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Structure of root nodules and nitrogen fixation in Egyptian wild herb legumes

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Abstract

Five wild herb legumes (*Trifolium resupinatum*, *Melilotus indica*, *Medicago intertexta*, *Trigonella hamosa*, and *Alhagi murarum*) were collected from cultivated lands of the Nile Valley, and compared with clover (*Trifolium alexandrinum*), a cultivated forage legume. The wild herb legumes exhibited great variation in nodulation percentage, nodule number, nodule mass and acetylene reduction activity with regard to locality. Nodulation of *T. resupinatum* and *M. indica* ranged between 50 - 100 % and 33 - 100 %, respectively, compared to 50 - 100 % for *T. alexandrinum*. The number of nodules formed on *T. resupinatum* was 9 - 128 and that of *M. indica* 6 - 39, compared to 13 - 122 nodule per plant for *T. alexandrinum*. Nodule mass was correlated with nodule number. In *M. indica*, a small number of nodules was compensated with high specific nitrogenase activity. The herb legumes formed nodules of small size, varying shape (globose, cylindrical, branched, *etc.*), and of different types (crotalaroid and astragaloid). Microscopic examination of root-nodules from *T. resupinatum*, *M. indica* and *M. intertexta*, showed that these legumes formed indeterminate and effective nodules, containing apical meristems, central symbiotic tissue with characteristic zonation and peripheral vascular bundles. The nodules harboured bacteroids with pleiomorphic morphology.

Additional key words: *Alhagi murarum*, bacteroids, *Medicago intertexta*, *Melilotus indica*, nodule ultrastructure, *Rhizobium*, symbiotic nitrogen fixation, *Trifolium alexandrinum*, *Trifolium resuspinatum*, *Trigonella hamosa*.

Introduction

Legumes are of considerable interest because of their multi-use potential for food, feed, fiber, and fuel and because of their nitrogen fixation capabilities, which could

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significantly reduce the level of synthetic fertilizer required for crop production. Leguminosae represents one of the largest plant families and contains about 20 000 species distributed from arctic to tropical latitudes. Leguminous plants are distinguished from other plant families by their ability to form nitrogen-fixing root-nodules in close cooperation with bacteria from the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* (Nap *et al.* 1989). The role of legumes in supplying nitrogen to vegetation in derelict land, clay waste and reclaimed lands has been investigated quantitatively (Skeffington and Bradshaw 1980, Palmer and Iverson 1983). The legumes, which perform well in arid ecosystems, may be adapted to fix more nitrogen under arid conditions than legumes grown in other habitats (Zahran 1991). Nodulated wild legumes have potential for nitrogen fixation, reforestration and to control soil erosion (Ahmad *et al.* 1984). Recently, it has been reported that novel, suitable wild legume-*Rhizobium* associations are useful in providing a vegetational cover in degraded lands (Jha *et al.* 1995).

The nodulation status, nodule ultrastructure and nodule nitrogen fixing activity of wild legumes are very important characteristics which should be determined when evaluating the contribution of these legumes to soil fertility. Only about one-sixth (less than 15 %) of known legume species have been examined for nodulation (Allen and Allen 1981). Adding new nodulating and nitrogen-fixing legumes to the list is of considerable importance. Another approach to use wild legumes for soil fertility is to isolate effective rhizobia from these legumes, which will be used for inoculation of other crop legumes. It has been reported that the rhizobial strains isolated from wild tree legumes (*e.g. Acacia*) form more efficient (nitrogen-fixing nodules) on cowpea and groundnut compared to the homologous (compatible) strains (Wange 1989).

In this study, the nodulation status, nodule type, structure and ultrastructure, symbiotic nitrogen fixation (acetylene reduction), and morphology of nodule bacteroids of some wild herb legumes, collected from different localities of cultivated lands at Beni-Suef district (Middle Egypt), were investigated. The rhizobia isolated from nodules of these legumes are being characterized (data not presented in this paper).

Materials and methods

Collection of plants: Five wild herb legumes were collected from four localities of cultivated lands of the Nile Valley at Beni-Suef district. These legumes were collected from wild plant populations on banks of small irrigation canals, road sides and from the boundaries of cultivated lands. Plants were collected at the end of the spring season (April - May 1997). The plant cover in these localities was mainly represented by two crops, clover (*Trifolium alexandrinum*) and wheat (*Triticum vulgare*). The following annual and perennial herbs were collected: *Trifolium resupinatum*, *Melilotus indica*, *Medicago intertexta*, *Trigonella hamosa*, and *Alhagi murarum*. When collected, the leguminous plants were in the flowering or fruiting stages and were identified after Täckholm (1974). For comparison, the forage crop *Trifolium alexandrinum* was also collected. Fifteen plants of each species were uprooted carefully from a fairly wet soil to avoid nodule damage, then plants were transported immediately to the laboratory in clean, sterilized and sealed plastic bags and kept at 4 °C until the time of examination.

Plant roots were cleaned from soil particles by gentle shaking or by washing under running tap water. The roots were examined for the presence of nodules and the percentage of the nodulated plants was calculated. The nodulated plants were examined further to determine the nodular type. The nodulated plants were divided into three groups, one used for acetylene reduction assay, the second for isolation of rhizobia, and the third for studying structure and ultrastructure of nodules.

Nitrogenase activity: Acetylene reduction assay was used to measure the nitrogenase activity of root-nodules. Nodulated roots or rootlets were placed in vials (30 cm^3) fitted with rubber septa to facilitate the addition and withdrawal of gases. The vials were tightly closed, then 3 cm^3 of acetylene were injected into each vial. This volume represents about 10 % of the vial volume. Before injection of acetylene, 3 cm^3 of air were withdrawn from the vials to adjust the internal pressure of gases. The injected bottles were incubated for two hours at 28 °C. For acetylene reduction assay, one cm^3 gas sample from each bottle was injected into an *ATI Unicome 610* (USA) gas chromatograph with a flame ionization detector and a 152.4 × 0.32 cm glass column with activated alumina (80 - 100 mesh) at a temperature of 150 °C. The carrier gas was nitrogen at pressure difference 0.07 MPa. After acetylene reduction assay, the nodules were detached from roots, counted and their fresh mass was determined. Oven dry mass of nodules (85 °C for 18h) was also determined.

Light and transmission electron microscopy: Whole nodules, approximately 1 - 4 mm in diameter were fixed overnight in a solution containing 4 % glutaraldehyde (m/v) and 4 % para-formaldehyde (m/v) in 100 mol m^{-3} Na-phosphate buffer, pH 7.3, at 4 °C for 72 h. The fixed materials were washed in the same buffer, post-fixed in 1 % OsO_4 (m/v) at 4 °C, dehydrated through an ethanol series at room temperature and embedded in *LR White* resin (Nap *et al.* 1989), then left overnight in an oven at 70 °C for polymerization. Semi-thin sections (1 - 4 µm in diameter) and ultrathin sections (less than 1 µm in diameter) were cut on a *Sorvall JB4* microtome (Norwalk, USA) from the fixed, dehydrated and polymerized nodule material, and stained at room temperature with uranyl acetate for 20 min and then with lead citrate for 40 s. The semithin sections were mounted in oil and examined and photographed in a *Carl Zeiss* (Jena, Germany) photomicroscope. The thin sections were then examined using a *Philips 201* (The Netherlands) transmission electron microscope operating at 80 kV of accelerating voltage.

Results and discussion

Nodulation of wild herb legumes: The percentage of nodulated wild legumes collected from cultivated lands was determined and compared to *T. alexandrinum*, a cultivated forage legume crop in the same localities (Table 1). The nodulation percentage was different from one location to another. Two of the five herb legumes, *T. resupinatum* and *M. indica*, formed relatively large number of nodules in the all localities.

Table 1. Nodulation status (% nodulated plants) of 5 wild herb legumes and cultivated forage legume crop *T. alexandrinum* from four locations of cultivated lands at Beni-Suef district (nf - not found).

Species	Location 1	Location 2	Location 3	Location 4
Trifolium resupinatum	80	86	100	50
Melilotus indica	50	83	100	33
Medicago intertexta	nf	nf	nf	30
Trigonella hamosa	nf	nf	0	nf
Alhagi murarum	0	50	0	0
Trifolium alexandrinum	100	100	50	100

M. intertexta was only found in the location 4 with nodulation percentage of about 30 %, *T. hamosa* was collected only in location 3 but plants were not nodulated. Although *A. murarum* was collected from four localities, only plants from location 2 formed nodules (percentage of nodulated plants about 50 %). The pecentage of

Table 2. Nodule number [plant^{-1}], nodule fresh and dry masses [mg plant^{-1}] and absolute [nmol(ethylene) plant^{-1} s^{-1}] and specific [nmol(ethylene) g^{-1}(nodule d.m.) s^{-1}] symbiotic nitrogen fixation (acetylene reduction) for wild herb and crop legumes collected from four localities of cultivated lands. Means ± SE, $n = 5$.

Species	Location	Nodule number	Fresh mass	Dry mass	Absolute activity	Specific activity
T. resuspinatum	1	70 ± 17.7	65.0 ± 29.3	28.0 ± 11.8	0.265	9.44
	2	128 ± 25.7	30.0 ± 4.6	19.0 ± 2.9	0.210	11.11
	3	9 ± 0.33	0.3 ± 0.03	0.2 ± 0.1	0.210	51.39
	4	12 ± 2.90	2.3 ± 0.7	1.3 ± 0.5	0.029	22.78
M. indica	1	39 ± 7.4	36.0 ± 12.1	12.0 ± 6.2	0.085	7.50
	2	17 ± 2.5	8.0 ± 1.6	3.0 ± 0.3	0.068	22.50
	3	6 ± 0.9	0.2 ± 0.03	0.1 ± 0.0	0.011	111.11
	4	6 ± 0.7	0.5 ± 0.05	0.2 ± 0.04	0.026	127.78
A. murarum	2	5 ± 0.7	5.6 ± 3.9	1.6 ± 0.9	0.050	31.39
T. alexandrinum	1	72 ± 6.9	141.0 ± 30.0	39.0 ± 8.4	0.328	8.38
	2	112 ± 25.0	50.0 ± 26.0	23.0 ± 8.5	0.076	3.33
	3	25 ± 8.3	2.9 ± 1.1	1.5 ± 0.5	0.006	3.89
	4	13 ± 2.7	5.0 ± 1.7	1.8 ± 0.5	0.016	9.17

nodulated plants in *T. alexandrinum* was markedly higher. *T. resupinatum* and *M. indica* were chosen to study the nodule number and mass (Table 2). The data showed great variation in different locations. Plants collected from location 1 and 2 showed the highest number of nodules (Table 2). The examined wild herb legumes formed effective *Rhizobium*-legume symbioses, as the number of nodules formed was similar to that in cultivated forage legumes such as clover (*T. alexandrinum*). *A. murarum* collected from location 2 only formed about 5 nodules per plant. Nodulation of legumes has been shown to be controlled by many environmental and nutritional factors (Zahran 1991).

Fresh and dry masses of nodules were of the same trend as nodule number. Plants from locations 1 and 2 had also the highest nodule mass (Table 2). Since the majority of young leguminous plants were usually collected from the boundaries of fields and road sides, which were relatively dry, the nodules contained little amount of water. Fresh mass/dry mass ratio of these nodules, as calculated from data in Table 2, ranged between 1.5 - 2.3, 2.0 - 3.0 and 2.0 - 3.6 for *T. resupinatum*, *M. indica* and *T. alexandrinum*, respectively. The water content in nodules of wild plants was about 50 % and that of *T. alexandrinum* was about 70 %. The highest content of water was in nodules of *A. murarum*, since the fresh mass/dry mass ratio was about 3.5. These plants were collected from a recently irrigated cultivated lands.

Nitrogenase activity: In general, nodules of the wild legumes showed a higher rate of specific nitrogenase activity (C_2H_4 production) compared to the cultivated legume (Table 2). It has been well established that some symbioses are more effective than others for a variety of legume species (Jordan 1974). In this study, nitrogenase activity correlate with nodule number and nodule mass. Plants from locations 1 and 2 formed effective symbiosis and showed 5 - 20 times higher nitrogenase activity than plants from other locations. Similarly *T. alexandrinum* showed great effect of locality on nitrogenase activity (Table 2). However, specific nitrogenase activity [nmol(C_2H_4) g^{-1}(nodule d.m.) s^{-1}], was higher in wild legumes collected from locations 3 and 4 (Table 2). The root nodules of these plants are usually very small. Nodules of *M. indica* showed the highest specific nitrogenase activity. In general, the wild herb legumes showed nitrogenase activity (absolute and specific) higher than clover (Table 2) or reported activities of tree legumes such as *Acacia cyanophylla* (Nakos 1977). This nitrogen-fixing ability is an important character, which presents the wild herb legumes as a good candidate for improving soil fertility. The wild herb legumes examined are promising for their nitrogen-fixing activity and ability to grow under drought conditions.

Type of nodules, nodule structure and ultrastructure, and bacteroid morphology: The root nodules were classified into three types according to morphological criteria. The nodules of *T. alexandrinum* and *T. resupinatum* were globose and elongated of the crotalaroid type (Fig. 1*A,B*) as suggested by Corby (1980, 1981). The nodules formed on *M. indica* and *M. intertexta* were elongated and sometimes with branches (Fig. 1*C,D*) of the astragaloid type (Corby 1980, 1981) while that of *A. murarum* were small and spherical to globose (pictures not shown). The nodules of wild herb legumes are relatively small, the nodule length ranged between 1 - 7 mm (Table 3).

The structure and ultrastructure of nodules from *T. resupinatum*, *M. indica* and *M. intertexta* were examined in a bright field microscopy (Figs. 2, 3) and transmission electron microscopy (Figs. 4, 5). The root nodules showed the characteristic features of the indeterminate nodule. In temperate legumes such as alfalfa, clover, field bean and pea, the nodules are described as indeterminate and show the following

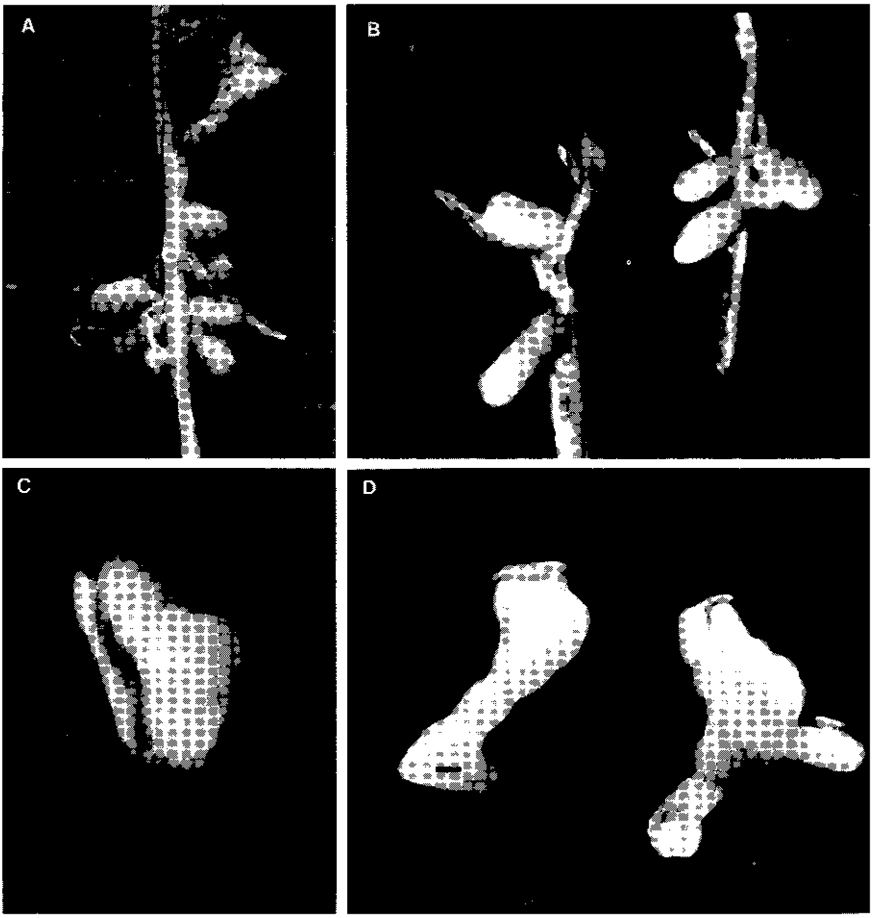

Fig. 1. Morphology of field collected nitrogen-fixing root-nodules of *Trifolium alexandrinum* (*A*), *T. resupinatum* (*B*), *Medicago intertexta* (*C*) and *Melilotus indica* (*D*). The nodules are elongate and corresponding to the crotalaroid type (*A,B*) or branched and corresponding to the astragaloid type (*C,D*); *bar* = 1 mm.

Table 3. Morphological features of root-nodules and bacteroids.

Species	Nodule shape	Nodule type	Length [mm]	Bacteroids
Trifolium resupinatum	globose, elongate	crotalaroid	4.0 - 5.0	irregular
Melilotus indica	elongate, branched	astragaloid	5.0 - 7.0	L,T,Y
Medicago intertexta	elongate, branched	astragaloid	5.0 - 7.0	Y
Alhagi murarum	spherical to globose	?	1.0 - 1.5	rods
Trifolium alexandrinum	globose, elongate	crotalaroid	1.5 - 2.0	Y

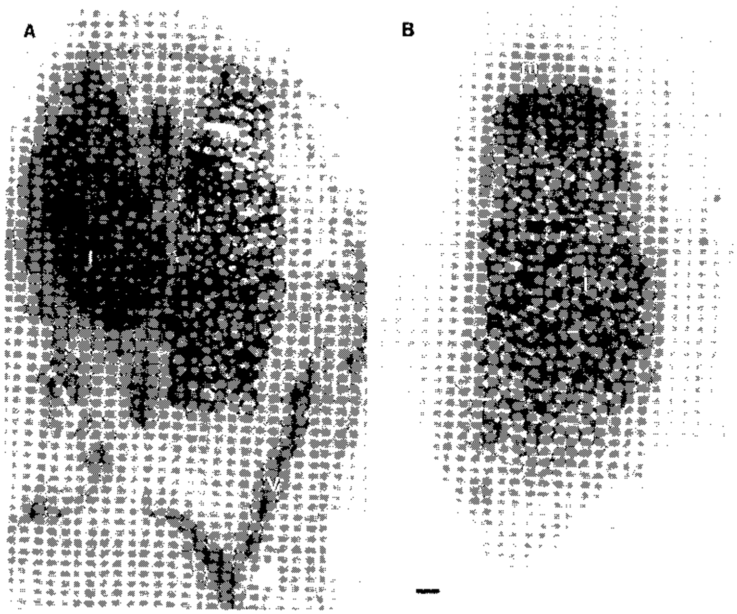

Fig. 2. Light microscopy micrographs (*bar* = 30 μm) to root-nodules. Longitudinal section in root-nodules of *Trifolium resupinatum* (*A*) showing branched nodules with two separate tissues containing infected tissues (i) and vascular bundles (v) extended from the root. Longitudinal section in root-nodules of *Melilotus indica* (B) showing various tissues of nodules; meristematic tissue (m) in the front of the nodule, infected tissue (i) occupying the whole area of the nodule, and a cortex (c) surrounding the central nodule tissues.

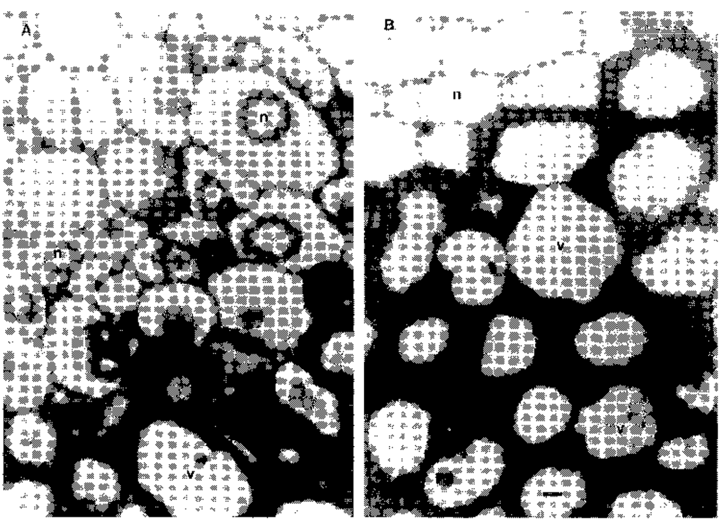

Fig. 3. Light microscopy micrographs (*bar* = 3 μm) to root-nodules. Meristematic cells of *T. resupinatum* (*A*) with prominent nuclei (n) and vacuolated infected cells (v). Non-infected cells (n) and vacuolated infected cells (v) of *M. indica* (*B*).

organization: an apical nodule meristem, a central nitrogen-fixing tissue (early and late symbiotic zone), peripheral vascular bundles interconnected with the plant vascular system, outer cortex, endodermis, nodule parenchyma (inner cortex) and boundary layer which surround the nodule (Zahran 1986, Sprent and Zahran 1988, Truchet *et al.* 1989).

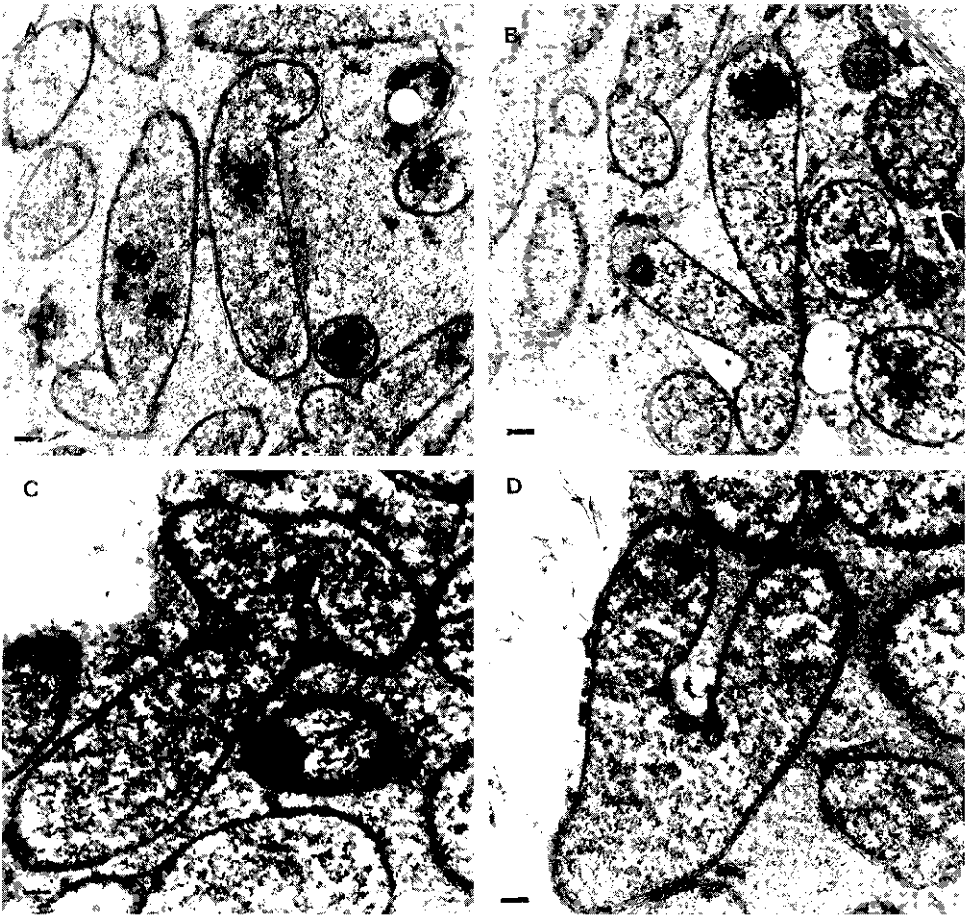

Fig. 4. Transmission electron microscopy (TEM) micrographs to ultrasections of root-nodules of infected cells of *M. indica* with pleiomorphic appearance: L shape (*A*), Y shape (*B*), T shape (*C*) and U shape (*D*); *bar* = 0.4 μm for *A,B* and 0.3 μm for *C,D*.

Root nodules of *T. resupinatum*, *M. intertexta* and *M. indica* contained bacteroids with pleiomorphic appearance (irregular and branched cells). The most common morphology was the T and Y shape or the cruciform shape. In addition, several irregular forms with L, S, and U shapes were also recognized (Figs. 4, 5). Bacteroid cells from *T. resupinatum* and *M. intertexta* contained globules of reserve food material such as poly-β-hydroxybutyric acid (Fig. 5*B,C,D*). Microscopic examination of bacteroids released from nodules, crushed on glass slides and Gram stained from nodules of various herb legumes, also revealed that the nodules are harboured bacteroids of variable morphological appearance (not shown). The bacteroids of these three legumes, in fact, are very similar to the bacteroids of *T. alexandrinum* nodules which also showed the typical pleiomorphic appearance (Table 3). The bacteroids of nodules from *A. murarum* were rods of different size (Table 3). The rod-shaped

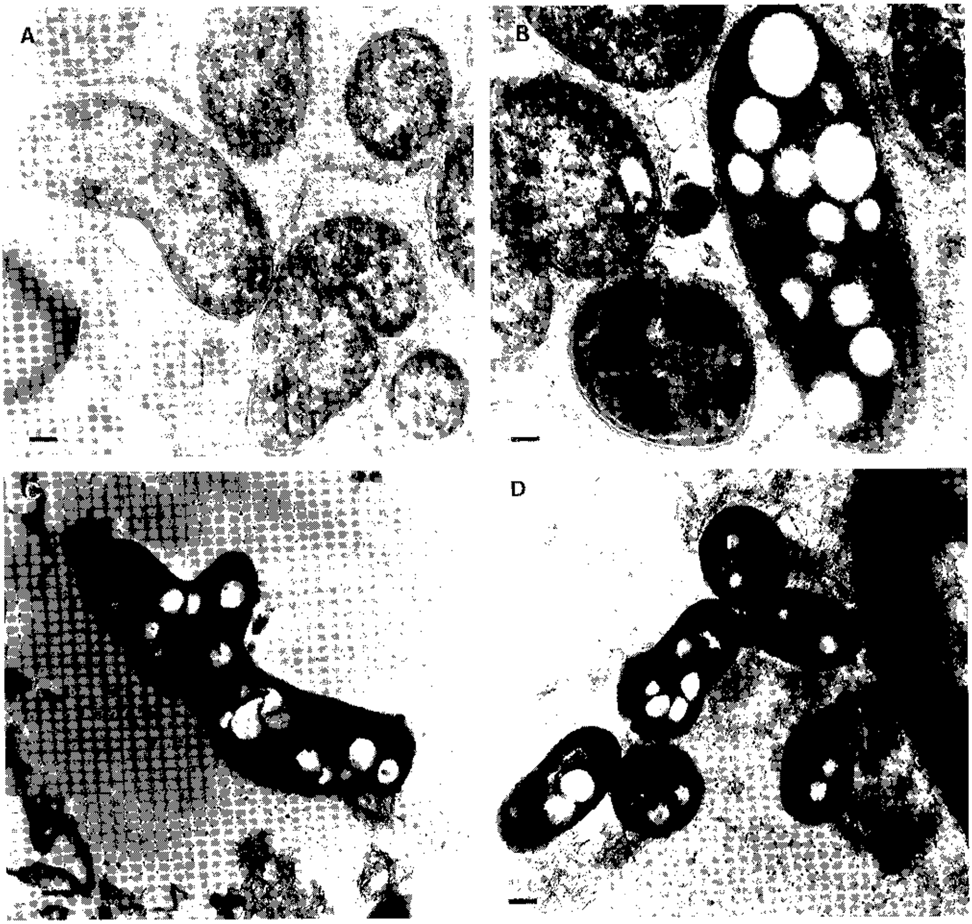

Fig. 5. TEM micrographs to ultrasections of root-nodules of infected cells of *T. resupinatum* (*A,B*) and *M. intertexta* (*C,D*) showing bacteroid morphology and ultrastructure. Irregular bacteroid cells (*A*; *bar* = 0.3 μm), cells containing reserved food materials (poly-β-hydroxybutyrate) in the form of white granules (*B*; *bar* = 0.2 μm), Y-shaped (*C*; *bar* = 0.3 μm) and rod-shaped (*D*; *bar* = 0.3 μm), bacteroids containing granules of reserved food materials.

rhizobia usually modified into enlarged and branched cells with pleiomorphic appearance when they become located intracellularly in nodules. It has been suggested (Gardiol *et al.* 1987) that differentiation and maintenance of bacteroids of symbiotic *R. meliloti* within alfalfa nodules require succinate dehydrogenase activity. Modification of rhizobial cells within nodules may be due in part to the high osmotic pressure in root or nodule cells compared to that of soil solution (Zahran 1986). It has been found that *in vitro* growth of rhizobia under extreme conditions of salt stress resulted in a modified cell morphology with the characteristic pleiomorphic appearance (Zahran *et al.* 1997).

In conclusion, the wild herb legumes form an effective symbiosis with *Rhizobium* strains, which is comparable with symbioses established between cultivated legumes and their rhizobia. These plants, which are adapted to wild life, can also be cultivated in reclaimed desert soil under arid conditions. Effective rhizobia, isolated from wild herb legumes will be used to inoculate other legume crops.

References

Ahmad, M.H., Rafique Uddin, M., McLaughlin, W.: Characterization of indigenous rhizobia from wild legumes. - FEMS Microbiol. Lett. **24**: 197-203, 1984.

Allen, O.N., Allen, E.K.: The Leguminosae. A Source Book of Characteristics, Uses and Nodulation. - University of Wisconsin Press, Wisconsin 1981.

Corby, H.D.L.: Rhizobial root nodules in the classification of the Leguminosae. - Ph.D. Thesis, University of Rhodesia, 1980.

Corby, H.D.L.: The systematic value of leguminous root nodules. - In: Polhill, R.M., Raven, P.H. (ed.): Advances in Legume Systematics. Part 2. Pp. 657-669. Royal Botanic Gardens, Kew - London 1981.

Gardiol, A.E., Truchet, G.L., Dazzo, F.B.: Requirement of succinate dehydrogenase activity for symbiotic bacteroid differentiation of *Rhizobium meliloti* in alfalfa nodules. - Appl. environ. Microbiol. **53**: 1947-1950, 1987.

Jha, P.K., Nair, S., Gopinathan, M.C., Babu, C.R.: Suitability of rhizobia-inoculated wild legumes *Argyrolobium flaccidum*, *Astragalus graveolens*, *Indigofera gangetica* and *Lespedeza stenocarpa* in providing a vegetational cover in an unreclaimed limestone quarry. - Plant Soil **177**: 139-149, 1995.

Jordan, D.C.: Ineffectiveness in the *Rhizobium*-leguminous plant association. - Proc. indian nat. Sci. Acad., Part B - Biol. Sci. **40**: 713-740, 1974.

Nakos, G. (1977) Acetylene reduction (N_2-fixation) by nodules of *Acacia cyanophylla*. - Soil Biol. Biochem. **9**: 131-133

Nap, J.-P., Van de Wiel, C., Spaink, H.P., Moerman, M., Van den Heuvel, M., Djordjevic, M.A., Van Lammeren, A.A.M., Van Kammen, A., Bisseling, T.: The relationship between nodulin gene expression and the *Rhizobium* nod genes in *Vicia sativa* root nodule development. - Mol. Plant Microbe Interact. **2**: 53-63, 1989.

Palmer, J.P., Iverson, L.R.: Factors affecting nitrogen fixation by white clover (*Trifolium repens*) on colliery spoil. - J. appl. Ecol. **20**: 287-301, 1983.

Skeffington, R.A., Bradshaw, A.Q.D.: Nitrogen fixation by plants grown in reclaimed China clay waste. - J. appl. Ecol. **17**: 469-477, 1980.

Sprent, J.I., Zahran, H.H.: Infection, development, and functioning of nodules under drought and salinity. - In: Beck, D.W., Materon, L.A. (ed.): Nitrogen Fixation by Legumes in Mediterranean Agriculture. Pp. 145-151. Martinus Nijhoff /Dr. W. Junk Publishers, Dordrecht 1988.

Täckholm, V.: Flora of Egypt. - Cairo University, Cooperative Printing Company, Beirut 1974.

Truchet, G., Camut, S., de Billy, F., Odorico, R., Vasse, J.: The *Rhizobium*-legume symbiosis. Two methods to discriminate between nodules and other root-derived structures. - Protoplasma **149**: 82-88, 1989.

Wange, S.S.: Response of groundnut (*Arachis hypogaea* L.) to inoculation with *Rhizobium* strains isolated from wild arboreal legumes. - World J. Microbiol. Biotechnol. **5**: 135-141, 1989.

Zahran, H.H.: Effects of sodium chloride and polyethylene glycol on rhizobial root hair infection, root nodule structure and symbiotic nitrogen fixation in *Vicia faba* L. plants. - PhD. Thesis, Dundee University, Dundee 1986.

Zahran, H.H.: Conditions for successful *Rhizobium*-legume symbiosis in saline environments. - Biol. Fert. Soils **12**: 73-80, 1991.

Zahran, H.H., Mohammad, E.M., Emam, M.M., Ismael, S.S.: The chemical composition, structure and ultrastructure of a halotolerant rhizobia isolated from Egypt. - In: Proc. 9th Microbiology Conference. Pp. 121-148. Egyptian Society of Applied Microbiology, Cairo 1997.