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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 2A-15
FINAL

Attachment

PN-ABA-523

2. A-15

FINAL REPORT

Rec'd In SCI: AUG 3 1988

FINAL REPORT

NEW VARIETIES OF RICE FOR SALINE AND ACID SOIL THROUGH TISSUE CULTURE

AID/SCI PROPOSAL 2A-15

GRANT NO. 936-5542-G-SS-3037-00

DECEMBER 31, 1987

U.S. INTERNATIONAL DEVELOPMENT
COOPERATION AGENCY
BANGKOK

DEPARTMENT OF BOTANY
CHULALONGKORN UNIVERSITY
BANGKOK

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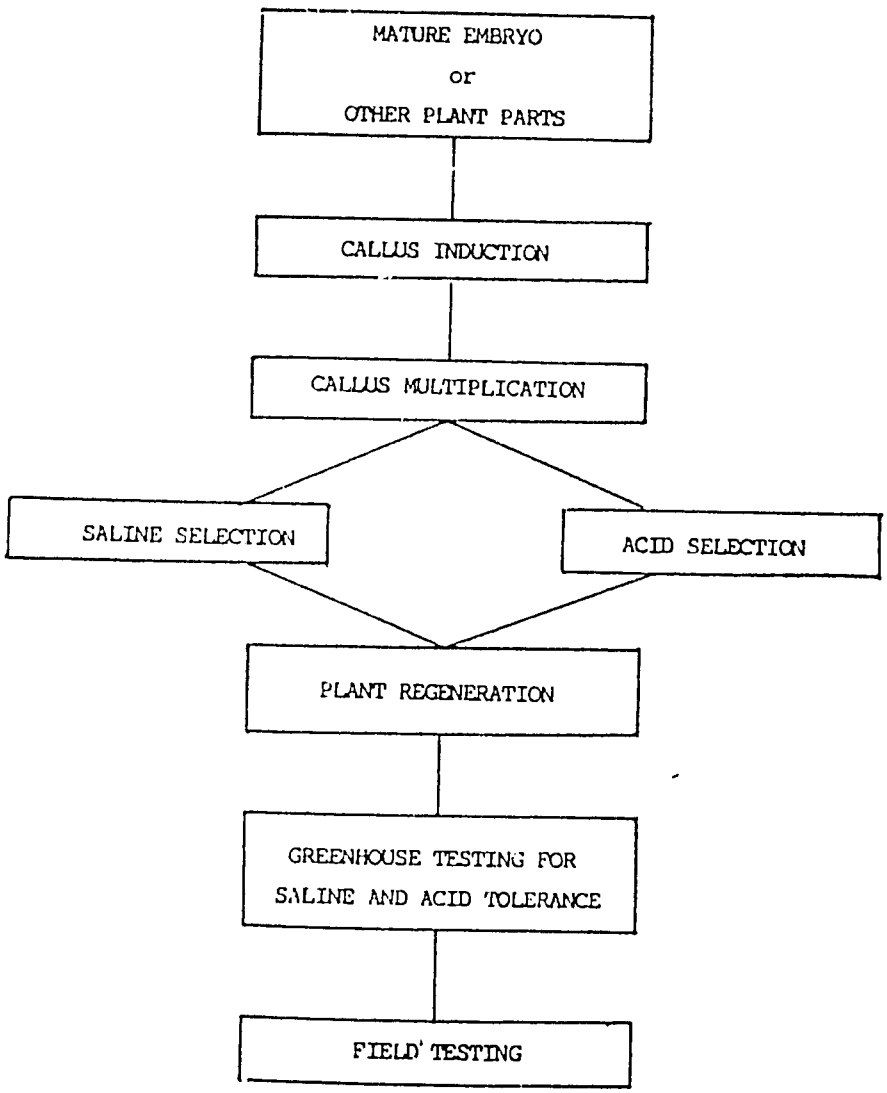
PROPOSED METHODS

FOR

PRODUCTION OF SALINE AND ACID TOLERANT RICE



PREFACE

This research project had been supported by the AID/SCI Grant No. 936-5542-G-SS-3037-00 from June, 1983 to June, 1986. A total of six progress reports have been submitted to U.S. International Development Cooperation Agency in Bangkok at six months interval. The summary of the work, results, problems and recommendation will be found in this final report. It is our intention to present the development of methodology in somatic rice tissue culture in our laboratory and elsewhere for anyone who wants to continue the work along this line. The methodology used in somatic tissue culture for indica rice is still in the infant stage comparing to techniques used on other crops. This still has much room for improvement. Screening of the tolerant individuals regenerated from callus was done under natural conditions in the house with simple methods, however several problems had arisen during the treatment which are worth noted.

Even the grant was terminated in June 1986, the research still continues up to the present time in screening of the tolerant individual. This may take another year or two before handing the selected lines to the Department of Agriculture and the Department of Land Development for field testing. The results obtained in the preliminary works indicates increase in salt tolerance in the descendance of the first and second generations of the treated individuals.

COOPERATION

The cooperation between our unit cell and other unit at other institutions for exchange of material, personel and technical know how or technical assistance is very important in working with rice tissue culture. In the rapid progress in the improvement of plant through tissue culture, one cannot rely solely on gathering information through published journals or short communication, a direct contact must be made to prevent repetition of the work and help each other through transferring of technology in the changing world.

The cooperation between TCCP at Colorado State University has been established since 1983 when this project was first started. Since then the principal investigators of both project have been visiting each other laboratories regularly approximately one a year for discussion on the problems and plan for the future. Besides this, the exchange of personels, materials and numerous correspondence have been made.

Other cooperation is being done with the IRRI in the Philippines, several visits of the personel from both institution have been made. IRRI also agrees to train some of our staff for various aspects of rice technologies.

Cooperation between government bodies specializing in different aspects of rice reseach has also been done. Our materials used in research are obtained from Rice Division, Department of Agriculture. An informal agreement was made with Dr. Somsri Arunin

the leader of the Soil Salinity Research Section at Land Development Department to field test our modified plants in different localities.

It is hoped that all activities that we have been doing would greatly help us to work efficiently in this project and the final outcomes (new varieties of rice and technologies for obtaining them) would be our contribution to the people who need them.

ACKNOWLEDGEMENT

This research project would not have been operated successfully without the help of organizations and people mentioned in the following parts. The university provided research fund for renovation and expansion of the laboratory and gave priority to plant tissue culture by establishing a research unit cell. Thank to Mrs. Sri Oonsiri for her generous gift added for the renovation of the laboratory. We thank the Rice Division of the Agriculture Department for providing us rice seeds for experiments and also their sugestion for the selection of varieties to suit the project.

The availibility of the USAID fund made it possible to increase resources used in the laboratory including a number of scientists and technicians working throughout this period. Special thanks to Mr. John Neave and Mr. Wanchai Jaisin who helped us at the very beginning of the contact with USAID and to Professor Ernest Briskey, Mr. Robert Barnes, Dr. Jaroon Kumnuanta for their interest, their advise and their help in all this period of funding.

Our cooperating investigator, Professor Murray W. Nabors, at the Tissue Culture for Crops Project; Colorado State University has been very helpful in the exchange of ideas in research especially in rice tissue culture. He giving us a wide perspective in dealing with various problems in rice. He initiated an agreement between Colorado State University and Chulalongkorn University in exchange of research student, this made possible for Miss Supaporn Watanaveeradej to spend six month in the TCCP laboratory to verify some of her works there.

As a result of the cooperation, Miss Pueng Petch Poonsupaya our laboratory manager was accepted as a Ph.D. student working under him in TCCP. We are not able to find appropriate written words to describe his special efforts to support this project. The authors sincerely thank him for his efforts for all those years.

Among the visiting scholars, some of them spent their valuable time discussing the specific technical aspects of rice tissue culture with us which lead to the improvement of our results. We like to express our gratitude to Dr. Javier F. Zapata of IRRI, Dr. Kiyoharu Ono of National Institute of Agrobiological Resources, Japan and Dr. Eizo Maeda of Nagoya University, Japan.

There are many more persons, whom their names are not listed here, have helped us through administrative works, acquiring materials or helped us in one way or another. The authors thank all of whom the names and their works are mentioned in all of the reports submitted to the USAID.

INTRODUCTION

The main problems on food production in the Developing countries are affected by unfavorable soil and water conditions. Drought and excess salts affect around 40% and 25% respectively of the world's arable land, in addition soil acidity which increases toxicity of aluminum, manganese and other elements, affects about 25% of the world's arable land.

The modification of environment to increase and sustain yield can be done with limitation and problems related to the modification seems to arise later. Irrigation systems, in many cases, cause salt build-up in such areas. Soil liming to reduce acidity neither practical logistically nor financially possible in many areas. The costs of modern technologies (including mechanized agriculture, pest control etc.) and energy are much too high for the farmers in the developing countries.

Thailand, is no exception, she has problems in low agricultural yield in most crops. For rice production, the average yield is only 707.5 kg. per acre, compared to the yield of 1250 kg. per acre in China, Indonesia and Malaysia, and 2250 kg. per acre in the United States, Japan and Korea. Some of the problems are certainly related to soil and water. It is estimated that there are one million acre of acid (and also phosphorus deficient) soil and 2.8 million acre of saline soil, representing 11% of the total rice-producing land. Irrigation is still not sufficient for agriculture, less than 20% of rice growing lands are under irrigation

the rest must depend on rainfall, which is not controllable and sometimes cause damage to the crops by either flooding the area before harvesting or sometimes a long dry period.

Besides the problems of soil and water other factors determine the low yield and low qualities. These are

1. Seeds: There are a number of high-performance cultivars available, but the seed production is inadequate, which the supply is only 4% of the total seeds used by the farmers. They have to rely on their own mediocre stocks.

2. Investment: Most of the farmers lack financial resources needed to pay for fertilizer, pesticides, soil conditioners, and farm machinery which would increase yield and qualities of the crops.

3. Management practices: A large proportion of the Thai farmers lack knowledge of proper cultivation and also lack of managerial skill to keep down cost of production.

4. Social Aspects: Some of the farming systems such as intercropping, crop rotation or twice yearly rice production are not well accepted even when all conditions are provided.

From the problems listed, it may become apparent that there are several cause of low yields and several plausible solutions. In some of these cases, the solutions must be applied concomitantly since the problems are inter-related. For example, the new high performance cultivars usually demand high fertilizer input and adequate irrigation and are less tolerant to adverse conditions.

This make the use of such cultivars impractical for farmers in poor soil areas, who depend solely on the unpredictable monsoon rains.

The present project intends to deal only with the problems related to salt and acid soils. Obtaining new varieties of rice for such conditions is an alternative approach to correct condition of soil by management which is less expensive.

The method employed in obtaining new varieties in our case is plant tissue culture selection. At present, plant tissue culture plays a very important role in plant breeding in that it enables workers to utilize more variants (resulting from somatic mutation) arising in culture in their breeding programs. Selection for such variants would be effective only when a large population is used due to the low mutation rate, in order of one or two per million cells and much less at the organism level. Using tissue culture techniques, the number of actively dividing cells in only a few small test tubes may exceed the number of dividing cells in the meristem of whole plants grown in one acre. Therefore, the appearance of chance variants resulting from spontaneous somatic mutation or segregation is more likely in tissue cultures than in the field selection. In tissue culture, both selected and non-selected variants are obtained with minimal commitments of space, time and other resources. A selected plant can either become the origin of an improved variaty suited to a specific condition directly or it can be used as a breeding stock for further improvement.

The purpose of this project is to accelerate the development of rice varieties which can be grown under adverse conditions (salt and acid soil) through tissue culture. This will certainly help small farmers in the marginal land to increare yield substantially without increasing cost.

IMPORTANCE OF TISSUE CULTURE IN RICE

Tissue culture of plant began over 50 years ago but its application to agriculture was first realized only during the last two decades. For rice, the history is even shorter due to difficulties in both callus culture and regeneration (Rush et al., 1982). Most advancement in rice tissue culture have been made in Japan, China and India. However, in 1977 IRRI began a comprehensive program for improvement through tissue culture. These include tissue culture of both from anther and from somatic isolates, screening and collection of germplasm amenable to tissue culture, studies on the improvement of rice tissue culture methods, studies of variation occurring during tissue culture.

Many problems restrict their use in rice improvement program. These are the poor production of callus and not sufficient regeneration in many varieties. The more advanced methods such as regeneration of plant from protoplast or transfering of foreign nucleic acid to an established variety have been claimed by some workers but these have yet to be verified.

Monoploid plants have been successfully obtained from either anther culture or isolated microspore culture. These plants may readily become diploid spontaneously or by colchicine treatment. By using this technique, it is possible to produce haploid or homozygous diploid from a single microspore. This reduce time for obtaining pure line to one year instead of five to seven years normally produced by conventional breeding method. When microspores from a hybrid of diverse origin are cultured, homozygosity is obtained in one step with no further segregation. If it is done, large number of homozygous variants could be produced from a single anther. Then, useful plants with varieties of beneficial characters can be selected for further breeding. These plants may posses certain characters such as specific disease resistant which otherwise does not occur during sexual or asexual propagation. Occurrence of two mutations in the same cell is, theoretically, a very rare incidence. Therefore the regenerates are almost identical to the original clones except the characters in question.

Rice protoplast have been obtained and some have developed callus. Unfortunately, regeneration has not been achieved sucessfully upto this moment, it may due to some unknown triggers to recover their totipotency. This technique, if success, may provide an important tool for genetic engineering in the future.

The ultimate goal of rice tissue culture is to produce new varieties which suit a given environment. These may be the resistance to fungal or bacterial diseases or adaptive to physical environments such as excess salts or acids in the soil or resistance

to drought. Therefore rice tissue culture is one of the modern effective methods in obtaining new variants useful for future breeding.

At present, rice tissue culture has come to the stage which one can apply to rice improvement program. Even some problems have been overcome but some major problems still exist. One of this is the lack of information concerning the reaction of most varieties in tissue culture. This causes either poor production of callus or regeneration of plants from callus in some varieties. It needs time and efforts to cope with each variety when the luck is not on your side. Therefore, the large scale culturing is extremely difficult to carry out. It is hope that in the future the techniques of culture will progress to the point that ones can rely on them.

RESEARCH IDEAS

I. The purpose is to determine whether the salt and acid tolerant plants resulting from spontaneous somatic mutation occurred in tissue culture can be selected.

II. The selected variants must be screened for heritable characteristics due to genotypic changes. These will be used in the future breeding programs.

III. It is expected that once the techniques for tissue culture and selection of the indica rice are established, they would be applicable to similar problems occurred elsewhere.

METHODS FOR PRODUCTION OF SALT AND ACID TOLERANT RICE

Three different methods have been employed for selection both in vitro and in natural conditions. Method A starts the treatment in callus stage while the method B begins shortly after shoot differentiation. The plants regenerated from the stressed tissues are grown to maturity and their seedlings are subjected to the treatment in natural conditions. One the contrary, Method for production of salt and acid tolerant rice Method C skips the treatment in vitro entirely, the regeneration takes place without stress and their seedlings are given the treatment in natural conditions for the first time. The question is, which method would have better chance of obtaining the wanted variants. At the first glance, plants regenerated under unfavorable condition seem to be better because the sensitive ones would be killed outright at the beginning leaving the ones that can tolerate such condition for further selections. It seems to have a major change in genetic make up of the survivors, if it is true we will be very lucky, but if it is just a kind of physiological adaptation or epigenetic change the plants will revert back to normal in the next generation. Another look into this problem is to assume that genetic of stress tolerance is rather complex and the chances are very romote to have just a simple gene responsible for a given characteristic. If this is the case, many useful variants occurring during callus growth would be lost through stressing when the surrounding cells are killed. Furthermore, if a gene in question is a recessive one it would not have chance to express itself, or

if an array of genes responsible for a specific character are of multiple type, this would further reduce chances of occuring down close to zero. By allowing regeneration to take its normal course and let the plants grow to maturity, this would facilitate gene recombination through sexual reproduction. Selection of seedlings from regenerated plants would be the combined results of gene mutation during tissue culture and gene recombination which finally express in the organism level, this seems logical and has a direct application. However, subsequent selections are still needed to fix gene combination in case of multiple gene and to select out the undesirable characters arising in the process.

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

THE RICE TISSUE CULTURE LABORATORY

The new tissue culture laboratory is on the second floor of Botany Building. It had toal floor area of 150 square meters (1614 sq. ft.) which half of the area is a preparation room and another half divides into two transfer rooms and two culture (incubation) rooms.

The preparation room is, almost self sufficient, equipped with a number of standard equipment and facilities necessary for plant tissue culture; excepting the autoclaves which are in separate compound. Nine transfer chambers are in a separate air conditioned rooms three of which are of laminar flow type and another six being

simple plexiglass chambers. The culture rooms comprise mainly of ten culture stacks, three cabinets and two shakers. Maximum shelf area is 80 square meters (860 sq. ft.), divided into 60% dark area which can accommodate 18,000 bottles and 40% light area for 12,000 bottles. These areas, have been used from 75% to near its maximum capacity most of the time since Febuary, 1984 which hold approximately 20,000 to 30,000 bottles at any time. Each week, two thousand bottles are replaced by the new culture bottles, the ones that are through with the experiments or are contaminated with microorganisms are washed and reused immediately.

A new section of the laboratory was added in February 1986 which enable to have nine persons working on transfering at the same time this is quite a relieve especially for graduate students who have only limited periods for laboratory work. The extra space also provides more space for culture room both for routine works on callus culture and plant regeneration, and for special problems dealing with nutrition, experiments on altering external environments and on manipulation of cells and tissues. Most of the latter are being worked out by graduate students either for their theses and special problems. All of the work in these areas are very valuable for the improvement of efficiency of regeneration.

The abovementioned operation have been made possible by a number of persons and organizations which we take this opportunity to express our gratitute to the following names. The conversion of the rooms into laboratory was made possible by Phya Anukul Siamkit Upaniksit Siamrath Fund, amount of US $ 13,050.00, donated by

Photo. 1 Preparation Room is, almost self sufficient, equipped with a number of standard equipment and facilities for plant tissue culture.

Photo. 2 Nine transfer chambers are installed in a tranfer room. Three of these are of laminar flow type and six being simple home-made type made of plexiglass.

Photo. 3 Two culture rooms comprise mainly of ten culture stacks, three cabinets and two shakers. Maximum shelf area is 80 square meters divided into 60% dark area which can accommodate 18,000 bottles and 40% light area for 12,000 bottles. These areas have been used between 75% to near its maximum capacity most of the time since 1985. Each week, two thousand bottles are replaced by the new culture bottles.

Mrs. Sri Oonsiri one of his descendants. Many of the equipment and glasswares, amount of US $ 21,740.00, used in this laboratory were donated by Khunying Chern Pisolyabutr. The research grant acquired from the USAID enable us to add more equipment, technical staff, and other expenses to the laboratory which facilitates our work greatly.

Recently, the university has recognized our laboratory as a unit cell for research and human resources development. To this end, the university granted us US $ 10,315 for the development of our laboratory by expansion of working space by 50%. This certainly improve our working condition, which in turn, will increase efficiency of our laboratory in the future. Last but not least, which it is the most important factor of all, is the generosity of the Head, Department of Botany and their staff for not only allocated the area for housing the new laboratory but also gave full support to our project.

METHODOLOGY

Many problems restrict the use of tissue culture in rice improvement program. Scientists working on rice tissue culture now a day are still facing with problems of callus culture and plant regeneration. These are due partly to erratic responses to the treatments among rice varieties or some unsolved biological factors of this particular group of plants.

Regeneration is the most difficult part of rice tissue culture. In screening of mutants with special characteristics a very large number of plants regenerated from callus is needed. This project would not have been materialized without having enough plants regenerated from callus. Even at the present time, regeneration is still fairly difficult, most calli develop to certain stages turn brown and eventually die. Even some problems have been overcome but some major problems still exist. One of these is the lack of information concerning the reaction of tissues in the culture. This causes either poor production of callus or low rate of regeneration of plant from callus in some varieties. The experiments have been designed to cover the problems arising during culture. It is hoped that, in the future, the techniques will progress to the point that ones can rely on them.

This part of report is an effort to provide information on techniques on rice tissue culture based mostly on our own experiments. Some experiments pointed toward some improvement whereas most failed to give any better result. If one wishes to work on rice

somatic tissue culture he should not be wasting time to repeat the fruitless experiments but should explore other possibilities.

I. MATERIALS AND METHODS FOR IN VITRO CULTURE

A. Materials for Callus Production

Eight rice varieties namely, RD23, RD25, KDML, KTH, LPT, NMS, RD8, and NSPT (the last two being glutinous rice) were used in the experiments; these are among the recommended varieties of the Rice Division, Department of Agriculture, and all the seeds were obtained from the Rice Division RD23 and RD25, the recently introduced varieties, are day neutral plants which their seeds can be harvested in only 100 to 130 days after sowings (annonymous, 1983). Both of which can be grown upto three crops a year. This definitely accelerates the work in the laboratory by having both continuous supply of materials and reduced generation time. But unfortunately, the RD23 and RD25 were among the most difficult varieties to handle in vitro. On the contrary, the KDML and LPT varieties, which are the improved strains of old established varieties, respond better in the in vitro method, but their harvest times are only in November and December when the night lengths are sufficiently long. So far, we have not yet found the ideal experimental materials.

Most of the seeds used in the experiments were the mature field-grown rice which carried lots of physical and biological conta inants which affected the callus production. Among these, fungal infection is the main problem. Screening of the infected

seeds were made on certain lots of seeds which proved to be high contamination, but that was not easy to spot the lesser affected ones. Whenever possible, the seeds harvested in the dry season were obtained, these proved to have lower fungal contamination. The seeds were stored in a refrigerator at 3 - 6 ^{o}C until used.

The seeds of later generations from the plants grown in the house were hand picked and air dried for one month before storing in refrigerator to overcome dormancy. It was found that the seeds of RD23 responded well to the following treatments in the raining season. After the seeds were harvested they were put in the heated chamber at 38 - 40 ^{o}C for five days to dry the seeds quickly, then they were put in a paper envelop leaving in the room for two weeks. The seeds germinated quickly after sowing (Progress Report 1 to 6).

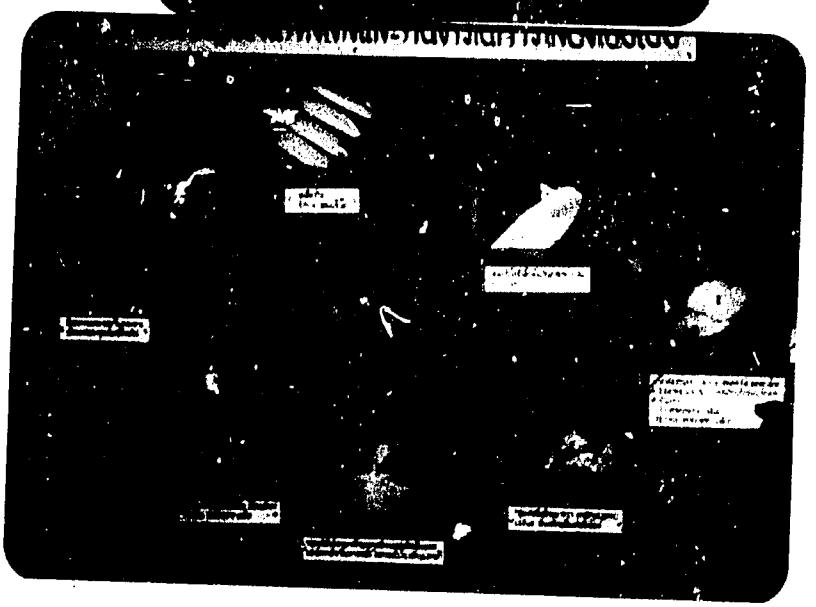
B. Method for callus induction

1. Surface sterilization

In callus culture from mature embryos most of the seeds are havested long time before use. The handling of seeds during harvesting and storage is very important to avoid contamination. Since we are not able to control the method of handling we have no alternative but to find effective way of cleaning the surface of the seeds. Bangkok is situated in the low land tropic the temperature and humidity are high all year round, which these conditions favor the growth of microrganisms. Some microorganisms are seed borne such as Pythium are commonly found but some attach only to the outer surface. The latter can be killed by proper methods of surface sterilization.

Photo. 4 Seeds of eight varieties were used in the experiments, these are among the recommended varieties of the Rice Division, Department of Agriculture. Most of the seeds were the mature seeds obtained directly from the Rice Division, but some were from our own plants collected at milky or soft dough stages.

Photo. 5 Steps of growth and development in laboratory: Starting from dehulled mature seeds, germination of embryo, callus initiation and growth, greenspot (rudimentary shoot apex) formation, shoot growth, and plantlet formation.



The problems occurred at IRRI are quite serious. The workers there treat the dehulled seeds with 1.0% mercuric chloride for 10 - 15 minutes, then they are rinsed with sterilized distilled water three times. This method is said to be very effective for disinfection. However, mercuric chloride is a very toxic substance which can easily damage embryos and it certainly is hazzard to the technicians if not handle properly. Therefore we decided to leave this for the last resource when it is really necessary.

In solving the problem of contamination by surface sterilization, we have tried different treatments during the course of experiment. A modified method used in Professor Nabors' laboratory at the Colorado Stated University was adopted. The modification is made by adding a detergent to water prior to the chemical treatments and each step the treatment is extended. The average contamination, using this method, is 8 to 10% which is considered to be satisfactory. These are the steps of the treatment at room temperature (27 - 30 ^{o}C).

1. Soak about 250 dehulled seeds in 100 ml. tap water with 6 drops of Tween 20. Shake continuously for 3 minutes and then wash in running tap water.

2. Soak the washed seeds in 90% ethanol for 1 minute

3. Transfer the seeds to 20% Clorox solution for 20 minutes

4. Transfer the seeds to 15% Clorox solution for 20 minutes

5. Transfer the seeds to 10% Clorox solution for 20 minutes

6. Wash the seeds with sterilized distilled water 3 times

7. Keep the surface sterilized seeds in sterilized Petri dish until use.

2. Callus induction

Callus induction of rice embryo is simple, the best two media for eight varieties studied have been derived from a series of experiments. The selected media induce the compact callus to form and still promote fairly good growth. Such callus composes mainly of isodiametric cells which is called embryogenic callus, it has high frequency of initiating shoot apex than that of non-embryogenic callus composed of loose tubular cells.

Table 1 Basic components of culture media for callus induction (Marashige and Skoog, 1962)

Constituent	Concentration (mg/l)
A. Macronutrients	
NH_4NO_3	1,650
KNO_3	1,900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
B. Micronutrients	
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Table 1 (cont.)

Constituent	Concentration (mg/l)
C. Vitamins	
thiamine.HCl	0.4
D. Others	
sucrose	30,000
agar	8,000
myo-inositol	100

Table 2 Standardized callus induction media

Constituents		Variety
A. Medium I		
basic components		RD8
from Table 1		RD23
		KDML
2, 4-D	1 mg/l	LPT
		NMS
kinetin	0.3 mg/l	NSPT
B. Medium II		
basic components		
from Table 1		RD8
		RD25
2, 4-D	2 mg/l	KTH
coconut water	100 ml/l	

Variations of the media were tried by adding tryptophan, coconut water at varying concentrations, or increasing the amount of 2, 4-D (Tables 1 to 8, Report 1; Table 1, Report 2). Only the compositions given above produced consistent results.

Four surface sterilized seeds were generally put, in a culture bottle. The culture bottles were kept in the dark for four weeks at 21 to 27 ^{o}C. A callus is visible in three days and it is cut off and transferred to a fresh medium at the age of one week. In most cases, calli at the age of one months were transferred to the regeneration media. Over ninety percent of the embryos produced large and healthy calli at the stage but unfortunately only few of them have been through complete process of regeneration. This has led to planning several experiments on regeneration.

Immature embryos in the late milk to soft dough stages were found to respond to callus induction better than the mature ones (Table 3 and 4, Report 2; Experiment 1, Table 4, Report 1V). Even the immature embryo of some varieties produces larger callus but the practical use of immature embryo for large scale callus production is questionable. First, inconsistency of callus growth has not been studied in detail; second, it involves growing own plants especially the photosensitive varieties that need photoperiodic treatments or collect mature seeds once a year; and third, regeneration of plants from an immature embryo is not better than the one from mature embryo even it is larger and healthier. On the other hand, mature embryos can easily be obtained all the time from the

seeds storing at 3 to 5 ^{o}C in a refrigerator. This saves time and efforts in acquiring materials prior to culture.

C. Method for plant regeneration

Plant regeneration from callus has been a major problem in tissue culure of Indica type rices. As reported earlier in the progress reports 3, 4 and 5 that the successful regeneration depended not only the techniques alone but the interaction between the materials and techniques. The response of Japonica type to the techniques used by many workers in the United States and Japan have not been successfully applied to the Indica type. Due to scarcity of the literatures on regeneration from Indica callus, much of the time has been spent during the pass three years to find basic causes of failure. The result obtained have been encouraging but we feel that there are still much room for improvement.

There are many different media used by tissue culturists around the world at present. Most of the recommendations are from workers that have worked out the methods for a specific group of plants and can be applied to other plants for different purposes (White, 1954; Murashige and Skoog, 1962). Some derived a formula from a series of tests in both monocotyledonous and dicotyledonous tissues (Schenk and Hildebrandt, 1972). Besides the inorganic compounds, the recommended organic compounds and the combination of them are numerous. That renders a systematic testing of the effects of these compound extremely difficullt. Therefore, we have selected only some combinations for experiments on plant regeneration from callus of Indica rices.

Photo. 6 Greenspot formation and shoot differentiation in vitro: Most of the calli developed greenspot in one or two weeks in a regeneration medium but only few of these developed into shoots. Thirty percent of LPT callus produced shoots but only 4.2% was obtained in RD23.

Photo. 7 Very few shoots were able to develop in the selection media containing 0.8, 1 or 2% NaCl. These selected plants were grown to maturity and their seedlings were screened for salt tolerance.

Photo. 8 Plants regenerated from callus at anthesis were grown in sand culture.

200 ml
150
100
NaCl 0.8%
NaCl 1%
200 ml
150
100
50
NaCl 2%

Due to some disagreement among the results reported by several workers and ours, which may be either the variation in responses of rice varieties or physical environment in the culture rooms besides the composition of media. This makes the problem even more complex. This report is base on the results obtained from the experiments which compare very well with the results of other workers and our previous results. After visiting to other laboratories in other cauntries, we have found that the results of regeneration of the indica rices are not any better than ours.

We have spent much affords on the fundamental works on regeneration of plant from somatic callus to establish the method for better regeneration. These works were not only to obtain a working method for our own use but for the benefit of all who wish to work on regeneration of the indica rices. A total of 14 experiments were performed inorder to obtain more plants regenerated from a callus. So far we can induce only five to thirty percent of the total calli to regenerate. There is still rooms for improvement.

The statements above are the critical problems which we are facing during the past period but these are only a fraction of the total works. All accomplishments including a series of preliminary tests cannot be all presented in the written form only positive results or negative results with special value are summarized in these reports.

1. Reference medium for plant regeneration:

All media for regeneration used in this report are based on white's macro-elements with some modifications e.g. the deletion of sodium sulfate or addition of ammonium sulfate. In addition, microelements (including iron) are used according to Murashige and Skoog (1962). Coconut water is the only undefined organic additive that gives consistent result for regeneration in many experiments done previously.

Table 3 Basic components of the culture medium for regeneration

Constituent	Concentration (mg/l)
A. Macronutrients	
$MgSO_4 \cdot 7H_2O$	720
$Ca(NO_3)_2 \cdot 4H_2O$	300
KNO_3	80
KCl	65
$NaH_2PO_4 \cdot H_2O$	16.5
$(NH_4)_2SO_4$	200
B. Micronutrients	
same as Table 1	
C. Vitamins	
Thiamine.HCl	0 or 0.4
D. Others	
IAA	1
kinetin	3
agar	8,000
coconut water	100 ml/l

2. Reference physical conditions.

a. Culture vessel:- Screw-cap bottle of 7 cm. high and 4.2 cm. wide containing 12 - 15 ml. of medium.

b. Light source:- Two Philips fluorescent tubes, TL 40 W/33, 23 - 30 cm. above callus.

c. Light regime:- 16/8 hrs. light/dark.

d. Temperature:- 21 - 27 ^{o}C

3. Formulation of the reference medium.

The above reference medium and physical conditions give consistent results on plant regeneration from callus. Other media may have some advantages in culturing callus of some varieties but they would have to be proven for their consistency. It is interesting to note that some changes in concentration of some components have much effect on regeneration but on the other hand the amount of some other components do not seem to have any apparent effect on regeneration over a wide range.

However, our key to success of the reference medium relies mainly on the supplementing 10% coconut water in the absent of sucrose.

Following are summaries of the results obtained from the experiments leading to the reference medium. It is hoped that these will be useful data for any one who wishes to do further experiment on plant regeneration.

a. Inorganic nutrition

1. Low and high salt formulae:

A modified white's macronutrients have been used as a standard medium for plant regeneration throughout the work.

It gives consistent results and is used as a reference medium in many experiments. Sodium sulfate is replaced by ammonium sulfate in the original formula, this tends to induce more regeneration. Other variations have been tried to obtain more information on inorganic nutrition but none of these produce much better result than our standard medium. The modern high salt formulae, like Murashige and Skoog (1962) and Schenk and Hildebrandt (1972), usually gave poorer results in several experiments (Report II, Exp. 2, Exp. 3; Report III, Exp. 2; Report V, Exp. 4, Exp. 5; Report VI, Table 11, 15).

2. Nitrogen level:

Ammonium nitrogen at 3.2 mM induces more regeneration than other media with lower or higher levels. (Report V, Exp. 5; Report VI, Tables 11, 15, Figs. 6, 7). On the contrary, a wide range of nitrate nitrogen (3.3 to 34.2 mM) gave similar results (Report III, Exp. 4; Report V, Exp. 4, Exp. 5; Report VI, Tables 11, 15).

3. Phosphorus, potassium, calcium and magnesium level:

Other elements, (phosphorus, potassium, calcium, and magnesium) did not give consistent results over a

wide range of concentrations in our experiments. As long as there were sufficient supply of these elements even at a bare minimum level and these elements did not reach toxicity level at highest concentrations tested. Therefore the wide range of these elements in the White's formula in combination of coconut water are sufficient for growth and development of callus, greenspot formation and shoot formation. (Report III, Exp. 3, 4; Report V, Exp. 4; Report V).

4. Microelement level:

Microelements used in the work were based on the formula of Murashige and Skoog (1962). When the amount of iron was reduced to half or increased to two and four times poorer regeneration resulted. Reduction of other microelements in half was also done and the result of regeneration did not change.

b. Growth regulators

A fairly high level of kinetin is used in addition to coconut water in the regeneration medium. In several plant species, only the presence of 10 to 15% autoclaved coconut water in the culture media give sufficient amount of cytokinin for plant regeneration. However, the result [illegible] experiments led us to conclude that addition of 3 ppm. kinet[illegible] 0.1 ppm. BAP gave slightly better regeneration than other concentrations. IAA was selected at 1 ppm. for the reference medium. A lower concentrations of NAA (0.1 ppm.) also gave fairly good results in some experiments (Report II, Exp. 1; Report III, Exp. 1 and 4; Report IV, Exp. 3).

c. Organic additives.

1. Vitamins

Thiamine have been considered to be essential for nearly all plant tissue cultures, whereas nicotinic acid and pyridoxine may stimulate growth (Gamborg et al., 1976). In our experiments only thiamine was effective in increasing regeneration in the presence of coconut water (Report V, Exp. 6, Tables 11, 12).

2. Complex organic supplements.

Only 10% coconut water used as supplement was found to be beneficial for plant regeneration. Coconut water is rich in organic compounds especially cytokinins which plays roles in both stimulation of nuclear division and retarding the breakdown of chlorophylls, nucleic acids and proteins (Salisbury and Ross, 1969). whenever the coconut water was used, other additives like vitamins (except thiamine), ammino acids, sugars were not effective. In addition, a combination of coconut water and sucrose gave worse results than the sucrose alone. This may be due to toxic substances forming during autoclaving at the temperature 120 ^{o}C (Puhan and Martin, 1970). Increasing the amount of coconut water to 20 and 40% to provide more cytokinins was not possible because it induces early death of the callus. (Report II, Exp. 2, Table 6; Report III, Exp. 2, Table 9; Report V, Table 9).

Casein hydrolysate, tryptophan and yeast extract tend to induce deleterious effects when combined with coconut water (Report II, Exp. 1, Table 1 to 8), but tryhtophan

acted like cytokinins in the absent of cocnut water (Report II, Exp. 1, Table 5).

d. Carbohydrates.

All media require the presence of a sugar as a carbon and energy source. Sucrose is usually added in concentrations of 2 to 4.5%. In the experiments on adding coconut water in a medium it was found that coconut water induced regeneration best in the abence of sucrose. This means that supply of different forms of sugars in coconut water is enough for the growth and differentiation in the early stages of differentiation. The reaction of sucrore and coconut water may give rise to toxic substances (as mentioned earlier), it is worth to study the carbohydrate nutrition in detail. This study may shed some light on the differentiation and subsequent development. (Report II, Exp. 2, Table 6; Report III, Exp. 2, Tables 9 and 10, Figs., 1 to 3; Report III, Exp. 2, Table 11 to 17, Figs. 4 and 5).

Adding Myo-inositol at the concentration of 100 to 1,000 mg/l gave slight improvement in regeneration inspite of its high content in coconut water (Report III, Exp. 2, Tables 9 and 10, Figs. 1 to 3; Report V, Exp. 6, Table 11 and 12).

Other forms of sugars were tried at different concentrations in place of sucrose, the results obtained were not consistent. However, a high sucrose medium is required for normal development of shoot and root after elongation of the shoot is observed. At this stage coconut water is left out and only a high salt inorganic formula with 6% sucrose is sufficient for the plantlet.

D. Method for selection of salt or acid tolerant individuals.

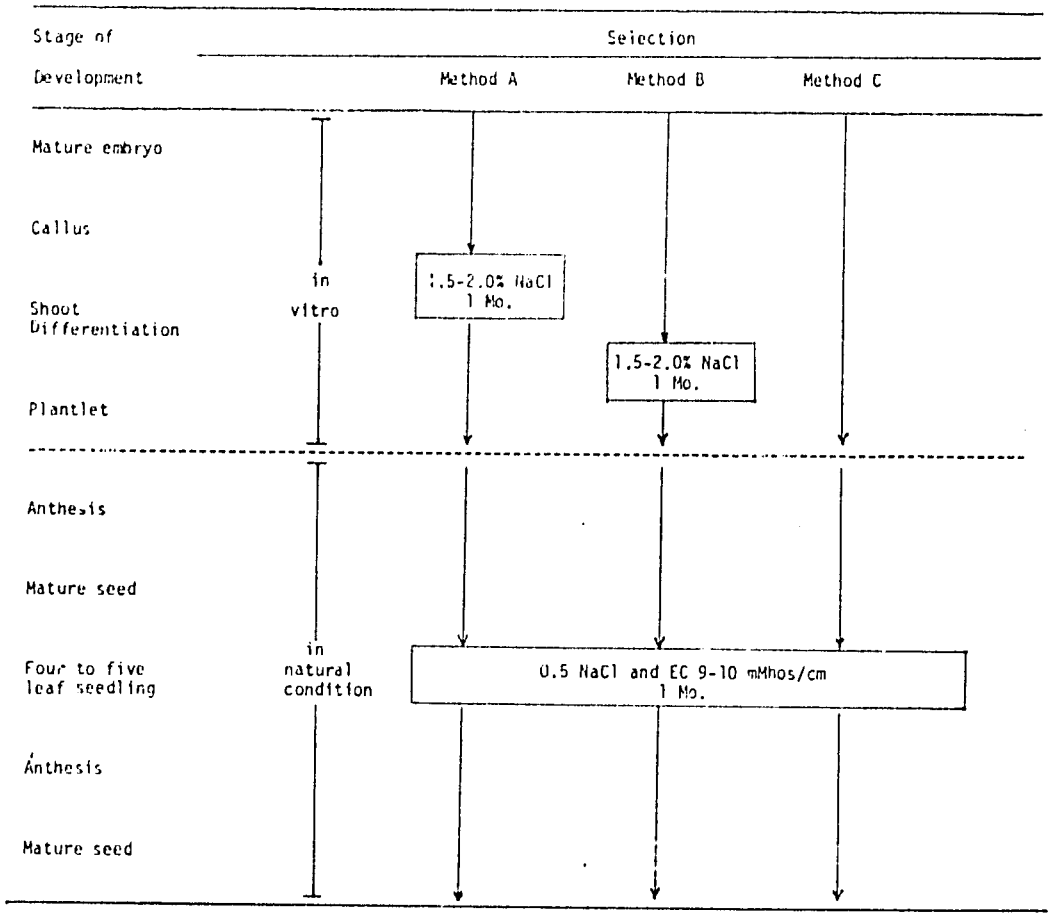
Preliminary studies were divided into two parts, one was made in vitro during callus growth and the other was made in natural condition when the plants produced four to five leaves. Treatment during growth would damage the sensitive cells, only the tolerant ones would continue to grow and differentiate into shoot and finally the whole plants. This method selects cell lines that tolerate high concentration of NaCl but it gives very low regeneration. Although salt tolerant cell lines have been selected in vitro, but it is no guarantee that tolerance selected in culture will be expressed at the whole plant level. The plants obtained by this method must be evaluated further for salt tolerance and genetic stability.

On the other hand, the treatment of plants at four to fiveleaf stages was also done in natural condition. This method had an advantage that the selection is done directly at the whole plant level which in some cases only the change in root functions can limit transportation of salt into inner tissues. In such treatment, if any favorable mutation should occur either in the cell lines or in root function the plant will tolerate high salinity. Such treatment was used in treating seedlings derived from plant regenerated in vitro.

1. Selection of salt tolerant plant.

After two weeks in the callus induction medium (Table 1), only the healthy calli were cut off from other tissue which they were originated from and were transfered onto media

Methods for Production of Salt or Acid Tolerant Lines



similar to the previous one except the presence of 1.5% or 2.0% NaCl in them. These culture vessels were then kept in the light for four weeks.

These treated calli were then transferred to a modified White's medium (Table 3) for regeneration. The culture vessels at this stage were moved to the shelves which the physical conditions were described in conjunction with Table 3). The regenerated plants from such a severe treatment are most likely to come from the tolerant cell lines resulting from somatic mutation occurring during callus growth and differentiation.

2. Selection of acid tolerant plant.

Acid selection is not as simple as one think, acid soils contain several toxic materials such as aluminum and iron compounds and at the same time they contain low level of phosphorus and calcium which both are essential for plant growth. Adding acid to culture medium or nutrient solution does not simulate the actual conditions in nature; putting soil extract in culture media increases the unknown up to a point that we cannot control the experiment. Only stabilizing the pH of culture media without changing the gelling property of agar took us a lot of time and materials for a series of experiments. However, after some experiments we were able to cope with the stabilization of pH in the media and could regenerate some plants from the treated calli.

The preparation of a medium with pH 4.0 is done by making a X2 buffer stock of acetic acid and sodium acetate which would gine 20 mM in the final solution. This stock is autoclaved

separately and then mixed with the X2 sterilized standard culture medium before pouring into sterile bottles. Some culture bottles are randomly chosen for checking the pH before inoculation, two weeks and four weeks after inoculation. Higher levels of acetic acid and sodium acetate (30 and 40 mM) were also tried in some experiments but found to have more deleterious effect.

II. MATERIALS AND METHODS FOR GROWING PLANT IN NATURAL CONDITION.

A. Materials for screening for stress tolerant plants.

Either seeds from regenerated plant or subsequent generations have been used directly for screening or as a stock of the next generation. Plantlets of both selected and unselected were grown for seeds, due to small sample sizes which were not suitable for direct screening.

B. Methods for culture.

1. Seed germination

Rice seeds are easy to germinate, they absorb water readily and begin to germinate almost immediately. The seeds were soaked in a small cup containing water at 50 ^{o}C and were left overnight. Then the seeds were sown on coarse river sand in a round plastic tray of 27 cm in diameter with 5 cm of sand covered with 5 mm of water above the surface. After one week to ten days when the seedlings had three leaves they were then tranferred to water or sand culture.

2. Preparation of the regenerated plantlets for outdoor culture.

Due to the extremely different conditions between in vitro and outdoor which caused many plants to die when they were moved to a new environment outside. It was necessary to harden the plants prior to transplantation to natural conditions by exposing them to more intense light on the laboratory shelf. In addition to this they were transferred to one of the high salt formulae with 6% sucrose for encouraging food storage.

3. Water and sand culture.

Water culture was the main culture used for growing seedlings for screening, however this method was also used for growing plant to maturity. The nutrition of the plant can be regulated easier than when they are growing in the soil. This can be done easily by changing nutrient solution frequently. In the meantime the electrical conductivity is monitored every few days which the adjustment can be made by either adding water or more nutrient to the solution when needed.

In most of the works we were using the following methods for growing rice seedling from two-leaf stage to maturity. They seemed to grow well but did not set seed well if more than two plant were grown in a 3 - 4 liter tray.

Two-leaf-stage Seedlings were planted on 25 holed round styrofoam raft 25 mm in diameters floating in plastic trays containing four liters of nutrient solution. Larger rafts were also used in some experiments. During the growth period, alternate

addition of fresh nutrient and water was regularly done to keep the water level high. Periodic measurement of conductivity was done and the nutrient solution was adjusted to 2.0 to 2.2 mMho/cm at 25 ^{o}C by adding the nutrient or water accordingly.

Some seedlings were planted in sand in deep clay jars filled with washed river sand. Nutrient solution was filled up between two to five centimeters above sand, and conductivity was adjusted as in water culture. This method is good for tall growing varieties which the plants will not topple easily.

The nutrient solution used in all cultures is that of a modified Wagner and Poesch's formula released since 1945 (Bentley, 1959), which all components can be mixed dry as the mixture will not deteriorate and can be stored and used as needed. The micro-elements are added according to Bentley's formula to insure the availability of micro-elements. Supply of iron is made by adding sodium ferric diethylenetriaminepentaacetate giving 5 ppm of available iron in working solution. This solution is quite satisfactory because of its simplicity in both mixing stock in dry form and mixing and storage of working solution. Some precipitation of calcium sulfate and triple superphosphate are quite high but these do not seem to affect the balance of elements in the solution. The plants do not exhibit any severe symptom of deficiency of any element except at the three-leaf stage. This stage often shows chlorosis due to lack of iron during the summer months, this can easily be corected by adding a soluble iron compound.

Photo. 9 A house which the roof was covered with clear plastic sheets to prevent rain was used to grow rice in water or sand culture. The side walls were covered with fish nets to prevent the birds from feeding on seeds.

Photo. 10 A dark frame was used to induce flowering of the short-day plant in summer. Another layer of black cloth was used under the dark blue cloth to insure darkness. The cloths were covered on the frame at 5 pm and taken of at 7 am next morning every day until seeing sign of flowering.

Table 4 Modified WP nutrient solution (Bentley, 1959)

Potassium nitrate	0.58	gram
Ammonium sulfate	0.10	"
Magnesium sulfate	0.45	"
Triple superphosphate	0.25	"
Calcium sulfate	0.50	"
Sodium ferric diethylenetriamine pentaacetate	0.05	"
Manganese sulfate	0.005	"
Boric acid	0.005	"
Zinc sulfate	0.0005	"
Copper sulfate	0.0005	"
Water	1	liter

4. Soil culture

Soil culture was made in a clay pot with the drainage hole sealed or in a plastic bucket. Each of these has capacity of 15 liters. A mixture of loose soil, coase sand, coconut peat at a ratio of 2:1:1 is satisfactory. This method is good for growing large plant to maturity. Generally one plant per pot was used but two to three plants of smaller varieties could be accommodated. Water level was kept at 1 to 5 cm above soil to insure that the plant had sufficient water for their normal growth. A 16-16-16 formula fertilizer was use at a rate of aproximately 5 gm per pot per week, this was also monitored by electrical conductivity measurement. The conductivity of the water above soil

was adjusted to 1 to 2 mMho/cm at 25 ^{o}C, this could alter fertilizing program when it was needed.

The plants grew well and set good seeds. Occasionally, some plant showed chlorosis which might due to diseases in the root system rather than mineral deficiency.

C. Method for salt and acid selection.

The selection of the whole plant in natural condition takes more time and space than the selection in vitro but the selective force is acted on the whole organism like plant growing in nature. Sometimes only a change in root function can limit transportation of salt into inner tissues which make the plant survive in high saline condition without having a change in cell or organs in other parts. Furthermore, any gene mutation or change in the function of some latent genes that arise during tissue culture will have a chance to express after gene recombination in the sexual phase.

1. Selection of salt tolerant individuals.

Seeds collected from plant regenerated from callus without treating with NaCl or seeds from salt selected plant were used in the treatment. Water culture was found to be more convenient to handle and easy to control salinity than the sand culture. Removal of the dead plants can be done without much disturbing others. When the seedlings reached 2-leaf stage they were transplanted to water culture trays with a modified WP nutrient formula (Bentley, 1959). When most of the seedlings

Photo. 11 Five-leaf stage seedlings were used for acid and salt selection in water culture. Twenty five plants were grown on a plastic foam raft floating in a four-liter round tray containing nutrient solution with either acid or salt at specified concentrations for one month.

Photo. 12 All of the plants died in two days when grown in a pH 4.0 buffer solution.

Photo. 13 After three weeks over ninety percent of the plants died leaving some tolerant ones growing until the end of fourth week. The selected ones would be grown to maturity in sand or soil culture for further evaluation.

RD 23 TC 22
CONTROL
RD 23 TC 22
pH 4.0
2 days

attained 5-leaf stage in appoximately 7 to 10 days, nutrient solution was drained off and a new solution with 0.5% NaCl was replaced. Electrical conductivity of the solution at the beginning was around 9 to 10 mMho/cm at 25 ^{o}C and tended to rise when day temperature was high due to evaporation. Whenever the conductivity was 10 mMho/cm or higher the level of nutrient was adjusted by adding tap water and if the conductivity was still lower than 10 mMho/cm, a nutrient solution with 0.5% NaCl was then added. Every week 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 happenned 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 or soil culture for seed production.

Two types of plastic tray were use, one type was a 5-liter rectangular 26 x 41 cm and another was a 3-liter round tray with 27 cm diameter and 10 cm height. A raft was made of styrofoam with 52 holes for a rectangular tray and [illegible] holes for a round one. The size of the raft is determined by the size the container, it should float freely on nutrient solution. Each plant was held in the hole by a piece of synthetic sponge wrapping around the stem. The 25-plant raft was selected for most of the salt selection because of its smaller size which could be handled more easily. Sand culture gave inconsistent electrical conductivity values.

Selection of acid tolerant individuals was done very much the same way as did the salt tolerant plant. Acid selection is complicated. A very low pH (4.0 or lower) soil will directly cause damage to root cells and other cells near vascular bundles which results in slowing down water uptake. Moreover, at a pH below normal range soils often release excess soluble toxic substances such as aluminum, iron, and copper compounds. At the same time, these soils are often found to contain a very low level of phosphorus and calcium both of which are essential for normal growth. Adding acid alone to an inert culture medium or nutrient solution may not be sufficient to upset physiological processes stated above because the avaiability of those elements mentioned above is limited. On the contrary, using extract of acid soil or using soil culture with acid added for testing may produce similar effect to acid soil in nature but the factors offecting the growth of the plant well not be discovered immediately unless soil analyses are readily available. In the experiments here the treatments of acid condition were limited to lowering the pH in the defined nutrient solution.

D. Environment of plant in natural condition.

Since Bangkok is in the humid tropic, the atmospheric controlled greenhouse can not be done easily. This due to excessive heat and humidity which make the adjustment costly.

In order to cope with the problem, the plastic protection was installed only on the wire roof only to prevent rain but not birds or insects. The side walls were covered with coarse

wire mesh to prevent birds but not insects. Sometimes a fish net could be used in place of wire mesh. Insecticides and fungicides were used only when necesscery. Light intensity was cut down as much as half by the plastic sheet when it was old. Such installments described above were satisfactory for our purpose.

RESULTS AND DISCUSSION

In this part only the results of salt and acid selection are reported, however the results of several experiments leading to the improvement of plant regeneration are summarized under the Materials and Methods heading. Even the results of salt and acid selection shown here are not the final and complete ones, the resultant materials indicate much variations occurred in the screening. Further screening of their offsprings in the subsequent generations are being continued both in pots and in field to confirm the results of the previous generations.

I. SALT SELECTION IN VITRO

Between August 1984 to October 1985 (15 months) 135,132 embryos from seven variaties were cultured in the standardized callus induction media for two weeks in the dark. At this stage, only the healthy calli were cut off from other tissues and were transferred onto the media similar to the previous ones except the presence of 1.5 or 2.0% NaCl in them. Calli used in different treatments were selected from only 30.9% of the embryos cultured, some embryos were inviable some were contaminated with microorganisms and some were too small or having undesirable characters. Therefore 41,821 selected calli were cultured in the NaCl added media in the light for four weeks. At the end of the treatment 38% of the treated calli survived. These calli were then transferred onto a regeneration medium, in this period almost all of the treated calli died quickly during the first two weeks and the rest continued to grow and produced greenspots and

some of them grew further into shoots. A total of 32 calli produced shoots which most of these produced roots and could be grown to maturity in natural condition by sand culture method.

Table 5 Tolerant plants regenerated under salt stress

Variety	No. of plantlets arising in vitro	No. of mature plants
KDML	2	1
KTH	0	0
LPT	19	16
NMS-4	0	0
NSPT	Not treated	-
RD8	2	1
RD23	16	12
RD25	1	1
Total	40	31

It is worth to note that once a plantlet arises in vitro it keeps on growing and responds well to the water culture method. Most of the plants (77.5%) reach maturity like the ones regenerated without high sodium chloride in the medium. It was a rare event that more than one shoot was formed on one callus, however the LPT and RD23 produced up to four shoots per one callus. Regeneration of the treated calli was 0.076% while the untreated calli of KDML normally give 8.3% regeneration, RD23 10%, and LPT ranging between 15 to 30% in the same regeneration medium.

The following Table covers only the work during April 1985 to October 1985 of salt selection in vitro just to demonstrate the results of treatment on seven varieties.

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 th. .atment. Two-week old calli were cultured in the regeneration medium with 1.5% NaCl for four weeks. The results are shown in Table 6

Table 6 Shoot formation from calli of seven rice varieties ofter 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
RD8	2050	1022	49.58	1	0.05	2	0.10
RD23	2138	951	44.48	1	0.05	4	0.19
RD25	2124	994	46.80	1	0.05	1	0.05
Total	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 greenspots but very few produced shoots.

Seven varieties, namely, KDML, KTH, LPT, NMS, RD8, RD23 and RD25 were used in the treatment. A total of 14,133 calli were selected for the treatment, fifty percent of these survived at the end of four week of 1.5% NaCl treatment. Then the living calli were transferred to a regeneration medium many of them stopped growing and died as a result of treatment. Some continued to grow and form greenspots but very few produced shoots. The LPT is still 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 treated with salt only 4 out of 3352 calli had shoots which is 150 to 300 times differences. 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 shows variability in calli in salt tolerance in the cell level, which only tolerant cells were able to survive and grow and later differentiated into shoots at later stages. It is interesting to note that the whole process of regeneration occurred after treatment, which presents us with a problem whether the whole plant obtained by this method would still be tolerant or not. This can be tested in the following generations.

II. ACID SELECTION IN VITRO

Preparation of media for acid selection are rather complex because of the interaction between acid and other components, especially the agar, in the medium. The differential absorbtion of ions by callus during the growth may change the balance of the ions in the medium. However, some successful results have been obtained after a series of experiments attempting to stabilize the pH during culture. This caused a delay in the selection for acid tolerant individual *in vitro*.

A total of 7880 calli of eight 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 ± 0.2 by buffer acetate for four weeks. Among these, 29 calli (0.37%) produced plantlets but only 7 plants (24.14%) reached maturity. This figure is only one third of the ones regenerated under salt stress.

Table 7 Tolerant plants regenerated under acid strees (pH 4.0 ± 0.2)

Variety	No. of plantlets arising *in vitro*	No. of mature plants
KDML	0	0
KTH	1	0
LPT	14	4
NMS-4	4	1
NSPT	1	0
RD8	2	1
RD23	7	1
RD25	0	0
Total	29	7

Table 8 Shoot formation on calli 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	1	0.35	1	0.35
LPT	1840	205	11.14	14	0.76	14	0.76
NMS-4	948	49	5.17	4	0.42	4	0.42
NSPT	765	126	16.47	1	0.13	1	0.13
RD8	1151	152	13.21	2	0.17	2	0.17
RD23	584	26	4.45	7	0.20	7	0.20
RD25	1232	117	9.50	0	0	0	0
Total	7880	865	10.98	29	0.37	29	0.37

Only ten percent of calli survived at the end of the fourth week which was five times lower than those in salt trealment. During treatment most calli turned white or very pale yellow instead of light yellow in the normal ones in the first two weeks. After transfering 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 RDML 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 KMS surprisingly produced two shoots in this acid treatment but never in salt treatment. This report will not be completed without mentioning about the steady RD23, the regeneration rate is around 8 - 10% with the method available and it tolerate not only salt and acid in these experiments, it also keep on regenerating in many experiments done previously.

The acid selection reported here is a priliminary work following the established method for stabilizing the pH. One should be aware of acid soils in the nature which are of several types, acid is the only a common factor among them other components of the soil vary greatly 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 be corrected by adding phosphate fertilizer, but toxicity due to aluminum, iron or other ions at low pH are probably the most difficult problems to deal with. However, acid selection method should be the first step to establish before tackle on the more complex treatments.

III. SALT SELECTION IN NATURAL CONDITION

Selection in natural condition has been done by using four to five-leaf stage seedlings of the regenerated plants or the seedlings obtaining from subsequent generations. These seedlings were grown in water culture containing a modified WP nutrient solution with 0.5% NaCl added. The electrical conductivity of the solution was read between 9 - 10 mMhos/cm. at 25 ^{o}C. Each of the treatment was kept for one month with frequent adjustment of the electrical conductivity. The dead plants were removed every week and the healthy plants were selected for growing to maturity at the end of the treatment. If the number of suruivors was more than two percent, only the best ones were chosen and discarded the badly affected plants. This number was further reduced by the loss of some plants which were never recover after transplanting into an ordinary water culture method. It is hoped that some of the tolerant plants are the result of real sometic mutation which would express themselves better after recombination in the next generation.

The salt selection began in January 1985 and still continues upto the time of writing but this report covers only the work upto December 1986. At this period, 24,567 seedlings of four varieties were treated with 0.5% NaCl in water culture. Among these, 11,666 were RD23, 7040 LPT, 5743 PDML and 176 RD15. Most of them were from the mother plants regenerated in normal regeneration medium, some of them were from the plants regenerated under sodium chloride stress, and some were from second generation of the selected individuals.

A. Seedlings of plant regenerated without stress

Following are some studies on the survival of seedlings of the KDML and RD23 during a one-month salt selection.

Table 9 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

Table 9 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 sommon 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 that the leafblades and leaf sheath have lost 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.

During the third and fourth weeks the weak plants with severe symptom died leaving the ones with little symptom growing. At the end of the fourth week 130 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 plant treated.

KDML is a selected variety for salt tolerance which is recommended by the Department of Agriculture to be grown in several locations in the country where soil salinity is high for 28 years. 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 a few may be a remote chance arising from somatic variations. Even 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.

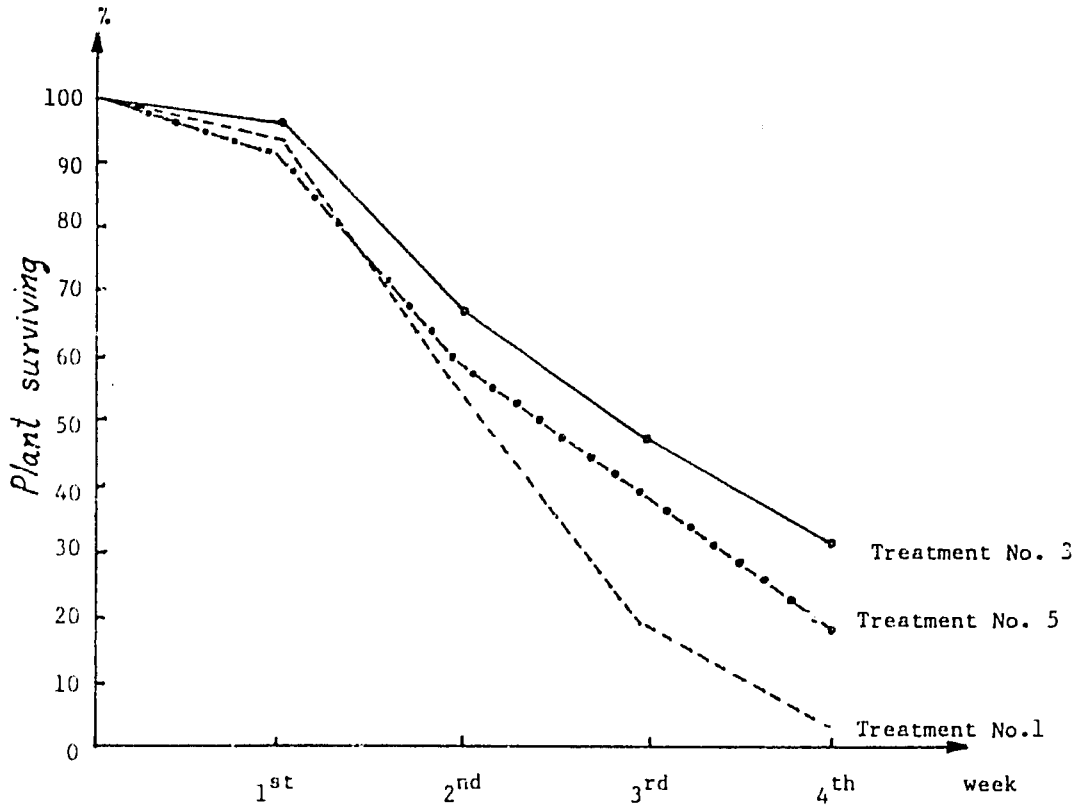


Figure 1 Responses of seedlings (generation R 1) of a selected regenerated plants (RO - TC 1), representing sensitive, intermediate and tolerant groups.

Re : Table 9 Electrical conductivity : 9-10 mMhos/cm

Variety : KDML Stage of seedlings at the beginning of treatment : Five-leaf stage

Table 10 Survival of four-leaf stage seedlings of 21 regenerated plants treated with 0.5% NaCl in nutrient solution for four weeks.

Variety : RD23 Electrical Conductivity : 9-10 mMhos/cm

Treatment		No of	Plant Surviving							
			1st wk.		2nd wk.		3rd wk.		4th wk.	
No.	Stock No.	Plant	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	56	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_1-10	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	00	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	42[illegible]	89.6	2220	46.7	809	17.0	202	4.2

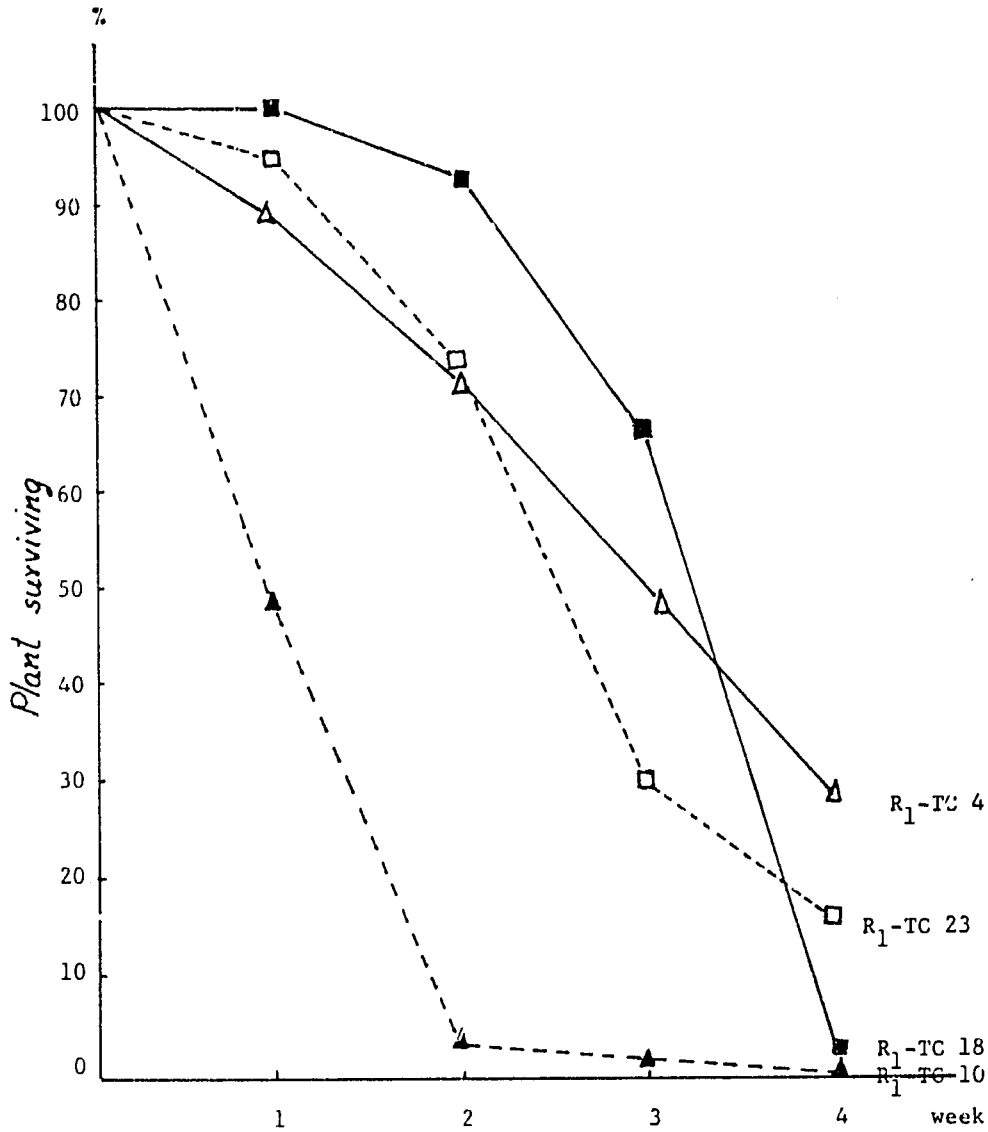


Figure 2 Responses of seedlings (generation R1) of four selected regenerated plant (RO-TC4, RO-TC10, RO-TCL8 and RO-TC23), representing sentitive, intermediate and tolerant groups

Re : Table 10 Electrical conductivity : 9-10 mMhos/cm.

Variety : RD 23 Stage of seedlings at the beginning of treatment : Five-leaf stage

A total of 4758 RD23 seedlings of 21 regenerates plants under in vitro condition were screened. The results were interestings 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 which the plant surviving rates drop sharply during the fourth week giving only 2.7 and 1.3% respectively.

RD23 is a new variety which has just been released by the Department of Agriculture in 1981. This is a very interesting variety for this experiment because it is a day neutral and short life cycle variety which can be grown up to three times a year. It is a short variety which can be grown all year round and it also has virus resistant character. If the salt tolerant character can be added to this variety it will be a very useful short-live variety for the rain fed localities.

B. Seedlings of plant regenerated under salt stress in vitro

Seedlings of the plants regenerated under salt stress in vitro are of interest. Generally, RD23 callus gives regeneration rates ranging from 8 to 10% but under 1 to 2% NaCl stress the regeneration rate is readily reduced to 0.14%. Furthermore, most of the plants regenerated under such method produce no viable seeds.

Only three mother plants produced viable seeds which could be germinated and used for screening.

Table 11 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 RD23 Electrical Conductivity : 9-10 mMhos/cm

*Na 1 = 1% NaCl in culture medium *Na 2 = 2% NaCl in culture medium

Treatment		No. of	Plant Surviving							
			1st wk.		2nd wk.		3rd wk.		4th wk.	
No.	Stock No.	Plant	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

The result of the screening is quite satisfactory (Table 11) the plant surviving rates are 14.4, 29.3 and 33.2 respectively. The average surviving rate of the seedlings from other mother plants is only 4.2% which is only $\frac{1}{6}$ of the figure obtained from the above population (Table 10).

It is interesting to note that survival 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 become malfunction and die which affect the tolerant cells. If the population

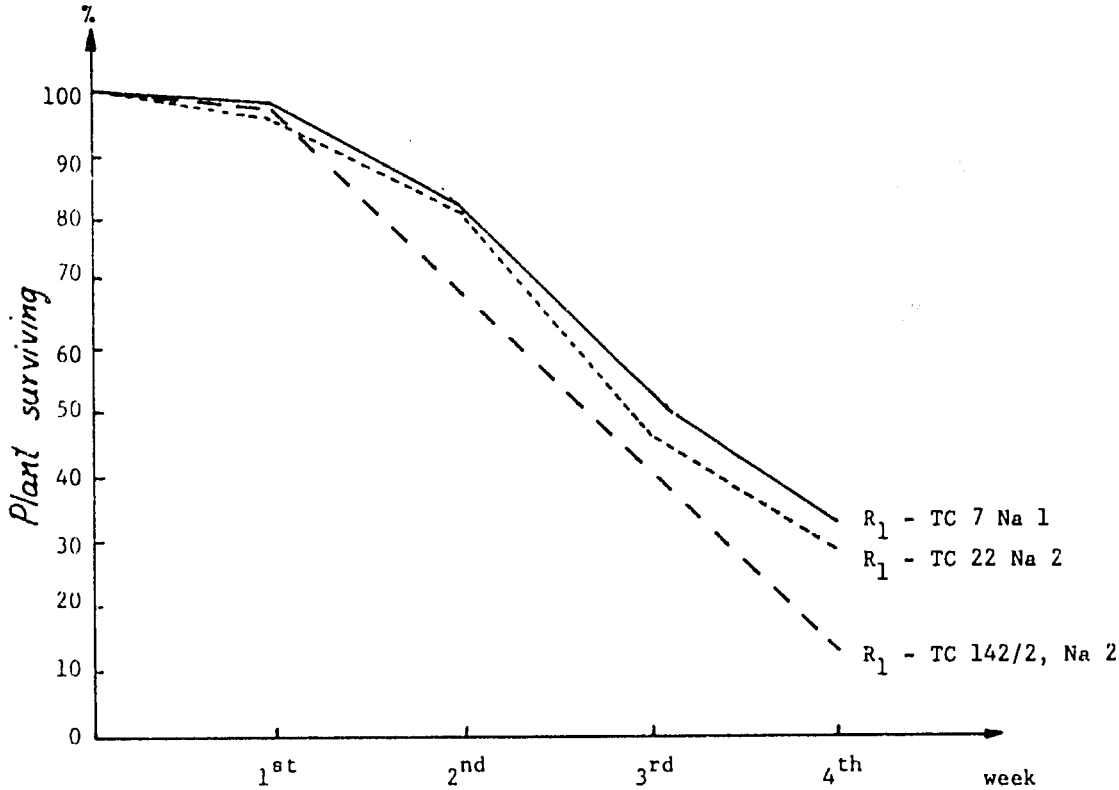


Figure 3 Responses of seedling of plants regenerated under salt stress in vitro to the treatment of 0.5 % NaCl in nutrient solution.

Re : Table 11 Electrical conductivity : 9-10 mMhos/cm

Variety : RD 23 Stage of seedlings at the beginning of treatment :Five-leaf stage

of tolerant cell cover large area they are likely to form shoot apex and finally become shoot. Therefore any shoot arising from such callus under stress should be tolerant.

The method of salt stress in vitro seems to be promising in 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.

C. Second generation seedlings of the individuals selected under natural condition

Table 12 Survival of four-leaf stage second generation of regenerated plants treated with 0.5% NaCl in nutrient solution for four weeks.

Variety : RD23 Electrical Conductivity : 9-10 mMhos/cm

Treatment		No. of	Plant Surviving							
			1st wk.		2nd wk.		3rd wk.		4th wk.	
No.	Stock No.	Plant	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	1100	877	79.7	532	48.4	249	22.6	116	10.5
3	R_2-Tc2	775	662	85.4	178	23.0	19	2.4	2	0.2
4	R_2-Tc3	500	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

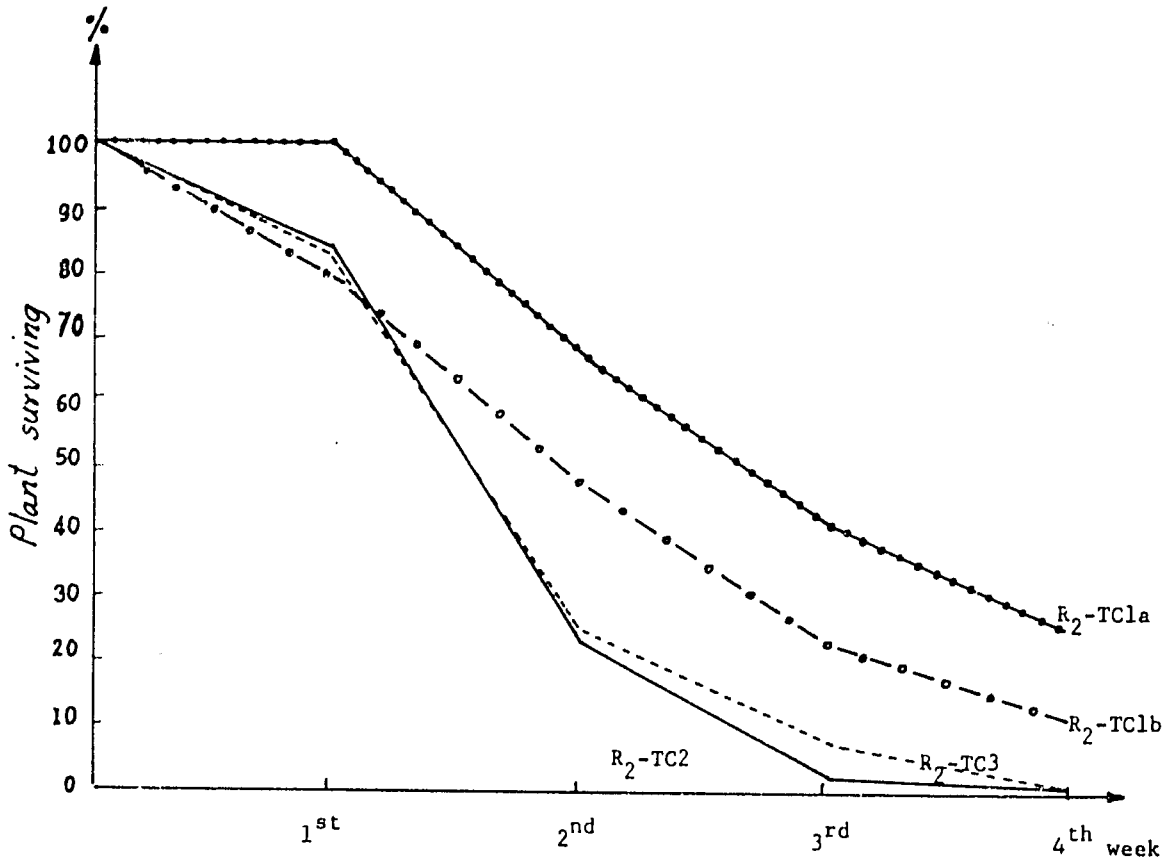


Figure 4 Responses of seedlings of the second generation (R2) of regenerated plants to the treatment of 0.5% NaCl in nutrient solution.

Re : Table 12 Electrical conductivity : 9-10 mMhos/cm

Variety : RD23 Stage of seedlings at the beginning of treatment : Four-leaf stage

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- Dr. HEBERD

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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 R1. However the percentage of the survivors were not better than the best of the R1 population (Table 13).

Table 13 Survival of four-leaf stage second generation of regenerated plants treated with 0.5% NaCl in nutrient solution for four weeks

Variety : RD25 Electrical Conductivity : 9-10 mMhos/cm

Treatment		No. of	Plant Surviving			
			1st wk.		4th wk.	
No.	Stock No.	Plant	No.	%	No.	%
1	R_2-Tc1	60	60	100.0	0	0
2	R_2-Tc2	16	13	81.2	3	18.80
3	R_2-Tc3	75	75	100.0	0	0
4	R_2-Tc4	25	20	80.0	5	20.00
Total		176	168	95.45	[illegible]	4.55

RD25 is another new hybrid released by the Department of Agriculture five years ago. It is a day neutral, short stature and short life cycle. This variety resists several pathogens and insects and can be grown three crops a year.

Unfortunately, this variety is a very difficult to handle both in the laboratory and in the field. Callus intiation seems to be normal but regeneration is extremely difficult. A callus with greenspots normally stops growing and dies. Alteration of culture media have been tried but the improvement of regeneration is still not satisfactory. However, some regenerates were obtained and their seedlings were treated with 0.5% NaCl during the R1 and R2.

The results of the treatment of the R2 were quite satisfactory upto the end of the fourth week. Two populations gave survivors upto 18.8 and 20.0 at the end of the experiment, but none of them survived when the plants were transferred to the standard nutrient solution. This variety has to be investigated in its physiology further in order to cope with the regeneration and methods of treatment.

D. Stages of selection in natural condition.

Two varieties namely LPT and RD23, are amiable to the culture in vitro, this results in high number of the seed stocks and advanced selection stages. Selected seed stocks have been used for further selection. Some stocks that responded well in the salt selection and some produced too few seeds in the early generations, these were continued for confirmation of the previous results. Such screening is being done at present and still continued until the uniform results are obtained before testing in the field.

Table 14 Seeds collected from tolerant plants selected by the treatment of 0.5% NaCl in nutrient solution in natural condition

Variety	No. of selected plant			
	R_0	R_1	R_2	R_3
KDML	-	8	2	-
KTH	-	1	-	-
LPT	-	76	6	-
NMS-4	-	2	-	-
NSPT	-	8	-	-
RD8	-	1	-	-
RD23	-	125	13	3
RD25	-	4	1	-
Total	-	225	22	3

R_0 = Seeds of the regenerated plant *in vitro* with or with out salt stressing. These are the beginning of second generation.

R_1 = Seeds of the first generation following the regenerated plants.

R_2 = Seeds of the R_1 plants.

R_3 = Seeds of the R_2 plants.

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 chances are remote to have just a simple gene responsible for a given characteristics. 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 chance to express it self, or if an array of genes responsible for a specific character are of multiple type, this would further reduce chance of occurring down 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, this would facilitate gene recombination through sexual reproduction. Selection of seedlings from regenerated plants would be the combined result of gene mutation during tissue culture and gene recombination which finally express in the organism level, this seems logical and has a direct application. However, subsequent select ons are still needed to fix gene combination in case of multiple gene.

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

IV. ACID SELECTION IN NATURAL CONDITION

Unlike the method of acid selection in vitro which the acetate buffer at low concentration is sufficient to keep the pH down around 4.0, but this buffer, if not used under sterile condition, it attracts microorganism which change not only pH but also other compositions of the medium. In the experiments on the buffer it was found that organic compounds are not suitable because they were easily affected by microorganism and all of the plants died quieckly in two days. The strong inorganic acids like hydrochloric acid or sulphuric acid were not stable enough in the uncontrolled environment.

Therefore the selected plants from acid treatments were not yet found.

SUMMARY

This final report is a condensed results of the works between June 1983 to December 1986 which most of the details appeared in any of the six progress report. It is particularly stressed on the recommended methods resulting from our experiments and the results obtained from salt and acid selections which some parts were not reported earlier.

1. Grand No. : 936-5542-G-SS-3037-00 amount of US $ 126,600.

2. U.S. Cooperating Investigator : Professor Murray W. Nabors, Colorado State University.

3. A method of callus induction was successfully established. Callus formation was found to be an average of 93.3 percent.

4. Many attempts were made on the improvement of plant regeneration, the results were not completely satisfactory. The percentage of callus that regenerate ranged from less than one to thirty percent depending on varieties. However this work has been carried out by using a fairly large number of embryos.

Over one million embryos were used for experiments on regeneration, for salt and acid selections and for straight regeneration without stress.

5. Methods of salt and acid selections <u>in vitro</u> were established and these were used throughout.

6. Methods of salt selection in natural conditions were worked out and finally decided on using water culture with 0.5% NaCl, added, this was monitored by checking electrical conductivity. The range of conductivity used was adjusted to 9 - 10 mMhos/cm at 25 ^{o}C.

7. Thirty one salt tolerant mature plants were obtained from the in vitro selection from 135,132 embryos (0.02%).

8. Seven acid toterant mature plants were obtained from the in vitro selection from a total of 25,502 embryos (0.03%).

9. A total of 249 seed stocks were obtained from salt selection in natural condition. Of these, 85 were from the mother plants regenerated in vitro, 139 from first generation, 22 from second generation and 3 from third generation.

10. None of the plants survived acid selection in natural conditon. This probably was caused by unsuitable buffer used in the treatment.

VISITORS

Since this grant has been awarded in June 1983 several important guests from governmental orgar.zations and scientists from oversea visited our laboratory. These include Deputy Prime Minister, Common House Speaker, Education Committee of the Senate, Education Committee of the House of Representative, Science and Technology Committee of the House of Representative and Minister of Science, Technology and Energy.

Our regular visitors during 1983 to 1987 were Professor E.J. Briskey, Director of the Office of Science and Technology US AID, Thailand and Professor M.W. Nabors, principal investigator of Tissue Culture for Crop Project and also our collaborating investigator. Professor Nabors had visited our laboratory approximately six days each year to discuss on the technical problems relating to tissue culture, including exchange of materials, personel development and organizing conferences.

Below is a list of scientists from different countries who visited our laboratory during 1983 to 1986.

LIST OF VISITORS DURING 1983 - 1987

AUSTRALIA : - Dr. Jen Mc COMB

Murdoch University

- Dr. Elizabeth S. WILLIAMS

Melbourne University

- Dr. Peter DART

Australian National University

BANGLADESH : - Prof. A.S. ISLAM

Dhaka University

CANADA : - Dr. J.P. KUTNEY

University of British Columbia

COSTA RICA : - Dr. MULLER

- Dr. Pierre DUBOIS

San Jose

FRANCE : - Dr. Coltte NITSCH
CNRS
Gif-sue-yuette

- Dr. Rachel RUDSTROM

UNESCO - Sc./SER.

- Dr. Phillippe SOUCAILLE

Laboratore de Genicbiochimgve

- Dr. Paul RITZENTHALER

Universite Paul Sabatier

GUATEMALA : - Dr. Carlos Rolz

Central American Research Institute

INDIA : - Prof. H.Y. MOHAN RAM

Delhi University

- Prof. G.M. REDDY

Osmania University

- Dr. S.R. SREE RANGASAMY

Tamil Nadu Agricultural University

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JAPAN : - Associate Prof. Michihiro HARA

Iwate University

- Prof. Gotaro TOMONABA

Tokai University

- Dr. Toyoki KOZAI

Chiba University

- Associate Prof. Nobumasa NITO

Saga University

- Dr. Kyoharu OONO

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Kyoto University

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- Prof. Hiroshi HARADA
  University of Tsukuba
- Dr. T. IRABU
  Okinawa Agriculture Experiment Station
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  Chief Advisor of R.F.D.
- Prof. Y. YAMADA
  Kyoto University

KOREA : - Im Ho LEE
- Hyung Soo NA
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- Hyung Kee LEE
  Korean Broadcasting System
- Changbok KIM

MALAYSIA : - Dr. S. KADZIMIN
  University of Agriculture
- Dr. David JONES
  University of Malaya

NETHERLAND : - Prof. H.J.J. NIJKAMP
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PHILIPPINES : - Dr. Aurora G. del ROSARIO

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IRRI

- Dr. N. TUYEN

Visayas State College of Agriculture

- Dr. S. YOSHIDA

IRRI

- Dr. F.J. ZAPATA

IRRI

POLAND : - Dr. B.A. MOLSKI

Director, Botanical Garden.

SINGAPORE : - Prof. A.N. RAO

National University of Singapore

- Dr. C.S. HEW

National University of Singapore

UNITED KINGDOM : - Dr. T. FLOWERS

University of Sussex

- Dr. M. TRIBE

University of Sussex

- Dr. J.M. WILSON

University of North Wales

- Dr. Sanga SUP-SRI

  Permanent Secretary,

  Ministry of Science, Technology and Energy
- Students from Faculty of Engineering
- Students from Faculty of Economics
- Students from King Mongkut Institute of Technology
- Students from Faculty of Agriculture
- Education Committee of the Senate
- Education Committee of the House of Representative
- Science and Technology Committee of the House of Representative

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