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SOLATION AND IDENTIFICATION OF SOME LOCAL ISOLATES OF *Bradyrhizobium* SP. (*LUPINUS*) USING SEROLOGICAL AND ANTIBIOTIC RESISTANCE MARKERS AND THEIR SYMBIOTIC PERFORMANCE UNDER DIFFERENT LEVELS OF N-FERTILIZATION

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ABSTRACT

From eight isolates of *Bradyrhizobium* sp. (*Lupinus*) (were selected from many isolates on the basis of their N_2-fixation efficient), four different groups were obtained and identified according to their sensitivity and resistance to antibiotics (IAR). On the basis of serological analysis, the representative isolates of IAR groups showed compatibility, indicating the possible grouping of indigenous isolates of lupin rhizobia into specific groups based on serological and IAR profiles of the isolates. Variations among indigenous rhizobial populations of the isolates from lupin was found, and showed that there is genetic potential to improve strain performance within rhizobial population. In order to evaluate the symbiotic performance of selected strains, a field experiment was conducted in two locations under different levels of N-fertilizer. The results showed that the *Rhizobium* inoculation and N-fertilizer had low effect in increasing the biomass and grain yield of white lupin cultivar. No interaction was observed between inoculation and N-fertilizer levels in all studied parameters. The biomass and seed yield tended to be greater with *Rhizobium* inoculation than with N-fertilizer. The results also indicated the potential for improvement of N_2-fixation by lupin through the application of efficient rhizobia strains such as ARC 401 and ARC 408.

Keywords: *Lupinus albus* L., Indigenous isolates, *Rhizobium* strains, N-Fertilizer, Competition.

INTRODUCTION

White lupin has been cultivate in Egypt for at least 4000 years. Lupin is well adapted to study soils conditions, and appears to be very efficient in N-assimilation (300 kg ha^{-1}) (Julier *et al.,* 1994). The fertilizers which supply nitrogen are very expensive especially, in the developing countries, where fertilizers are imported. Thus, alternate sources of N-fertilizer need to be studies. 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 or cultural features and biochemical tests. However, *Rhizobium* serology has useful in the evaluation of the taxonomic relatedness among *Rhizobium* sp. (Vincent and Humphrey, 1970 ; Abd El-Rhim *et al.,* 1978) and their identification when isolated from the nodules (Ghobrial *et al.,* 1991; 1992). The parallel use of antibiotic marker and serodignosis 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_2-fixing rhizobia specific to this cultivars. In this study, we

assessed the diversity within indigenous rhizobial isolates from different locations in Egypt, using IAR 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-fertilizer on growth of white lupin under different field conditions. It should be stated that the highly effective, competitive and adapted strains from this study was chosen to strength the *Rhizobium* culture collection and consequently for production of high quality legume inoculants.

MATERIALS AND METHODS

Isolation of the *Rhizobium* strains :

During season (2003/2004) root nodules were collected from lupin plants grown in two governorates (Sharkia and Ismaillia). Nodules preservation and isolation of rhizobia were carried out according to Vincent (1970). All the obtained isolates were subjected to purity tests and all the recommended methods used for identification of rhizobial strains, including morphological, cultural and biochemical tests (Somasegaran and Hoben, 1994). The bacterial isolates were stored on YEMA slant tubes for further investigation. Each culture passed the purity tests was considered as individual *Rhizobium* isolate.

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, □g ml^{-1} were ampicillin sulphate (AMP, 20), ceentamycin (CN, 10), ciprofloxcin (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), penicillin (PN, 30) and teramycin (TE, 30). The antibiotic solutions were filter sterilized (0.20 mm). YEMA (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 YEMA plates supplemented with each antibiotic tested. Three plates of each antibiotic were used for each rhizobial isolate. Antibiotic and control (without antibiotic) plates were 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 Phaseolus medium in 10 liters flasks and aerated by bubbling sterilized air, or in 500 ml conical flasks placed on rotary shaker. Cultures were harvested after 10 days by centrifugation at 8000 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 vivo* 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 colorimetrically by a Biuret reagent (Kabat and Mayer, 1971).

Immunization :

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 precipitin 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. Rabbits immunized by the tested Bradyrhizobia 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 performance of Egyptian white lupin with four selected rhizobial strains (represent 4 serogroup) under different levels of N-fertilization. Two field experiments were carried out at the El-Tahrir and Ismailia regions in Egypt during the winter season 2003/2004. A factorial experiment (split plot design) with three replicates was used. Main plots were assigned to the N-fertilizer levels, while subplots were devoted to four randomized selected inoculation treatments: noninoculated, inoculated with strain ARC 400, ARC 401, ARC 412 or ARC 408. The main physical and chemical properties of the tested soil at the two experimental sites are given in Table (1). *Rhizobium* inoculations were prepared using solid carrier containing rhizobial population (1×10^8) cfu g^{-1}. Seventy days after planting, five plants where uprooted to record nodule numbers, dry weight of nodules 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 2004 in both locations. Seed and straw yield were recorded in kg ha^{-1}. The data were analyzed statistically using the SAS system software (SAS, 1988).

It is worthy to mention that the second experiment was inoculated with strain 408 which achieved high productive symbiosis in the first experiment.

Table (1): The main physical and chemical properties of the used soils.

Soil analysis	Values	
	Ismailia	El-Tahrir
Sand (%)	91.00	67.96
Silt (%)	3.63	26.80
Clay (%)	5.57	5.24
Texture grade	Sandy	Sandy loam
$CaCO_3$ (%)	1.78	4.5
pH (soil paste)	7.52	7.55
E.C. (dS/m at 25°C)	0.30	0.32
Soluble cations (meq/L)		
Ca^{++}	0.72	1.85
Mg^{++}	0.50	0.82
Na^{+}	1.60	0.64
K^{+}	0.14	0.18
Soluble anions (meq/L)		
$CO^{=}_3$	--	--
HCO^{-}_3	1.77	1.62
Cl^{-}	0.60	0.76
$SO^{=}_4$	0.59	1.84
Total N (%)	0.020	0.033
Total soluble-N (ppm)	22.10	38.00
Available P (ppm)	5.90	9.40

RESULTS AND DISCUSSION

Morphological, cultural and biochemical tests of all the tested isolates gave positive results and showed uniform reactions, confirming its relation to *Bradyrhizobium* as defined in Bergy's Manual.

Antibiotic marker:

All of the tested isolates were intrinsically sensitive to clindamycin (2 mg ml^{-1}). On the other hand, the three isolates (ARC 408, ARC 410 and ARC 411) representing group D were intrinsically sensitive to all antibiotics tested. The pattern of the intrinsic antibiotic resistance showed that the isolates were divided into four groups (Table, 2). The isolates of each group showed the same pattern towards IAR. However, group A includes (ARC 400 and ARC 403) showed resistance to eleven antibiotics out of the twelve antibiotics tested. The only isolate ARC 401 (group B), showed resistant to all antibiotics except ciprofloxcin and clindamycin. Two isolates ARC 409 and ARC 412 showed sensitivity to clindamycin, neomycin and penicillin and resistant to the rest of antibiotics which can be considered as a separate group (C). 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 and Brockaman *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 complementary tools in conjunction with other serological methods to identify *Bradyrhizobium* strains.

Serodiagnosis:

This experiment was carried out to investigate the efficiency of serological methods in differentiation between different isolates of *Bradyrhizobium* sp. (*Lupini*) which behaved differently in their response to antibiotics resistance. Therefore, immunization of animals with antigens extracted from four isolates (represent 4 IAR group)may be used 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. Data presented in Table (3) indicate that the antigens tested gave positive reaction with homologous antigens, while other ones tested, including heterologous antigens, gave negative results. The precipitin patterns obtained from double diffusion tests conducted between the antisera of the tested isolates and their respective and irrespective antigens are presented in Table (4). When the precipitin bands are matched, the following could be concluded: (a) enumeration of the precipitin band formed revealed that the highest number of precipitin lines were developed when every antisera 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 precipitin lines, when every antisera was subjected to react with the heterologous antigens of other tested isolates and (c) the common precipitin band developed in the heterologous reactions ranged from 3-9 lines as shown in Table (4).

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 tests.

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

Table (2): Variation in intrinsic antibiotic resistance (IAR) among isolates of rhizobia nodulating lupin.

Groups	Rhizobial isolates	AMP 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}	PN 30 µg ml^{-1}	TE 30 µg ml^{-1}
A	ARC 400	+	+	+	-	+	+	+	+	+	+	+	+
A	ARC 403	+	+	+	-	+	+	+	+	+	+	+	+
B	ARC 401	+	+	-	-	+	+	+	+	+	+	+	+
C	ARC 409	+	+	+	-	+	+	+	-	-	+	+	+
C	ARC 412	+	+	+	-	+	+	+	-	-	+	+	+
D	ARC 408	-	-	-	-	-	-	-	-	-	-	-	-
D	ARC 410	-	-	-	-	-	-	-	-	-	-	-	-
D	ARC 411	-	-	-	-	-	-	-	-	-	-	-	-

Antibiotics used : ampicillin sulphate (AMP), ceentamycin (CN), ciprofloxcin (CP), clindamycin (CD), rifampicin (RF), topramycin (TP), colistin sulphate (CT), neomycin (N_1), amoxycillin (AM), naladixic acid (NA), penecillin (PN) and teramycin (TE).

Table (3): Cross-agglutination tested between the isolates of *Bradyrhizobium* sp. (*Lupini*).

Isolates	ARC 400 (A)	ARC 401 (B)	ARC 412 (C)	ARC 408 (D)
ARC 400	+	-	-	-
ARC 401	-	+	-	-
ARC 412	-	-	+	-
ARC 408	-	-	-	+

(A) Isolate ARC 400, (B) Isolate ARC 401, (C) Isolate ARC 412 and (D) Isolate ARC 408.

Table (4): The number of precipitin band 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 ARC 401, (C) Isolate ARC 412 and (D) Isolate ARC 408.

Field experiments:

The nodulation status of inoculated treatments (Tables 5 and 6) showed unexpected results, as the most inoculated strains achieved less number of nodules/plant than the uninoculated control at the two experimentation sites except that strain ARC 408 at Ismaillia, as recorded 59.0 nodule/plant with increasing percentage over control reached to 62.98%. These findings are completely opposite to those reported for dry weight of nodules and shoots and total nitrogen content (Table 5). This differences could be attributed to the higher N_2-ase activity of the bacteroides in the nodular tissue.

Inoculation effect on shoot dry weight and total nitrogen accumulation were 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 have a high capacity to fix nitrogen and was able to compete against native soil rhizobia (Table, 5). At the reclaimed desert area (El-Tahrir), 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 El-Tahrir indicates the presence of inefficient lupin specific *Rhizobium*.

Application of N-fertilizer at Ismailia gave responses on shoot dry weight and N-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 El-Tahrir, particularly with 140 kg N ha^{-1} inhibited nodulation and decreased the biomass and seed yield. Similar findings were

reported by Vargas *et al.* (2000). In both sites no effect on yield and biomass was observed from N-application while, increasing N fertilizer level had a strong negative effect on the nodulation status and failed to increase yield (Table, 6). There were no interaction between inoculation and N-fertilizer levels in all characters studied.

Results indicated that application of N-fertilizer have little effect on yield of the Egyptian white lupin which in both sites showed an efficient nodulation (Ayisi *et al.*, 1992). In agreement with earlier findings (Pate *et al.*, 1979 and Larson *et al.*, 1989) N-fertilizer inhibited nodulation. In this study, the inoculant strains were not able to outcompete indigenous rhizobia and therefore 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). Also, the present results suggests that N-fertilizer 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. Further trials should assess if gains are sufficient to cover the extra effort.

The obtained results indicated that lupin crop growing in traditional (Ismailia) or in newly reclaimed soil (El-Tahrir) are well nodulated and this mean that soil have adequate number of lupin rhizobia. These local strains seems to be poor nitrogen fixers. These could be clarified from the data of seed yield, compared to the control plants, although they bear a reasonable number of nodules, but they achieved less yield as compared with inoculated treatments (Table, 4). Furthermore, as these rhizobia have the capacity to establish themselves in the soil in the absence of the host legume, (so-called saprophytic competence), their abundance in the rhizosphere will have competitive advantage against introduced strains. For these reasons Ghobrial *et al.* (1992) mentioned that inoculation in those areas must be carried with effective strains of rhizobia that can survive in soil, multiply in the rhizosphere, compete successfully with native root nodule bacteria faraway from sites of roots and resist the antagonistic action of soil is very important in nodulation process.

Table (5): Effect of inoculation on the growth of white lupin under different field conditions.

Treatment	Location 1 (Ismailia)						Location 2 (El-Tahrir)					
	Nodule number	Nodule D.Wt. (mg/plant)	SW (g/ plant)	N (mg/ plant)	Biomass (kg/ha)	Seed (kg/ha)	Nodule number	Nodule D.Wt. (mg/plant)	SW (g/ plant)	N (mg/ plant)	Biomass (kg/ha)	Seed (kg/ha)
Control	36.2	316	8.9	258.3	5950	1885	14.2	229	8.5	208.4	3532	883
ARC 400	39.5	463	10.9	278.8	6805	2181	13.5	311	10.0	260.5	3610	995
ARC 401	37.1	491	10.7	260.4	6840	2305	15.4	365	9.3	239.0	3583	1098
ARC 412	33.9	511	10.5	269.1	6971	2241	16.9	393	9.5	252.0	3138	916
ARC 408	59.0	498	10.9	286.3	7583	2406	16.2	390	9.8	249.7	2860	835
L.S.D 0.05)	19.3	5.8	2.3 ns	89.1 ns	1271	383	4.1 ns	4.6	1.4	48.0	860 ns	235 ns

SW = Shoot dry weight (g/plant) , N= Nitrogen content (mg/plant).

Table (6): Effect of N-fertilizer on the growth of white lupin inoculated with strain ARC 408 under different field conditions.

Treatment	Location 1 (Ismailia)						Location 2 (El-Tahrir)					
	Nodule number	Nodule D.Wt. (mg/plant)	SW (g/ plant)	N (mg/ plant)	Biomass (kg/ha)	Seed (kg/ha)	Nodule number	Nodule D.Wt. (mg/plant)	SW (g/ plant)	N (mg/ plant)	Biomass (kg/ha)	Seed (kg/ha)
0 kg N/ha	58.8	521	8.6	199.0	6766	2116	19.8	378	9.8	243.7	3710	1108
35 kg N/ha	44.9	512	11.6	307.8	7216	2351	16.5	361	9.6	254.2	3910	1048
70 kg N/ha	30.5	394	10.6	298.0	6821	2128	12.5	358	9.6	250.4	3321	966
140 kg N/ha	30.4	311	10.8	278.0	6516	2221	12.2	216	8.8	219.5	2243	655
L.S.D (0.05)	17.3	4.6	2.1	79.7	1136 ns	341 ns	3.6	6.0	1.3 ns	42.9 ns	768	210

SW = Shoot dry weight (g/plant) , N= Nitrogen content (mg/plant).

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تعريف وتصنيف عزلات من البرادى ريزوبيم باستخدام طريقتى التصنيف السيرولوجى والمقاومة للمضادات الحيوية ومدى تأثر عملية التثبيت الحيوى للأزوت على نمو نباتات الترمس فى وجود مستويات مختلفة من التسميد النيتروجينى

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تم اختبار أربعة عزلات مختلفة تبعا للمقاومة الذاتية للمضادات الحيوية (IAR) من البرادى ريزوبيا ودراسة العلاقة السيرولوجية عن طريق اختبارات التجمع والانتشار المزدوج فى أطباق الجيل وأوضحت الدراسة وجود أربع مجاميع سيرولوجية مختلفة وهذه النتائج كانت متطابقة مع اختبارات المضادات الحيوية. كما وجد أن هناك أختلافا فى المجموعات بين السلالات المتوطنة فى التربة وبين سلالات الترمس تحت الدراسة. كما أوضحت النتائج وجود تحسن وراثى للسلالات المختبرة داخل تجمعات الريزوبيا. أيضا أوضحت النتائج إمكانية تقسيم العزلات الى مجاميع سيرولوجية مختلفة وهذه المجاميع متوافقة مع المضادات الحيوية.

ولدراسة كفاءة تثبيت الأزوت الجوى للسلالات المختبرة ، أجريت تجربتين حقليتين فى موقعين مختلفين من الأراضى حديثة الاستصلاح (الأسماعيلية وجنوب التحرير) فى وجود مستويات مختلفة من التسميد النيتروجينى المعدنى. وقد أشارت النتائج الى ان كل من التلقيح بالريزوبيا والتسميد النيتروجينى المعدنى كان لهما تأثير قليل على زيادة محصول المادة الجافة ومحصول الحبوب لصنف الترمس تحت الدراسة. كما أشارت النتائج الى عدم وجود تأثير للتداخل ما بين التلقيح ومستويات التسميد النيتروجينى على جميع الصفات المدروسة. بينما أظهرت النتائج الى أن تأثير التلقيح كان أكبر من تأثير التسميد النيتروجينى على كل من محصول المادة الجافة والحبوب. أيضا تشير النتائج الى أن زيادة كفاءة نباتات الترمس فى تثبيت نيتروجين الهواء الجوى كان من خلال استخدام سلالات عالية الكفاءة من الريزوبيا مثل ARC 401 و ARC 408.