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INCREASING BIOLOGICAL NITROGEN FIXATION OF PEANUTS IN DEVELOPING COUNTRIES

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Thailand

This is a continuation from the first report (January - June, 1988). Works done in this phase were site selection, soil sample collection, determination of rhizobial population, isolation and authentication.

Computation of MPN-plant infection data had been finished and they are presented in Table 1. Results in the Table 1 indicated that the number of peanut rhizobia varied from 0 to > 10^3 cells per gram soil. The population of rhizobia are always high in the peanut cultivated areaes. Interestingly, we found that most noncultivated areas especially in the fallow, there were high population of peanut rhizobia in soils. The finding may against the concept that none or a low population of rhizobia is expected in the noncultivated areas. The presence of high population of rhizobia in these areas might be due to the native legumes carrying the same rhizobia infecting peanut. In constrast, we did not find any rhizobia in many locations which have been cultivated with monocrop, such as sugar cane, casava and pineapple for a long period of time. This might be due to a continuouse cultivation of monocrop did not allow leguminous weeds to grow resulted in indigenous rhizobia could not be maintained.

High population of rhizobia found in the noncultivated areas may or may not be effective enough for increasing yield of peanut. If the population are not effective, inoculation response will be expected eventhough a soil contains high number of rhizobia. Thus, it is nessessary to

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determine the effective of peanut rhizobial population in the different sites. This task has been being carried out in our glasshouse. The details of the procedure were given in the first report. Following the glasshouse experiments, field experiments will be carried out in a selected site depending on the result from glasshouse trials. We plan to conduct some field experiments in the raining season starting from June to July.

Development of antisera of standard strains of peanut has been being conducted. Standard strains are strains having found high effective on our recommended peanut cultivars grown in Thailand and they are used in our inoculant production for peanut. The inoculants will be made from these strains to be used as a positive control and from the superior strains found in this study. The superior strains will be also used for antisera production.

Table 1. Number of rhizobia in soil collected from different locations as determined by MPN-plant infection technique.

Sites*	Descriptions	No. of rhizobia (cells/g soil)
E-1	Skeleton soil, peanut croping	2.88×10^1
E-2	Fallow, having native legumes	1.60×10^3
E-3	Casava plantation	$<2.82 \times 10^1$
E-4	Fallow, having few native legumes	7.04×10^1
E-5	Noncultivated, forest shrub	0
E-6	Corn cultivation	0
E-7	Corn cultivation rotation with mungbean	5.70×10^2
E-8	Peanut cultivation area	3.26×10^3
E-9	Noncultivated, shrub	0
E-10	Falllow, having native legumes	2.82×10^1
E-11	Casava, pineaple, rubber trees	0
E-12	Sugar cane plantation	4.61×10^1
E-13	Pineaple intercroping between rubber tree	0
E-14	Fallow, having native legumes	2.24×10^3
E-15	Fallow, having native legumes	2.24×10^3
E-16	Fallow, skeleton soil having native legumes	6.40×10^3
E-17	Eucalyptus plantation	1.15×10^3
E-18	Sugar cane plantation	0
E-19	Sweet potato cultivation	0

Sites*	Descriptions	No. of rhizobia (cells/g soil)
E-20	Casava plantation	0
N-21	Peanut cultivation	2.05×10^2
N-22	Shrub, teak trees and mimosa	2.88×10^2
N-23	Peanut cultivation	1.41×10^2
N-24	Peanut cultivation	1.41×10^2
N-25	Cotton and corn cultivation	4.61×10^1
N-26	Shrub with some mimosa	1.02×10^2
N-27	Corn cultivation	0
N-28	Peanut cultivation	4.03×10^2
N-29	Peanut cultivation	1.15×10^3
N-30	Soybean cultivation	2.82×10^1
N-31	Casava having mimosa weeds	1.02×10^2
N-32	Shrub	$<2.82 \times 10^1$
NE-33	Corn rotation with peanut	4.61×10^3
NE-34	Yard long bean (cowpea) cultivation	4.61×10^1
NE-35	Peanut cultivation	3.26×10^3
NE-36	Cover legumes cultivation	1.15×10^3
NE-37	Casava cultivation	2.82×10^1
NE-38	Sugar cane having native legumes	8.32×10^2
NE-39	Fallow, having many native legumes	9.60×10^3
NE-40	Shrub	$<2.82 \times 10^1$
NE-41	Corn cultivation	6.40×10^3
NE-42	Peanut cultivation	5.70×10^2
NE-43	Peanut cultivation	1.15×10^3

Sites*	Descriptions	No. of rhizobia (cells/g soil)
M-44	Peanut cultivation	2.24×10^3
M-45	Corn rotation with peanut	8.32×10^2
M-46	Fallow	2.05×10^2
M-47	Corn rotation with peanut	4.61×10^3
M-48	Corn cultivation	0
M-49	Cotton cultivation	0
M-50	Sugar cane cultivation	0
M-51	Casava cultivation	0
M-52	First year of peanut cultivation, peanut did not nodulate	0
M-53	Fallow	0
M-54	Sugar cane cultivation	0
M-55	Coconut and casava cultivation	0

* E = Eastern region

N = Northern region

NE = Northeastern region

M = Central plain region

Israel

Objectives

The specific objectives of the work carried out in Israel during the period under review were as following:

1. Studies on the relationship of somatic antigens among peanut Rhizobia isolates collected in Thailand.

2. Research on identification techniques that would provide a sensitive and precise recognition of Rhizobium strains in culture and root nodules.

Work plan of specific objective 1

Task 1

Isolation of single colonies

19 peanut Rhizobium isolates collected from 3 sites of Thailand (Table 1) by Dr. Boonrerd were stricted on yeast-mannitol agar (YMA) plates and incubated at 28°C per 10 days. Typical isolated colonies of slow-growing rhizobia which usually appeared 6-7 days after planting were picked up along the lines of spreading transferred to YMA slopes in tubes, additionally incubated for 7 days at 28°C and maintained at room temperature for further studies. The differences in growth on YMA among the isolates tested are presented in Table 1.

Task 2

Serological screening of indigenous peanut rhizobia

a) Antigens

Rhizobial strains are characterized by their genic heterogeneity. Thus it is common to use serological tests for the identification of these bacteria. 19 isolates listed in Table 1 were chosen at this stage for the determination of their antigenic properties. Bacteria were cultured at

28°C for 6 days in Erlenmayer flasks contaianing 50 ml of YM brobh. The bacteria were then centrifuged at 1000 rpm for 5 min. and washed three times in phosphate-buffered saline (PBS) to diminist extracellular polysacharide slime. The sediment of cells was suspended in PBS to get a density of antigenic suspension of 10^7 cells/ml. This suspension was homogenized and boiled for 30 min in water bath. The somatic antigens thus obtained were stored at +4°C and used for ELISA tests.

b) Antisera

So far no antisera against the isolates collected in Thailand have been prepared. Therefore it was decided that antisera against serologically un-related peanut *Rhizobium* strains from our culture collection will be used for a preliminary screening of the isolates. Four antisera against the following strains were used: 280A, 2212A are usually used in commercial inoculants for peanut inoculation in Israel; CB756 - is a well known inoculant strain from Australia; strain XAX6 is designated as ineffective.

c) ELISA assay

Reactions were carried out in polystyrene microtitre plates (Nunc) and the ELISA values are expressed as the absorbance at 405 nm, which was read in a Microelisa reader. The indirect ELISA assay followed the protocol outlined in Fig. 1.

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Test sample in PBS, overnight, RT

Strain-specific - globulin in PBS-Tween,1h, RT

Alkaline phosphabase-labelled Protein A
PBS-Tween,2h,RT

Substrate solution,1h,RT

Stop reaction with 0.05 ml 3N NaOH

Measure absorbance at 405nm

Fig.1. Sequence of operations in indirect ELISA procedure. Each well contains 0.2 ml.
RT - room temperature

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d) Results

All four antisera were used in indirect cross-ELISA test with somatic antigens of 19 peanut Rhizobium isolates (Table 2). The results indicated that there is no antiserum reacting with all of the isolates tested. Moreover, two antisera (2212A and XAX6) did not react with any of the isolates examined. Among 19 isolates tested 11 (57.8%) were serologically related exhibiting strong ELISA reactions with antiserum 280A. There were also weak (A_{405} = 0.20 - 0.26) 280A reactions with two isolates (13 and 3) collected in the peanut cultivation area of Thailand. It should be indicated that among 6 isolates collected from the peanut cultivation area these two isolates were the only ones reacting with any of the antisera tested.

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Conclusion

The results obtained at this stage provide a relaianie information regarding the serological properties of the peanut rhizobia isolates collected in Thailand. These data will be useful in selection if isolates for antisera preparation.

Work Plan of Specific Objective 2

Task 1

Enzyme-linked immunosorbent assay (ELISA) on nitro-cellulose membranes (Immunobloting) in identification of peanut Rhizobium colonies.

Recently, nitrocellulose membranes have been found to be an acceptable and often superior alternative to polystyrene plates commonly used as solid surfaces in ELISA. The applicability of immunobloting system for Rhizobium strain analysis was recently compared with conventional ELISA methods by Olsen and Rice (1987) who found it most useful for identification of R. melloti strains either as colonies on YEMA plates or as inhabitants in nodules. Although immunoblotting procedures have been developed for use with fast-growing rhizobia the utility of this method for detection and identification of slow-growing peanut Rhizobium strains has not yet been examined. This report presents an immunoblotting procedure as it was applied for identification of peanut Rhizobium colonies growing on YEMA plates.

Bacteria

Three peanut Rhizobium strains (280A, CB756 and XBL6) belonging to different serological groups were used in this study. Strains (single or mixed) were grown for 10 days to 1-2 mm colonies on YEMA. Plates containing 50-70 colonies were used in this assay.

Immunoblotting procedure

The protocol of Olsen and Rice (Publication No. N.R.G-88-07, 1988) with some modifications developed in our laboratory was used. For

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slow-growing peanut Rhizobia the test was carried out as follows: (1) Nitrocellulose membrances (NCM) (Schleicher and Sohuell, Dassel 2A-85, West Germany, 0.45 mm pore size) were cut to the size required with a razor blade and one side of the membrane was marked with a pen. The dry membrane was layed, marked side down onto the surface of media with colonies and lightly pressed onto colonies to ensure good contact. The membrances were processed through all succeeding steps with the marked side up (2). The colonies were washed off membrane with a gently stream of running oxygenized water to the point of invisibility to the naked eye. The membrances were then given two washes each of 5 min. in PBS (20% of normal concentration. At this stage the membranes were air dried for 24 hours.(3) The membranes were immersed in 20% (v/v) methanol containing 10 mM HCl for 1 h. in order to inactivate endogenous alkaline phosphase, and then washed twice (each of 5 min.) in PBS-Tween. (4) The surface dry membrane was put into 0.5% blocking reagent (Boehinger-Mannheim, Cat. 109o1/6) in Tris-HCl, 100 mmol/1, 150 mmol/1, pH 7.5 buffer for 30 min. with occasional agitation. Blocking reagent does not dissolve very rapidly. Therefore the solution was prepared at least 1 h. in advance and dissolved at 60°C in water bath. The membranes were then washed for 5 min. in PBS-Tween by skaing and placed in specific antibody solution (rabbit antistrain IgG at a concentration of 1 mg/ml) in PBS-Tween for 2 h. with occasional agitation at room temperature. This was followed by 2 washes, each of 5 min, in PBS-Tween. (5) The membranes were placed in alkaline-phosphatase conjugated-protein. A solution diluted at 1:4000 in PBS-Tween and then kept at room temperature with occasional agitation for 1.5 h. This was followed by 3 washes in PBS-Tween and 2 additional washes in PBS without Tween and then in water to remove unbound conjugate. (6) Enzyme activity was detected by covering the membranes with a solution of substrate composed of X-phosphate (0.175 mg/ml) and nitroblue tetrazolium salt (0.337 mg/ml) (both from Boehringer-Mannheim) in 0.1M Tris -HCl buffer, pH 9.5, containing 50 mm $MgCl_2$. The colour reaction was staged by washing the membranes for 5 min. in 10 MCl Tris-HCl containing 1 mM EDTA, pH-8.0.

An incubation with the precipitating substrate yields visable purple spots on the membranes in conformity with the location of Rhizobium colonies. This indicates that even small colonies (1 mm diam.) contain enough antigen for immunoblotting detection. Since rhizobia produce

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alkaline-phosphotase it was necessary to include a control membrane which got all the washing steps, but were not treated by the first antibody. Lack of colour development on this membrane confirms that the coloured spots are due to a specific antigen-antibody reaction. In our experiments the endogenous alkaline phosphatase of all strains tested was completely denaturated during the acid methanol incubation step. We have tried skim milk powder (Oxoid) and blocking reagent (Boehringer-Mannheim) as blockers and settled on the blocking reagent since it provides no background and very clear spots.

It is likely that these methods can be useful for specific identification and numeration of rhizobia in multi-strain inoculants.

Table 1: Population and growth characteristics of peanut rhizobia collected from soils at 3 sites in Thailand.

Sites	Number of* rhizobia (g^{-1} soil)	Isolate	Growth characteristics**	Previous history
E-7 (East)	5.92×10^2	2	slight to moderate	Corn cultivated area
		6	" "	"
		17	" "	"
		18	" "	"
		20	" "	"
N-21 (North)	2.05×10^2	8	" "	Commonly cropped to uninoculated peanuts
		2	" "	"
		12	" "	"
		3	very little growth	"
		13	" "	"
		20	" "	"
N-31 (North)	4.02×10^2	4	" "	Sugar cane cultivation
		14	" "	"
		11	" "	"
		19	slight to moderate growth	"
		2	" "	"
		7	" "	"
		17	" "	"
		9	abundant growth	"

* Determined by Dr. N. Boonkerd (Thailand)

** Tested on yeast-mannitol agar at 26-28°C, during 10 days

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Table 2: The heterologous ELISA reactivity of same peanut Rhizobium strains isolated in Thailand

Site	Strain*	Indirect ELISA (A_{405nm}) values obtained with antibodies**			
		280A	XAX6	2212A	CB756
E-7	2	0.73	-	-	-
(Corn	6	0.66	-	-	-
cultiv-	17	0.93	-	-	-
	18	0.82	-	-	-
	20	0.53	-	-	-
N-21	8	- ***	-	-	-
(Peanut	2	-	-	-	-
	12	-	-	-	-
	3	0.26	-	-	-
	13	0.20	-	-	-
	20	-	-	-	-
N-31	4	0.82	-	-	0.28
(Sugar	14	-	-	-	-
cane	11	0.68	-	-	0.13
cultiv-	9	-	-	-	0.51
ation	2	0.43	-	-	-
	7	0.75	-	-	0.16
	17	0.38	-	-	-
	19	0.51	-	-	-

* Antigens heated cell suspensions at 10^7 bacteria/ml

** Concentration of heterologous anti-strain -globulin: 8mg/ml for antibodies to strains 280A and XAX6; 16 mg/ml for antibodies to strain 2212A; dilution of XS21 unfractionated antisera 1:500.

Dilution of enzyme-labelled protein A = 1:2000.

*** ELISA readings (A_{405} = ≤ 0.1 considered as negative).

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