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EFFECT OF INOCULATION OF INDIGENOUS EGYPTIAN *Bradyrhizobium* sp. *Lupini* ON N UPTAKE AND YIELD OF Lupin

S. RAZA and B. JØRNSGÅRD
The Royal Veterinary and Agricultural University, Department of Agricultural Sciences, HØjbakkegård Alle 9, DK-2630 Taastrup, Denmark

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ABSTRACT

Eight indigenous rhizobial isolates obtained from nodules of non-inoculated white lupins from different climatic regions in Egypt could according to their pattern of intrinsic antibiotic resistance (IAR) to 12 antibiotics be clustered into 4 groups. Antigens from rabbits raised with antigenic material from one isolate of each of the four IAR group gave positive reaction with homologous antigens, while other combinations gave negative results. Agglutination and double diffusion tests confirmed the genetic separation into 4 different groups according to their antigenic structure and the possible grouping of indigenous isolates into specific groups. In a field experiment with inoculation in the traditional lupin growing area in Ismailia, the inoculation with most efficient strain resulted in increased nodulation status, N uptake and yield. At the reclaimed desert area Tahrir the strain ACR 401 gave the highest seed yield, however, the non-inoculated control had also abundant nodulation. At both locations, the seed yield tended to be greater with *Rhizobium* inoculation than with N fertiliser, which indicated a potential for improvement of N fixation by lupin through the application of efficient rhizobial strains such as ARC 401 and ARC 408. The results from this study suggested that variation in N fixing capacity is due to clear genetic differences among the isolates.

Key Words: Characterisation, competition, indigenous isolates, *Lupinus albus* L., N-fertiliser

RÉSUMÉ

Huit isoles indigènes de rhizobium obtenus des nodules de lupins blancs non inoculés des différentes régions climatiques de l'Egypte pourraient être regroupées selon leur tendance de la résistance antibiotique intrinsèque (RAI) de 12 a 4 groupes. Les antigènes obtenus des lapins avec des matériels antigéniques d'un isole pour chaque groupe RAI donna une réaction positive avec les antigènes homologues, alors que les autres combinaisons donnèrent des résultants négatifs. Les tests d'agglutination et de double diffusion ont confirme la séparation génétique dans quatre groupes différents selon leur structure antigénique et les groupements possibles des isoles locales en des groupes spécifiques. Dans des expériences de terrain avec inoculation, dans la zone traditionnelle de Lupin de Ismailia, l'inoculation avec les strains les plus efficaces entraînant l'augmentation des statuts de nodules, le prélèvement de l'azote et le rendement. Dans le désert réclamé de Tahrir le strain ACR 401 donna le rendement le plus élevé, cependant, les contrôles non inocules avaient aussi une nodulation abondante. Aux deux endroits, le rendement en grains avait tendance a augmente avec l'inoculation au *Rhizobium* par rapport à l'engrais d'azote, qui indiquait la potentialité d'augmenter la fixation d'azote par le Lupin a travers l'application des strains efficaces de rhizobium tel que ARC 401 et ARC 408. Les résultats de cette étude suggèrent que la variation de capacité de fixation d'azote est due à des différences génétiques marquées parmi les isolés.

Mots Clés: Caractérisation, compétition, isolés indigènes, *Lupinus albus* L., engrais azote

INTRODUCTION

White lupin (*Lupinus albus* L.) has been cultivated in Egypt for at least 4.10^3 years (Gladstones 1970). Lupin is well adapted to sandy soil conditions, and appears to be very efficient in N-assimilation (300 kg N ha^{-1}/year) (Julier *et al.*, 1994). The fertilisers which supply nitrogen are very expensive especially, in the developing countries, where fertilisers are imported. Thus, alternate sources of N need to be studied. One such alternative is biological nitrogen fixation. Strains of *Rhizobium* or *Bradyrhizobium* that have dramatic differences in such important traits as host specificity, ineffectiveness and effectiveness are indistinguishable from each other under microscopic observation, cultural features and biochemical tests. However, *Rhizobium* serology has been useful in the evaluation of the taxonomic relatedness among *Rhizobium sp.* (Vincent and Humphrey, 1970; Abd-El-Rhim *et al.*, 1978) and their identification has been possible when isolated from the nodules (Ghobrial *et al.*, 1991; 1992). The parallel use of antibiotic marker and serodiagnosis, both relatively stable in themselves, provide a means of confirming the stability of each marker independently in ecological research. Little information is available on the population diversity of symbiotic N-fixing rhizobia specific to Lupinus (L.). In this study, we assessed the diversity within indigenous rhizobial isolates from different locations in Egypt, using intrinsic antibiotic resistance marker and serological diagnosis to follow up their persistence and behavior when introduced into the soil. The study also includes the potential of effective selected rhizobial strains and N-fertiliser on growth of white lupin under different field conditions.

MATERIALS AND METHODS

Isolation of the *Rhizobium* strains. Eight rhizobial isolates obtained from root nodules of white lupin grown in different climatic regions in Egypt were included in the study. Healthy nodules were separated from the lupin root, immersed for 10 seconds in 70% ethanol, then soaked in 93% sodium hypochlorite solution for 3 minutes and rinsed several times with sterilised distilled water. Individual nodules were crushed in sterile distilled water (1 ml). One loopful of each nodule suspension was streaked into plates of yeast extract mannitol agar (YEMA) medium (Vincent, 1970) containing congo red at a final concentration of 25 mg/l^{-1}. Sub-culturing occurred only once during the experiment and single colonies were selected and each isolate was restreaked to purification (Somasegaran and Hoben, 1994). The bacterial isolates were stored on YEMA slant tubes for further investigation. Multiplication of *Rhizobium* was performed in 500 ml conical flasks, containing 20 ml YEMA medium autoclaved at 121 °C for 3 days in the case of fast-growing *Rhizobium strains,* and for 6 days for slow-growing *Bradyrhizobium* strains, to give a final concentration of about 10^8 celles ml^{-1}.

Antibiotic marker and serodiagnosis. Eight rhizobial isolates, which formed nodules on lupin *Bradyrhizobium* sp. *(Lupini)* were characterized using intrinsic antibiotic resistance (IAR) patterns. The antibiotics used and their concentrations (µg ml^{-1}) were ampicillin sulphate (AP, 20), crentamycin (CN, 10), ciprofloxacin (CP, 5), clindamycin (CD, 2), rifampicin (RF, 5), topramycin (TP, 10), colistin sulphate (CT, 50), neomycin (N, 30), amoxycillin (AM, 25), naladixic acid (NA, 30), ampicillin (AN 30) and teramycin (TE, 30). The antibiotic solutions were filter sterilized (0.20 mm) on yest extract mannitol agar (Vincent, 1970), plates separately containing the antibiotics tested were used to determine the resistance level of the tested isolates to each of the antibiotics under investigation. Each rhizobial isolate was streaked on yest extract mannitol agar plates supplemented with each antibiotic tested.Three plates of each antibiotic were used for each rhizobial isolate and incubated for 7 days at 30 °C, and the growth was scored by visual inspection as (+) for growth and (-) no growth.

Preparation of *rhizobium* cell suspensions and antigens. Four isolates (ARC 400, ARC 401, ARC 412 and ARC 408) representing the IAR group of a bradyrhizobial culture was maintained on 7 liters of common bean (*Phaseolus vulgaris* L.) medium in 10 liters flasks and aerated by bubbling sterilized air, or in 500 ml conical flasks

placed on a rotary shaker, to give a final concentration of about 10^8 cells ml^{-1}. Cultures were harvested after 10 days by centrifugation at 8.000 r.p.m, the precipitated cells were washed several times in a sterilized physiological solution (0.85% NaCl), then stored at freezing temperature until usage. Antigens applied for in vitro immunization were prepared by carefully adding 10 ml of Freund's complete adjuvant drop to heavy cell suspension (Kabat and Mayer, 1971). The mixture was continuously stirred in one direction until a white colloidal paste was obtained. Antigens for in vitro serological reaction were obtained by adding 16 grams of fine washed sand to 8 grams of washed cells. The mixture was then crushed thoroughly in a mortar submerged in an ice box, then centrifuged at 4000 r.p.m., and subsequently the precipitates were discarded. The antigens were kept in tubes at 0°C. Before use the antigens protein content was determined calorimetrically by a Biuret reagent (Kabat and Mayer, 1971).

Immunisation. Rabbits 2-3 kg. in weight were immunized by a weekly injection with 1 ml, of the antigens. A blood sample was taken from the lateral ear vein, 7 days after each injection, and the antiserum was tested against the homologous antigens by the matrix technique (Tokay and Karczage, 1968). When the maximal precipition bands were obtained, a cell suspension in a physiological solution without adjuvant was injected subcutaneously. Bleeding was carried out 8 days after the last injection, and the blood was incubated for 3 hours, then stored overnight at 4 °C and the separated antiserum was kept in vials at 0 °C. Once a week, rabbits immunized by the tested bradyrhizobia and totally received 12 to 14 injections. The tested isolates that represent IAR groups designated A, B, C, and D were allowed to react with their homologous as well as their heterologous antigens through agglutination and a double diffusion test via matrix technique (Tokay and Karczage, 1968).

Field experiments. To evaluate the symbiotic nitrogen fixation of white lupin with four selected rhizobial strains and applications of N-fertiliser, two field experiments were carried out at the Tahrir (29.34.04 N & 31.14.42 E) and Ismailia regions (30.33.24 N & 32.05.07 E) in Egypt during the winter season 1999/2000. The main climatic and growing conditions of the experimental sites are given in Table 1. A factorial experiment (split plot design) with three replicates was used. Main plots were assigned to the N-fertiliser levels, while subplots were devoted to four randomized selected inoculum treatments: noninoculated, inoculated with strain ARC 400, ARC 401, ARC 412, or ARC 408. Each plot consisted of three rows of 2.5 m length with a row spacing of 0.7 m, i.e., the plot size was 5.25 m^2. Seeds were sown in hills 20 cm apart with 2 seeds per hill. *Rhizobium* inoculations were prepared using solid carrier containing rhizobial population (1×10^8) cfu g^{-1}. Seventy days after planting, five plants were uprooted to record nodule number, dry weight of nodule and dry weight of shoots. The total nitrogen content was determined by the micro Kjeldahl method (Bremner, 1965). Harvesting was carried out at full maturity for each plot of three rows on 15 May 2000 in both locations. Seed and straw yield were recorded in kg ha^{-1}. The data were analysed statistically using the SAS system software (SAS 1988).

TABLE1. Location and growing conditions at the study site

Location	Climatic characteristics			Soil characteristics				
	Rainfall mm	Temperature °C	Sunshine %	Clay %	pH	CaCO3 %	O.M %	E.C
Ismailia	38	20.7	77	7.8	7.8	7.2	0.02	1.4
Tahrir	20	24.1	81	7.5	8.1	8.5	0.22	2.4

Rainfall (average yearly precipitation). Temp. (daily mean temperature °C). Sunshine (hours in pct. of potential)
O.M. (organic matter). PH (1:2.5, soil: water). E.C. (electric conductivity)
CaCO3 (calcium content)

RESULTS AND DISCUSSION

Antibiotic marker. All of the tested isolates were intrinsically sensitive to clindamycin (2 mg ml^{-1}). On the other hand, the three solates (ARC 408 and ARC 410 and ARC 411) were intrinsically sensitive to all antibiotics tested. However, the pattern of the intrinsic antibiotic resistance showed that the isolates could be divided into four groups (Table 5). The isolates of each group showed the same pattern as IAR. However, group A showed resistance to eleven antibiotics out of twelve antibiotics tested. These results may indicate the similar genetic background of the isolates of each group irrespective of their difference in site of isolation and climatic region. Intrinsic antibiotic resistance profile was used to identify strains of *R. leguminosarum* bv. *viceae* (Josey *et al.*, 1979; Brockman *et al.*, 1989). On the other hand, Young and Chao (1989) reported that both the fast and slow growing strains of rhizobia showed wide variability in resistance to antibiotics. Our results suggests that IAR characteristics can be used as a complementary tools in conjunction with other serological methods to identify *Rhizobium* strains.

Serodiagnosis. This experiment was carried out to investigate the efficiency of serological methods in differentiation between different isolates of *Bradyrhizobium* sp. *lupini* behaved differently in their response to antibiotics resistance. Therefore, immunization of animals with antigens extracted from four isolates (representing 4 IAR groups) to raise antibodies, and the qualitative analysis of the cross reactive antigens of the antibodies, so raised, with antigenic materials of the whole cell as well as their extractions. This was accomplished by agglutination and double diffusion test. Cross agglutination and number of precipitin bands formed in double diffusion gave positive reactions with homologous antigens, while other ones tested, including hetrologous antigens, gave negative results.

Double diffusion testes. The precipitation patterns obtained from double diffusion tests conducted between the antisera of the tested isolates and their respective and irrespective antigens are presented in Table 2. When the precipitation bands are matched, the following could be concluded: (a) enumeration of the precipitin band form revealed that the highest number of precipitin lines were developed when every antiserum was allowed to react with its homologous antigens as they gave 10, 12, 14 and 13 bands for isolates ARC 400, ARC 401, ARC 412 and ARC 408, respectively, (b) occurrence of certain common antigens between all the tested isolates, as they shared common precipitation lines, when every antiserum was subjected to react with the heterologous antigens of other tested isolates and (c) the common precipitin band developed in the homologouse reactions ranged from 3-9 lines as shown in Table 2.

Results obtained from agglutination and double diffusion tests indicated that the tested isolates could be related to four different serogroups, according to their antigenic structure. Such results are compatible with those obtained from the IAR testes.

Therefore, the results of serological tests could be considered, as additive criterion strengthen the aforementioned results obtained from the IAR pattern. The results also proved that certain common antigens existed in all the tested isolates as they belong to one species of *Bradyrhizobium* sp. *(lupini)*.

Such findings also correlated with that found by

TABLE 2. The number of precipitin bands formed in cross reactions between the tested isolates of *Bradyrhizobium* sp. *(lupini)*

Isolates	ARC 400 (A)	ARC 401 (B)	ARC 412 (C)	ARC 408 (D)
ARC 400	10	6	7	4
ARC 401	6	12	8	5
ARC 412	9	8	14	6
ARC 408	5	4	3	13

(A) Isolate ARC 400, (B) Isolate ARC401, (C) Isolate ARC 412, (D) Isolate ARC 408

Chanway and Holl (1986) who showed that serology is less variable than IAR when strains of *R. trifolii* are identified.

Field experiments. Both at the site in the main growing area (Ismailia) and in the reclaimed desert area (Tahrir) moderate effect was observed on yield, biomass production and nodulation following artificial inoculation. However at Ismailia, lupin plant in non-inoculated formed 36 to 14 nodules plant^{-1} and the shoot dry weight ranged from 8.5 to 8.9 g plant^{-1} (Table3). This result suggests the present of indigenous *Rhizobium* strains of lupin in the soil. The above characters were increased with inoculations, which were also influenced by the competitive ability of the inoculation and indigenous *Rhizobium* strains (Graham and Temple 1984). Yield characteristics was correlated to nodules number, to dry weight of nodules, to dry weight of shoot and to total N accumulation. A similar result was reported from a previous investigation (Howieson *et al.*,1994) although this has not always been observed (Lange and Parker, 1961).

Inoculation effect on shoot dry weight and total nitrogen accumulation was not significant at Ismailia. However, the most efficient strain, ARC 408 resulted in increased nodulation status, N uptake biomass production and seed yield. These responses indicated that the strain has a high capacity to fix nitrogen and was able to compete against native soil rhizobia (Table 3).

At the reclaimed desert area (Tahrir), yields were lower and the effect of inoculation less, but strain ARC 401 showed a significantly higher seed yield. Perhaps this lack of response of lupin to inoculation is due to the low competitive ability of inoculant strains. The abundant nodulation in non-inoculated plots at Tahrir indicates the presence of efficient non lupin specific rhizobium.

Application of N fertiliser at Ismailia gave positive responses on shoot dry weight and N

TABLE 3. Effect of inoculation on the growth of white lupin

Treatment	Location 1 (Ismailia)					Location 2 (Tahrir)				
	Nodule number	SW	N	Biomass kg ha^{-1}	Seed kg ha^{-1}	Nodule number	SW	N	Biomass kg ha^{-1}	Seed kg ha^{-1}
Control	36.2	8.9	258.3	5950	1885	14.2	8.5	208.4	3532	883
ARC 400	39.5	10.9	278.8	6805*	2181*	13.5	10.0**	260.5**	3610	995
ARC 401	37.1	10.7	260.4	6840**	2305**	15.4	9.3*	239.0*	3583	1098
ARC 412	33.9	10.5	269.1	6971**	2241*	16.9	9.5*	252.0*	3138	916
ARC 408	59.0**	10.9	286.3	7583**	2406**	16.2	9.8*	249.7*	2860	835
LSD(0.05)	19.3	2.3 ns	89.1 ns	1271	383	4.1 ns	1.4	48.0	860 ns	235 ns

SW=Shoot dry weight (g plantTreatment). N=Nitrogen content (mg plantTreatment)
Means values marked with * and ** are significant at 0.05, 0.01

TABLE 4. Effect of N-fertiliser on the growth of white lupin

Treatment	Location 1 (Ismailia)					Location 2 (Tahrir)				
	Nodule number	SW	N	Biomass kg ha^{-1}	Seed kg ha^{-1}	Nodule number	SW	N	Biomass kg ha^{-1}	Seed kg ha^{-1}
0 kg N ha^{-1}	58.8	8.6	199.0	6766	2116	19.8	9.8	243.7	3710	1108
35 kg N ha^{-1}	44.9	11.6**	307.8*	7216	2351	16.5	9.6	254.2	3910**	1048
70 kg N ha^{-1}	30.5	10.6**	298.0	6821	2128	12.5	9.6	250.4	3321*	966
140 kg N ha^{-1}	30.4	10.8**	278.0	6516	2221	12.2	8.8	219.5	2243	655
LSD(0.05)	17.3	2.1	79.7	1136 ns	341 ns	3.6	1.3 ns	42.9 ns	768	210

SW=Shoot dry weight (g plant^{-1}). N=Nitrogen content (mg plant^{-1})
Means values marked with * and** are significant at 0.05, 0.01 probability

TABLE 5. Variation in intrinsic antibiotic resistance (IAR) among isolates of rhizobia nodulating lupin

Groups	Rhizobial isolates	AP 20 µg ml^{-1}	CN 10 µg ml^{-1}	CP 5 µg ml^{-1}	CD 2 µg ml^{-1}	RF 5 µg ml^{-1}	TP 10 µg ml^{-1}	CT 50 µg ml^{-1}	N 30 µg ml^{-1}	AM 25 µg ml^{-1}	NA 30 µg ml^{-1}	AN 30 µg ml^{-1}	TE 30 µg ml^{-1}
A	ARC400	+	+	+	-	+	+	+	+	+	+	+	+
A	ARC403	+	+	+	-	+	+	+	+	+	+	+	+
B	ARC401	+	+	-	-	+	+	+	+	+	+	+	+
C	ARC 409	+	+	+	-	+	+	+	-	-	+	+	+
C	ARC412	+	+	+	-	+	+	+	-	-	+	+	+
D	ARC408	-	-	-	-	-	-	-	-	-	-	-	-
D	ARC410	-	-	-	-	-	-	-	-	-	-	-	-
D	ARC411	-	-	-	-	-	-	-	-	-	-	-	-

Antibiotics used: ampicillin sulphate (AP), crentamycin (CN), ciprofloxacin (CP), clindamycin (CD), rifampicin (RF), topramycin (TP), colistin sulphate (CT), neomycin (N), amoxycillin (AM), naladixic acid (NA), ampicillin (AN) and teramycin (TE)

uptake but, there were no significant differences between biomass and seed yield compared to the non-inoculated controls. Increasing nitrogen level in the soil at Tahrir, particularly with 140 kg N ha^{-1} inhibited nodulation and decreased the biomass and seed yield. A similar finding was reported by (Vargas *et al.*, 2000). In both sites no effect on yield and biomass was observed from N application but, increasing N fertiliser level had a strong negative effect on the nodulation status and failed to increase yield. (Table 4). There was no interaction between inoculation and N fertiliser levels in all characters studied.

Results indicate that application of N-fertiliser has little effect on yield of the Egyptian white lupin which in both sites showed an efficient nodulation. In agreement with earlier findings (Pate *et al.*, 1979, Larsen *et al.*,1989) N fertiliser inhibited nodulation. In this study, the inoculant strains were not able to outcompete indigenous rhizobia and therefor no large variation in growth of white lupin was observed between inoculated and non-inoculated plants. These findings are in agreement with the findings of Ghobrial *et al.* (1991).

Present results suggest that N-fertiliser should not be recommended to Egyptian farmers. Increases from inoculation were relatively low and inconsistent but improvement through development of highly effective and competitive rhizobia strains such as ARC 401 and ARC 408 may be possible and offer security for nodulation and can be used as inoculants for successful lupin growth. Further trials should assess if gains are sufficient to cover the extra effort.

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