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Full Length Research Paper

Ecological studies on nitrogen fixing bacteria from leguminous plants at the north of Jordan

Abu-Zaitoon Yousef* and Sallal Abdul-Karim

Department of Applied Biology, Faculty of Science, University of Science and Technology, Irbid, Jordan.

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The rates of nitrogen fixation by symbiotic rhizobia are often two to three times higher than the ones exhibited by free-living nitrogen-fixing bacteria in soil. In this study, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Rhizobium loti* and *Rhizobium ciceri* were isolated and identified from leguminous plants grown at the north of Jordan. The aim of this study was initially to determine the most rhizobial species resistant to salt and heat stress. Nitrogenase activity and ammonium production were then used to select the most active bacterial species. Nitrogenase activity, using acetylene reduction technique, was 0.763 and 0.475 µmol C_2H_4 / mg protein/ h for *R. leguminosarum* and *R. meliloti* respectively. However, those two species growing on a nitrogen-free media produced 2 to 3.4 and 2.8 to 5.0 µg ml^{-1} of ammonium with glutamate or histidine, as nitrogen sources respectively. The specific association between rhizobia and plant species was revealed under the transmission electron microscopy. The effect of salt on the growth of *Rhizobium* appeared to depend on the strains themselves, as the responses of the strains were disparate in the same soil and region.

Key words: Jordan, nitrogen fixation, nitrogenase, *Rhizobium.*

INTRODUCTION

The symbiotic fixation of nitrogen is of extreme importance for the maintenance of soil fertility and, in agricultural practices, it is utilized to increase crop yields. Nitrogen fixation is ecologically important because some of the fixed nitrogen is likely to become available to plants. The best characterized symbiotic association involving nodules is the one occurring on the roots of leguminous plants. Members of the family Leguminosae (Fabaceae) are the only plants capable of forming nitrogen-fixing nodules with rhizobia, with the single nodule exception of the nonlegume genus *Parasponia*, a tropical tree (Atlas and Bartha, 1987).

The largest contribution of combined nitrogen in terrestrial habitats is due to the symbiotic nitrogen-fixation by rhizobia. However, the rates of nitrogen fixation by symbiotic rhizobia are often two to three times higher than the ones exhibited by free-living nitrogen-fixing bacteria in soil (Dalton, 1974). The five rhizobial genera, *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* (Chen et al., 1988), *Azorhizobium* (Dreyfus et al., 1988) and *Photorhizobium* have been grouped for many years with the *Agrobacteria* and *Phyllobacteria* into one family, the Rhizobiaceae (Jordan, 1984). Each of these genera has close relatives, that are not plant symbionts , placed in different families. In this study, various species of rhizobia were isolated from leguminous plants grown at the north of Jordan.

*Corresponding author. E-mail: yousefaz@yahoo.com.

MATERIALS AND METHODS

Sample collection

Fresh nodules from different leguminous plants were collected from Irbid district at the north of Jordan (Table 2) and transferred to the laboratory for immediate testing.

Isolation of nitrogen - fixing bacteria

Healthy root nodules (10 to 15), depending on the relative nodule size, were placed in a small tube and washed with running tap

Table 2. The ability of isolates of Rhizobia to nodulate with listed host plant species indicated as (+) signs.

Host plant	*R. leguminosarum*	*R. ciceri*	*R. meliloti*	*R. loti*
Cicer arietinum		+		
Lathyrus aphaca	+			
Lathyrus gorgonei	+			
Lupinus pilosus				+
Onobrychis spinacrisbi		+		
Ononis nabrix				+
Ononis viscose				+
Psoralea bituminosa			+	
Tetragonolobus palaestinus	+			
Trifolium clypeatum	+			
Trigonella kotchyi			+	
Vicia faba	+			
Vicia palesbina	+			
Vicia peregrine	+			

water for few min. Then they were immersed in 95% (v/v) ethanol for 5 to 10 seconds and then subjected to 5% H_2O_2 for 1 to 4 min depending on its relative size. This treatment was followed by eight changes in sterile water. Nodules were homogenized in 2 ml sterile water. Serial 10-fold dilutions of the nodule homogenate were made and 0.1 ml of the dilutions was spread on yeast extract mannitol agar medium (YEM) containing 0.25 mg (mgL^{-1}) bromothymol blue. All plates were incubated at 28°C for 35 days (until visible growth was observed). For each nodule isolate, a single colony was selected and restreaked on YEM agar plates and checked for purity. When pure cultures had been confirmed, each isolate was streaked on YEM agar slopes incorporating 3 g $CaCO_3$ L^{-1} and stored at 5°C, for further identification and nodulation experiments (Brown and Dilworth, 1975; Date and Halliday, 1987; Gao and Yang, 1995).

Identification of bacterial isolates

Various types of bacteria were streaked on YEM agar plates containing Congo red (25 µg L^{-1}) as a presumptive test for purity of cultures (Tas et al., 1996). The motility, catalase and growth in litmus milk biochemical tests were done. Additionally, fermentation was tested on carbohydrate sources including: arabinose, rhamnose, xylose, mannose, maltose, sucrose, glucose, fructose, lactose and ribose. Different species of bacteria were tested for their sensitivity to nalidixic acid of 80 mg ml^{-1} final concentration in YEM agar. On the other hand, the effect of 1 to 6% w/v concentration of NaCl was experimented in YEM agar plates and the growth at (31, 36, 38 and 42°C) temperatures tested on YEM agar plates too. The pH tolerance of bacteria was determined by using YEM agar plates in which the pH values were adjusted to 4.0, 5.0, 6.0, 8.0, 9.0 and 10 by adding 1 N HCl or 1N NaOH. Control culture was grown under pH = 6.8. The presence or absence of growth scored in inoculated YEM agar plates after 3 to 5 days.

Plant nodulation

To determine species of *Rhizobium*, number of seeds of *Trifolium clypeatum, Trigonella foenum, Chickpea cicer*, and *Lupinus pilosus* (1 to 4 depends on the seeds size) that represent the major inoculation plant groups were immersed in tubes for 10 sec in 95% ethanoland then in 1.12% (w/v) $HgCl_2$ solution for 3 to 5 min. After decanting, the seeds were rinsed 8 to 10 times with sterile water. The surface sterilized seeds were then spread onto 0.9% water agar in Petri dishes and incubated at 25°C in an inverted position to provide uniform seedlings with straight radicles. Tubes containing seedlings nutrient agar with Fahraeus solution (Gibson, 1980) and radicles 1 to 2 cm long were inoculated with 4 ml of a 72 h old *Rhizobium* culture (Dunfield et al., 1999; Tas et al., 1996; Somasegaran and Hoben, 1994). Uninoculated plants served as negative control. Then, tubes were placed under controlled conditions (at 37°C, 16 hours of light and 8 hours of dark) (Olivaris et al., 1980). Plants were harvested at 6 weeks after planting, nodulation determined and species identity was confirmed from nodule crushes according to Ronson and Primrose (1979).

Nitrogenase enzyme assay

Nitrogenase enzyme was performed to the two isolates of *R. leguminosarum* and *R. meliloti* that isolated from *Trifolium clypeatum* and *Psoralea bituminosa* respectively. Two McCartney bottles (29 ml) contained 12 ml broth media composed of ((g L^{-1}): KH_2PO_4, 1.1; $K_2HPO_4.3H_2O$, 2.2; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; $FeCl_2.6H_2O$, 0.015 and $(NH_4)6MoO_{24}.4H_2O$, 0.002 (Pagan et al., 1975). The media was first autoclaved at 121°C for 15 min and then 1 ml of the filtrate growth factor solution was added (10 mg of biotin and 20 mg pyridoxin hydrochloride prepared in 100 ml distilled water), pH 5.9 . Different types of rhizobia were incubated in that media at 25°C for 10 days. C_2H_2 was injected to give a final concentration of 10%. Bottles were incubated for 1 h at 28°C. Gas samples were assayed chromatographically for acetylene reduction using a gas chromatograph model 437 A (Chrompack Packard, Raritan, NJ) fitted with a dual flame ionization detector (FID) and Porapak N columns (2 m × 2 mm). The oven temperature was kept at 60°C. Nitrogen as carrier gas, and H_2 as fuel gas, were used at 30 to 35 ml and 20 to 25 ml min^{-1}, respectively. To determine the amount of ethylene produced, 0.2 ml of gas sample from each bottle was injected (Parmar and Dadarwal, 1999).

Estimation of NH_4^+ concentration

Different species of rhizobia were grown in the following medium

Table 1. Carbohydrate utilization by different *Rhizobium* species. (+) sign indicates the ability of the isolates to grow on the type of sugar indicated in comparison with the reference positive control (YEM agar).

Rhizobium species	*R. leguminosarum*	*R. meliloti*	*R. ciceri*	*R. loti*
Fructose	+	+	+	+
Lactose	+	+	+	+
Ribose	+	+	+	+
Arabinose	+	+	+	+
Rhamnose	+	+	+	+
Xylose	+	+	+	+
Mannose	+	+	+	+
Maltose	+	-	+	+
Sucrose	-	+	+	+
Glucose	+	+	+	+

(g/L): mannitol, 1.0; KH_2PO_4, 0.3; Na_2HPO_4, 0.3; $MgSO_4.6H_2O$, 0.1; $CaCl_2$, 0.05; and trace elements (mg/L) H_3BO_3, 10.0; $ZnSO_4.7H_2O$, 1.0; $CuSO_4.5H_2O$, 0.5; $MnCl_2.4H_2O$, 0.5; $Na_2MoO_4.2H_2O$, 0.1; $FeCl_3$, 1.0; Biotin, 0.2; pH 6.8. Glutamate or histidine (1 mg/ml) was added as two separate nitrogen sources. NH^{+4} was determined colorimetrically at 410 nm using Nessler's reagent (Somasegaran and Hoben, 1994) on cell supernatants clarified by centrifugation at 10 000 × g for 15 min. Absorbance was converted into µM of NH^4 from a calibration curve prepared using 1 to 5 mg of NH_3 per ml (Adams, 1990).

Preparation of plant nodules for electron microscopic studies

Due to the high cost of examining samples under the electron microscope, only tissues of *Lupinus pilosus* nodules obtained from the plant nodulation experiment were investigated. Three samples each include 3 to 5 nodules were initially fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 hours, then rinsed with the same buffer for 3 times at 5 min intervals. After that, the tissues were fixed in 1% osmium tetroxide (OSO_4) in 0.1 M sodium cacodylate buffer. The resultant fixed tissue was washed 3 times of 5 min each with the same buffer. Dehydration was done in ascending series of acetone starting with 30, 50, 70 and 90% for 10 min each and 100% 4 times of 10 min each. Different mixture of acetone and Spurr's resin were used to infiltrate tissues as the following 1:3, 1:1 and 3:1 for 20 min each in a rotator. Subsequent infiltration occurred in pure resin for 48 h with a change every 12 h. Finally, the tissues embedded at 65°C for 8 h. The diameter of silver gold sections was 90 nm and the electron microscope used was Zeiss EM 10 CR.

RESULTS AND DISCUSSION

R. leguminosarum, R. meliloti, R. loti, and *R. ciceri* were isolated from leguminous plants at the north of Jordan, They were Gram negative, rod curved or straight, occurring singly and grew well under aerobic conditions. They grew on YEM agar plates containing bromothymol blue turning the indicator to yellow. Although it provides a useful indicator of relatively small changes in acidity, many non-rhizobial contaminants can be revealed in this medium by their more drastic effect on pH turning the medium to more dark color (Vincent, 1977). Only the isolates of *R. meliloti* absorbed Congo red strongly. Most isolates of rhizobia lack the ability to absorb Congo red from a yeast extract mannitol salt medium containing 0.0025% final concentration of this dye, resulted in colorless or faintly pink colonies, whereas contaminant colonies absorb the dye and become a deep red.

Most of the rhizobial isolates were able to grow rapidly on minimal salts agar containing yeast extract and fructose, glucose, ribose, arabinose, rhamnose, xylose and mannose as the sole carbon source similar results to that obtained in Graham and Parker (1964) and Glenn and Dilworth (1981). In addition, they grew on lactose, maltose and sucrose as presented in Table 1. Carbon utilization patterns (Bouzar et al., 1995) are an important method used for identification purposes, which is very accurate and can be achieved through short times comparing with conventional nodulation assay (Dunfield et al., 1999). The carbon sources utilized by *Rhizobium* have been reviewed (Elkan, 1984) and studies of 95 strains of *Rhizobium* in different species showed that 36 out of 40 utilized lactose, maltose and sucrose. While mannitol is the carbon source routinely used for in vitro cultivation of *Rhizobium*, a number of strains do not utilize mannitol.

Rhizobia were identified by the ability to nodulate legumes (Table 3). Each isolate was also authenticated by their ability to nodulate the plant species from which it was originally isolated. Unfortunately, seeds from a given legume species were not available, so other host from the same cross-inoculation group was used as presented in Table 2.

R. leguminosarum has a unique characteristic among other rhizobial species concerning its resistance to grow at high concentration of nalidixic acid (80 µg/ml) compared to other isolates. This is similar to Soberon-Chavez and Najera (1988) report on the growth of all strains (more than 150 strains of different geographical origins) on the same growing conditions.

Bacterial isolates varied in their response to salt stress, which range from 1-5% NaCl. *R. meliloti* was

Table 3. Ammonium concentrations in the supernatant of bacteria grown on media with a glutamate or histidine as a nitrogen source.

Rhizobium species	NH_4^+ concentration (µg/ml)	
	Glutamate	Histidine
R. leguminosarum	3.4	5.0
R. ciceri	2.6	3.8
R. meliloti	2.0	2.8
R. loti	2.8	4.0

found to have greater resistance compared with other rhizobial species reaching to about 5% NaCl. It has been reported that tolerance of different species of *Rhizobium* to NaCl varies from 0.1 to 0.75 mol 1^{-1} (0.6 to 4.4%) (Tu, 1981; Le Rudulier and Bouillard, 1983; Bernard et al., 1980). Graham and Parker (1964) showed that fast-growing acid producing rhizobia were generally more salt tolerant than the slow-growing, alkaline-producing strains. Rhizobia show marked variation in salt tolerance, a number are inhibited by 100 mmol (0.6%) salt (Zhang et al., 1991) but growth at salt concentrations of more than 500 mmol L^{-1} (3%) has been reported (Graham and Parker, 1964).

All isolates in this study grew at pH range 6-8. *R.meliloti* is known to be adapted to alkaline conditions. All isolates of this species grew at pH 9, while *R. loti* was the most acidic tolerant species. These results were similar to that obtained by Graham and Parker (1964).

Relative to the growing temperature, Rhizobial isolates grew at 36°C, however, the maximum growth temperature for *R. leguminosarum* and *R. ciceri* was 38°C, while *R. meliloti* and *R. loti* had the ability to grow at 42°C. Maximal growth temperature for a large collection of rhizobia of tropical and temperate legumes has been determined (Zhang et al., 1991). Among temperate strains those of *R. meliloti* were the most tolerant (36.5 to 42.5°C) being 8°C higher on the average than those of *R. leguminosarum* (31 to 38°C). Graham and Parker (1964) examined the ability of 79 strains from different rhizobial species to grow at 39°C and found that 9 strains 8 of them from *R. meliloti* grew at that temperature.

Nitrogenase activity of *R. leguminosarum* and *R. meliloti* was 0.763 and 0.475 µmol C_2H_4 / mg protein/h respectively. Ammonium concentration detected in the supernatant in the presence of glutamate varied from 2 to 3.4 µg/ml depending on nitrogen-fixing microorganism but these values were higher when histidine was used as a nitorgen source (Table 3). The demonstration of the relatively higher NH^{4+} enrichment in the cell supernatants indicates that a major portion of the fixed nitrogen was exported by these cultures. O'Gara and Shanmugam (1976) found that the majority of the nitrogen fixed in liquid cultures of free-living rhizobia is exported into the supernatant as NH_4^+. The buildup of glutamate and glutamine that known to inhibit the assimilation of NH_4^+ in free living *Rhizobium* species may function to repress the NH_4^+ assimilation pathways. Brown and Dilworth (1975) as well as Kurz et al. (1975) have reported that several key rhizobial enzymes involved in NH_4^+ assimilation may be switched off during symbiotic nitrogen fixation, allowing fixed nitrogen to be excreted as NH_4^+.

Transmission electron microscopy used to examine samples of *Lupinus pilosus* nodules obtained from plant nodulation experiment. Sections through these nodules (Figure 1) showed that many host cells in the central tissue were invaded by bacteria forming pleomorphic endosymbiotic bacteroids within symbiosomes. Bacterial suspensions prepared from the crushed nodules revealed a variety of bacterial cell types ranged from spherical, oval to elongated cells. Cells of nodular tissue were filled with bacteroids and the cytoplasm occupied a limited volume of the cell. Granules of poly-b-hydroxy butyrate were common in the bacteroids as shown in Figure 1.

The association between rhizobia and plant root is very specific. The mutual recognition between the two compatible partners is based upon chemotactic response and specific binding to the root hair. The legume plant root recognizes the right population of *Rhizobium*, which in turn recognizes the right kind of leguminous root. Establishment of an adequate rhizosphere population of *Rhizobium* is an absolute prerequisite for infection (Atlas and Bartha, 1987). The process of nodule formation is the result of a complex sequence of interactions between the rhizobial species and the plant roots (Solheim, 1984; Nutman, 1965).

Concluding remarks

Rhizobium generally does not absorb Congo red when plates incubated in the dark, but it absorbs this dye in light conditions. The fate of NH_4^+ may be determined by the organic nitrogen source such as L-glutamate and L-histidine available to the cell. *Rhizobium* may export an excess of fixed N_2 as NH_4^+ by simultaneously derepressing their *Nif* genes while repressing genes of NH^+_4 assimilation, thus free-living *Rhizobium* utilizes relatively little of their fixed N_2 in the presence of glutamate. It appears that the effect of salt on the growth of *Rhizobium* depends on the strains themselves, as the

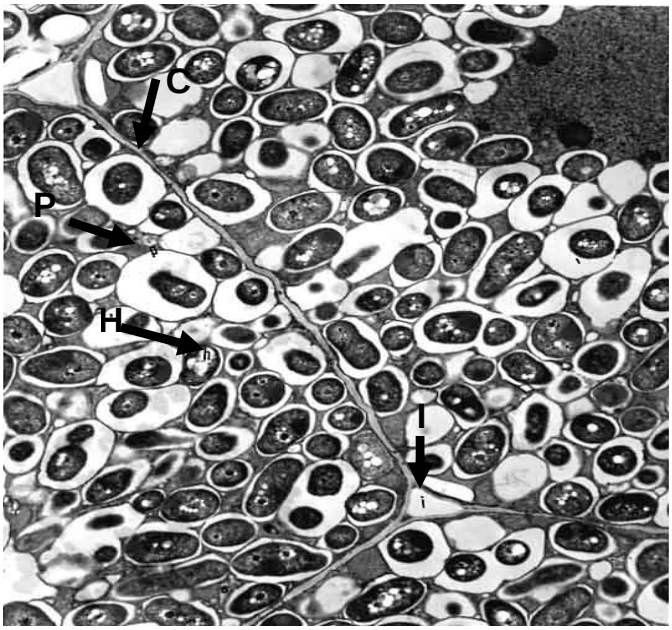

Figure 1. Root nodule of *Lupinus pilosus* infected with *R. loti* examined by transmission electron microscopy (X 6250). Observe a portion of an infected host cell with numerous symbiosomes containing pleomorphic bacteroids surrounded by peribacteroid membranes. P, peribacteroid membrane; H, poly-β-hydroxybutyrate granule; C., cell wall; I, intercellular space.

responses of the different strains used were disparate, even with plants collected from the same soil in the same region.

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