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# INCREASING BIOLOGICAL NITROGEN FIXATION OF PEANUTS IN DEVELOPING COUNTRIES

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SUMMARY: One of the objectives of this research was to assess the number and the effectiveness of peanut rhizobia native to tropical soils of four major peanut-growing regions in Thailand: the Central Plain, the East, the Northeast and the North. Three cropping areas: 1) continuously cropped with peanuts, 2) continuously cropped with non-legumes, and 3) non-cultivated fields were chosen in each region. Peanut rhizobia were found in the soil at 38 of 55 sites sampled. Only one among 15 peanut fields tested did not contain rhizobia specific to peanuts. In contrast no peanut rhizobia were found in approximately 50% of fields continuously cropped with nonlegumes. Cultivated fields with a peanut cultivation history contained (as estimated by MPN) an average of $1.6 \times 10^3$ cells $g^{-1}$ of soil. The number of peanut rhizobia in most of the fallow fields and some of the non-cultivated shrub or forest locations were much the same as at the sites where Arachis hypogaea was cultivated. In contrast there were no or few (28 - 46 cells $g^{-1}$ of soil) of peanut rhizobia in the majority of fields which were continuously cultivated with sugar cane, cassava, corn and pineapple. It appears that in these areas the indigenous peanut rhizobial populations are not adequate in number for a maximal nodulation of peanuts.

A total of 343 Bradyrhizobium isolates were tested for effectiveness and found to vary widely in their ability to fix $N_2$. In some areas the majority of rhizobia were quite effective while in others they were less effective than the inoculum strain THA 205 recommended in Thailand.

In order to determine the diversity of natural peanut rhizobia population they were characterized by serological properties, electrophoretic protein profiles and response to selected antibiotics. Rhizobial strains are characterized by their antigenic heterogeneity. Thus it is common to use serological tests for the identification of these bacteria. ELISA reactions and antibody adsorption tests were applied to determine the minimal somatic antigen constitution of 243 strains of Bradyrhizobium sp. (Arachis) using 12 antisera. The 243 indigenous bradyrhizobial isolates were from 15 sites in four regions of Thailand.

A total of 29 serogroups were identified. 80% of the isolates tested had at least one heat-stable antigen in common with strain

280A, forming a so-called 280A serocluster. At 11 of 15 sites tested, 53 to 100% of the isolates fell into one or two predominant serogroups that individually or jointly were superior at one or several sites. The serological properties of the indigenous bradyrhizobia were not related to the cropping history of the cultivated fields from which they were isolated.

Many of the isolates within individual serogroups had identical or similar SDS-gel protein profiles whereas others were distinct and hence could be distinguished from one another by this method. The electrophoretic patterns of all strains tested were found to be reproducible.

To differentiate the isolates on the basis of antibiotic response they were typed according to their intrinsic resistance to chloramphenicol (97.2%) and neomycin (97.2%). The percentage of resistant isolates was lower on media containing penicillin, kanamycin, spectinomycin, and rifampicin: 64.9, 61.0, 51.0, and 38.6 respectively. Eighty five percent of the isolates were sensitive to streptomycin.

The symbiotic performance of 12 peanut Bradyrhizobium strains and their interaction with 6 peanut cultivars were examined in two nethouse experiments under controlled conditions. A considerable variability in symbiotic performance among peanut cultivars was observed. There was a significant effect of plant cultivar and Bradyrhizobium strain on dry matter and the amount of total nitrogen accumulated in the tops of peanuts. Cultivars Tainan 9 and KK 60-3 were the worst regarding the top dry weight and accumulation of total nitrogen.

Three field experiments were carried out in the Eastern and Northeastern parts of Thailand to determine the symbiotic performance of peanut cultivars and Bradyrhizobium strains on soils containing native rhizobia specific to peanut. At Prachinburi better seed quality was obtained from inoculation. A very low recovery of inoculant strains in peanut nodules, which was observed in Sakol Nakorn and Udorn Thani trials indicated that the effective inoculant strains could not compete successfully with indigenous rhizobia. The results indicate the problem of obtaining a response to inoculation when native rhizobia are present in the soil.

INTRODUCTION

Nitrogen is one of the most commonly encountered limiting factors in agricultural production of developing countries. As the population of these countries is ever growing at an extremely high rate, the availability of fixed nitrogen to agricultural crops becomes more important for continued productivity. Nitrogen fertilizers are extremely expensive and, therefore are not an acceptable solution in developing countries. Among the various biological systems, which are able to fix atmospheric nitrogen the symbiosis of legumes with nitrogen-fixing bacteria (Rhizobium or Bradyrhizobium) seems to contribute the most nitrogen to the ecosystem and to food production. The cultivated peanut (Arachis hypogaea L.) is an important farm crop in Thailand (Arwooth Na Lampang, et al, 1980) and is one of the most widespread and potentially very important food legumes in the world (Gibbons, 1980). Being a legume peanut is able -due to its symbiosis with rhizobia- to supply most of the nitrogen required to obtain a large yield making it unnecessary to add nitrogen in the form of organic or mineral fertilizer. The process requires that the peanut plant be adequately nodulated by Bradyrhizobium spp. bacteria effective in $N_2$ fixation. These rhizobia are henceforth also referred to as 'peanut rhizobia'. In rhizobia-free soils all of the root nodules are formed by Bradyrhizobium spp. strain(s) applied as an inoculant. However, in soils containing indigenous rhizobia an inoculant strain may be outcompeted by indigenous strains. The indigenous rhizobia are often of mediocre effectiveness (Schiffmann 1982) and, therefore, will not provide the same quantity of nitrogen as when peanut is nodulated by highly effective strains. It can be assumed, therefore, that the substitution of nodules, that are expected to be occupied by indigenous rhizobia, with nodules from a more effective inoculant strain may increase the symbiotic performance of inoculated plants.

To approach this problem, a better understanding of the nature of native rhizobia populations was necessary. Considering the high adaptation of native rhizobia to local soil conditions, it was important to characterize the indigenous strains so as to identify the most effective ones for use in inoculant production. Rhizobial strains are characterized by their antigenic heterogeneity. Thus it is common to use serological tests for the identification of these bacteria

(Dudman, 1977). Although the antigenic specificity of peanut rhizobia has been demonstrated by several authors (Van der Merve & Strijdom 1973; Dadarval et al. 1974), no data are available on the serological distribution of peanut Bradyrhizobium strains (Cowpea spp.) in different soils.

There is a great variability in the effectiveness of different Bradyrhizobium strains specific for peanuts ( Law and Strijdom, 1974). Therefore in the framework of this project a special attention was paid to selection of Bradyrhizobium strains highly effective in supplying N during vegetative and reproductive plant growth. It was suggested that the first screening of Bradyrhizobium strains for effectiveness in $N_2$ fixation can be carried out with plants grown under controlled conditions (Wynne et al, 1980; Schiffman and Lobel, 1981). An additional evaluation of a strain superiority in a field environment was performed in Thailand.

The objectives of research were as following:

1. Assessment of the number and the effectiveness of peanut rhizobia native to tropical soils of the major peanut-growing regions in Thailand.
2. Characterization of the indigenous rhizobial strains in terms of their serological properties, protein profiles, and response to selected antibiotics.
3. Determination of host plant and Bradyrhizobium strain effects on growth and nitrogen fixation of peanuts and selection of rhizobia highly effective in supplying N to peanut cultivars recommended in Thailand.
4. Symbiotic performance of selected Bradyrhizobium strains under field conditions in Thailand.

**OBJECTIVE 1:** ***Assessment of the number and effectiveness of peanut rhizobia in the major peanut-growing areas of Thailand.***

MATERIALS AND METHODS

**Location of sites and soils.** The numbers of peanut rhizobia were determined in four regions of Thailand: The Central Plain, the East, the Northeast, and the North. According to the FAO-UNESCO Soil Map of the World (volume 9, Southeast Asia, 1979) the sites were the samples were collected are located: in the North and East mostly on acrisols of continental Southeast Asia (dark greyish brown fine sandy loam to fine sandy clay loam), in the Northeast on gleyic acrisols (light

yellowish brown and very pale brown loam) and in The Central Plain on acrisols and nitosols (dark brown sandy clay loam). The physical and chemical properties of the soils are described in the above-mentioned map. The sites altitudes in the North, Northeast, East and the Central Plain are respectively 380 m, 165 m, 5-10 m, and 1-10 m. The mean annual rainfall over the four regions, which ranges from 1000 to 1500 mm is distributed evenly in the North, East and The Central Plain regions,while dry periods are pronounced on the Northeast Korat plateau. Rice, cassava, sugar cane and various legumes are extensively planted in all of the regions listed above.

**Soil sampling.** Three different cropping areas: 1) continuously cropped in peanuts, 2) continuously cropped in non-legumes and 3) non-cultivated fields were chosen in each region. The survey included 55 sites: 15 uninoculated peanut fields, 34 fields cropped with non-legumes and 6 uncultivated fields where to the best of our knowledge peanuts had never been grown.

Before sampling, all the equipment and containers used were cleaned to remove rhizobia contaminant. Spades and hoes were washed with water, soaked in alcohol and then flamed. This was done for each sample collected. The top 2 cm of soil was removed from a sample site and the soil was collected to a depth of 20 cm. Soil samples were obtained by randomly collecting 10 core samples from each field. All cores from one site were placed on a clean plastic sheet, mixed together, passed through a 6.5-mm sterile sieve and a sample of 3 kg was taken. The sample was then immediately put into an ice box and kept at $4^0$C until
used (for at most 7 days).

**Quantification of rhizobia.** For most probable number (MPN) counts of rhizobia (Weaver and Frederick, 1972) a 50-g sub-sample of each soil was diluted in 150 ml of sterile water and placed on a wrist-action shaker for 15 min. Seven additional four-fold dilutions were made and 1 ml of each dilution was applied to inoculate surface-disinfected (in 2% of sodium hypochlorite for 2 min) peanut seeds. The inoculum placed near the seeds was followed by 100 ml of water to distribute the inoculum in the sand.Four replication plants grown in modified Leonard jars (Swedish Match - Helleshog) plastic tubes were inoculated with each dilution. Uninoculated control plants were included to determine

if cross-contaminations of jars occurred. Control plants were inoculated with sterile water.Plants were grown in an air-conditioned lighted room equipped with a Sun-Brellar light source which provides a flux density of light of about 450/uEs$^{-1}$m$^{-2}$ with a 12 hr - 12 hr light-dark regime. Plants were harvested and the roots were scored for nodulation at 21 days after inoculation.

**Isolation of indigenous rhizobia.** A portion of 2 kg from each sieved soil sample collected as already described was mixed with sterilized sand at a 1:1 ratio. Clean plastic bags were filled with the soil sample:sand mixtures and put in 1-liter plastic containers.

Peanut seeds were surface-sterilized in 2% sodium hypochlorite for 2 min, rinsed six times with sterile water and pregerminated in an agar water plate. The germinated seeds were planted into plastic containers, six seeds per container. All pots were placed in a greenhouse.

About 30 days after planting, the plants were harvested and the nodules were collected. Isolation from randomly selected nodules was made by surface sterilizing nodules in 2.75 % sodium hypochlorite for 1 to 3 min (depending on nodule size ) and rinsing them in sterile water about 6 times. Nodules were crushed in a drop of sterile water and streaked onto yeast extract mannitol agar (YEMA) plate ( Weaver and Frederick, 1982) containing congo red indicator and incubated for 10 days at 26$^0$C. Single colonies were picked from plates, numbered and stored on YEMA slants. Isolates were authenticated on siratro.

**Effectiveness of rhizobia.** The method for growing plants utilized sterile plastic containers filled with sterilized sand. Four peanut seeds (cv. Tainan 9) were sown in each pot and the plants were grown under greenhouse conditions . After addition of inoculum in water suspension ( 5 ml of the appropriate strain containing 5 x 10$^8$ cells ml$^{-1}$ to each pot) the soil was covered with sand-coated paraffin as

described by Vincent (1970). An uninoculated control was included in each test. Seedlings were thinned to one per pot seven days after germination. N-depleted plant nutrient solution was added to each container. Completely randomized design with three replications for each of the isolates tested was used. Acetylene reduction rates were determined according to established procedures (Hardy et al. 1973) and the results were calculated as umol $C_2H_4$/plant/h (total activity). The plant tops were dried and used for N-content determinations by the Kjeldahl method. Duncan's New Multiple Range Test was used to compare the treatments tested.

**Effect of inoculum level on nodulation and $N_2$ fixation of peanut.** An experiment was conducted in the nethouse of the Volcani Center (Bet Dagan, Israel) to examine the response of peanuts (Virginia type, cv.Shulamit) to the number of rhizobia added at planting time. Two effective strains of Bradyrhizobium from the Volcani Center culture collection were grown in broth of yeast extract mannitol (YEM) medium (pH 7) on a gyrotary shaker (New Brunswick Scientific Instrument, Co., New Jersey, U.S.A.) at 140 rev. $min^{-1}$ and utilized as an inoculum. The number of rhizobia was initially determined by direct microscopic counts and verified by a plate counting procedure on YEM agar plates (Vincent, 1970). Tin-coated containers of 3-liter capacity were utilized in this experiment. An amount of 3.5 kg of sterilized sandy soil was placed in each container. Three peanut plants, which were grown in each container, were inoculated with one of four rates of inoculum ranging from $1.4 \times 10^3$ to $1.4 \times 10^9$ rhizobia per seed ($1 \times 10^4$ to $1 \times 10^{10}$ rhizobia per container. The plants were grown during two months (May-June) at maximum day temperatures of 25°C (May) and 28°C (June). The minimum night temperatures were respectively 14°C and 17.5°C. The experiment was conducted using a completely randomized design with 5 replications for each inoculum level of each strain.

RESULTS

Peanut (cowpea group) rhizobia were found in the soil at 38 of 55 sites sampled (Table 1). Only one among 15 peanut fields tested did not contain rhizobia specific to peanuts. In contrast, no peanut rhizobia were found in approximately 50% of fields continuously cropped with non-legumes. The numbers of rhizobia in Arachis hypogaea fields (soils which were under peanut production at least twice during the last five years) ranged from 29 to 4600 per g of soil with an

average of 1.6 x $10^3$ rhizobia $g^{-1}$ of soil (Fig 1). The effect of the number of indigenous rhizobia on nodulation and $N_2$ fixation was not determined in this study. However, the results of a pot experiment carried out on autoclaved sandy soil indicated that the number of rhizobia applied for inoculation had a significant effect on the nodulation rate of peanut plants, dry matter and N accumulation in peanut foliage. As shown in Fig 2 not less than 1.4 x $10^7$ rhizobia cells per seed (1 x $10^8$ rhizobia per pot) were required to provide maximal N accumulation and shoot weight. Nodule development was affected in the same manner (Fig. 3).

The rhizobial isolates obtained from the plants grown on soil samples collected in different regions of Thailand varied greatly in their total nitrogenase activity. Of the 300 isolates collected from 15 fields (20 isolates from each field) 60% were considered as effective ($C_2H_4$ $plant^{-1}$ $h^{-1}$ values not significantly different from those produced by strain THA 205 recommended for inoculation of peanuts in Thailand) and 40% as mediocre and noneffective (Table 2). Bradyrhizobium isolates which differ in their $N_2$ fixing activity were isolated at each of the sites tested. Assuming that the ratio between effective and ineffective bacteria isolated from each field reflects their quantitative relation in soil at each site tested, various sites differ from one another by their effective:ineffective isolates ratio. As shown in Table 2, among the 15 fields tested, 4 in the Northeast region (NE-41, NE-38, NE-39, NE-37), and one in the Central plain (M-53) were inhabited mostly by ineffective or mediocre rhizobia while 55-90% of isolates obtained from 10 other fields showed high acetylene-reduction rates.

The results were even more prominent in an additional greenhouse experiment where the effect of 43 randomly selected Bradyrhizobium isolates (collected from seven fields) on N accumulation in peanut plants was tested. As shown in Table 3 only two of seven isolates collected at site E-10 may be considered as effective. The Bradyrhizobium populations at other sites were mostly effective. At present, the reasons for such differences among sites are not clearly understood. It should be emphasized, however, that most of the fields investigated in the Northeastern region (mostly poor soils and a bad rainfall distribution) contained a high percentage of mediocre and ineffective peanut rhizobia isolates. Further field inoculation tests

are needed to check whether an inoculation response can be obtained: a) in fields inhabited mostly by ineffective rhizobia; b) when Bradyrhizobium strains comprising a range of effectiveness are present in soil.

DISCUSSION

The numbers of peanut rhizobia found at different sites in Thailand are similar to those of Mahler and Wolum (1981), showing that populations of cowpea rhizobia in cultivated peanut fields in California ranged from 2 to 10000 rhizobia $g^{-1}$ of soil. Significantly higher counts than these were reported by these authors for R.meliloti and R. trifolii in fields planted with their respective hosts. Interestingly, the number of rhizobia in most of the fallow fields and some of the non-cultivated shrub or forest locations were much the same as in the sites where Arachis hypogaea was cultivated. These results can probably be explained by the presence at these sites of native legumes nodulating with the same cross-inoculation group of rhizobia infecting peanut. In contrast, there were no or few (28-46 cells $g^{-1}$ of soil) of peanut rhizobia in the majority of fields which were continuously cultivated with non-legumes such as cassava, sugar cane, corn and pineapple. The non-occurrence or negligible amounts of rhizobia in these cropping systems might be due to the absence of their host legume. Intensive land preparation practices associated with wind or water erosion of the surface soil may also result in a significant decrease in the numbers of rhizobia nodulating different legumes (Habte and El-Swaify 1991). It was previously reported (Mahler and Wolum, 1981) that the appearance of peanut in the cropping history greatly influenced the number of cowpea rhizobia in soil. According to Wilson and Trang (1980) and Boonkerd and Weaver (1982) MPN counts of cowpea rhizobia in soil are usually underestimated. Therefore the counts of rhizobial populations in the soils tested should probably be increased by a factor of at least ten to get their true number.

The results obtained in this study confirm those of Nambiar et al.(1983) showing the significance of a high Bradyrhizobium inoculum level for a maximal nodulation and $N_2$ fixation by peanuts. Assuming that in our experiments the rhizobia cells applied to each pot are dispersed evenly in 3.5 kg of soil the number of rhizobia cells required for maximal nodulation and $N_2$ fixation would result in $3 \times 10^4$ rhizobia $g^{-1}$ of soil. Although it is difficult to relate this data to

field conditions, it is anticipated that the rhizobial population of some soils in Thailand may be not adequate for maximum nodulation and plant growth. It has been recently reported by Kucey and Toomsan (1988) that field inoculation of different peanut cultivars in northeastern Thailand resulted in increased seed yields and the amount of N fixed in the plants.

**OBJECTIVE 2:** *Ecological characterization of peanut Bradyrhizobium strains*

Peanut (Arachis hypogaea L.) is naturally nodulated by the slow-growing rhizobia (Bradyrhizobium spp.) that are widespread in tropical soils. It was shown (see objective 1), that the native peanut rhizobia isolates collected in the main peanut growing areas of Thailand range widely in their ability to fix $N_2$. In some areas, the majority of bradyrhizobia isolated from peanut nodules were less effective than the inoculum strains THA 205 and 280A that are recommended respectively in Thailand and Israel for peanut inoculation. Substitution of ineffective nodules, formed by indigenous peanut rhizobia, with nodules from a more effective inoculant strain should therefore increase the symbiotic performance of inoculated plants. To approach this problem, a better understanding of the nature of native peanut rhizobial populations is necessary. Although the antigenic specificity of peanut rhizobia has been demonstrated by several authors (Dadarval et al. 1974), no data are available on the serological distribution, intrinsic antibiotic resistance and electrophoretic protein profiles of Bradyrhizobium sp. (Arachis) in different soils.

MATERIALS AND METHODS

**Isolation of indigenous rhizobia**

Soil samples were collected from four regions of Thailand: North, Northeast, East and Central Plain. Three different cropping areas were chosen at each region: 1) continuously cropped with peanuts; 2) continuously cropped with non-legumes and 3) non-cultivated fields. Soil samples were collected at 15 sites: one uncultivated field, two fields cropped with nonlegumes, three fallow fields and nine fields cropped with legumes: peanuts (5), mungbean (1), cowpea (1), soybean (1), and a mixture of different legumes(1). Soil samples were obtained by collecting randomly 10 core samples from

each field. All cores from one site were mixed together, passed through a 6.5 mm sterile sieve and a sample of 3 kg was taken. The sample was then put immediately into an ice box and kept at $4^0$C until use (Seven days at the most).

A portion of 2 kg of each soil sample was mixed with autoclaved sand at a 1:1 ratio and the sand/soil mixtures were put in clean plastic containers. Peanut seeds (cv. Tainan 9) were surface-sterilized in 2% sodium hypochlorite and pregerminated on agar water plates. The germinated seeds were planted in plastic containers, six seeds per container. The seedlings were thinned to three per container seven days after germination. All pots were placed in a glasshouse that was cooled artificially to maintain a 27-$32^0$C temperature range under sunlight conditions. Thirty days after inoculation, randomly-selected nodules (10 per plant) were detached from peanut plants and used for isolation of rhizobia according to the procedure described by Vincent (1970). Isolates were authenticated under bacteriologically controlled conditions on Macroptilium atropurpureum (Siratro). In all, 243 Bradyrhizobium Cowpea spp. isolates obtained from 15 fields were used in this study.

**Serological procedures**

As somatic antigens of rhizobia were reported to be more specific than flagellar and internal antigens (Vincent,1982), they have been used in this study to define strains in terms of their antigenic structure and to separate peanut Bradyrhizobium isolates into different serological groups. For the preparation of culture antigens, the bacteria were grown at $28^0$C to early stationary phase in yeast-mannitol broth (YMB). Numbers of rhizobia in pure cultures were estimated by using a plate-counting procedure (Vincent, 1970). Cultures were centrifuged and washed three times in phosphate -buffered saline (PBS). The final cell pellet was resuspended in PBS to a final concentration of $10^8$ cells/ml and the antigen suspension was heated at $100^0$C for 30 minutes to inactivate the flagellar antigens. Three of the antisera used (280A, 2212A, and CB756) were produced from strains isolated in Israel (280A and 2212A) and Australia (CB756) and kept in the Volcani Center Rhizobium Collection. Nine antisera: 7/20E, 31/14N, 31/9N, 22/6N, 30/4N, 34/6NE, 25/3N, 28/15N, and 10/14N were produced from Bradyrhizobium isolates obtained in Thailand during the course of this study. The antisera were prepared according to the schedule adopted in

previous work (Kishinevsky and Gurfel, 1980). The indirect enzyme-linked immunosorbent assay (ELISA) of Engwal and Perlmann (1971) with the modifications of Kishinevsky and Maoz(1983) and Fuhrmann and Wollum II (1985) employed for Bradyrhizobium cultures followed the protocol described by Kishinevsky et al. (1984). For adsorption of antibodies cells from 6-day old liquid culture containing approximately $2x10^9$ Bradyrhizobium cells/ml were packed by centrifugation. The pellet was resuspended in 1/100 diluted heterologous antiserum and the adsorption was carried out at 37°C for one-hr with an additional two-hr agitation of the mixture at four °C. Complete adsorption was verified by ELISA testing of absorbed antiserum with a fresh preparation of absorbing antigen. The minimum number of antigenic determinants occurring in a given strain was determined as follows: Cross-reacting strains shared a common antigen. If the antiserum continued to react with the homologous strain after adsorption of antibodies by a heterologous strain, two antigenic determinants were assigned to the homologous strain. A homologous reaction of each strain after a heterologous adsorption indicated that besides the common antigen(s), each strain bore specific antigens. As in the case with R.japonicum strains (Robert & Schmidt 1985), Roman numerals were adopted for designation of the antigens found.

**Determination of antibiotic resistance**

Fresh solutions of antibiotics were added to melted and cooled to $50^0$C yeast- mannitol agar to give a final concentration of 50 ug $ml^{-1}$. The following antibiotics were used: penicillin( benzylpenicillin), kanamycin monosulfate, neomycin sulfate, streptomycin sulfate, chloramphenicol, rifampicin and spectinomycin - all from Sigma.

To differentiate the isolates on the basis of antibiotic response an exponential phase cultures were diluted optically to $2x10^7$ cells $ml^{-1}$ and 10 ul of each isolate (in duplicates) were dropped on the antibiotic plates. The plates were incubated for 12 days at $28^0$ C. Growth on the antibiotic plates was compared with a YMA control plate and the isolates were scored as sensitive (no growth) or resistant (moderate growth).

**SDS-PAGE Analysis**

Bradyrhizobium isolates were grown in 50 ml of yeast - mannitol broth (YMB) medium (Vincent, 1970) at 28°C to the late exponential phase of growth (1 X $10^8$ cells/ml) with shaking. Cultures were harvested by

centrifugation for 20 min at 12000 g and washed twice in ice-cold 10 mM tris (hydroxymethyl) aminomethane (Tris)-hydrochloride, pH 7.6. The final cell pellet was resuspended in 0.5 ml of the same Tris-HCl buffer. After ultrasonic disruption on ice for 60 s with a microtip probe at 50 W, 0.5 ml of the SDS buffer (Dughri and Bottomley, 1983) was added. The extracts were stored at - 20°C prior to analysis by electrophoresis. Stacking and resolving gels containing respectively 3 per cent and 12.5 per cent of acrylamide were cast between two glass plates: 16 x 20 cm (inner plate) and 18.3 x 20 cm (outer plate). The thickness of the gel slabs was 1 mm. The gels were polymerized chemically by the addition of 0.05 per cent by volume of tetramethylethylenediamine (TEMED) and 0.03 percent of ammonium persulphate. The final concentrations in the resolving gel were as follows: 0.375 M Tris-HCl (pH 8.8) and 0.1 per cent SDS. The stacking gel contained 0.125 M Tris-HCl (pH 6.8) and 0.1 per cent SDS. The proteins were dissociated by immersing the samples for 1 min in boiling water. The gels were electrophoresed at 4°C with a current of 0.025 A until the bromophenol blue marker reached the bottom of the gel (about 6 h). The proteins were fixed and stained by a consecutive immersion of each gel into three solutions: 1) 0.0035 per cent (wt/v) Coomassie brilliant blue, 25 per cent (v/v) isopropanol and 10 per cent (v/v) glacial acetic acid for 2 h; 2) 0.0035 per cent (wt/v) Coomassie brilliant blue, 10 per cent (v/v) isopropanol and 10 per cent (v/v) acetic acid (overnight); 3) 0.0035 per cent wt/v) Coomassie brilliant blue and 10 per cent acetic acid for 2 h and destained for 2 h in 10 per cent acetic acid. Then the gels were recorded on photograph.

RESULTS

**ELISA reactions of Bradyrhizobium strains used for preparation of antisera**

The results of the indirect cross-ELISA tests using antigens and antisera of 12 Bradyrhizobium strains are presented in Table 4. The ELISA readings for homologous reactions were significantly higher than the heterologous ones, and ranged from as low as $A_{405}$= 0.64 for strain CB756 to $A_{405}$=1.94 for strains 280A and 31/14N. Most of the homologous readings were in the range of 1.10 to 1.20. Strain 280A was serologically- related individually to each of the following strains: 2212A, 7/20E, 31/14N, 22/6N and CB756, that showed no cross-reaction

among them. As strains 2212A, 7/20E, 31/14N, 22/6N and CB756 reacted with antiserum to 280A it is reasonable to postulate that strain 280A possesses five different antigens: I, II, V, VII, and IX, respectively, common to each of these strains (Table 4). Reciprocal adsorption tests (Table 5) showed that strains 7/20E, 2212A, and 31/14N, applied individually or as a mixture, did not adsorb all antibodies from antiserum 280A, as adsorbed antiserum still reacted strongly with homologous antigen. Thus, strain 280A contains at least one additional antigen (antigen XI), as indicated in Table 4. Similarly, antisera 7/20E, 2212A, and 31/14N, adsorbed by strain 280A continued to react with homologous antigens, indicating that each of these strains contains a second antigen (III, IV, and VI respectively). Five further strains: 30/4N, 34/6NE, 25/3N, 28/15N, and 10/14E appeared to be unique in their antigenic make-up, reacting only with homologous antisera.

There were erratic ELISA reactions in some strain-antiserum systems (Table 4). Strain CB756 reacted with antiserum 280A, while antiserum developed against strain CB756 failed to react with 280A antigen(s). No ELISA reaction was observed between strain 22/6N and antiserum 280A, whereas strain 280A reacted positively with antiserum 22/6N (Table 4).

**Serogrouping of indigenous populations**

Twelve antisera described above were used to examine the serological properties of indigenous peanut rhizobia of 15 origins in four different regions. Serological grouping of indigenous Bradyrhizobium isolates in this study was based on the assumption that nodules of greenhouse and field plants grown in a given soil are formed by identical population(s) of rhizobia (Johnson & Means, 1963). From 243 isolates collected, only 13 ( about Five % ) did not react with any of the antisera used (Table 6). About 80% of the 243 isolates collected in Thailand were found to have at least one heat-stable antigen in common with strain 280A forming what might be called a 280A serocluster (Fig. 4). Based on the pattern of their ELISA reactions, the 280A positive isolates were distributed into 18 serogroups. A serogroup was outlined by isolates reacting with the same antiserum orantisera tested. Sixty five isolates that reacted with antiserum 280A only, formed the first serogroup. Antigen XI (Table 4) or another hypothetical antigen may be involved in the 280A reaction of this

serogroup. Four following serogroups (two to five) were composed of 80 isolates reacting with antibodies 280A and one of the following antisera: 2212A, 31/14N, 7/20A, and 22/6N respectively. At least four different antigens (probably II, V, I, and IX) took part in these reactions. About 48% of the 280A positive isolates tested fell into the first two groups. Twelve isolates belonging to serogroup six (Table 6) reacted with two antisera: 280A and 30/4N, having no antibodies in common (Table 4). A minimum of two determinants should form the antigenic constitution of these isolates. Serogroups seven through-11 and 12-18 comprised, respectively, 25 and 13 isolates that were characterized by positive ELISA reactions with different combinations of three (serogroups seven through-11 ) and four to six (serogroups 12-18) antisera having 280A in common. Thirty five (15%) of the isolates reacting mostly with single antisera (Table 7) were distributed over 11 additional serogroups (19-29). None reacted with antiserum 280A.

The serogroups distribution of indigenous rhizobia was not related to the cropping history of the fields from which they had been isolated. Thus, serogroup two that was supreme in peanut grown fields N-21, N-23, N-28 in the North, was also abundant at a fallow field (E-16) in the Eastern part of Thailand (Table 6). Although identical serological groups were found in different soils, the frequency of a particular serogroup varied from field to field. For example, only two serogroups were found at site E-16 in the eastern region, whereas rhizobia, isolated from nodules of peanuts grown in E-7 soil of the same area, were more heterogenic and could be divided, therefore, into five serogroups. No serogroup common to all fields has been detected. At 11 of 15 sites tested, 53 to 100% of the isolates fell into one or two predominant serogroups, that individually or jointly were superior at one or several sites (Table 6). Serogroups one and two (singly or in combination) dominated the rhizobial population in eight fields: E-16, N-21, N23, N-28, NE-33, NE-36, NE-39, and M-47 located in the eastern, northern, northeastern, and central parts of Thailand. The peanut rhizobia populations at sites E-7 and N-30 belonged mainly to serogroups one plus four and one plus six respectively. One additional serogroup, lacking antigens in common with 280A, formed the majority of peanut rhizobia at site N-22 (Table 7).

At least 17 somatic antigenic determinants were found among 12

strains that were used for antisera preparation. There was a close antigenic similarity between a well-known inoculant strain CB756 from Australia and strain 31/9N isolated in Thailand. In our experiments, strain 280A was found to have the most complex antigenic constitution, bearing at least six antigenic determinants as compared with one and two determinants detected in the other eleven strains used for antisera preparation.

**Intrinsic antibiotic resistance in Bradyrhizobium sp.(Arachis)**

To differentiate the isolates on the basis of their intrinsic antibiotic resistance (IAR) they were screened for their response to the following antibiotics: neomycin, chloramphenicol, penicillin, kanamycin, rifampicin, spectinomycin and streptomycin. These were selected since they are the major antibiotics which were used to demonstrate a heterogeneity in natural population of other Rhizobium and Bradyrhizobium species (Beynon and Josey, 1980). The responses of 251 isolates of Bradyrhizobium sp.(Arachis) to seven antibiotics are presented in Table 8. The vast majority of the isolates collected at different sites were resistant to chloramphenicol and neomycin. The percent of resistant isolates was lower (in succession) on media containing penicillin, kanamycin, spectinomycin, rifampicin and streptomycin. Isolates resistant to streptomycin were found only in the soil at 5 of 15 sites sampled. The great majority (87%) of the isolates collected at two sites (fallow and peanut fields in the eastern and northern regions) grow on YMA media containing streptomycin. The number of streptomycin resistant strains in the rest three fields ranged from 25% to 27%.

When groups of intrinsic antibiotic resistance were examined rather than resistance to a single antibiotic, twenty five patterns of resistance were established for 251 isolates (Table 9). Only 4 isolates had unique fingerprints (Groups No. 11, 20, 22 and 23). Twenty one other fingerprints were represented by more than one isolate. As shown in Table 10 nine of these IAR groups contain 76% of the isolates examined. It is obvious that many strains may have been in each field examined. The number of IAR groups in individual fields ranged from 2 to 10 and the number of sites where isolates of a particular IAR group was detected varied from 1 to 12 (Table 11). The isolates collected at sites N-25, N-22 and NE-39 formed respectively 2, 3 and 3 fingerprints only. As shown in Table 12 the indigenous

field isolates forming a particular serogroup were distributed through different IAR groups. For example, 110 and 45 isolates belonging to two dominating serogroups (1 and 2 respectively) could be subdivided into 20 (serogroup 1) and 17 (serogroup 2) IAR fingerprints. On the other hand two groups of isolates (N-22 and NE-39) which differ from each other by antigenic structure (Table 6) were mostly similar in their antibiotic resistance patterns. Individual IAR groups were specific for a particular serogroup, e. g., all 106 isolates of 14 IAR groups (1, 2, 4, 6, 8, 9, 10, 11, 13, 16, 18, 22, 23) were of serocluster 280A (Fig. 5). Serocluster 280A dominated the 55 isolates of five additional IAR groups (5, 7, 12, 14 and 24). Isolates lacking antigens in common with 280A formed the majority of three IAR groups: 3, 17 and 19.

**SDS - PAGE characteristic of proteins**

Sixty four Bradyrhizobium spp. isolates obtained from plants grown on soil samples collected at 7 sites in different regions of Thailand were analyzed by SDS - PAGE. ELISA analysis applied to all sixty four isolates indicated that most of them belong to the 280A serocluster. Close inspection of the SDS-PAGE results (Table 13) showed that in most cases isolates within individual serocluster could be distinguished from one another by electrophoretic analysis. For example, the isolates collected at E-7 site and placed in the 280A serocluster formed three gel types (Table 13 and Fig. 6). Also isolates belonging to one serogroup, but collected at different sites were dissimilar in their SDS-gel protein patterns (Fig. 7). Close inspection of protein profiles of nine isolates collected at site N-23 in the Northern part of Thailand reveals three gel types that make up two serogroups (Table 13, Fig. 8). On the other hand, isolates belonging to different serogroups have identical protein profile patterns (Table 13, locations N-30, N-28 and N-22). According to the electrophoretic profiles three to five gel types were found to be present at each location. The electrophoretic patterns of all strains tested were found to be reproducible.

DISCUSSION

To our knowledge, this is the first report to indicate the abundance of certain serogroups within indigenous soil populations of Bradyrhizobium spp. (Arachis). These findings are in agreement with the results obtained for B.japonicum, reporting the well-known

phenomenon of a high occurrence of serogroup 123 in different soils of soybean fields in Iowa, Indiana, Illinois and Nebraska (Damirgi et al. 1967, Weber et al. 1989). One can only speculate about the reasons leading to dominance of a certain serogroup in a given soil. It was shown, however, for Rhizobium leguminosarum bv. Viceae (Brockman & Bezdicek 1989) that soil microclimate and topography may affect the serological distribution of indigenous rhizobia. The role of the soil microclimate in serogroup distribution of peanut rhizobia is yet to be investigated.Vincent (1982) reported that antigenically-similar strains of Rhizobium were obtained from widely separated regions of the world. The present results confirm those of Dadarval et al. (1974) showing that the spectrum of serological reactions of peanut rhizobia varies among strains.

It can, therefore, be assumed that more strains may react with 280A antiserum than with antisera against strains endowed with a unique antigenic make-up. The reasons underlying the inadequate ELISA reactions of some antigen-antiserum combinations (e.g. strains CB756 and 22/6N with antiserum 280A) is still not clear and needs further clarification. In agglutination tests similar failures are supposed to be due to the masking of certain antigens (Date and Decker, 1965). Perhaps the most significant result of this study is the finding that 80% of the 243 isolates collected in Thailand were found to have at least one heat-stable somatic antigen in common with strain 280A, forming what might be called a 280A serocluster. Moreover, the serogroups reacting with antiserum 280A dominated the peanut rhizobial population in most of the fields tested. It can, therefore, be expected that if strain 280A were used as an inoculant strain in such fields, many nodules on the uninoculated control plants would react with the 280A antiserum. In our previous experiments (Kishinevsky et al. 1987), such non-specific cross-reactions were eliminated selectively by using inoculant strain gamma-globulin pretreated with bulk nodule material from uninoculated plots. The adsorbed gamma-globulin still reacted strongly with the inoculant strain.

The antigen(s) involved in the reaction of isolates with 280A antiserum cannot be determined with certainty from our data. To figure out the antigenic structure of the isolates, antisera against each of them must be prepared; nevertheless, the differences in extinction values among isolates within a given serogroup (data are not

presented) indicate the absence of a full identity in their antigenic make-up. Isolates belonging to the same serogroup should be investigated further to identify and characterize their intraserogroup diversity.

Our results show the suitability of the indirect ELISA for serological typing of strains of Bradyrhizobium. The simplified indirect ELISA procedure (Kishinevsky and Maoz, 1983; Fuhrmann and Wollum II, 1985) allow to adapt this reliable technique to variously equipped laboratories.

In spite of the fact that the Bradyrhizobium spp. (Arachis) isolates examined in this work were isolated in different areas most of them were similar in their resistant to chloramphenicol and neomycin and many were also resistant to penicillin, kanamycin and spectinomycin. The general resistance of the isolates to chloramphenicol and neomycin was similar to results reported by Cole and Elkan (1979) for Bradyrhizobium japonicum. The variation in intrinsic resistance of Bradyrhizobium isolates enabled us to distinguish a range of strains present in an indigenous population. According to the fingerprinting data a substantial heterogeneity exists among isolates belonging to the same serogroup. On the other hand individual IAR groups were highly specific for a particular serogroup. Similar results for Rhizobium leguminosarum were reported by Brockman and Bezdicek (1989).

The electrophoretic protein profile analysis used as supplementary to the serological test also showed that rhizobia within a serogroup are not necessarily identical. An intraserogroup diversity revealed by electrophoretic typing was reported by several authors( Noel and Brill, 1980; Dughri and Bottomley, 1983; Bottomley, 1992).

**OBJECTIVE 3:** ***Cultivar and Bradyrhizobium strain effects on growth and nitrogen fixation of Arachis hypogaea***

Peanut cultivars of the Virginia and Valencia genotypes occupy most of the area assigned to peanut growing in Thailand and Israel. In the legume-Rhizobium symbiosis both plant cultivars and Rhizobium strains can affect nodulation and accumulation of nitrogen in the vegetative and reproductive tissues (Roskotten, 1989; Wynne et al. 1983). The symbiotic performance of peanut Bradyrhizobium strains and their interaction with Arachis hypogaea cultivars were examined in two nethouse experiments under controlled conditions in Israel.

MATERIALS AND METHODS

*Nethouse studies.* For both experiments metal pots of 3.5 liter capacity were filled with 3.5 kg of sandy nitrogen poor soil and sterilized in an autoclave for 2 h at 120°C. The inoculant strains were grown on yeast-mannitol broth medium (YMB) for seven days at 28°C with agitation. Seven peanut seeds were sown in each pot and after adding the inoculum in water suspension the soil was covered with paraffin-coated sand as described by Vincent (1970). Seven days after emergence plants were thinned to three plants per pot. Daily irrigation of plants was based on the weight of the pots in each treatment. An uninoculated treatment was included for each cultivar. The experimental layout was a complete randomized block design with 6 replicates and 6 treatments for each cultivar. Bradyrhizobium strains: 280A usually used as an inoculant strain in Israel), E-22/7, E-7/20, N-31/14 and N-21/3 (all isolates collected by Dr. Boonkerd in various regions of Thailand) and peanut cultivars Tainan 9 (the most commonly grown in Thailand Virginia type cultivar), Shulamit and Hanoch (both Virginia type cultivars usually grown in Israel) and Valencia improved (Israel) were used in the first experiment.

Bradyrhizobium strains: 280A, N-22/13, N-31/19, NE-39/12, NE-39/16, N-25/2, N-25/9, N-25/7 and four peanut cultivars of bunch Virginia: Tainan 9, KK 60-3 and KK 60-1 (all seen in Thailand) and Shulamit (the most commonly grown cultivar in Israel) were used in the second experiment.

RESULTS AND DISCUSSION

*First pot experiment.*

A great variability in the effectiveness of Bradyrhizobium strains, as measured by plant dry weight and total nitrogen accumulated in tops, was found already 48 days after planting. The most effective strains on four peanut cultivars were 280A, E-22/7 and E-7/20, which accumulated respectively 410.4, 351.0 and 331.5 mg of N per pot as compared with 130 and 199 mg of N in plants inoculated with the ineffective strains N31/14 and N21/3 (Table 14). There were also significant effect of plant cultivar and Bradyrhizobium strain on dry matter, nitrogen content and total nitrogen in the tops of peanuts. The highest values of top dry matter and total nitrogen were observed in cv. Hanoch: 17 g and 318 mg per pot respectively compared to 6.8 g dry matter and 162.2 mg N in cv. Thainan. The cv. Shulamit / 280A

combination exceeded four times the top N of the poorest symbiotic combination: Tainan 9\ N31/14 (Table 14). Similar results were obtained with 73 days old plants. A considerable variability in symbiotic performance among Virginia types of peanut has been reported from studies in North Carolina (Wynne et al., 1978).

Strain 280A was proved to be the best regarding the top dry weight and accumulation of total N (Table 15).

Nitrogen fixation and yield of Tainan 9 could probably be increased by selection of segregates from Shulamit (or Hanoch) x Tainan 9 crosses, all Virginia type peanuts.

*Second pot experiment.*

As in the first experiment, both the host genotype and the strain treatments influenced the effectiveness of the symbiotic association (Table 16). No significant host genotype - strain interaction was detected. Similar results with different peanut cultivars were obtained by Elkan et al.,(1980). The peanut genotypes were significantly different (0.05 level of probability) for top dry weight nitrogen content and the amount of symbiotic nitrogen accumulated in peanut tops. Cv. Shulamit and KK 60-1 were better developed than Tainan 9 and KK 60-3. The highest dry weight of shoots and amount of total N in tops were obtained from plants inoculated with strains 280A and N-22/13 followed by NE-31/19.

The results obtained in the pot experiments demonstrated the importance of considering both the host plant cultivar and the Bradyrhizobium strain in the selection programs for higher $N_2$- fixation in peanuts and there is a scope for obtaining promising host-rhizobia associations.

**OBJECTIVE 4.** ***Symbiotic performance of different peanut cultivars and Bradyrhizobium strains under field conditions***

Although the first screening of Bradyrhizobium strains for effectiveness and nitrogen fixation can be carried out with plants grown under controlled conditions, an additional evaluation is needed of a strain's superiority in a field environment. Field experiments are essential in studies designed to evaluate previously greenhouse - tested strains for nodulation and nitrogen - fixing activity in soils containing indigenous rhizobia. In such fields an introduced Rhizobium strain used as an inoculant for a legume crop has to compete with the

established rhizobia for nodule sites on the roots. The problem is of agronomic importance in soils harboring rhizobia which are less effective than the desired inoculant strain.

MATERIALS AND METHODS

Three field experiments were conducted at: a) Prachinburi in the Eastern part of Thailand; b) Sakol Nakorn and c) Udorn Thani (both in Northeastern part of Thailand) in soils which had been planted with peanuts before.

**Prachinburi trial**

A split plot design consisted of three peanut cultivars: Tainan 9, KK 60-1 and KK 60-3 as the main plots treatments. Sub-plot treatments were (a) N fertilizer application at 75 kg/ha; (b) inoculation with strain THA 205; (c) no inoculant. The plot size was 3x6 m. The experiment was carried in four replications. All treatments were applied with optimum rate of P and K. The soil at the experimental site contained an estimated $10^3$ cells$^{-1}$ of Bradyrhizobium spp. capable of nodulating peanuts.

**Sakol Nakorn and Udorn Thani trials**

Each of eight strains of Bradyrhizobium spp. highly effective on the local peanut cultivar Tainan 9 were used in two field trials. Strains 280A and 2212A were from the Volcani Center, Israel. Strain THA 205 is currently used for inoculant preparation in Thailand. Five other strains were local isolates. Peanut seeds (cv.Tainan 9) were planted in (a) uninoculated soil, (b) soil inoculated with each of the eight strains of Bradyrhizobium spp. and (c) uninoculated and N-fertilized at 75 kg/ha. The experimental layout was a randomized block design with four replicates per treatment. The plot size was 3 x 5 m.

The indirect ELISA method was used for indirect serological typing of inoculant strains in nodules (Kishinevsky et al., 1984). Nodule contents were treated with antiserum prepared against the inoculant strain involved; nodules of plants in uninoculated plots were tested against antiserum of each of the inoculant strains. The age of the plants taken for determination of nodule number, nodule weight, nitrogen-fixing activity and strain recovery was 55 days. Four plants were randomly selected from each replicate, nodules from tap and secondary roots were pooled together and 50 nodules from each plot were randomly collected for typing. Finally 100 day-old plants were harvested and the pods removed by hand.

RESULTS AND DISCUSSION

**Prachinburi trial**

Application of N fertilizer had a tendency to reduce nodulation in peanut cultivars Tainan 9 and KK 60-1, but this reduction was not statistically significant (Tables 17 and 18). This was probably due to the rate of N used which was not high enough. The specific ARA (Table 19) was also not different among all treatments. Plant shoot dry weight at 60 days after planting and their percentage of nitrogen content in tops did not differ significantly among treatments and cultivars (Tables 20 and 21). The N- fertilized treatments produced the highest pod dry weight of peanut cultivars KK 60-1 and KK 60-3. The lowest yield was obtained in the uninoculated and none N-fertilized plots of Tainan 9 (Tables 22, 23). Results from this study indicated that inoculation in this area could not meet the needs of peanuts for N. This was probably due to some factors limiting $N_2$ fixation in rhizobia or effective rhizobia from inoculant could not compete with the indigenous rhizobia. Though inoculation could not increase seed yield of peanuts better seed quality was obtained by inoculation. The results presented in Table 24 showed that shelling percentage was increased by inoculation and N- fertilizer application.

**Sakol Nakorn and Udorn Thani trials**

The soil at Sakol Nakorn contained an estimated 250 cells/g soil Bradyrhizobium spp. capable of nodulating peanuts. The inoculant strains did not differ significantly with regard to their effects on numbers and mass of nodules and top dry weight (Table 25). The performance of strains E-7/20 and N28/12 tended to be inferior with regard to nodule mass and nitrogenase activity. No significant differences in pod yield at harvesting were obtained with any of the treatments. The percentage of nodules occupied by each of the seven inoculant strains was very low and varied between 7 and 31. Strain E-7/20 was not detected in any of the nodules.

The soil at Udorn Thani contained an estimated 3600 cells/g soil capable of nodulating peanuts. Most of the inoculant stains increased the number and mass of nodules on the tap root but did not affect the nodulation of the secondary roots (Table 26). There were no significant differences in the total number and mass of nodules among the various different treatments, even though a tendency towards poor inoculation was found in uninoculated and N-fertilized plants (Table

26). As indicated in Table 27 the percentage of nodules which gave strong positive ELISA reactions with homologous antisera varied between 6 (strain N-28/12) and 56 (strain 280A).

In both experiments the lack of yield response to inoculation was probably due to a very low recovery of inoculant strains in peanut nodules or to unfavorable soil or climatic factors, others than nitrogen shortage which limited yields. It was shown (objective 3 of this report) that, even under favorable conditions, the performance of Tainan 9 tended to be inferior with regard to the amount of nitrogen accumulated in the peanut shoots. It is assumed, therefore, that mediocre rhizobia, which comprise often a large part of indigenous populations, are able to provide the demand of Tainan 9 plants for nitrogen. In our experiments a comparatively higher than other strains percentage of nodules formed by strain 280A did not contribute to the end-product of nodule efficiency: pod and kernel yields. The results indicate the problem of obtaining a response to inoculation when native rhizobia are present in the soil.

**CONCLUSION**

In the course of this research an insight was gained into the population size, composition, diversity and symbiotic performance of indigenous Bradyrhizobium sp. (Arachis) in major peanut-growing areas of Thailand. These factors are known to influence the outcome of peanut inoculation. The data provide guidelines for the assessment where peanut inoculation is necessary and what are the difficulties in obtaining a response to inoculation on soils harboring indigenous rhizobia. A number of strains of Bradyrhizobium sp. (Arachis) highly effective on local peanut cultivars were selected in the framework of this project. Our finding, that the symbiotic performance of Tainan 9 (the most commonly grown cultivar in Thailand) tended to be inferior with regard to the amount of nitrogen accumulated in peanut shoots indicates that this cultivar should be engaged in a breeding program for improving $N_2$ fixation.

**PROJECT ACTIVITIES**

Drs. B. Kishinevsky and N. Boonkerd participated and present papers at the Networking Workshop held in Banff, Canada in September 1991.

The results of this research were also presented at the 13th North American Symbiotic Nitrogen Fixation Conference, held in Canada in 1991 and at the 9th International Congress on Nitrogen Fixation held

in Mexico in 1992.

In the framework of this project the team in Thailand became familiar with the direct and indirect ELISA techniques including a relatively new modification by using enzyme-labeled protein A as a very sensitive conjugate for rhizobial antigen determination.

**PUBLICATIONS**

1. Boonkerd, N., Wadisirisuk, P. and Thananusont, V. 1991. Effectiveness variation of indigenous rhizobia under different cropping system in Thailand. In *Proceedings of 13th North American Symbiotic Nitrogen Fixation Conference, August 1991, Banff, Canada, p. 46.*

2. Boonkerd, N. and Kishinevsky B. D. 1991. Ecological characterization of peanut Bradyrhizobium strains isolated in Thailand. In *Abstracts Biological Nitrogen Fixation Networking Workshop, September 1991, Banff, Canada, p. 48.*

3. Boonkerd, N., Wadisirisuk, P., Meromi, G. and Kishinevsky B. D. 1992. Quantity, symbiotic performance and serological properties of native peanut rhizobia in soils of Thailand. In *New Horizons in Nitrogen Fixation. Proceedings of the 9th International Congress on Nitrogen Fixation, Cancun, Mexico, December 6-12, 1992, p. 598.*

4. Boonkerd, N., Wadisirisuk, P., Meromi, G. and Kishinevsky B. D. 1993. Population size and $N_2$-fixing activity of native peanut rhizobia in soils of Thailand. Biol. Fertil. Soils, 15: 275-278.

5. Kishinevsky, B. D., Gurfel, D., Boonkerd,N. and Nemas, C. 1993. Serological grouping of indigenous Bradyrhizobium sp. (Arachis) isolated from various soils of Thailand. World Journal of Microbiology and Biotechnology, 9, 635-640.

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Table 1. Populations of peanut rhizobia in soil samples collected from 55 sites in Thailand.

| Regions[a] | Cropping History | Number of Sites Tested | Percent of fields containing: 0 | $10^2$ | $10^2$ - $10^3$ | $10^3$ or more |
|---|---|---|---|---|---|---|
| | | | cells/g of soil | | | |
| N,NE,E,C | Peanut cultivation | 15 | 7 | 7 | 40 | 46 |
| C,E,NE | Fallow having native legumes | 9 | 11 | 22 | 11 | 56 |
| C,N,E,NE | Fields continuously cropped to non-legumes | 24 | 54 | 25 | 13 | 8 |
| E,N,NE | Non-cultivated shrub or forest | 7 | 29 | 29 | 29 | 13 |

[a] E-Eastern; N-Northern; NE-Northeastern; C-Central Plain

Table 2. The distribution of 300 peanut rhizobia isolates in accordance with their nitrogenase activity (µmol $C_2H_4$/plant/h)*

| Site** | Cropping History | Percent of isolates | | | Total of mediocre & ineffective strains (%) |
|---|---|---|---|---|---|
| | | Effective+ | Mediocre | Ineffective | |
| NE-41 | corn | 5 | 65 | 30 | 95 |
| NE-38 | sugar cane | 20 | 45 | 35 | 80 |
| NE-39 | fallow | 20 | 60 | 20 | 80 |
| NE-37 | cassava | 30 | 15 | 55 | 70 |
| C-53 | fallow | 40 | 20 | 40 | 60 |
| C-50 | sugar cane | 55 | 5 | 40 | 45 |
| NE-34 | cowpea | 60 | 35 | 5 | 40 |
| NE-42 | peanut | 70 | 10 | 20 | 30 |
| N-25 | corn | 75 | 15 | 10 | 25 |
| NE-33 | peanut | 80 | 5 | 15 | 20 |
| N-23 | peanut | 85 | 5 | 10 | 15 |
| N-22 | shrub | 85 | 5 | 10 | 15 |
| N-29 | peanut | 85 | 15 | 0 | 15 |
| E-12 | sugar cane | 85 | 5 | 10 | 15 |
| N-21 | peanut | 90 | 5 | 5 | 10 |

* Separate experiments were carried out for each group of 20 isolates and each isolate was tested in 3 replicates. The acetylene reduction values (µmol $C_2H_4$/plant/h) of plants inoculated with an inoculant strain THA205 were different in each experiment and varied between 2.04 and 15.3.

**Regions: NE - Northeastern; C - Central plain, N - Northern; E - Eastern.

\+ The designations "effective", "mediocre" and "ineffective" describe isolates which display nitrogenase activity: a) higher than or not significantly inferior; b) up to 50% below; and c) of values lesser than 50% of that of the recommended strain THA205.

Table 3. Symbiotic performance of 43 Bradyrhizobium isolates collected from seven sites in Thailand.

| Site* | Cropping history | No. of strains tested | Total N (mg/pot) accumulated in peanut shoots by each of the strains tested |
|---|---|---|---|
| N-21 | Peanut cultivation | 5 | 102.6 ± 14.2**; 109.5 ± 8.5; 128.5 ± 3.0 133.8 ± 13.1; 135.3 ± 19.5 |
| N-22 | Non-cultivated shrub | 7 | 86.0 ± 13.1; 121.1 ± 10.6; 121.8 ±16.7; 125.6 ± 9.1; 133.3 ± 13.2; 150.7 ± 4.7; 153.2 ± 7.7. |
| N-31 | Non-legumes | 10 | 69.2 ± 0.9; 79.7 ± 1.2; 101.6 ± 4.3; 102.1 ± 20.8; 126.8 ± 7.4; 127.1 ± 4.3; 128.2 ± 4.0; 143.4 ±13.5; 148.1 ±13.4; 154.4 ± 2.3. |
| E-16 | Fallow having native legumes | 5 | 123.7 ± 10.0; 125.7 ± 11.7 136.1 ±11.9; 139.3 ± 14.2; 141.6 ± 8.9. |
| E-17 | Non-legumes | 4 | 127.9 ± 10.8; 129.2 ± 10.2; 135.2 ± 8.7; 146.0 ± 5.4. |
| E-7 | Mungbean cultivation | 5 | 111.4 ± 7.1 ; 116.3 ± 3.4 ; 118.1 ±12.0; 129.9 ± 10.3; 155.8 ± 13.4. |
| E-10 | Fallow having native legumes | 7 | 72.7 ± 10.3; 92.5 ± 4.3; 92.7 ±13.3 97.7 ± 1.6; 99.6 ± 16.3; 120.7 ± 4.0; 151.0 ± 18.7. |
| Inoculum strain 280A | | 1 | 136.4 ± 3.2 |
| Uninoculated | | | 70.9 ± 6.2 |

* N - North; E - East.

** Means ± S.E.

Table 4 Somatic ELISA cross reactions of *Bradyrhizobium* sp. (*Arachis*)

| | Indirect ELISA absorbance values ($A_{405}$ nm) with antisera | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antigen | 280A | 7/20E | 2212A | 31/14N | CB756 | 31/9N | 22/6N | 30/4N | 34/6NE | 25/3N | 28/15N | 10/14E | Minimal antigenic constitution+ |
| 280A | 1.94* | 0.17 | 0.36 | 0.17 | - | - | 0.39 | - | - | - | - | - | I,II,V,VII,IX,XI |
| 7/20E | 0.53 | 1.69 | - | - | - | - | - | - | - | - | - | - | I,III |
| 2212A | 0.45 | - | 1.28 | - | - | - | - | - | - | - | - | - | II,IV |
| 31/14N | 0.29 | - | - | 1.94 | - | - | - | - | - | - | - | - | V,VI |
| 22/6N | - | - | - | - | - | - | 1.10 | - | - | - | - | - | VII,VIII |
| CB756 | 0.31 | - | - | - | 0.64 | 0.20 | - | - | - | - | - | - | IX, X |
| 31/9N | - | - | - | - | 0.51 | 0.72 | - | - | - | - | - | - | X, XII |
| 30/4N | - | - | - | - | - | - | - | 0.94 | - | - | - | - | XIII |
| 34/6NE | - | - | - | - | - | - | - | - | 1.12 | - | - | - | XIV |
| 25/3N | - | - | - | - | - | - | - | - | - | 1.4 | - | - | XV |
| 28/15N | - | - | - | - | - | - | - | - | - | - | 1.10 | - | XVI |
| 10/14E | - | - | - | - | - | - | - | - | - | - | - | 1.20 | XVII |

-, No antigen-antibody reaction

* Values are means of duplicate wells, less the negative control mean; results for homologous reactions are underlined. Dilutions of antisera, 1:500 (antiserum 30/4N, 1:100). Dilutions of enzyme-labeled protein A, 1:2000. Antigen -$10^8$ cells/ml.

\+ Based on data from Tables 1 and 2.

Table 5. Antigenic heterogeneity among peanut *Bradyrhizobium* strains as revealed by cross-adsorption of antibodies

| Adsorbed antibodies | ELISA reactivity* with antigens | | | | | |
|---|---|---|---|---|---|---|
| | 280A | 7/20E | 2212A | 31/14N | CB756 | 31/9N |
| 280A(unadsorbed) | +++ | ++ | ++ | ++ | ++ | - |
| 280A(7/20E)+* | ++ | - | | | | |
| 280A(2212A) | +++ | | - | | | |
| 280A(31/14N) | +++ | | | - | | |
| 7/20E(unadsorbed) | + | +++ | - | - | - | - |
| 7/20E(280A) | - | +++ | | | | |
| 2212A(unadsorbed) | ++ | - | +++ | - | - | - |
| 2212A(280A) | | | +++ | | | |
| 31/14N(unadsorbed) | ++ | - | - | +++ | - | - |
| 31/14N(280A) | - | | | +++ | | |
| CB756(unadsorbed) | - | - | - | - | ++ | ++ |
| CB756(31/9N) | | | | | + | - |
| 31/9N(unadsorbed) | - | - | - | - | + | ++ |
| 31/9N(CB756) | | | | | - | +++ |

* Visual assessment after incubation for 30 min with the enzyme substrate: +++, bright yellow; ++ yellow; + very pale yellow; - not detectable. For concentrations of antisera enzyme-labeled protein A and antigens, see footnote to Table 1.

\+ Adsorbing antigen is indicated in parenthesis.

Table 6 Indirect ELISA analysis of somatic antigens of 243 isolates of peanut *Bradyrhizobium* collected in Thailand.

| Field† | Cropping history | No. of isolates evaluated | Not reacting with any antisera | Per cent of isolates reacting (A405>0.20) with antisera of serogroups*: 1 280A only | 2 280A 2212A | 3 280A 31/14N | 4 280A 7/20E | 5 280A 22/6N | 6 280A 30/4N | 7 280A 31/14N 22/6N | 8 280A 2212A 7/20E | 9 280A 22/6N 2212N | 10 280A 2212N 31/14N | 11 280A 2212A 30/4N | 12-18 4 or more antisera including 280A | 19-29 Other sero groups |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E-7 | Mungbean cultivation | 17 | - | 18 | - | 18 | 46 | 12 | - | - | 6 | - | - | - | - | - |
| E-10 | Fallow with native legumes | 16 | 18 | - | - | 6 | - | - | - | 12 | - | - | - | - | 12 | 52 |
| E-16 | Fallow with native legumes | 13 | - | 46 | 54 | - | - | - | - | - | - | - | - | - | - | - |
| N-21 | Peanut cultivation | 18 | - | 12 | 70 | - | - | 6 | - | - | - | - | - | - | 6 | 6 |
| N-23 | Peanut cultivation | 14 | 13 | - | 53 | 7 | - | - | - | - | 20 | - | - | - | 7 | - |
| N-28 | Peanut cultivation | 13 | - | 35 | 44 | - | - | - | - | - | 14 | - | - | - | 7 | - |
| N-25 | Cotton and corn cultivation | 16 | 19 | - | - | 12 | - | - | - | 19 | - | - | - | - | 7 | 43 |
| N-30 | Soybean cultivation | 16 | - | - | - | - | - | - | 76 | - | - | - | - | 6 | 6 | 12 |
| N-22 | Shrub, teak trees, mimosa | 15 | - | 19 | 14 | - | 7 | 7 | - | - | - | - | - | - | - | 53 |
| N-31 | Cassava having mimosa weeds | 14 | 6 | 19 | 25 | 6 | 6 | - | - | 6 | 6 | - | - | 6 | - | 20 |
| NE-33 | Corn rotation with peanuts | 18 | - | 56 | 6 | 6 | - | - | - | - | - | - | - | - | 32 | - |
| NE-34 | Cowpea cultivation | 20 | 20 | 20 | 10 | 5 | - | - | - | 15 | - | - | - | - | - | 30 |
| NE-36 | Cover legumes | 19 | - | 64 | 31 | - | - | - | - | - | - | - | 5 | - | - | - |
| NE-39 | Fallow with native legumes | 18 | - | 52 | 18 | 6 | - | - | - | 18 | - | - | 6 | - | - | - |
| M-47 | Corn rotation with peanuts | 16 | - | 38 | 25 | - | - | 25 | - | - | - | 12 | - | - | - | - |

* The antisera displayed in a column indicate that the given isolate(s) reacted with all the antisera in the serogroup

† Regions: E-Eastern; N-Northern; NE-Northeastern; M-Central Plain.

Table 7 Serological distribution of peanut Bradyrhizobium isolates from seven soils in which additional serogroups were detected besides the 280A serocluster.

| Site* | Per cent of isolates reacting with 280A antisera | Per cent of isolates reacting ($A_{405} \geq 0.20$) with antisera of serogroups: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
| | | 31/14N | 30/4N | 31/9N | 22/6N | 25/3N | 10/14E | 34/6NE | 2212A 10/14E | 10/14E CB756 | 31/9N CB756 | 30/4N 22/6N |
| E-10 | 48 | 7 | - | - | - | - | 24 | - | 7 | 14 | - | - |
| N-21 | 94 | - | 6 | - | - | - | - | - | - | - | - | - |
| N-25 | 57 | 13 | - | - | - | 30 | - | - | - | - | - | - |
| N-30 | 88 | - | 12 | - | - | - | - | - | - | - | - | - |
| N-22 | 47 | - | - | - | 53 | - | - | - | - | - | 7 | - |
| N-31 | 80 | 7 | - | - | - | - | - | - | - | - | - | 6 |
| NE-34 | 70 | - | 22 | 8 | - | - | - | - | - | - | - | - |

* The location and cropping history of the fields are indicated in Table 3

Table 8. Response of peanut Bradyrhizobium isolates to selected antibiotics*

| Site | Cropping History | No. of isolates | Resistant to antibiotics: Chl | Neo | Pen | Kan | Spe | Rif | Str |
|---|---|---|---|---|---|---|---|---|---|
| N-21 | Peanuts | 17 | 16 | 16 | 13 | 8 | 4 | 5 | 0 |
| N-28 | Peanuts | 15 | 15 | 15 | 15 | 7 | 11 | 9 | 0 |
| N-23 | Peanuts | 15 | 15 | 15 | 15 | 15 | 15 | 9 | 13 |
| N-31 | Cassava | 16 | 14 | 14 | 8 | 5 | 4 | 4 | 0 |
| N-30 | Soybeans | 16 | 16 | 15 | 14 | 2 | 5 | 1 | 4 |
| N-25 | Cotton, corn | 16 | 15 | 15 | 0 | 0 | 0 | 0 | 0 |
| N-22 | Shrub | 15 | 15 | 15 | 14 | 15 | 11 | 4 | 4 |
| NE-39 | Fallow | 18 | 18 | 18 | 16 | 18 | 18 | 4 | 0 |
| NE-36 | Cover legumes | 19 | 19 | 19 | 5 | 16 | 13 | 13 | 0 |
| NE-34 | Cowpeas | 19 | 19 | 19 | 15 | 4 | 5 | 6 | 0 |
| NE-33 | Peanut | 19 | 19 | 19 | 5 | 19 | 7 | 11 | 0 |
| E-10 | Fallow | 17 | 16 | 17 | 7 | 2 | 5 | 2 | 0 |
| E-7 | Mungbean | 18 | 18 | 18 | 11 | 16 | 13 | 7 | 0 |
| E-16 | Fallow | 15 | 15 | 15 | 15 | 14 | 5 | 15 | 13 |
| M-47 | Peanuts, corn | 16 | 14 | 14 | 10 | 12 | 12 | 7 | 4 |
| | Total | 251 | 244 | 244 | 163 | 153 | 128 | 97 | 38 |
| | % | | 97.2 | 97.2 | 64.9 | 61.0 | 51.0 | 38.6 | 15.1 |

*The concentration of each of the antibiotics tested was 50 ug/ml. Isolates were scored as resistant based on their ability to grow on YMA containing antibiotics: Chl, chloramphenicol; Neo, neomycin; Pen, penicillin; Kan, kanamycin; Spe, spectinomycin; Rif, rifampicin; Str, streptomycin.
Isolates were scored for resistance after 12 days of growth at 28°C.

Table. 9. Antibiotic resistance patterns of field isolates of Bradyrhizobium sp (Arachis).

| Group no. | No. of isolates in group | Antibiotics | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Chl | Neo | Pen | Kan | Spe | Rif | Str |
| 1 | 13 | +* | + | + | + | + | + | + |
| 2 | 25 | + | + | + | + | + | + | - |
| 3 | 6 | + | + | + | + | + | - | + |
| 4 | 13 | + | + | + | + | - | + | + |
| 5 | 40 | + | + | + | + | + | - | - |
| 6 | 10 | + | + | + | + | - | + | - |
| 7 | 3 | + | + | + | - | + | + | - |
| 8 | 13 | + | + | - | + | + | + | - |
| 9 | 3 | + | + | + | - | + | - | + |
| 10 | 2 | + | + | + | - | - | + | + |
| 11 | 1 | + | + | - | + | + | - | + |
| 12 | 6 | + | + | + | + | - | - | - |
| 13 | 12 | + | + | + | - | - | + | - |
| 14 | 3 | + | + | + | - | + | - | - |
| 15 | 15 | + | + | - | + | + | - | - |
| 16 | 5 | + | + | - | + | - | + | - |
| 17 | 30 | + | + | + | - | - | - | - |
| 18 | 6 | + | + | - | + | - | - | - |
| 19 | 4 | + | + | - | - | + | - | - |
| 20 | 1 | + | + | - | - | - | + | - |
| 21 | 30 | + | + | - | - | - | - | - |
| 22 | 1 | + | - | + | - | - | - | - |
| 23 | 1 | + | - | - | - | - | - | - |
| 24 | 3 | - | + | - | - | - | - | - |
| 25 | 5 | - | - | - | - | - | - | - |

* +, resistant, i.e. growth; - sensitive, i.e. no growth.

Table 10. The most commonly observed groups of antibiotic resistance in 251 Bradyrhizobium sp. (Arachis) strains.

| IAR group no. | Resistant to: | % Occurrence |
|---|---|---|
| 1. | Chl, Neo, Pen, Kan, Spe, Rif, Str | 5 |
| 2. | Chl, Neo, Pen, Kan, Spe, Rif | 10 |
| 4. | Chl, Neo, Pen, Kan, Str. Rif | 5 |
| 5. | Chl, Neo, Pen, Kan, Spe | 16 |
| 8. | Chl, Neo, Spe, Kan, Rif | 5 |
| 13. | Pen, Neo, Chl, Rif | 5 |
| 15. | Neo, Kan, Chl, Spe | 6 |
| 17. | Pen, Neo, Chl | 12 |
| 21. | Neo, Chl | 12 |

Table 11. Distribution of isolates from different regions of Thailand within each intrinsic antibiotic resistance cluster (IAR).

| IAR group no. | Antibiotic resistance patterns | Site* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N21 | N28 | N23+24 | N31 | N30 | N25 | N22 | NE39 | NE36 | NE34 | NE33 | E10 | E7 | E16 | M47 |
| 1. | Chl+,Neo+Pen+,Kan+,Spe+,Rif+,Str+ | | | 8 | | | | | | | | | | | 4 | 1 |
| 2. | Chl+,Neo+Pen+,Kan+,Spe+,Rif+(Str-) | | 6 | 1 | 2 | 1 | | | 4 | 1 | 1 | 1 | 2 | 1 | 1 | 4 |
| 3. | Chl+,Neo+Pen+,Kan+,Spe+,Str+(Rif-) | | | 5 | | 1 | | | | | | | | | | |
| 4. | Chl+,Neo+Pen+,Kan+,Rif+,Str+(Spe-) | | | | | | | 4 | | | | | | | 9 | |
| 5. | Chl+,Neo+Pen+,Kan+,Spe+,(Rif-Str-) | 2 | 1 | 1 | 1 | | | 10 | 12 | | 2 | | | 6 | | 5 |
| 6. | Chl+,Neo+Pen+,Kan+,Rif+,(Spe-Str-) | 2 | | | 1 | | | | | | | 3 | | 4 | | |
| 7. | Chl+,Neo+Pen+,Spe+Rif+(Kan-, Str-) | | 2 | | | | | | | | 1 | | | | | |
| 8. | Chl+,Neo+Spe+,Rif+Kan+(Pen-,Str-) | | | | | | | | | 7 | | 4 | | 2 | | |
| 9. | Chl+,Neo+Spe+,Pen+,Str+(Kan-,Rif-) | | | | | 3 | | | | | | | | | | |
| 10. | Chl+,Neo+Pen+,Rif+,Str+(Kan-,Spe-) | | | | | | | | | | | | | | | 2 |
| 11. | Chl+,Neo+,Kan+ Spe+,Str+(Pen-,Rif-) | | | | | | | | | | | | | | | 1 |
| 12. | Chl+,Neo+,Kan+,Pen+(Spe-,Rif-,Str-) | 3 | | | 1 | | | | | 1 | | 1 | | | | |
| 13. | Chl+,Neo+,Pen+,Rif+(Kan-,Spe-,Str-) | 2 | 1 | | 1 | | | | | 3 | 4 | | | | 1 | |
| 14. | Chl+,Neo+,Pen+,Spe+(Kan-,Rif-,Str-) | | 2 | | | | | | | | | | 1 | | | |
| 15. | Chl+,Neo+,Kan+,Spe+(Pen-,Rif-,Str-) | | | | | | | 1 | 2 | 5 | 1 | 2 | | 3 | | 1 |
| 16. | Chl+,Neo+,Kan+Rif+(Pen-,Spe-,Str-) | | | | | | | | | 2 | | 3 | | | | |
| 17. | Chl+,Neo+,Pen+(Kan-,Spe-,Rif-,Str-) | 4 | 3 | | 4 | 8 | | | | | 7 | | 4 | | | |
| 18. | Chl+,Neo+Kan+(Pen-,Spe-,Rif-,Str-) | 1 | | | | | | | | | | 5 | | | | |
| 19. | Chl+,Neo+Spe+(Pen-,Kan-,Rif-,Str-) | | | | 1 | | | | | | | | 2 | 1 | | |
| 20. | Chl+,Neo+,Rif+(Pen-,Kan-,Spe-,Str-) | 1 | | | | | | | | | | | | | | |
| 21. | Chl+,Neo+(Pen-,Kan-,Spe-,Rif-,Str-) | | | | 2 | 2 | 15 | | | | 3 | | 7 | 1 | | |
| 22. | Chl+,Pen+(Neo-,Kan-,Spe,Rif-,Str-) | | | | | 1 | | | | | | | | | | |
| 23. | Chl+(Neo-,Kan-,Spe-,Rif-,Pen-,Str-) | 1 | | | | | | | | | | | | | | |
| 24. | Neo+(Chl-,Pen-,Kan-,Spe-,Rif-,Str-) | 1 | | | 1 | | | | | | | | 1 | | | |
| 25. | (Chl-,Neo-,Pen-,Kan-,Spe-,Rif-,Str-) | | | | 2 | | 1 | | | | | | | | | 2 |

Regions: N-Northern; NE - Northeastern; E - Eastern; M - Central plain

Table 12. Distribution of isolates within each intrinsic antibiotic resistance fingeprint by their serogroup identity.

| IRA group* | Number of isolates reacting with antisera of serogroups**: 280A only | 280A 2212A | 280A 31/14N | 280A 7/20E | 280A 22/6N | 280A 30/4N | 280A 31/14N 22/6N | 280A 2212A 7/20E | 280A 22/6N 2212A | 280A 2212A 31/14N | 280A 2212A 30/4N | 4 or more antisera including 280A | Other sero-groups |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 1 | 5 | 1 | - | - | - | - | - | 6 | - | - | - | 1 | - |
| 2 | 16 | 6 | 1 | - | - | - | - | - | - | - | - | 2 | - |
| 3 | 1 | 1 | - | - | - | - | - | - | - | - | - | - | 4 |
| 4 | 11 | 2 | - | - | - | - | - | - | 1 | - | - | - | - |
| 5 | 21 | 6 | 1 | 3 | - | - | - | - | 4 | - | - | - | 8 |
| 6 | 3 | 4 | - | - | - | - | - | 1 | - | - | - | 2 | - |
| 7 | - | 2 | - | - | - | - | - | - | - | - | - | - | 1 |
| 8 | 8 | 1 | - | - | 2 | - | - | - | - | - | - | 2 | - |
| 9 | 3 | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | 1 | 1 | - | - | - | - | - | - | - | - | - | - | - |
| 11 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| 12 | 1 | 3 | - | - | - | - | - | - | - | - | - | 1 | 1 |
| 13 | 7 | 5 | - | - | - | - | - | - | - | - | - | - | - |
| 14 | 1 | - | - | - | - | - | - | 1 | - | - | - | - | 1 |
| 15 | 3 | 5 | - | 3 | - | - | - | - | 1 | 1 | - | 1 | 1 |
| 16 | 4 | 1 | - | - | - | - | - | - | - | - | - | - | - |
| 17 | 4 | 4 | - | - | - | - | 1 | - | - | - | - | 1 | 20 |
| 18 | 6 | - | - | - | - | - | - | - | - | - | - | - | - |
| 19 | - | - | 1 | - | - | - | - | - | - | - | - | - | 3 |
| 20 | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| 21 | 12 | - | - | - | - | 2 | 2 | - | - | - | - | - | 14 |
| 22 | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| 23 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| 24 | 1 | 1 | - | - | - | - | - | - | - | - | - | - | 1 |
| 25 | 1 | - | - | - | - | - | - | - | - | - | - | - | 4 |

* The antibiotic resistance patterns of each group are indicated in Table 11.

** The antisera displayed in a column indicate that the given isolate(s) reacted with all the antisera in the serogroup.

Table 13. Relationship between serological properties and SDS-gel protein patterns of 64 Bradyrhizobium spp. (Arachis) isolates.

| Origin | Number of isolates tested | Gel type | Isolates belonging to: Sero-cluster 280A | Other sero groups |
|---|---|---|---|---|
| N-30 | 9 | (1) | | 1 |
| | | (3, 5, 7, 8) | 5,7,8 | 3 |
| | | (4, 12) | 12 | 4 |
| | | (19) | 19 | |
| N-28 | 10 | (15, 16, 20) | 16, 20 | 15 |
| | | 13 | 13 | |
| | | (7, 8, 3) | 7, 8, 3 | |
| | | (4, 5, 12) | 4, 5 | 12 |
| N-25 | 9 | 2 | | 2 |
| | | (1,3,7,10,11,19) | | 1,3,7,10,11,19 |
| | | (4, 17) | 4, 17 | |
| N-23 | 8 | (1,3,6,10,12,15,1) | All | |
| | | 5 | 5 | |
| | | 16 | | 16 |
| N-22 | 9 | (6, 7, 10, 12) | | 6, 7, 10, 12 |
| | | (13, 20) | 13 | 20 |
| | | (5) | 5 | |
| | | (17, 18) | 17, 18 | |
| | | | 1, 5 | |
| | | | 7, 11 | |
| | | | 16,18,19 | |
| NE-39 | 10 | (1, 5) | 1, 5 | |
| | | (7, 11) | 7, 11 | |
| | | (16, 18, 19) | 16,18,19 | |
| | | (4, 20) | 4, 20 | |
| E-7 | 9 | 17 | 17 | |
| | | (1,8,10,13,19,20 | All | |
| | | (2, 6) | 2, 6 | |

Table 14 . Analaysis of variance for top dry weight, nitrogen content and total nitrogen for four peanut cultivars inoculated by different Rhizobium strains (48 day old plants).

| Source of variation | df | Mean squares: Top dry weight (g/pot) | N content (%) | Total N (mg/pot) |
|---|---|---|---|---|
| Cultivar | 3 | 331.5* | 0.75* | 79658* |
| Rhizobium | 5 | 116.5* | 3.20* | 187506* |
| Cultivar x Rhizobium | 15 | 7.9 | 0.12 | 8047 |
| Error | 46 | 6.4 | 0.06 | 7500 |

* Significant at P = 0.05

| Cultivar | Mean | | |
|---|---|---|---|
| Hanoch | 17.04 A | 1.78 B | 317.78A |
| Shulamit | 13.95 B | 1.86 B | 280.71 A |
| Valencia | 12.61 B | 1.96 B | 262.69 A |
| Tainan 9 | 6.80 C | 2.24 A | 162.17 B |
| **Strain** | | | |
| 280A | 15.6 A | 2.35 A | 410.4 A |
| E22/7 | 15.3 A | 2.27 B | 351.0 A |
| E7/20 | 15.1 A | 2.22 B | 331.5 A |
| N27/3 | 11.4 B | 1.85 C | 199.2 B |
| N31/14 | 9.2 B | 1.50 D | 130.2 BC |
| Control | 9.0 B | 1.27 E | 112.7 C |

Table 15. Analysis of variance for top dry weight, nitrogen content and total nitrogen for four peanut cultivars inoculated by different Rhizobium strains (73 day old plants).

| Source of variation | Degrees of freedom | Mean squares: Top dry weight (g/pot) | N content (%) | Total N (mg/pot) |
|---|---|---|---|---|
| Cultivar | 3 | 362.1* | 1.55* | 10680 |
| Rhizobium | 5 | 1281.3* | 2.40* | 825724* |
| Cultivar x Rhizobium | 15 | 76.9 | 0.28 | 50883 |
| Error | 46 | 19.0 | 0.06 | 12144 |

*Significant at P = 0.05

| | Mean | | |
|---|---|---|---|
| Cultivar | | | |
| Valencia | 25.5 a | 1.6 b | 428.9 a |
| Hanoch | 23.9 a | 1.6 b | 415.6 a |
| Shulamit | 23.1 a | 1.6 b | 419.4 a |
| Tainan 9 | 16.7 b | 2.1 a | 380.7 a |
| Strain | | | |
| 280A | 33.5 a | 2.2 a | 730.0 a |
| E7/20 | 28.4 b | 1.9 b | 517.5 b |
| E22/7 | 27.5 b | 1.9 b | 513.9 b |
| N21/3 | 19.7 c | 2.0 b | 395.3 c |
| N31/14 | 13.9 d | 1.5 c | 194.6 d |
| Control | 10.7 e | 1.1 d | 115.6 e |

Table 16 Analysis of variance for top dry weight, nitrogen content and total nitrogen for four peanut cultivars inoculated by different Rhizobium strains (60 day old plants).

| Source of variation | Degrees of freedom | Mean squares | | |
|---|---|---|---|---|
| | | Top dry weight (g/pot) | N content (%) | Total N (mg/pot) |
| Cultivar | 3 | 984.0* | 1.5* | 500944* |
| Rhizobium | 8 | 344.4* | 2.5* | 450024* |
| Cultivar x Rhizobium | 24 | 44.9 | 0.4 | 68464 |
| | 105 | 15.7 | 0.08 | 15732 |

*Significant at P = 0.05

| | | | |
|---|---|---|---|
| Cultivar | | | |
| KK 60-1 | 24.4b | 2.3a | 568.7a |
| KK 60-3 | 15.7d | 1.9b | 316.1c |
| Shulamit | 28.1a | 1.9b | 562.9a |
| Tainan 9 | 21.7c | 2.2a | 496.7b |
| Strain | | | |
| 280A | 27.0a | 2.4a | 673.6 a |
| N-22/13 | 27.0 a | 2.5 a | 655.3a |
| N-31/19 | 26.8a | 2.4 a | 640.9 a |
| NE39/12 | 22.6b | 2.1 bc | 513.2 b |
| NE39/16 | 22.5b | 2.3 cb | 517.9 b |
| N-25/2 | 22.4b | 1.8 d | 424.0 b |
| N-25/9 | 21.1b | 2.0 cd | 409.6 b |
| N25/7 | 20.1b | 1.9 d | 397.8 b |
| Control | 12.1c | 1.2 e | 142.5 c |

Table 17 Effect of inoculation and N application on nodule number (/pl) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 36 | 27 | 55 | 39 |
| -N -R | 39 | 37 | 26 | 34 |
| +R | 56 | 50 | 32 | 46 |
| Avg. | 44 | 38 | 37 | |

CV: Main plot = 82.5% ; sub-plot = 45.8%

Table 18. Effect of inoculation and N application on nodule dry weight (mg/pl) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 29.4 | 25.7 | 59.7 | 38.2 |
| -N -R | 44.4 | 45.4 | 65.5 | 51.7 |
| +R | 52.7 | 51.5 | 30.8 | 45.0 |
| Avg. | 42.2 | 40.9 | 52.0 | |

CV: cultivars = 168.5% ; treatment = 77.5%

Table 19. Effect of inoculation and N application on specific ARA (Umole $C_2H_4$/g.ndwt/h) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 115.7 | 146.3 | 54.4 | 90.7 |
| -N -R | 81.7 | 80.3 | 45.2 | 64.6 |
| +R | 71.4 | 77.0 | 97.9 | 79.6 |
| Avg. | 85.3 | 92.8 | 57.8 | |

CV: Cultivars = 81.6% ; Treatment = 30.2%

Table 20 Effect of inoculation and N application on shoot dry weight (g/pi) at 60 days of planting of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 13.8 | 19.8 | 17.8 | 17.2 |
| -N -R | 20.9 | 16.8 | 16.2 | 18.0 |
| +R | 19.1 | 17.2 | 17.1 | 17.8 |
| Avg. | 18.0 | 17.9 | 17.1 | |

CV: cultivars = 77.4% ; treatment = 29.3%

Table 21. Effect of inoculation and N application on plant N (% N) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 2.9 | 3.1 | 3.1 | 3.0 |
| -N -R | 2.7 | 2.8 | 2.8 | 2.7 |
| +R | 2.8 | 2.9 | 2.8 | 2.8 |
| Avg. | 2.8 | 2.8 | 2.8 | |

CV: Cultivars = 17.9% ; Treatment = 9.1%

Table 22. Effect of inoculation and N application on pod fresh weight (kg/ha) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 2600 | 3300 | 3150 | 3016 a |
| -N -R | 2125 | 2675 | 2500 | 2433 b |
| +R | 2325 | 2575 | 2425 | 2441 b |
| Avg. | 2350 | 2850 | 2691 | |

CV: cultivars = 24.4% ; treatment = 10,3%

Table 23. Effect of inoculation and N application on pod dry weight (kg/ha) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 1425 bc | 1950 a | 1900 a | 1758 a |
| -N -R | 1300 c | 1625 b | 1525 bc | 1483 b |
| +R | 1450 bc | 1550 b | 1475 bc | 1492 b |
| Avg. | 1392 | 1708 | 1633 | |

CV: Cultivars = 23.3% ; Treatment = 8.8%

Table 24. Effect of inoculation and N application on shelling percentage (%) of 3 peanut cultivars

| Treatments | Peanut cultivars | | | Avg. |
|---|---|---|---|---|
| | Tainan 9 | KK 60-1 | KK 60-3 | |
| +N | 58.5 | 63.5 | 58.7 | 60.2 a |
| -N -R | 56.5 | 56.5 | 57.2 | 56.7 b |
| +R | 60.0 | 61.2 | 61.2 | 60.8 a |
| Avg. | 58.3 | 60.4 | 59.0 | |

CV: cultivars = 7.5% ; treatment = 4.9%

Table 25. Effect of selected effective rhizobia on peanut grown in Sakol Nakorn.

| Treatment | Inoculant strain (%) | Nod wt mg/pl | Nod no /pl | ARA µmole/pl | Top dry wt g/pl | Pod dry wt kg/ha | Shell % |
|---|---|---|---|---|---|---|---|
| Control | - | 35 b-d | 71 | 21.05 a-d | 7.48 | 741 | 62.98 |
| THA 205 | 7 | 49 a-c | 68 | 26.13 a-c | 7.36 | 854 | 70.25 |
| 280 A | 31 | 43 a-d | 76 | 26.68 ab | 9.33 | 455 | 66.49 |
| 2212 A | 27 | 62 a | 96 | 28.62 a | 8.12 | 633 | 63.69 |
| E-16-7 | 21 | 46 a-c | 81 | 18.03 b-d | 7.00 | 638 | 64.57 |
| E-7-20 | - | 33 cd | 59 | 16.16 d | 6.90 | 566 | 59.45 |
| NE-36-19 | 24 | 64 a | 108 | 25.48 a-c | 8.39 | 591 | 68.67 |
| M-47-12 | 17 | 56 ab | 112 | 26.39 a-b | 8.00 | 536 | 62.79 |
| N-28-12 | 23 | 37 b-d | 67 | 17.70 cd | 8.00 | 480 | 66.45 |
| N-Fert. (75 kg/ha) | - | 22 d | 68 | 14.97 d | 7.99 | 783 | 61.58 |
| Mean | | 45 | 81 | 22.12 | 4.5742 | 653 | 64.69 |
| F-test | | ** | NS | ** | NS | NS | NS |
| CV.(%) | | 30.70 | 32.30 | 24.00 | 22.80 | 33.40 | 13.60 |

Table 26. Effect of selected effec 've rhizobia on peanut grown in Udorn Thani.

| Treatment | ARA (µmole/pl/h) | Primary root Nod wt (mg/pl) | Primary root Nod no /pl | Secondary root Nod wt (mg/pl) | Secondary root Nod no /pl | Total Nod wt (mg/pl) | Total Nod no /pl |
|---|---|---|---|---|---|---|---|
| Control | 11.51 | 35 | 29 | 83 | 168 | 118 | 197 |
| THA 205 | 9.47 | 37 | 46 | 106 | 246 | 143 | 292 |
| 280A | 11.79 | 67 | 70 | 84 | 155 | 151 | 225 |
| 2212A | 14.53 | 90 | 88 | 98 | 200 | 188 | 288 |
| E-16/7 | 11.08 | 52 | 50 | 122 | 234 | 174 | 284 |
| E-7/20 | 12.10 | 61 | 62 | 137 | 275 | 198 | 337 |
| NE-36/19 | 11.40 | 60 | 67 | 77 | 158 | 137 | 225 |
| M-47/12 | 10.93 | 63 | 65 | 113 | 249 | 176 | 314 |
| N-28/12 | 13.00 | 51 | 53 | 92 | 227 | 143 | 280 |
| N-Fert | 6.77 | 27 | 32 | 61 | 142 | 88 | 174 |
| F-test | NS | * | * | NS | - | - | NS |
| CV(%) | 34.4 | 44.5 | 40.2 | 39.2 | - | - | 33.4 |

Table 27. Effect of selected effective rhizobia on peanut grown in Udorn Thani.

| Treatment | Inoculant strain (%) | Pod dry weight (kg/ha) | Shelling (%) | 1000 pods | | |
|---|---|---|---|---|---|---|
| | | | | Pod weight (g) | Seed weight (g) | Seed no. |
| Control | - | 2115 | 75.4 | 984.4 | 742.0 | 2010 |
| THA205 | 34 | 2052 | 74.5 | 957.7 | 713.6 | 1942 |
| 280A | 56 | 2209 | 75.6 | 968.8 | 732.4 | 1927 |
| 2212A | 32 | 2271 | 75.8 | 982.2 | 745.2 | 1990 |
| E-16/7 | 33 | 2119 | 75.1 | 958.3 | 719.9 | 1924 |
| E-7/20 | - | 2063 | 75.2 | 971.0 | 730.7 | 1986 |
| NE-36/19 | 23 | 2260 | 75.2 | 965.6 | 726.6 | 1920 |
| M-47/12 | 15 | 1915 | 75.4 | 945.4 | 712.7 | 1927 |
| N-28/12 | 6 | 1890 | 75.2 | 947.7 | 712.7 | 1914 |
| N-Fert. | - | 1966 | 75.2 | 948.0 | 712.9 | 1702 |
| F-test | | NS | NS | - | - | - |
| CV(%) | | 14 | 0.8 | - | - | - |

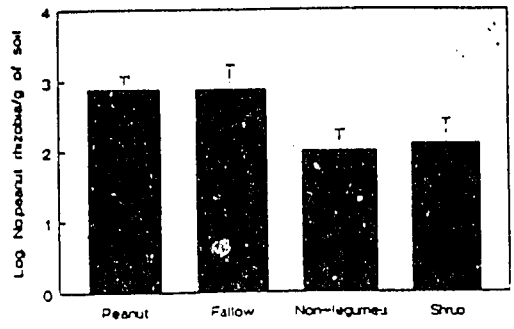


Fig.1. Peanut rhizobia populations in fields with different cropping history in various regions of Thailand.

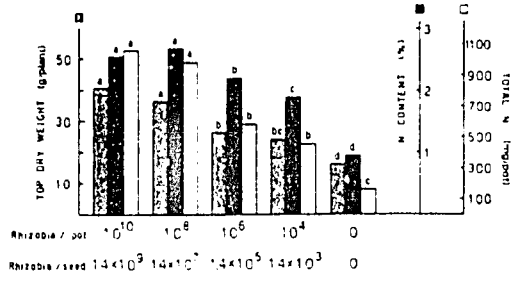


Fig.2. Influence of Bradyrhizobium cell concentration on growth and nitrogen accumulation in peanut plants.

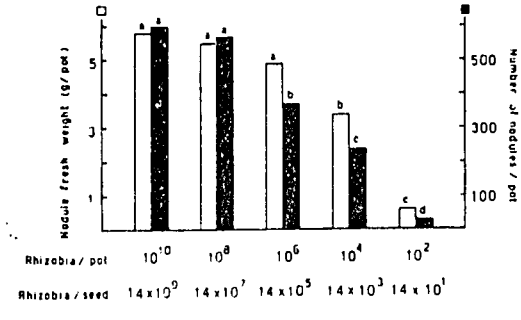


Fig.3. Influence of Bradyrhizobium cell concentration on the rate of peanut plant nodulation.

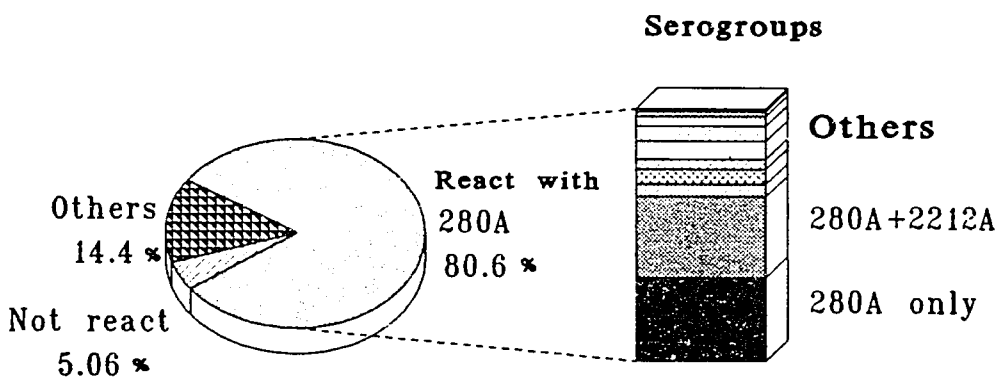


Fig. 4. Serogrouping of indigenous rhizobia based on indirect ELISA analysis of 243 peanut Bradyrhizobium isolates.

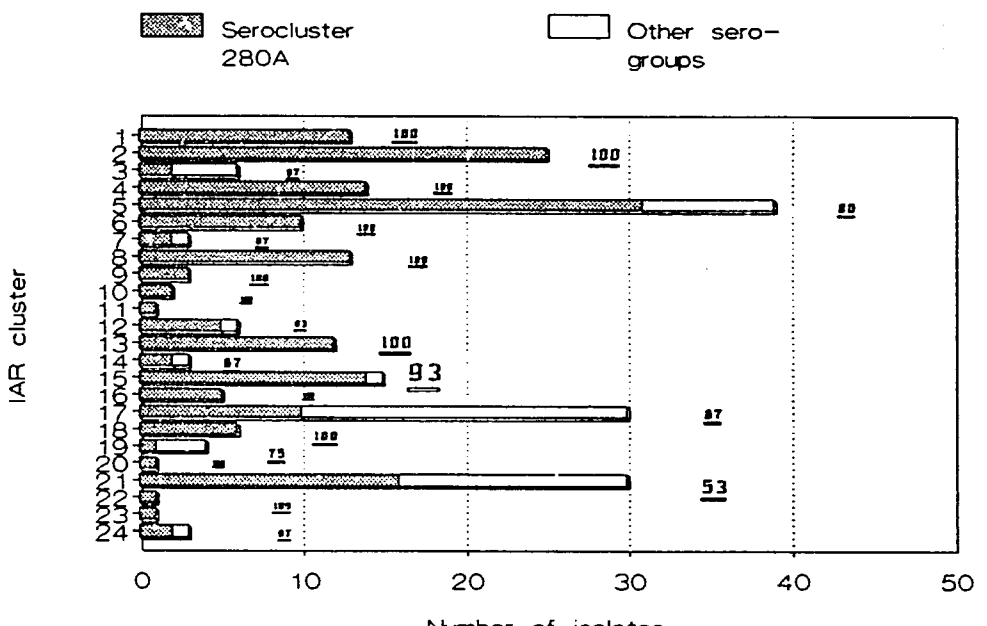


Fig. 5. Serological distribution of isolates of Bradyrhizobium spp. (Arachis) within each IAR group. Numbers to the right of bars shows the percentages of isolates in the dominant serocluster of each IAR group.

## LEGENDS

Fig. 6. Acrylamide gel (12%) showing the protein profiles of ten isolates collected at site E-7 (mungbean cultivation) in the Eastern part of Thailand. Protein standards (PS) are displayed in the far left and two right lines. These are identified in order of decreasing molecular weight (in kilodaltons) from top to bottom of gel: Phosphorylase b, 94.4 kd; BSA 66.2 kd; ovalbumin, 42.7 kd; carbonic anhydrase, 31 kd; soybean trypsin inhibitor, 21.5 kd; lysozyme, 14.4 kd.

Fig. 7. Protein profiles of seven isolates belonging to one serogroup as displayed on 12% acrylamide gel. Protein standards (P. st.) are the same as in Fig.6. Isolate 10/19 from site E-10 (fallow with native legumes), isolates 16/1, 16/6 and 16/16 from site E-16 (fallow with native legumes) and 7/17 from site E-7 (mungbean cultivation) were collected in the Eastern region of Thailand. Isolates 21/12 from site N-21 (peanut field) and 31/11 from site N-31 (non-legumes) were collected in the Northern region.

Fig. 8. Acrylamide gel (12%) showing the protein profiles of ten isolates collected at site N-23 (peanut field) in the Northern part of Thailand. Protein standards (PS) are the same as in Fig.6.