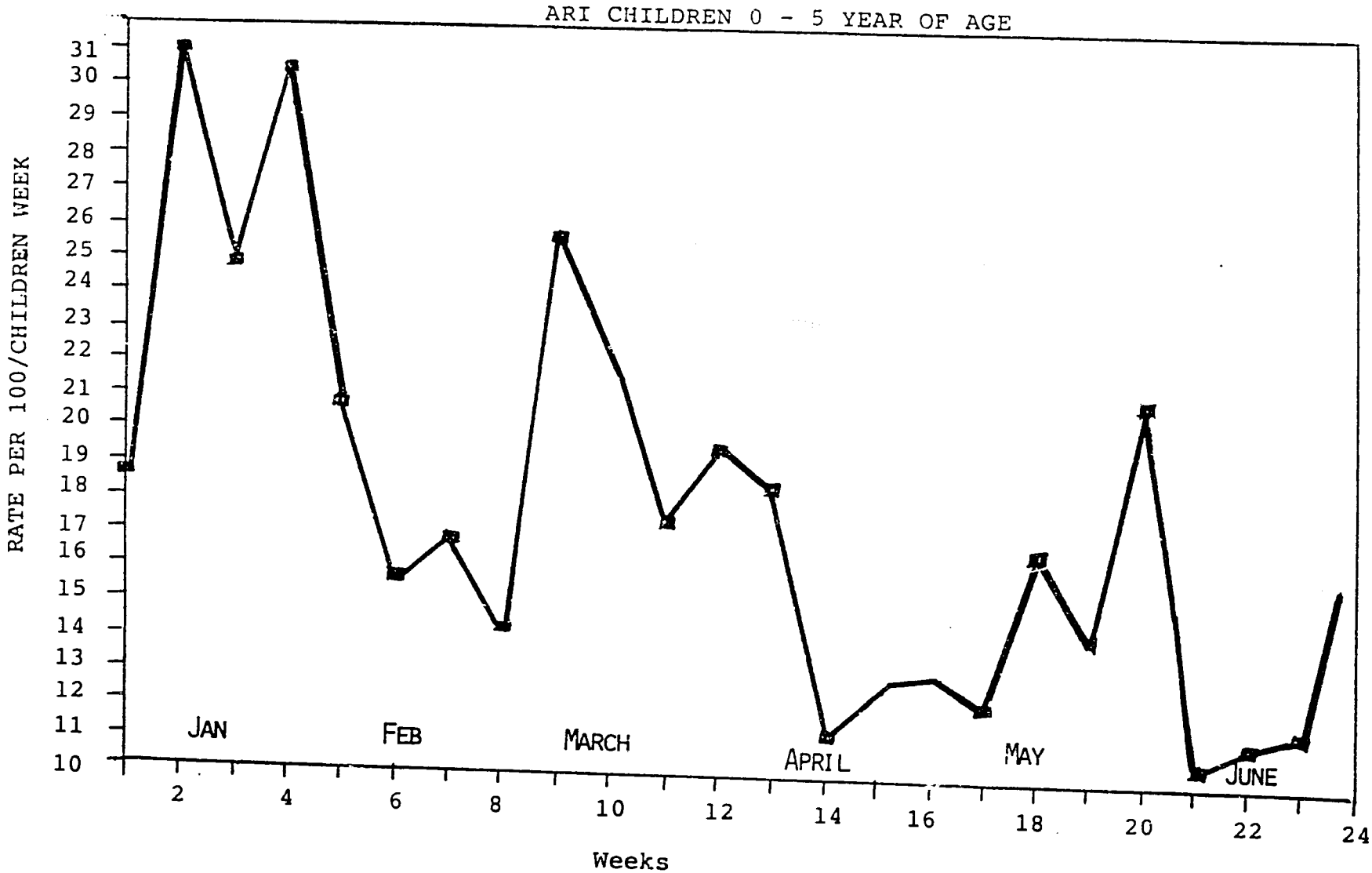
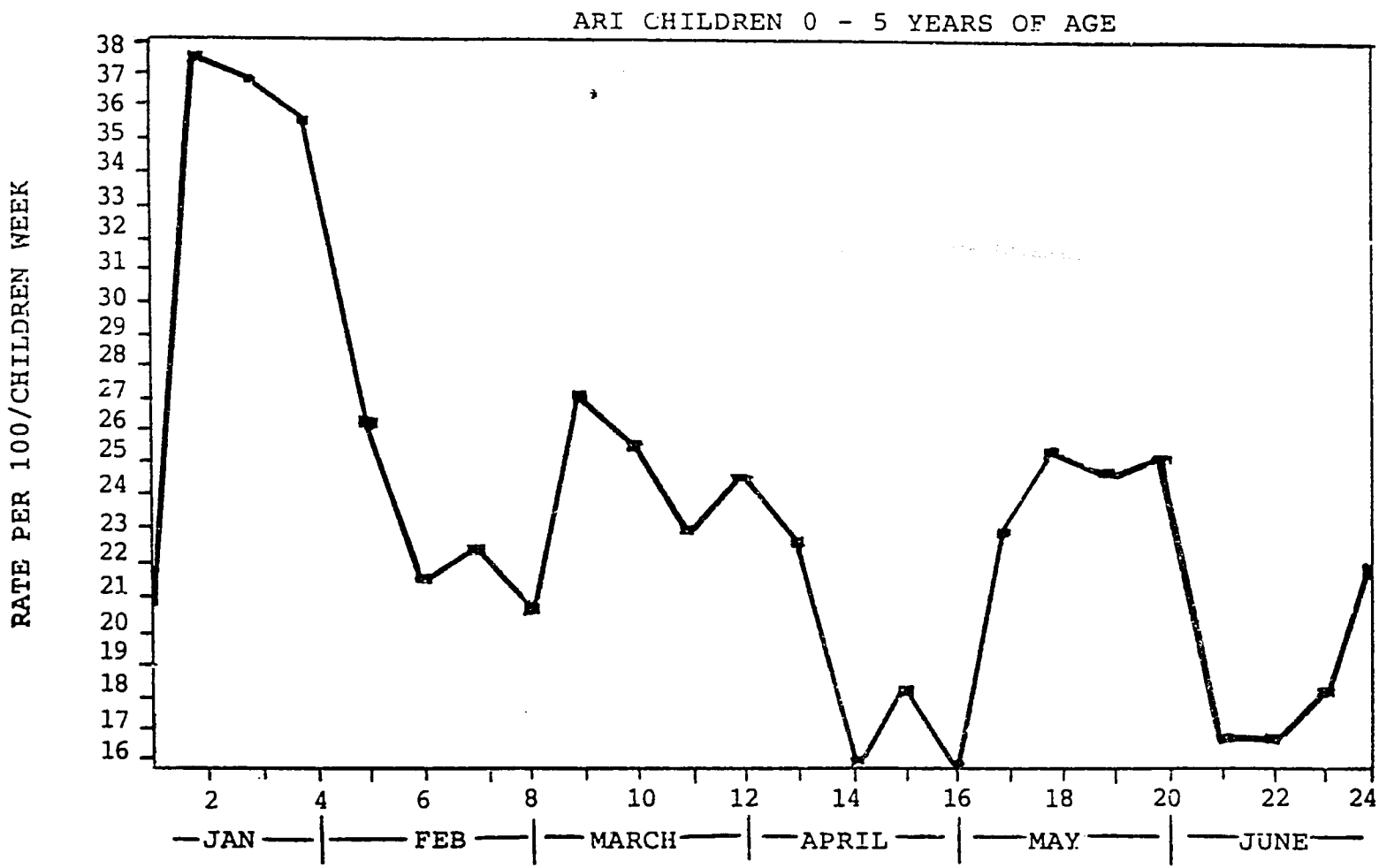


Figure 3

THE WEEKLY PREVALENCE RATE
IN JANUARY - JUNE 1986



FY 1986

COMPARATIVE LONGITUDINAL STUDIES OF MALARIA VECTORS INFECTION RATES IN DIFFERENT ECOLOGICAL AREAS IN THAILAND (MVR-TH-9-87-66)

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BACKGROUND

One of the most critical problems currently confronting the THAI Anti-Malaria Program is a lack of reliable scientific information characterizing the site of malaria transmission as being inside or outside houses; within specific habitats such as the village itself or in nearby forests or rubber plantations. Current information, based on malaria case investigation indicates that up to 89% of cases are contracted in forested areas. If this is in fact the case, residual spraying in villages and other vector control measures in many localities may be inefficient. However, to desist in these activities is to risk a resurgence of malaria transmission, so this decision must be made on the basis of reliable data concerning the site of transmission. Presently the means of determining this information is through case investigation which is, at best, speculative. In Thailand we have identified three major ecologies in which the putative principal vectors are most frequently found : forests, rubber plantations and rice cultivation fields including villages located near any of these three. it is in these three ecologies that we wish to examine vector sporozoite rates in an attempt to characterize areas of high malaria transmission and to study which vectors are primarily responsible for this transmission at different times of the year.

HYPOTHESIS

1. The relative importance of anopheline vector species will vary in accordance with seasonal an ecological factors. This can be evaluated by longitudinal studies of vector infection rates in selected geographical areas.

2. Malaria transmission occurs outdoors rather than indoors, and most cases are contracted in heavily forested areas as compared to other ecological settings.

OBJECTIVES

1. To correlate P. falciparum and P. vivax infection rates in anopheline mosquitoes with seasonal gametocyte infections in humans in different ecological settings.

2. To determine the prevalence of P. falciparum and P. vivax in different ecological settings.

3. to confirm the presence of the known primary, secondary and/or suspected anopheline vectors of human malaria parasite in different ecological settings and to compare the vectorial capacities of these species.

4. To investigate daily and seasonal variations in the nocturnal biting habits of anopheline vectors and determine the period of maximum risk of contracting malaria indoors and outdoors in different ecological settings.

5. To determine the feasibility of integrating the ELISA method of sporozoite analysis into routine malaria epidemiological field studies.

STUDY AREAS

Three different ecological settings were selected in Chantaburi Province southeastern Thailand, as follows:

1. Rice Field Village - Takrid a hamlet in Village No. 2, Tambon Pattawee Makham District.

2. Rubber Plantation - Khoa Sung, another from Takrid hamlet in Village No 2, Tambon Pattawee of Makham District. it is approximate by 3-4 kilometers.

3. Forest - Takree, one hamlet in Village No. 8, Tambon Pattawee, Makham District.

Each hamlet has approximately 50-60 houses and 250-300 inhabitants. Annual malaria incidence is approximately 30/1000 population. All three hamlets are about 20-30 km. from the provincial capital of Chantaburi.

METHODS

The field study will require 2 years, from July,. 1987 to June, 1989. This phase will see the first use of the ELISA technique as a research tool in the Anti-Malaria program in Thailand. During these two years of field studies we will be particularly interested in determining which anopheline mosquito species are natural malaria vectors in the study area, investigating specific ecological habitats and sites of malaria infection, i.e., indoors or outdoors, villages or surrounding environments (forest, rubber plantations, rice field). We will observe Anopheles and Plasmodium species interrelationships as well as seasonal variations in malaria transmission. The first year's study will be conducted to obtain basic information as stated above. The second year's study will be carried out to determine any possible annual variation and to confirm the first year's study results. Moreover, any statistical study such as this requires as broad a data base as possible, especially if we hope to use the information gained as a model on which control measures may be planned.

Three field teams will be used during the study, one for each of the ecological settings. Each team will consist of a team leader, a driver (both from the Malaria Division), and 12 collectors (four Malaria Division staff members and 8 locally hired and trained collectors). A Regional Entomologist will be responsible for the overall supervision of the three field teams.

Human biting mosquito collections will be conducted each month, during the same phase of the lunar cycle, to determine the identity and density of vectors species infected with human malaria sporozoites. Collections will be made between dusk and dawn by two men serving as bait inside and two men outside study houses located in each of the ecological settings (i.e,. the rice field, forest and rubber plantation). Houses used during the study will have at least three walls. The men will work in shifts from dusk until midnight and midnight until dawn (8 men/night/setting = 24 men). Collections will also be made by two men in the forest and two men in the rubber plantation away from dwellings. These men will be relieved at midnight (4 men/night/setting = 8 men). Additionally, two men working in shifts will collect mosquitoes for 20 minutes each hour from a water buffalo or a cow (or other suitable non-human host) located in the vicinity of the study house in the rice field setting (2 men/shift = 4 men). Collections will be made on each of five consecutive nights. All anopheline mosquitoes collected on humans will be captured in vials and the time of collection recorded. Suction tubes will be used to collect specimens from the walls of a net enclosing a cow or water buffalo. The net will hang about 0.33 meters above the ground to allow mosquitoes to enter. it will be cleared of all mosquitoes remaining uncollected after the 20-minute collection period. Anopheline mosquitoes will be screened using a dissection microscope and standard morphological characteristics. Currently available morphological keys for the subgenus of (Cellia) and Anopheles e.g. Harrison an B.A. Harrison and J.E. Scanlon will be used for identification. Those mosquitoes belonging to the Anopheles dirus complex, Anopheles maculatus complex and An. philippinesis / An. nivipes, and Ar. barbirostris group will be identified based on the morphological characteristics elaborated by AFRIMS, the Faculty of Science (Mahidol University) and the Smithsonian Institution (Washington, D.C.) researchers. A key for the An. maculatus complex has been published by P. Rattanarittikul and C.A. Green. A key to the An. dirus complex is being prepared for publication by E.L. Peyton. If this key is not available, the voucher specimen will be examined for the presence or absence of the enzyme glyceral dehydrogenase. This will be tested by C.A. Green and V. Baimai of Mahidol University. Each mosquito will be given an accession number. Duplicate numbers will be used, one for the abdomen, the other for the head and thorax. Mosquitoes will be first dissected and the ovaries examine for parity under a compound microscope. Parity data will be used to estimate mosquito longevity (See diagram). parous mosquitoes will then be dissected in accordance to direction from Dr. R. A. Wirtz, WRAIR and the two halves placed in separate labelled Eppendorf tubes and stated above for testing by ELISA. Eppendorf tubes containing the samples will be placed, with their lids open in an incubator containing copper sulfate till the next day's samples are ready to be dried. At this time the previous day's samples will

be sealed and packed for transportation and the next samples placed in the incubator for drying. Each month's samples will be transported to Bangkok in a padded packing box for ELISA testing. All dissection and identification data will be correlated with the accession number of mosquitoes and the results of ELISA tests will be recorded following the same procedure. One Investigator, two Research Assistants and two Laboratory Assistants will be at the study area in each month and will be responsible for scoring the identification and dissection of mosquitoes including labelling the tube with the accession numbers and performing other related field activities. Sporozoite species analysis will be performed using the ELISA method described by Wirtz.

Anopheline biting activity will be related to human infection. The area will be mapped and a census taken before the onset of collections. Approximately 200 individuals (approximately 30-50% of population) representative of the age structure and proportion of individuals living and/or working in each of the ecological situations will be selected for monthly evaluation. Malaria prevalence will be determined by taking thick blood smears from these individuals. The slides will be fixed, stained, and examined by a Malaria Division microscopist according to established procedures (examination of 200 microscopic fields will be carried out later in order to confirm these initial results). Parasitemia, Plasmodium species, and stages of infection will be determined. Malaria positive cases will be treated according to the policy established by Malaria Division through the local Malaria Office. Records of patient history including age, sex and occupation will be maintained. These investigations will be performed by a Research Assistant and a laboratory Assistant. Meteorological data for the study areas will be collected monthly. Average temperature, humidity and rainfall will be recorded from meteorological stations established near the collection sites.

PROGRESS

Study areas have been selected as mentioned before. Two Research Assistants and two Laboratory Assistants were recruited, and they have been trained at AFRIMS, Bangkok on mosquito identification, dissection for gut, glands and ovaries as well as ELISA techniques. We are at the beginning of the establishment of an ELISA Laboratory at the Malaria Division with assistance from Dr. J.G. Olson and Dr. R. A. Wirtz.

FY 1986

MANAGEMENT AND PRODUCTION OF SELECTED NITROGEN-FIXING TREES (FGT-TH-10-87-72)

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ABSTRACT

Sixteen nitrogen-fixing tree species of the genera Acacia, Albizia, Calliandra, Casuarina, Enterolobium Gliricidia, Leucaena, Pithecellobium, Samanea, Sesbania and 2 non-nitrogen-fixing species of Cassia and Eucalyptus were evaluated for site adaptabilities and multipurpose potential:

In non-acid soil conditions at Nakhon Ratchasima Province, the species used in the trials which grew well were Acacia mangium, Albizia Falcataria, Eucalyptus camaldulensis, Gliricidia sepium and Leucaena leucocephala. Recently, however, the Leucaena trials have been damaged by a psyllid (Heteropsylla cubana) infestation.

In acid soil conditions at Chumphon Province, the most adaptable species were Acacia mangium, Acacia auriculiformis, Eucalyptus camaldulensis, Albizia falcataria and Casuarina equisetifolia.

Heights and diameters at breast height of most tree species increased with increased spacing. Total biomass was generally highest at the closest spacings.

Cassia siamea, Eucalyptus camaldulensis, Leucaena leucocephala, Gliricidia sepium and Calliandra calothyrsus coppiced well compared to other species used in the trial.

Among the species tested, Leucaena leucocephala, Cassia siamea, Gliricidia sepium and Sesbania grandiflora were identified as suitable for animal feed. Acacia mangium, Acacia auriculiformis and Eucalyptus camaldulensis were identified as having exceptional promise, particularly for fuelwood, pulp, wood products and high yielding biomass.

Overall, the results indicate the greatest multiuse potential of Acacia mangium and suggest this species warrants further research and development for cultivation in Thailand and in other humid tropical areas.

INTRODUCTION

The problem of rapidly depleting forests is of great international concern. In Thailand, the annual depletion of forest resources during 1982-1986 was estimated at 5,339 square kilometers or about 0.21 million hectares[2]. Shifting cultivation, illegal logging and the increasing

need for wood have resulted in this loss. Much of the reforestation has involved slow-growing tree species, often requiring 50-60 years before economic harvest.

Research and development of fast growing, multipurpose, nitrogen-fixing trees are feasible ways to approach the problem of decreasing forest resources.

Recognizing the need for solutions to the problem of rapidly depleting forest reserves, the Thailand Institute of Scientific and Technological Research (TISTR), the Ministry of Science, Technology and Energy has conducted research and development projects on fast growing, nitrogen-fixing trees (FGNFT) started with Leucaena leucocephala, a fodder tree which has proved widely adapted in Thailand for its multiple uses.

From 1982-1985 TISTR has conducted the research project entitled "Field Trials and Testing of Selected Species of Fast-Growing, Nitrogen-Fixing Trees" under support of the Board on Science and Technology for International Development (BOSTID), U.S. National Academy of Science, to test 18 species of FGNFT. The objectives were to identify tree species most appropriate for the locations (Chumphon and Nakhon Ratchasima Provinces), to determine cultural and management techniques and to investigate the properties of the trees and their potential uses. The results of this project (briefly presented in this report) has led to the on-going research and development project entitled "Management and Production of Selected Nitrogen-Fixing Trees" with the overall objectives to evaluate the relationships of outstanding tree species and provenances (Acacia sp.) with the climatic and soil variables, to identify significant genetic variability and develop high yielding composites. These project trails have been established in 1987 at Chiang Mai, Chonburi, Chumphon and Suratthani Provinces. It is expected that the research results will not only benefit the agro-forestry activities of Thailand but should provide knowledge of international importance in exploiting these significant multipurpose nitrogen-fixing tree species.

MATERIALS AND METHODS

Experiments at Chumphon

1. Species trial of 18 FGNFT species (Table 1): The augmented design, which consists of species No. 1-10, augmented with species No. 11-18, with six replications was employed. The trees were planted at 1 m x 1 m spacing.

2. Spacing trial: The split plot design with two replications was employed. Species No. 1-10 were the main plots and the spacing of 0.5 m x 1 m; 1 m x 1 m; 1 m x 1.5 m; 1 m x 2 m; 1 m x 3 m; and 1 m x 4 m were the subplots.

3. Fertilizer trial, coppiced regrowth study

   The split plot design with four replications was employed. Species No. 1-10 were the main plots and four

levels of phosphate fertilizer (18.75; 37.50; 75.00; 150.00 P_2O_5/ha) were the subplots. A basal fertilizer dressing of ammonium sulphate to give 28 KgN/ha and potash to give 75 Kg K_2O/kha was applied. The plant spacing was 1 m x 1 m. One half of each subplot of each species was coppiced to allow collection of data on coppicing and biomass determination.

Experiment at Nakhon Ratchasima

1. Species trials of six FGNFT species: Acacia mangium, Albizia falcataria, Eucalyptus camaldulensis, Gliricidia sepium, Leucaena diversifolia and Leucaena leucocephala were used. The experiment was a randomized complete block design with 3 replications. Trees were planted at 1.5 m x 1.5 m spacing.

2. Spacing trial

   The same six tree species utilized in the species trial were used. A complete randomized design with 3 replications and 3 spacing treatments: 1.5 m x 2 m; 1.5 m x 3 m; and 1.5 m x 4 m was employed.

Analysis and Testing

Leaf tissue was analysed chemically for animal feed characteristics and in vitro dry matter digestion. Physical properties of woods (moisture content, heating value, density) and pulping and paper making characteristics were determined.

RESULTS AND DISCUSSION

On the coastal sandy acidic soil at Chumphon, the most adaptable species were Acacia mangium, Acacia auriculiformis, Eucalyptus camaldulensis, Albizia falcataria and Casuarina equisetifolia, which appear to have a higher tolerance for acid soil conditions. On the non-acidic soil at Nakhon Ratchasima, the most adaptable species included Leucaena leucocephala, Leucaena diversifolia, Eucalyptus camaldulensis, Albizia falcataria, Acacia mangium and Gliricidia sepium.

Most of the tree species grew taller and had greater diameters at breast height as spacings increased. In contrast, the biomass and stem volumes were highest at the closest spacings. In this trial, weeds, particularly Imperata cylindrica were predominantly found in plantings with wider spacings. Higher density plantings allowed early canopy closure which suppressed weeds.[1]

In considering of biomass production at different tree densities, it should be noted that selection of the optimum planting density should ultimately depend on the purpose and scale of planting, labour supply and the species to be used. For small scale plantings, the use of 1m x 1m or 1m x 1.5m spacings could be employed since they allow the use of labours

or small tillers for maintenance. The trees could be thinned at each suitable stage for specific wood products and to allow the remaining trees to grow larger for other wood uses. In a commercial scale plantings, the use of wider spacing may be necessary to allow full mechanization.

Among the adaptable species, Albizia falcataria grew well in both acidic and non-acidic soils and appears useful as a source of paper pulp. The wood, however, has a relatively low specific gravity and is unsuitable for other wood uses.

Leucaena leucocephala and L. diversifolia grew and yielded well in the non-acid soil and seem to possess many useful qualities. In Thailand, Leucaena leucocephala has been widely cultivated in small farm plots and boarder rows for fodder/fuelwood and increasingly for commercial fodder production. However, its inability to grew in acid soils and susceptability to psyllid (Heteropsylla cubana) infestation[6,7] suggust that its cultivation should be limited until these problems are solved[3]. Recent research has reported some success in identifying psyllid tolerant leucaena.[5]

One of the interesting species was Acacia auriculiformis, which could tolerate the arid and acid soil conditions, and several months of permanent flooding. It was highly productive, yielding wood of high calorific value with good wood property for paper pulp. Its use as lumber could be limited due to its crooked stem and branching. However such disadvantages may be overcome through the selection and breeding of straight trunk trees.

Another one of the best adapted species was Eucalyptus camaldulensis, a well-known fast-growing tree, which is being cultivated throughout Thailand and in many countries. It produced the highest biomass as well as good quality wood for the paper pulp. In the other hand, it has been suggested that its cultivation may pose environmental and econological problems[4]. Thus, research to determine the environmental effects of this specific problem seems warranted.

Among all tree species tested, Acacia mangium was considered the most interesting both for its site adaptability and multipurpose uses. The average biomass produced by Acacia mangium was close to the biomass produced by Eucalyptus camaldulensis. Its wood has the highest calorific value of all species tested, and is suitable for paper pulp production as well as for lumbering for furniture and plywood. In Thailand, it has been found to grow well not only in the acid soil at Chumphon, or on the non-acid soil of Nakhon Ratchasima but also in less fertile soils in disturbed sites.

In consideration of the properties of the tree species tested, we conclude that Acacia mangium and Acacia auriculiformis warrant further research and development for cultivation in Thailand and in other humid tropical areas.[6]

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Table 1 Fast Growing Tree Species Used in Experiments

	Species	Seed Source
1.	*Acacia auriculiformis* A. Cunn. ex Benth.	Local seed, Nakhon Ratchasima province
2.	*Acacia mangium* Willd.	Forest Research Center Sabah, Malaysia
3.	*Albizia falcataria* (L.) Fosberg.	NFTA. Hawaii
4.	*Calliandra calothyrsus* Meissin.	Indonesia
5.	*Cassia siamea* Lam.	Local seed, Nakhon Ratchasima, province
6.	*Casuarina equisetifolia* Forst. & Forst.	Prachuab Khiri Khan, Royal Forest Department
7.	*Eucalyptus camaldulensis* Dehnh.	Australia.
8.	*Gliricidia sepium* (Jacq.) Steud.	ASEAN Canada Forest Tree Seed Center Muak Lek, Saraburi Province
9.	*Leucaena leucocephala* K8 Lam. de Wit	NFTA, Hawaii
10.	*Sesbania grandiflora* (L.) Poir.	Local seed, Chachoengsao province
11.	*Albizia lebbek* (L.) Benth.	Local seed, Nakhon Ratchasima province
12.	*Albizia procera* (Roxb.) Benth.	Local seed, Saraburi province
13.	*Causuarina junghuhniana* Miq.	Local vegetative plants
14.	*Albizia Lebbekoides* Benth.	Pak Thong Chai, Nakhon Ratchasima province
15.	*Enterolobium cyclocarpum* (Jacq.) Griseb	Kasetsart University, Bangkok
16.	*Leucaena diversifolia* (Schlecht) Benth.	NFTA, Hawaii
17.	*Pithecellobium dulce* (Roxb.) Benth.	Local seed, Bangkok
18.	*Samanea saman* (Jacq.)	Kasetsart University, Bangkok

APPENDICES

H.M. Fifth Cycle Commemorative Conference of
USAID Science Research Award Grantees
Whale Hotel, Nakorn Pathom
July 24-26, 1987

AGENDA

Friday, July 24, 1987

Opening Ceremonies

(Venue - Conference Room, Faculty of Education, Silpakorn University, Tabkaew Campus)

09:00	Registration and Coffee
10:00	Audience takes seats in Conference Room
10:15	Professor Dr. H.R.H. Princess Chulabhorn arrives
10:30	- Minister of Science, Technology and Energy reports to H.R.H. Princess Chulabhorn on Purpose of the Conference - H.R.H. Princess Chulabhorn formally opens the Conference - U.S. Ambassador's address on Thai-U.S. Collaboration in S&T Research
11:00	Professor Dr. H.R.H. Princess Chulabhorn reviews Exhibits on Research Activities
11:30	Professor Dr. H.R.H. Princess Chulabhorn meets with dignitaries to discuss S&T issues
12:00	Professor Dr. H.R.H. Princess Chulabhorn departs
12:15	Lunch for conference participants at Whale Hotel hosted by Conference Organizing Committee

Balance of Conference

(Venue - Conference Room, Whale Hotel, Nakorn Pathom)

14:30	Coffee is served in the large conference room at the Whale Hotel, where conference continues after lunch.

15:00	Plenary session convened by Dr. Yongyuth, who makes brief opening remarks and introduces speakers and panel.
15:15	Overview of USAID support for research projects. - Program for Science and Technology Cooperation (PSTC) - Dr. Irvin Asher - Board on Science and Technolohy in International Development (BOSTID) - Dr Judith Bale - Cooperative Development Research (CDR) - Mr. Ariel Kerem - Science and Technology Development Board (STDB) - Dr. John R. Eriksson
16:00	Panel Discussion of centrally funded research grant programs by university representatives. Representative of Ministry of University Affairs in the chair. Representatives of Mahidol University, Chiang Mai University, Kasetsart University, and Khon Kaen University constitute the panel. General discussion follows individual presentations.
17:30	Afternoon session concludes
18:30	Reception hosted by USAID.

Saturday, July 25, 1987

At this point, presentations and discussions on each of the project begin. Projects are divided into three areas - Health and Life Science Projects, Agriculture and Natural Resources Projects, and Engineering Related Projects. Presentations on projects from each of the three areas will occur concurrently. Presentations on Health and Life Science Project will be in Room A, presentations on Agriculture and Natural Resources Projects will be in Room B, and presentations on Engineering Related Projects will be in Room C.

As indicated below, in some cases reports on projects will occur as part of Panel Discussions while in other cases, project reports will be presented individually as Short Papers. In panel discussions each panel member will make a presentation on his/her project. Questions and discussion on the projects will occur after all panel members have made their presentations, and hopefully, will serve to highlight similarities and differences in approaches taken by the different panel members in carrying out their projects.

In the case of "short paper" presentations, questions and answers sessions will occur immediately following the presentation.

Health and Life Science Projects : Room A

08.30 Panel Discussion on Immunodiagnosis

Part 1

"Development of an Immunoperoxidase Test for Early Diagnosis of Acute Reactional States in Leprosy Patients"
Dr. Choti Theetranont, Chiang Mai University

"Evaluation In Vitro of Interleukin 1 and Interleukin 2 as Possible Immunotherapeutic Agents in Leprosy"
Dr. Sanit Makonkawkeyoon, Chiang Mai University

"Immunodiagnosis and Seroepidemiology of Tuberculosis"
Dr. Sanit Makonkawkeyoon, Chiang Mai University

"Development of Immunodiagnosis and Improvement of Immunotherapy of Snake Venom Poisoning"
Dr. Kavi Ratanabanangkoon, Mahidol University

10.00 Coffee Break

Part 2

10.30 "Production of Monoclonal Antibodies to *Salmonella* That Cause Enteric Fever"
Dr. Suthipant Sarasombat, Mahidol University

"Application of Monoclonal Antibodies Against *Entamoeba histolytica* in Tropical Medicine Research"
Ms. Nitaya Thammapalerd, Mahidol University

"Development of Methods for Immunodiagnosis of Liver Fluke Infection"
Dr. Stitaya Sirisinha, Mahidol University

"Identification and Cloning of Surface Antigens and Genes of *Schistosoma japonicum*"
Dr. Prasert Sobhon, Mahidol University

12.00 Lunch

13.00 Short Papers

"Immunopathogenesis of Dengue Hemorrhagic Fever/Dengue Shock Syndrome"
Dr. Vinai Suvatte, Mahidol University

"Biological Control of Schistosomiasis Transmitting Snails in Southeast Asia"
Dr. Suchart Upatham, Mahidol University

"Etiology and Development of Acute Respiratory Infections in an Urban Community and Hospital"
Dr. Subharee Suwanjutha, Mahidol University

"Sulfonamid Drugs as a Male Antifertility Agent"
Dr. Choempol Pholpramool, Mahidol University

15.00 Coffee Break

15.30 Plenary Session : Financial Management and Grant Administration

18.00 Hospitality Hours

Agriculture and Natural Resources Projects : Room B

08.30 Panel Discussion on Plant Genetic Resources

Part 1

"Yield Improvement, Agronomy and Local Use of Amaranth"
Dr. Chuckree Senthong, Chiang Mai University

"Species Selection and Potential Uses of Grain Amaranth As an Introduced Crop"
Mr. Soonthorn Duriyaprapan, TISTR

"Collection and Utilization of Tamarinds Germplasm"
Dr. Sumrit Feungchan, Khon Kaen University

10.00 Coffee Break

Part 2

"Enhancing Genetic Variability and Breeding Improved Sesame Cultivars"
Dr. Vichitr Benjasil, Department of Agriculture

"Fiber Farming Through Weed Utilization"
Dr. Thanakorn Uan-On, Mahidol University

"Management and Production of Selected Nitrogen Fixing Trees"
Dr. Kovith Yantasath, TISTR

12.00 Lunch

13.00 Panel Discussion on Biofertilizer and Nitrogen Fixation

"Culture, Maintenance and Propagation of Azolla Under Tropical Conditions"
Dr. Nantakorn Boonkerd, Department of Agriculture

"Ri-Plasmid Induced Root Culture As a Potential Substrate for In Vitro Propagation of Mycorrhizal Fungi"
Dr. Malee Suwana-adth, Kasetsart University

"Role of VA Mycorrhiza in the Phosphorus Nutrition of Economic Leguminous Crops in Thailand"
Ms. Yenchai Vasuvat, Department of Agriculture

"Interactions in Legume - Nonlegume Intercropping Systems"
Dr. Benjavan Rerkasem, Chiang Mai University

15.00 Coffee Break

15.30 Plenary Session : Financial Management and Grant Administration

18.00 Hospitality Hours.

Engineering Related Projects : Room C

Short Papers

08.30 "Heat Sterilization and Accelerated Drying of High Moisture Rice for Safe Storage"
Dr. V. K. Jindal, Asian Institute of Technology

"Improved Producer Gas Cleaning System"
Ms. Pojanie Khummongkol, KMIT Thonburi

"Cashew Nut Compact Sized Sheller"
Mr. Thavachai Thivavarnvongs, Khon Kaen University

10.00 Coffee Break

10.30 "Soil Salinity Control for the Growth of Plants Which Can Serve As Energy Source"
Ms. Benjamas Chattong, KMIT North Bangkok

"Hydrological Study of Rainfed Agricultural Watersheds"
Dr. Sacha Sethaputra, Khon Kaen University

12.00 Lunch

13.30 Plenary Session : Financial Management and Grant Administration

Sunday, July 26, 1987

Health and Life Science Projects : Room A

08.30 Panel Discussion on Malaria

Part 1

"Studies on the Bionomics of *Anopheles maculatus* and Its Role in Malaria Transmission"
Dr. Suchart Upatham, Mahidol University

"Development of DNA Probes to Differentiate Sibling Species of *Anopheles dirus* and *An. maculatus*"
Dr. Sakol Panyim, Mahidol University

"Comparative Longitudinal Studies of Malaria Vector Infection Rates in Different Ecological Areas"
Dr. Chusak Prasittisuk, Department of Communicable Disease

10.00 Coffee Break

Part 2

10.30 "Comparison of Two In Vitro Techniques for Culture of *Plasmodium falciparum*"
Dr. Udom Chitprarop, Malaria Center, Chiang Mai

"Antigenic Diversity of Plasmodium Vivax"
Dr. Srisin Khusmith, Mahidol University

"Improvement of Bacterial Agents for Control of Mosquito Vectors"
Dr. Somsak Pantuwatana, Mahidol University

12.00 Lunch

Agriculture and Natural Resources Projects : Room B

08.30 Panel Discussion on Tissue Culture

Part 1

"New Variety of Rice for Saline and Acid Soil Through Tissue Culture"
Ms. Montakan Vajrabhaya, Chulalongkorn University

"In Vitro Conservation and Germplasm Exchange of Bamboo"
Dr. Paiboolya Gavinlertvatana, Kasetsart University

"Development of Methodologies for Cowpan Tissue Culture"
Dr. Ponpimon Suriyajantratong, Khon Kaen University

10.00 Coffee Break

Part 2

10.30 "Biotechnology Application for Characterization and Selection for Better Yielding Rubber Clones"
Dr. Rapepun Wititsuwannakul, Prince of Songkla University

"Tissue Culture of Teak"
Dr. Apichart Kaosa-ard, Teak Improvement Center, Lampang

12.00 Lunch

Short Papers

13.00 "Natural Insecticides for *Spodoptera litura*"
Dr. Pichaet Wiriyachitra, Prince of Songkla University

"Changes of Cyanogenic Glycosides and Related Enzymes in Cassava Roots During Storage and Processing"
Dr. Montri Chulavatnatol, Mahidol University

"Utilization of Cassava and Cassava Waste Through Fermentation Technology"
Ms. Napha Lotong, Kasetsart University

"Hybridization of Halotolerant Yeast for Alcohol Fermentation"
Ms. Savitree Limtong, Kasetsart University

LIST OF PARTICIPANTS

H.M. Fifth Cycle Commemorative Conference of
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Invited Guests

1. Dr. Udom Dechmani, Department of Agriculture, Ministry of Agriculture and Cooperatives
2. Dr. Nipon Wongvisetsirikul, KMIT-L, Bangkok
3. Dr. Thira Sutabutra, Kasetsart University, Bangkok
4. Dr. Thiraphan Bhukaswan, Department of Fisheries, Ministry of Agriculture and Cooperatives, Bangkok
5. Dr. Terdthai Vatanatham, Prince of Songkla University, Songkla
6. Dr. Jariya Boonjawat, Chulalongkorn University, Bangkok
7. Dr. Sumalee Pichyangkura, Chulalongkorn University, Bangkok
8. Dr. Maitree Duangsawasdi, Department of Fisheries, Ministry of Agriculture and Cooperatives, Bangkok
9. Dr. Phanna Waikakul, Khon Kaen University, Khon Kaen
10. Dr. Intarachai Hovichitr, Khon Kaen University, Khon Kaen
11. Dr. Uthai Charanasri, Bangkok Flowers Center Co., Bangkok
12. Dr. Kamphol Adulavidhya, Kasetsart University, Bangkok
13. Dr. Morakot Tanticharoen, KMIT-T, Bangkok
14. Dr. Makumkrong Poshyachinda, Chulalongkorn University, Bangkok
15. Dr. Avudh Srisukri, Chiang Mai University, Chiang Mai
16. Dr. Thiraphat Vilaitong, Chiang Mai University, Chiang Mai
17. Dr. Narongchai Akrasanee, IFCT, Bangkok
18. Dr. Tipaporn Limpaseni, Chulalongkorn University
19. Dr. Thalerng Thamrong-Nawasawat, STDB
20. Dr. Yongyuth Yuthavong, NCGEB, Ministry of Science, Technology and Energy
21. Dr. Chuckree Senthong, Chiang Mai University, Chiang Mai
22. Dr. Serene Piboonniyom, Mahidol University
23. Dr. Chuvit Sukprakarn, Department of Agriculture, Ministry of Agriculture and Cooperatives
24. Dr. Patcharee Chaisith, Khon Kaen University, Khon Kaen
25. Dr. Thavon Vajrabhaya, Graduate School, Chulalongkorn University, Bangkok
26. Dr. Tavee Lertpanyavit, Chulalongkorn University, Bangkok
27. Dr. Swang Wangboonkong, Department of Agriculture, Ministry of Agriculture and Cooperatives
28. Dr. Pachernchai Chaiyasith, KMIT-L, Bangkok
29. Dr. Suthat Julsrikraival, Mahidol University, Bangkok
30. Mr. Ariel Kasem, Israel Embassy, Bangkok

Agriculture and Natural Resources

1. Dr. Thipmani Paratasilpin, Chiang Mai University
2. Dr. Soonthorn Duriyaprapan, TISTR, Bangkok
3. Dr. Panas Buranasilpin, TISTR, Bangkok
4. Dr. Sanyan Tanpanich, TISTR, Bangkok
5. Dr. Sumrit Feungchan, Khon Kaen University, Khon Kaen
6. Mr. Taweekiat Yimsawat, Khon Kaen University, Khon Kaen
7. Mr. Sorot Chindaprasert, Khon Kaen University, Khon Kaen
8. Dr. Vichitr Benjasil, Department of Agriculture, Ministry of Agriculture and Cooperatives
9. Mr. Thanom Dao-ngarm, Department of Agriculture, Ministry of Agriculture and Cooperatives
10. Dr. Kovith Yantasath, TISTR, Bangkok
11. Dr. Suthijed Chandrasiri, TISTR, Bangkok
12. Dr. Winai Supatnakul, TISTR, Bangkok
13. Dr. Nantakorn Boonkerd, TISTR, Bangkok
14. Dr. Prayoon Swatdee, TISTR, Bangkok
15. Dr. Somporn Choonluchanon, TISTR, Bangkok
16. Dr. Malee Suwana-adth, Kasetsart University, Bangkok
17. Dr. Poonpilai Suwannarit, Kasetsart University, Bangkok
18. Dr. Yupa Mongkolsuk, Kasetsart University, Bangkok
19. Dr. Rapeepun Chewatanasuk, Kasetsart University, Bangkok
20. Dr. Tanusara Vinotai, Kasetsart University, Bangkok
21. Dr. Benchai Vasuvat, Department of Agriculture, Ministry of Agriculture and Cooperatives
22. Dr. Omsub Nopamornbodi, Department of Agriculture, Ministry of Agriculture and Cooperatives
23. Ms. Supaporn Thamsurakul, Department of Agriculture, Ministry of Agriculture and Cooperatives
24. Dr. Benjawan Rerkasem, Chiang Mai University, Chiang Mai
25. Dr. Sakarindr Bhumiratana, NCGEM/KMITT, Bangkok
26. Dr. Paiboolya Gavinlertvatana, Kasetsart University, Bangkok
27. Dr. Pranom Prutpongse, Kasetsart University, Bangkok
28. Dr. Ponpimon Suriyajantratong, Khon Kaen University, Khon Kaen
29. Dr. Sumonthip Boonnag, Khon Kaen University, Khon Kaen
30. Dr. Wanwali Athiwatpong, Khon Kaen University, Khon Kaen
31. Dr. Rapepun Wititsuwannakul, Prince of Songkla University, Songkla
32. Dr. Lerluck Buranakarl, Kasetsart University, Bangkok
33. Dr. Apichart Kaosa-ard, Teak Improvement Center, Lampang
34. Dr. Pimchai Apavatjrut, Chiang Mai University, Chiang Mai
35. Dr. Pichaet Wiriyachitra, Khon Kaen University, Khon Kaen
36. Dr. Montri Chulavatnatol, Mahidol University, Bangkok
37. Dr. Somsak Ruchirawat, Mahidol University, Bangkok
38. Dr. Savitree Limtong, Kasetsart University, Bangkok
39. Dr. Wichien Yongmanichai, Kasetsart University, Bangkok
40. Dr. Pramote Tammarate, Kasetsart University, Bangkok
41. Dr. Manee Tantirungkit, Kasetsart University, Bangkok
42. Ms. Napha Lotong, Kasetsart University, Bangkok
43. Dr. Busaba Yongsmith, Kasetsart University, Bangkok
44. Dr. Vichien Kitpreechavanich, Kasetsart University, Bangkok
45. Dr. Prasartporn Smitamana, Chiang Mai University, Chiang Mai

46. Dr. Renu Pinthong, Chiang Mai University, Chiang Mai
47. Dr. Aran H. Kittikun, Chiang Mai University, Chiang Mai
48. Dr. Niphone Thaveechai, Kasetsart University, Bangkok
49. Dr. Jiamjit Boonsom, Department of Fisheries, Ministry of Agriculture and Cooperatives
50. Dr. Shabtay Dover, Israel
51. Dr. Chuntana Suwanthada, Chiang Mai University, Chiang Mai
52. Dr. Sacha Sethaputra, Khon Kaen University, Khon Kaen
53. Dr. Amnat Apichatwallop, Khon Kaen University, Khon Kaen
54. Dr. Somchet Tinapong, Khon Kaen University, Khon Kaen
55. Dr. Tipvadee Attathom, Kasetsart University, Nakorn Pathom
56. Dr. Sudawan Chaeychomsri, Kasetsart University, Nakorn Pathom
57. Dr. Ed Sarobol, Kasetsart University, Bangkok
58. Dr. Chareinsak Rojanaridpiched, Kasetsart University, Bangkok
59. Dr. Thanakorn Uan-on, Mahidol University, Bangkok
60. Dr. Jantip Janprasert, Khon Kaen University, Khon Kaen
61. Ms. Thidarat Eksittikul, Mahidol University, Bangkok

Health and Life Sciences

1. Dr. Choti Theetrancnt, Chiang Mai University, Chiang Mai
2. Dr. Kamthorn Thamprasert, Chiang Mai University, Chiang Mai
3. Dr. Lertlakana Bhoopat, Chiang Mai University, Chiang Mai
4. Dr. Sanit Makonkawkeyoon, Chiang Mai University, Chiang Mai
5. Dr. Kavi Ratanabanangkoon, Mahidol University, Bangkok
6. Dr. Suthipant Sarasombat, Mahidol University, Bangkok
7. Dr. Napatawn Banchuin, Mahidol University, Bangkok
8. Dr. Nitaya Thammapalerd, Mahidol University, Bangkok
9. Dr. Savanat Tharavanij, Mahidol University, Bangkok
10. Dr. Stitaya Sirisinha, Mahidol University, Bangkok
11. Dr. Surasakdi Wongratanacheewin, Mahidol University, Bangkok
12. Dr. Prasert Sobhon, Mahidol University, Bangkok
13. Dr. Vithoon Viyanant, Mahidol University, Bangkok
14. Dr. Maleeya Kruatrachue, Mahidol University, Bangkok
15. Dr. Thanit Kusamran, Mahidol University, Bangkok
16. Dr. Vinai Suvatte, Mahidol University, Bangkok
17. Dr. Chantapong Wasi, Mahidol University, Bangkok
18. Dr. Subharee Suwanjutha, Ramathibodi Hospital, Bangkok
19. Dr. Kanda Vathanopas, Ramathibodi Hospital, Bangkok
20. Dr. Pilaipan Puthavathana, Ramathibodi Hospital, Bangkok
21. Dr. Chumpol Pholpramool, Mahidol University, Bangkok
22. Dr. Sakol Panyim, Mahidol University, Bangkok
23. Dr. Sumalee Tungpradabkul, Mahidol University, Bangkok
24. Dr. Chusak Prasittisuk, Ministry of Publich Health
25. Dr. Sukkid Yasothornsrikul, Ministry of Public Health
26. Dr. Somthas Malikul, Malaria Division, Ministry of Public Health
27. Dr. Malinee Prasittisuk, Malaria Division, Ministry of Public Health
28. Dr. Nilobol Therasilp, Malaria Division, Ministry of Public Health
29. Dr. Somsak Krachaikun, Malaria Division, Ministry of Public Health
30. Dr. Suchart Puatipongse, Malaria Division, Ministry of Public Health
31. Dr. Srisin Khusmith, Mahidol University, Bangkok

32. Dr. Somsak Pantuwatana, Mahidol University, Bangkok
33. Dr. Amaret Bhumiratana, Mahidol University, Bangkok
34. Dr. Prapon Wilairat, Mahidol University, Bangkok
35. Dr. Vina Churdboonchart, Mahidol University, Bangkok
36. Dr. Wanlop Chusattayanond, Mahidol University, Bangkok
37. Dr. Chantakorn Shutidamrong, Mahidol University, Bangkok
38. Dr. Somphong Sahaphong, Mahidol University, Bangkok
39. Dr. Vicharn Vithayasai, Mahidol University, Bangkok
40. Dr. Dhirayos Wititsuwannakul, Mahidol University, Bangkok
41. Dr. Udom Chitprarop, Malaria Center, Chiang Mai
42. Dr. Supaporn Ratanatnam, Mahidol University, Bangkok
43. Dr. Pornchai Matangkasombut, Mahidol University, Bangkok
44. Dr. Watchara Kasinrerk
45. Dr. Chakrit Hirunpetcharat
46. Dr. Sakchai Dettrairat

Engineering Science

1. Dr. V.K. Jinda, Asian Institute of Technology, Bangkok
2. Dr. V.G. Reyes, Asian Institute of Technology, Bangkok
3. Dr. Pojanie Khummongkol, KMIT, Bangkok
4. Dr. Damrong Khummongkol, KMIT, Bangkok
5. Dr. Thavachai Thivavarnvongs, Khon Kaen University, Khon Kaen
6. Dr. Winit Chinsuwan, Khon Kaen University, Khon Kaen
7. Dr. Suchart Ngaecharoenkul, Khon Kaen University, Khon Kaen
8. Dr. Benjamas Chattong, KMIT, Bangkok
9. Dr. Bundit Fungtammasan, KMIT
10. Dr. Sacha Sethaputra, Khon Kaen University, Khon Kaen
11. Dr. Amnat Apichatwallop, Khon Kaen University, Khon Kaen
12. Dr. Somchet Tinapong, Khon Kaen University, Khon Kaen

AID/SCI, Washington, D.C.

1. Dr. Howard Minners

USAID, Bangkok

1. Dr. John R. Eriksson
2. Mr. Robert F. Barnes
3. Dr. Gordon L. Hiebert
4. Dr. Jaroon Kumnuanta
5. Mr. Ronald Hammersley
6. Ms. Vimol Thammongkol
7. Ms. Veraanong Na Songkla

PROGRAM GUIDELINES

The Different Programs:

Three different programs are supported by A.I.D.'s Office of the Science Advisor:

Advisor

- The Program in Science and Technology Cooperation (PSTC), initiated in 1981, is a Congressionally-mandated activity of the U.S. Agency for International Development (AID). It seeks to stimulate new and innovative research on problems that confront developing countries, and is currently implemented through a system of highly competitive research grants which undergo scientific peer review.

- U.S.-Israel Cooperative Development Research (CDR) Program is new and separate AID initiative began in 1985. For several decades, Israel -- a small developing country with few natural resources - has sought rapid growth and rural development based largely on technological innovation. Scientists from less developed countries (LDCs) have expressed interest in Israeli technology and in collaborating with researchers in Israel. The U.S.-Israel CDR Program seeks to facilitate access to Israeli scientific and technological capability to help solve significant problems that confront LDCs. It provides funding for Israeli and LDC scientists to cooperate in joint research, thereby strengthening the ability of LDC scientists to do such research themselves. This program is also characterized by intensive competitive, and rigorous peer review.

- The Research Grants Program of the Board on Science and Technology for International Development of the National Academy of Sciences (BOSTID Grants) - In 1969 the National Research Council (NRC) established BOSTID to help developing countries tap the scientific expertise of their own and U.S. scientists. In the 1970s it organized a number of studies and workshops involving both U.S. and less-developed country (LDC) scientists. The BOSTID Research Grants Program supports research carried out by developing country institutions in areas important for economic development. Using AID funds, grants are made for specific research projects in selected areas--grants of a general support nature are not considered. The Committee on Research Grants (CRG) determines project areas and approves grants.

Areas of Research Emphasis

PSTC	U.S.-Israel CDR	BOSTID
Major Emphasis		
Biotechnology/Immunology	Arid Land Agricultures	Grain Amaranth
Plant Biotechnology	Plant Biotechnology	Tropical Trees
Chemistry for World Food Needs	Chemistry for World Food Needs	Biological Nitrogen Fixation
Biomass Resources and Conversion Technology	Biotechnology Related to Disease	Mosquito Vector Field Study
Biological Control	Biological Vector Control	Rapid Epidemiological Assessment
Diversity of Biological Resources	Genetic Diversity	Acute Respiratory Infections in Children
Less Emphasis	Earth Sciences	
Engineering Technology	Engineering	
Atmospheric, Ocean and Earth Sciences		

Proposal Development

Both the PSTC and the U.S.-Israel CDR Programs utilize a two-stage review and approval process, involving, first, a preproposal, and then a full proposal. Only those scientists whose preproposals, after review, have been determined to be potentially promising are invited to develop and submit full proposals.

The BOSTID Grants Program, however, does not involve a pre-proposal stage. Only fully developed proposals are accepted under this program.

Grant Amounts

The maximum grant allowed under the PSTC program is US$ 150,000, and projects are expected to be completed within three years.

The maximum grant allowed under the U.S.-Israel CDR Program is US$ 250,000. Projects may last up to five years.

BOSTID Grants usually have a ceiling of US$ 150,000, but, occasionally, grants may be higher. Projects usually are expected to be completed with three years.

There is no minimum size to grants under any of the programs.

Collaboration

All three programs strongly encourage collaboration in the research work with foreign scientists and institutions where appropriate. In the PSTC and BOSTID programs, collaboration with American scientists is encouraged, but not actually required. In the U.S.-Israel CDR Program, collaboration with Israeli scientists and institutions is required.