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US-ISRAEL CDR PROGRAM

INCREASING BIOLOGICAL NITROGEN FIXATION OF PEANUTS IN DEVELOPING COUNTRIES

CDR PROJECT NO. C7-034

First Progress Report

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Objectives:

The overall aim of this research is to increase the rates and efficiency of N_2 fixation in order to increase production of protein per unit area and to reduce nitrogen fertilizers.

Efficient nitrogen fixation by using effective peanut inoculants may improve the quantity and quality of peanut production.

Specific objectives of the project are as following:

1. Population and effectiveness of peanut rhizobia in peanut - growing and other croping areas of Thailand.
2. Basic research on identification techniques that would provide a sensitive and precise recognition of peanut Rhizobium strains in culture and root nodules.
3. Response of peanut to inoculation with selected effective strains.
4. Enhancement of nitrogen fixation and yield increase by inoculated strains.

Work plan of specific objective 1.

"Population and effectiveness of peanut rhizobia in peanut - growing and other croping areas of Thailand"

Task 1

Site selection

Experimental sites in each of the four parts; the Central plain, the East, the Northeast and the North of Thailand were selected. In each site soil samples were collected to represent different types of cropping management. Three different cropping areas, 1) continuously cropped in peanuts, 2) continuously cropped in non-legumes, and 3) non-cultivated areas were chosen. In each cropping area, the specific sites were selected for soil sample collection. At each site, where soil were colleced, we

took notes of soil temperature, moisture, legume plants within that area, recent cropping practice, and rainfall records from the meteorological station near by. Thus, for each part of Thailand nine specific sites were chosen (3 cropping x 3 soil sampling). Then total sites were 36.

Task 2

Soil Sampling

Before sampling, all equipment and containers used were cleaned to remove rhizobial contaminant. Especially spade or hoe was washed with water and soaked in alcohlo then flamed. This has to be done every time a sample was collected. The top 2 cm of soil was removed from a sample site, and the soil was mined to a depth of 20 cm and passed through a 6.5 mm screen. Making pooled sample from 10 cores within the area of 5 x 5 m on a clean plastic sheet, a subsample of 3 kgs was taken. The sample was then immediately put into an ice box and kept at low temperature until used. Each soil sample was used for - (1) determination of rhizonial population (2) isolation and determination of their effectiveness.

Task 3

Determination of Rhizobial Population

About 500 g of each soil sample from task 2 was used for determination of rhizobial populations by most probable number (MPN) - plant infection technique (Weaver and Frederick, 1974).

Because peanut does not nodulate well in plastic growth pouchs we used a modified Leonard's jar, the Swedish Match-Helleshog plastic tubes which have been used for growing peanut as MPN - plant infection count with succesful in our laboratory.

50 g (oven dry basis) subsample of each soil was diluted in 150 ml of sterile water and placed on a wrist-action shaker for 15 minutes. This is designated as part of a serial dilution. Seven additional four-fold dilutions of the initial dilution were made.

A 1ml portion of each dilution was applied to four replication plants. The plants were placed in an air conditioning light room equipped with Sun-Brellar light source which provide flux density of light about 450/uEs^{-1} m^{-2} with a 12 hr-12hr light-dark regime. Plants were scored for nodulation at 21 days after inoculation.

Task 4

Isolation and Characterization of Indigenous Rhizobia

A portion of 2 kgs of each soil sample from task 2 was mixed with sterilized course sand at the 1:1 ratio. The samples soil mixture were put in clean plastic bags and in plastic containers.

Seed of peanuts were surface sterilized in 2% sodium hypochlorite for 2 min, rinsed 6 times with sterile water and pregerminated in the agar water plate. The germinated seeds were planted into plastic containers, 6 seeds per container. All pots were placed in glasshouse.

About 30 days after planting, 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 a plate of YEM containing congo red indicator. Single-colony was picked from plates, numbered and stored on YEM slants. Isolates were authenticated on siratro. Randomly selected of 20 isolates of each legume were used as effectiveness test in comparison to the known effective strain.

Effectivenss tests will be carried out in Leonard's jar suppleminted with N-depleted plant nutrient solution. Completely randomized block design will be used as statistical analysis. Plants will be placed in a glasshouse until flowering stage then weight and acetylene reduction activity (ARA).

Results

Determination the population of peanut rhizobia in soil samples was completed. Analysis of data are being processed. Isolation and authentication of rhizobia from soil samples were also done about 50% of the samples. Isolates will be tested for effectiveness in our glasshouse in Thailand. The duplication of isolates will be sent to Dr. Kishinevsky for further study at the Voleanic Center.

Research work on specific objective 2

"Basic research on identification techniques of peanut Rhizobium strains"

Task 1

The use of amplified ELISA for the detection and identification of Rhizobium strains

Enzyme-linked immunosorbent assays (ELISA) which are commonly used for the detection, and identification of Rhizobium strains (Kishinevsky and Jones, 1987) offer a number of advantages over the agglutination and immuno-diffusion techniqes (Dudman, 1977)).

However, since ELISA enables the detection of Rhizobium in suspensions containing not less than 10 to 10^5 cells/ml (if the cells are heat treated) it may be considered that the technique has not sufficient sensitivity for legume/Rhizobium systems in which the nodules may contain lower cell numbers (Renwick and Jones, 1985). The same limitation features the ELISA system used for routine determination of legume inoculant quality (Lochner et al, 1988) or enumeration of cell numbers in the soil (Nambiar and Anjaiah (1985).

Demands for increased sensitivy stimulated Morley and Jones (1980) to develop a more sensitive modified ELISA technique in which the p-nitrophenyl phosphate substrate of the conventional ELISA technique was substituted by 3-O-methylfluorescein phosphate, which degrades to be a fluorescent product and can be precisely measured. This method has been used successfully in several ecological experiments (Jones and Morley, 1984) but it requires an expensive plate reading spectrofluorimeter.

Recently a new technique using enzyme labels catalizing the formation of a trigger molecule was described.

In amplified ELISA the primary substrate is NADP which is dephosphorilated to give NAD. The latter takes part in a second, cyclic, enzyme mediated reaction including alcohol dehydrogenase/ethanol and diaphorase (NADH dye oxidoreductase)/p-iodonitro-tetrazolium violet. During each turn of the cycle a molecule of coloured formasan is formed, while a molecule of NAD is reduced to NADH which triggers the next cycle (Johanson et al, 1985).

Since this method has proved extremely sensitive in several immunological systems (Stanley et al. 1985) an attempt was made in the frame work at this project to compare the relative merits of the conventional an amplified ELISA tests used for serologic [illegible] ification of Rhizobium strains.

Materials and Methods

Bacteria: Strains used in this study were isolated from root nodules of Arachis hypogaea and kept in te culture collection of the Department of Legume Inoculation, Agricultural Research Organization, The Volcani Center, Bet Dagan. All strains were grown on yeast extract mannitol agar (YMA), derived from simple colony isolates and tested for their ability to nodulate the host legumes under bacteriologically controlled conditions.

Materials Microplates were Dynatech M29AE 96 well Bovine [illegible] albumin - fraction V (BSA), cat. No. A-4503; NADP monosodium salt [illegible]. no. 0505 alcohol dehydrogenase cat. no. A3263 diaphorase NADH: dye [illegible]doreductase, cat no. D-2331; paranitrophenyl phosphate (pNPP) and p-iodonitratetrasolium violet (INT). cat no. I-8377, were all obtained from Sigma Chemical Co. St. Louis. U.S. Antibodies to Rhizobium strains were raised in rabbits (Dudman,

1977). Alkaline phosphatase-labeled protein A for the indirect ELISA was prepared by a two-step glutaraldehyde conjugation procedure (Engvall, E. 1978) as described in our previous work (Kishinevsky and Maoz, 1983).

Conventional and amplified ELISA tests

For Rhizobium assays the samples were added to the microplates so that each half plate was replicated in the second half and the plates were processed by the indirect or direct conventional ELISA methods (Clark and Adams, 1977, Kishinevsky and Maoz, 1983) up to including the conjugate step after which half plate was incubated with pNPP solution for 30-40 min. while the other half was processed according to the Enzyme-Amplified system:

NADP was dissolved to a final concentration of 0.1 mM in a substrate buffer consisting of 50 mM diethanolamine, 1 mM MgCl2 0.1 mM ZnCl2, 15 mM NaN3, pH adjusted to 9.5 with HCl (Johansson et al. 1986). The solution was filtered through a 0.22 um filter, dispensed in 10 ml ali[illegible] and kept at -20°C. 100 ul of this solution were added to microplate we[illegible] using the alkaline phosphatase conjugate, and plates were incubated at room temperature for 20 min, after which 'amplifier' was added.

Amplifier buffer was 20 mM Na-phosphate buffer. pH 7.2 (Johansson et al., 1986). 40 mg alcohol dehydrogenase. 30 mg diaphorase, and 55.6 mg INT were each dissolved in separate volumes of 64 ml amplifier buffer, and frozen at -20°C in 6.4 ml aliquots. Immediately before use reagents were thawed and one aliquot of each enzyme and of INT were mixed and 0.8 ml absolute ethanol added to give 20 ml 'amplifier'. Amplifier, 200 ul/well was added on top of the NADP and plates were incubated at room temperature for 3-10 min. after which the reaction was stopped by 0.4 N HCl, 50 ul/well. Absorbance was read at 490 nm over an 'amplifier' blank.

Controls and Background

In both ELISA systems of at least 6 wells/plate were loaded with serologically non-related peanut/Rhizobium strains and served as controls. The background was calculated as the averaged OD of all serologically non-related wells in a plate + 2 standard deviations. Since the wells of the edge of microtitre plates were usually given inconsistent reading they were not used in the tests.

Results

Results of the sensitivity of the direct and amplified ELISA methods applied to detection of culture antigens of three peatnut Rhizobium strains (280A, R283A, and 944C) are presented in Table 1. Both assays showed a quantitative response over the concentration range for the strains tested and allowed the detection of peanut rhizobia down to 10^5 cells/ml (DAS) and 10^4 cells/ml (amplified test). On the other hand the amplified ELISA did not show a greater sensitivity than the direct assay for the detection of strain 944C (Table 1). Preliminary studies using individual nodules from peanut plants inoculated with 280A and R283A, strains showed that the sensitivity of the amplified ELISA was approximately ten times greater than that of the direct ELISA.

The amplified test, however, did not show a greater sensitivity than the indirect assay for the detection of culture antigens of all three strains tested (Table 2). Similar results were observed for nodule antigens and are therefore not reported. It should be emphasized that an extremely sensitive enzyme-labeled prpotein A (Kishinevsky and Maoz, 1983) was used in both indirect and amplified ELISA assays.

Although a close agreement between the conventional and amplfied ELISA was obtained with the strains tested, we have some probleems with high

background reactions, which have usually been inherent in the amplified direct and indirect systems (Table 1 and 2).

Conclusions

Comparison of the conventionl indirect enzyme-linked immunosorbent assay with the amplified test for the detection and determination of peanut Rhizobium culture and nodule antigens gave comparable results. The indirect ELISA system using enzyme-labeled protein A with and without amplification reagents/NADP, alconol dehydrogenase, diaphorase and p-iodonitrotetrazolium violet) were found to be more sensitive than the direct double-antibody sandwich ELISA.

Task 2

Production of monoclonal antibodies to identify serologically related peanut Rhizobium strains.

Four female mice were immunized three times at 10 days intervals by intraperitaneal injections of 0.2 ml of emulsion of equal parts of Freund's complete adjuvant (Difco) and a culture suspension containing approximately 10^9 cells/ml. Ten days later each of the immunized mice was tested for antibody poduction by the indirect ELISA procedure. The mouse that showed the strongest antibody response was selected as the lymphocycle donor.

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Table 1. Detection of a range of peanut Rhizobium cell concentrations by conventional direct ELISA and amplified ELISA.

Antigen concentration	Antiserum to strains: 280A				R283A				944C			
	DAS*		AMP**		DAS		AMP		DAS		AMP	
	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het
10^7	1.61	0.11	>2.0	0.12	1.54	0.04	>2.0	0.20	1.10	0.09	1.60	0.20
10^6	0.82	0.11	1.18	0.09	0.95	0.08	1.20	0.18	0.55	0.07	0.78	0.17
10^5	0.40	0.13	0.[illegible]	0.06	0.38	0.10	0.51	0.12	0.27	0.04	0.32	0.14
10^4	0.17	0.10	0.42	0.11	0.15	0.07	0.38	0.11	0.12	0.04	0.11	0.13
10^3	0.14	0.07	0.12	0.08	0.11	0.06	0.14	0.11	0.10	0.03	0.09	0.13

* Conventional indirect ELISA A_{405} values measured after incubattion of substrate for 40 min. Homologous (Hom) and Heterologouss (Het) reactions.

** Amplified ELISA.

Table 2. Comparison of conventional indirect ELISA with amplified ELISA in the detection of peanut Rhizobium strains

Antigen concentration	Antiserum to strains: 280A IND*		280A AMP**		R283A IND		R283A AMP		944C IND		944C AMP	
	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het	Hom.	Het
10^7	>2	0.09	>2.0	0.24	1.80	0.05	>2	0.32	>2	0.12	>2	0.32
10^6	1.26	0.07	1.32	0.20	1.20	0.03	1.53	0.24	1.40	0.10	1.50	0.20
10^5	0.82	0.07	0.84	0.17	0.69	0.01	0.88	0.18	0.65	0.03	0.80	0.22
10^4	0.20	0.03	0.22	0.14	0.28	0.01	0.40	0.20	0.20	0.04	0.30	0.16
10^3	0.21	0.01	0.15	0.10	0.13	0.00	0.20	0.14	0.18	0.02	0.16	0.14

* Conventional Double-antibody sandwich ELISA A_{405} values measured after incubation of substrate for 30 min. Homologous (Hom) and heterologous (Ho) reactions.

** Amplified ELISA.

Thailand

Financial Report on CDR Project No. C 7-034

Received

st 1	check in April	$7,650 =	191,048.55	bahts
nd 2	check in July	$4,290 =	108,710.45	bahts
	Total	$11,940 =	299,759.00	bahts

Expenditure (Bahts)

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	TOTAL
Salary	-	-	-	-	-	-	1,688.30	19,033.32	8,895.00	29,616.62
Materials & Supplies	-	-	-	-	3,780	14,500	19,828	21.625	17,340	77,073.00
Equipment	-	-	-	-	-	20,000	-	-	-	20,000.00
Travel	-	-	-	-	5,298	-	2,146	6,601	7,428	21,473.00
			Total		9,078	34,500	23,662.3	47,259.32	33,663	148,162.62
										$5,933.63

Balance 299,759.00 - 148,162.62 = 151,596.38
= $6,071.14

(1 US$ = 24.97 bahts)