Symbiosis, 15 (1993) 227–238
Balaban, Philadelphia/Rehovot

The Root Nodule Symbiosis between *Rhizobium 'hedysari'* and its Drought-Tolerant Host *Hedysarum coronarium*

ANDREA SQUARTINI[1]*, FRANK B. DAZZO[2], SERGIO CASELLA[1] and MARCO P. NUTI[1]

[1] *Dipartimento di Biotecnologie Agrarie, Università degli Studi di Padova, via Gradenigo 6, 35131 Padova, Italy Tel. +39 49 8071442, Fax: +39 49 8070517*

[2] *Department of Microbiology and Public Health and Center for Microbial Ecology, Michigan State University, East Lansing, MI 48823*

Received March 10, 1993; Accepted May 24, 1993

Abstract

The symbiotic interaction occurring between the legume *Hedysarum coronarium*, a drought-tolerant forage crop relevant for the Mediterranean basin, and its natural host-specific, nitrogen-fixing bacterial symbiont, *Rhizobium 'hedysari'*, was studied by combined light and transmission electron microscopy. Several unusual morphological features of this root nodule symbiosis were found: in nature the plant is able to form modified pseudo-roots called 'shovels'; we observed that these structures can form independently from the presence of bacteria. Computer-assisted image analyses suggested three size classes of root hair lengths, one of which was longer than 600 μm. The primary infection route is through infection threads in deformed root hairs. Transmission electron microscopy of infection threads within the nodule revealed an atypical double layered wall. Nodules were indeterminant with an apical meristem, infection zone, and central bacteroid tissue. The predominant shape of differentiated *R. 'hedysari'* bacteroids is cruciform.

Keywords: *Rhizobium 'hedysari'*, *Hedysarum coronarium*, host-specificity, infection, root nodule structure

* To whom correspondence should be addressed.

1. Introduction

Hedysarum coronarium L. (sulla, Spanish sainfoin, Spanish esparcet, French honeysuckle) is a perennial leguminous plant widely used as a forage crop in the Mediterranean basin. Its area of cultivation includes Portugal, Spain, Morocco, Algeria, Tunisia, Egypt, Italy, Yugoslavia and Greece. The plant is also found in Israel, Lebanon and Turkey. The agronomical features of this legume, specifically its resistance to drought and to alkaline conditions (up to pH 9; Lupi et al., 1988), make it a suitable choice for highly calcareous and semiarid soils. Its strong root apparatus is also appreciated for anti-erosion properties and reclamation of marginal areas. When grown as a rainfed crop, a yield of 60 tons of green forage per hectare can be harvested annually (Sarno and Stringi, 1981). The leaf protein content reaches 24% of the dry weight (Ballatore, 1963). Due to its showy, red fragrant flowers, sulla is also exploited for honey production in regions of Italy and in the Balearic islands.

A unique morphological feature of this plant is the production, observed both in natural and laboratory conditions, of elongated ribbon-shaped structures called "shovels" which branch from the primary root. The length and frequency of shovels are variable and their physiological significance has not been elucidated (Nuti and Casella, 1989). Calcium oxalate has been reported to accumulate in shovels. Uptake and immobilization would reduce the calcium concentration in the rhizosphere, possibly leading to an increased solubility of phosphates and other ions.

Sulla participates in a host-specific nitrogen-fixing symbiotic interaction with soil bacteria which have been referred to as *Rhizobium 'hedysari'*. Cross inoculation with this bacterial symbiont revealed a specificity for *Hedysarum coronarium* (Cabrera et al., 1989). Cultivation of the plant in Australian soil, away from its natural habitat, required inoculation with the proper symbiont to ensure nodulation, indicating that the biogeography of this microsymbiont is somewhat restricted (Casella et al., 1984). *R. 'hedysari'* can also nodulate other species of *Hedysarum* and occasionally *Onobrychis viciaefolia*, a member of the same tribe (*Hedysareae*), though rhizobia specific for the latter cannot nodulate *Hedysarum* (Casella et al., 1984). Glatzle et al. (1986) reported that in Morocco strains having a *nod+ fix+* phenotype on *Hedysarum coronarium* are *nod+ fix–* on *Hedysarum flexuosum*. Genetic studies (Espuny et al., 1987, Mozo et al., 1988; Ollero et al., 1989) also demonstrate a marked host-specificity of *R. 'hedysari'* and the presence of large symbiotic plasmids.

Little information is available on the infection process and nodule development in this bacterial-plant symbiosis. Our aim therefore was to uncover the

morphological details and highlight the structural peculiarities of this unique, drought-tolerant root nodule symbiosis.

2. Materials and Methods

Bacterial strains and culture conditions

Rhizobium 'hedysari' strain IS 123 and CC1335 were isolated from root nodules of *H. coronarium* in Spain, the former was kindly provided by Dr. F.J. Ollero, University of Seville. Bacteria were grown at 28° C in YMB (Vincent, 1970), TY (Beringer, 1974), or BIII (Dazzo, 1984) media.

Plant material and nodulation tests

Seeds of *H. coronarium* ecotype Villamagna, obtained from the Dept. of Agronomy, University of Pisa, were surface sterilized by immersion in 95% ethanol for 25 sec, and in 0.1% mercury chloride for 7 min; rinsed 7 times in sterile distilled water, and germinated in the dark on inverted TY agar plates at 22° C. Plants were grown in a vermiculite-perlite substrate (1:1) in styrofoam cups watered with 1/8 strength nitrogen-free Fahraeus solution under a 14 hr light/10 hr dark photoperiod at 22° C/18° C in a growth cabinet. For nodulation tests 1 ml of a BIII-grown bacterial culture (ca. 5×10^8 cells/ml) was applied to the crown of each plant and nodules were scored after 25 days. Shovels were observed on plants grown as previously described (Casella et al., 1984).

Light and transmission electron microscopy

Root hairs were photographed using brightfield microscopy and subsequently measured by image analysis with a digitized morphometry system (Dazzo and Petersen, 1989). Free bacteroids in squash mounts of 25 day-old nodules were examined by phase contrast microscopy. For sectioning, nodules were fixed in 4% glutharaldehyde, 1% paraformaldehyde, 1 mM $CaCl_2$ in 50 mM Na cacodylate buffer (pH 6.8) under vacuum for 2 hr, washed in the same buffer 4 times for 15 min each, and post-fixed in 1% OsO_4 in 100 mM Na cacodylate buffer (pH 6.8) for 2 hr. Fixed nodules were rinsed 3 times with water (15 min each), dehydrated through an acetone series and embedded in Spurrs firm epoxy media. Two μm sections were stained with alkaline toluidine blue solution and examined by brightfield microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Philips CM-10 transmission electron microscope.

3. Results and Discussion

Shovels

The size and frequency of these modified roots exhibit a wide range of variation. Both sparse formations and dense clusters (Fig. 1A and B) are produced. Occasionally, extreme elongated shovels developed (Fig. 1C). A study of the

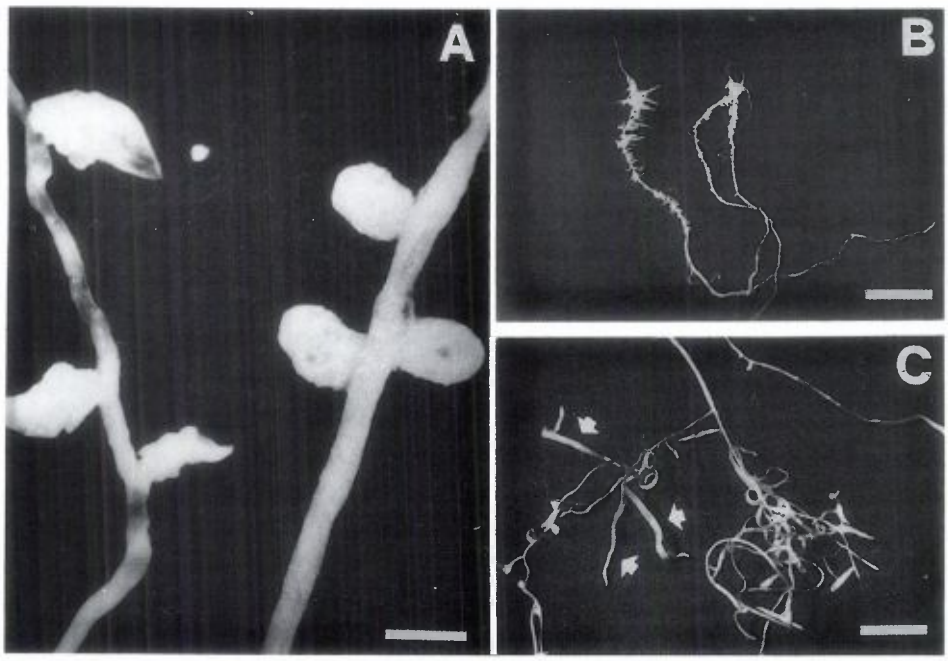

Figure 1. Roots of *Hedysarum coronarium* with shovels of different size and frequency; in (A) a comparison of shovels (left) with nodules (right) is shown. (B) dense shovel formation (C) elongated shovels (arrows). Bar = 3 mm in (A), 10 mm in (B), C).

ultrastructure of shovels by electron microscopy is in progress. A correlation between their variable morphology and different culture conditions such as relative humidity, nutrient availability, salinity, penetrability of rooting support, could not be drawn. Moisture level and calcium concentration though, seem to play a role in the formation of shovels. It is not known whether these structures can assist the plant in the drought tolerance mechanisms. Presence of rhizobia or other organisms is not a prerequisite for this process since shovels can be produced by axenically-grown *H. coronarium* plants. Hedysarum coronarium *can produce unusually long root hairs.*

Figure 2 depicts the typical morphology of deformed root hairs on sulla inoculated with strain CC1335. A prominent feature was the wide range of root hair length, including some which were usually long (see arrows in Fig. 2A). A

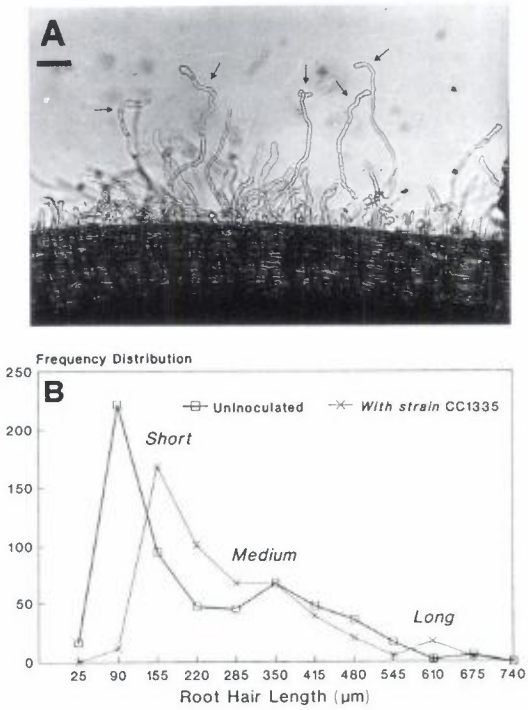

Figure 2. (A) Deformed root hairs in the mature root hair region of *H. coronarium* inoculated with strain CC1335. Arrows indicate unusually long root hairs; bar = 100 μm. (B) Frequency distribution of root hair lengths on inoculated and uninoculated roots.

detailed evaluation of the size distribution of root hairs by computer-assisted image analysis (n=600 hairs per sample) revealed that their length distribution clustered into three overlapping classes, with peaks at approximately 90–155 μm, 350 μm, and 610–675 μm. The peaks of medium and long root hairs were more prominent under axenic rather than inoculated conditions (Fig. 2B). Other leguminous species normally present root hairs not longer than 400 μm. It is possible that the unusually long root hairs may in part contribute to tolerance to dry soil conditions typical of *Hedysarum coronarium*.

Infection process and nodule structure

In the *Rhizobium*-sulla symbiosis the infection process involves entry and dissemination of the bacteria through infection threads that originate in deformed root hairs. Nodules are of the indeterminant type with developmentally distinct zones, resembling the *R. leguminosarum* bv. trifolii-white clover symbiosis, which also originates in the Mediterranean. Following inoculation with

Figure 3. Phase contrast micrograph of a segment of the root epidermis of *H. coronarium* showing a deformed root hair infected with *R. 'hedysari'*. Note the refractile intracellular infection thread (arrows). Bar = 25 μm.

infective strains of the rhizobial symbiont, root hair deformation was observed. Phase-contrast microscopy of roots stained with methylene blue revealed infection threads which had developed in root hairs and extended into the cortex (Fig. 3). A nodulated root and different nodule shapes are shown in Fig. 4. Brightfield (Fig. 5) and transmission electron microscopy of nodule sections (Figs. 6–9) offered a comprehensive view of dissemination of the bacterial symbiont within root nodules (see figure legends for details). A peculiar feature was observed in the ultrastructure of the infection thread wall which encases the lumen matrix and itself is surrounded by the host ensheathed membrane. In cross section (Fig. 6), the infection thread wall was composed of two distinct layers of different electron density, i.e. anamorphous outer layer surrounding a more dense fibrillar layer. In contrast, the differentiation of an additional outer amorphous layer is not apparent in the ultrastructure of infection thread walls in *Rhizobium*-clover and *Rhizobium*-pea symbioses (Napoli et al., 1975; Rae et al., 1992).

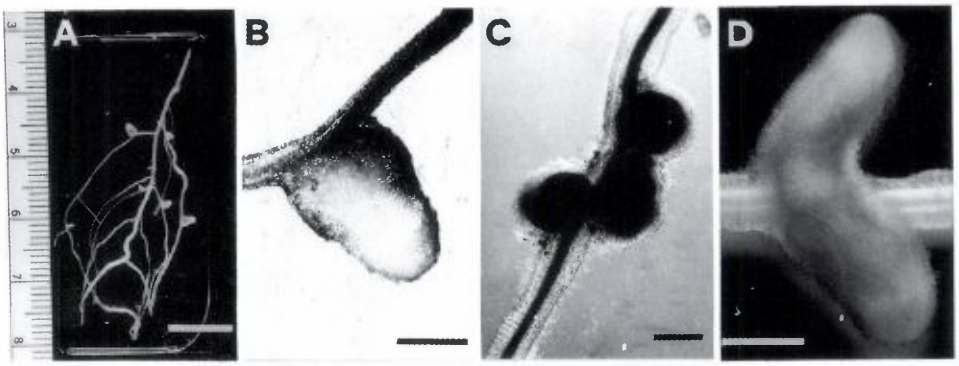

Figure 4. (A) Excised root system of *H. coronarium*, and (B–D) indeterminant nodules of different size and shape. Bar = 1 cm in (A) and 1 mm in (B), (C), (D).

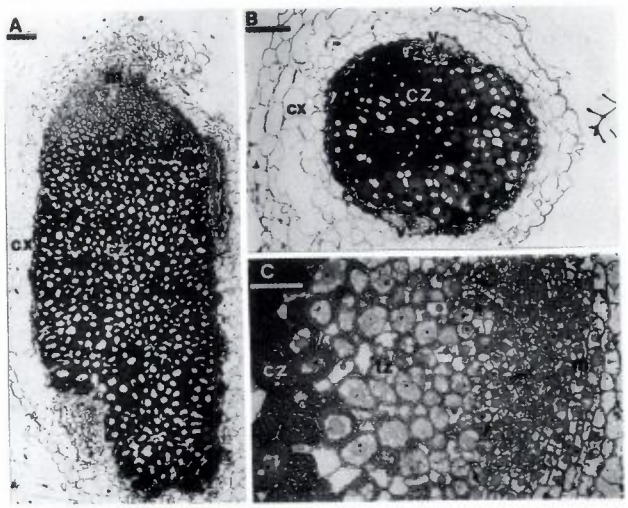

Figure 5. Light micrographs of the histology of *Hedysarum coronarium* indeterminant root nodules in (A) longitudinal section and (B) cross-section. Note the enlarged central zone of infected host cells and the peripheral uninfected cortex (cx) with vascular (v) elements. (C) A longitudinal section at the distal end of the nodule revealing host cells at different stages of symbiotic development, including the uninfected nodule meristem (m), infection zone containing several infection threads (arrows), a transition zone (tz) where rhizobia have begun endosymbiotic colonizationat the periphery of the host cell protoplast, and the distal border of the central zone (cz) of enlarged host cells filled with endosymbiotic bacteroids. Note the gradient of host cell size which increases towards the central bacteroid zone. Bars = 100 μm in (A, B) and 50 μm in (C).

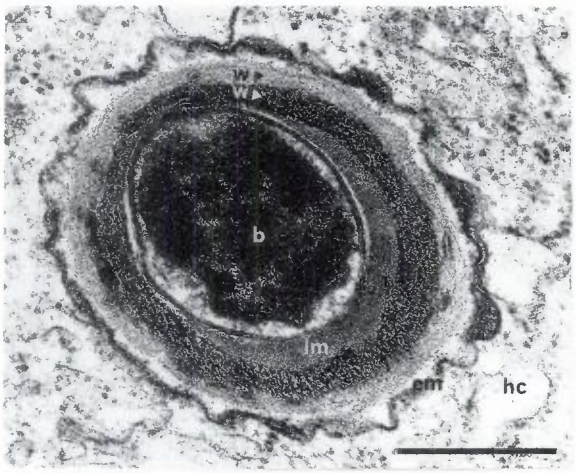

Figure 6. Transmission electron micrograph showing part of a *H. coronarium* cell with an infection thread in cross section; host cytoplasm (hc), the ensheathed membrane (em), a double-layered wall (w with arrows), lumen matrix (lm), and bacteria (b). Bar = 0.05 μm.

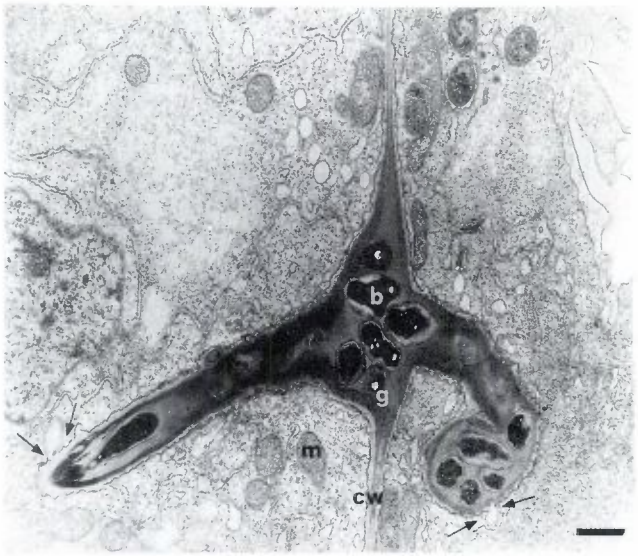

Figure 7. Transmission electron micrograph showing a longitudinal section of an infection thread traversing adjacent cell walls (cw) of neighbouring host cells, an abundance of neighbouring membrane-enclosed vesicles including some which have fused with the ensheathed membrane (arrows), mitochondria (m), bacteria (b). Electron-transparent granules (g) are probably formed by poly-β-hydroxybutyrate (PHB). Bar = 1 μm.

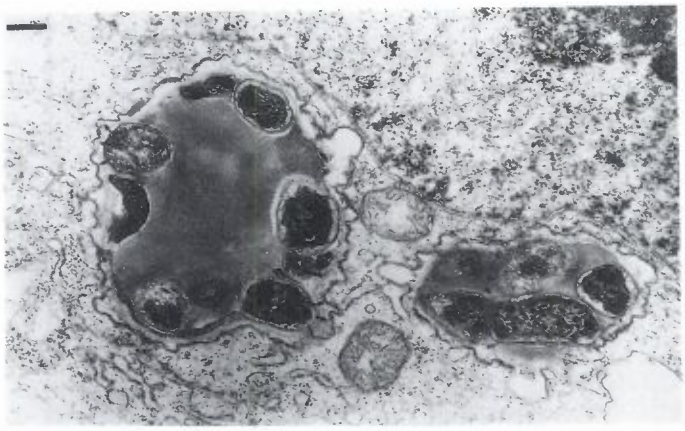

Figure 8. Transmission electron micrograph of bacteria which are partially released from an infection thread whose wall is thin, fragmented and discontinuous, and are still confined to a membrane-enclosed droplet within a nodule host cell of *H. coronarium*. Bar = 5 μm.

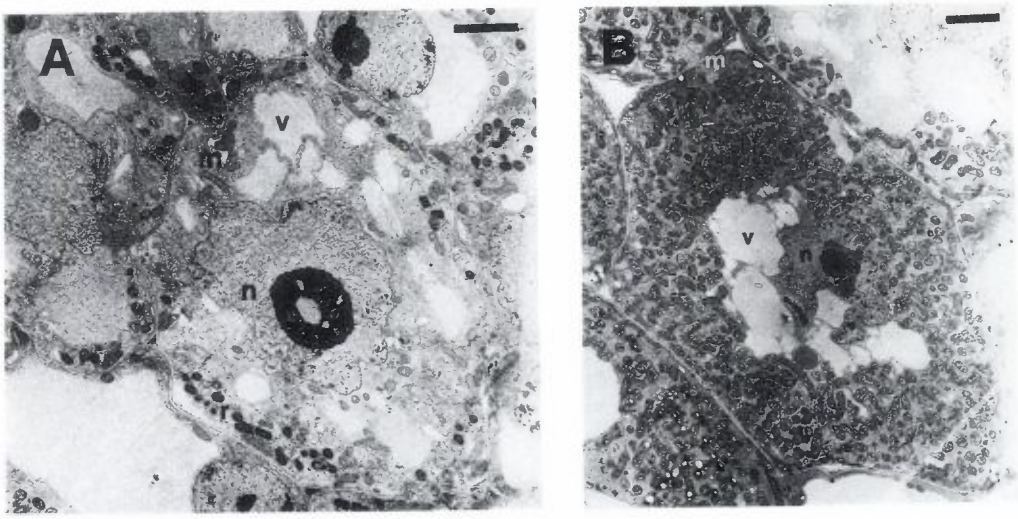

Figure 9. Transmission electron micrograph of nodule cells of *H. coronarium* at early (A) and advanced (B) stages of infection with *Rhizobium*. Nucleus (n), vacuole (v), rhizobia (r), mitochondria (m). Bar = 5 μm.

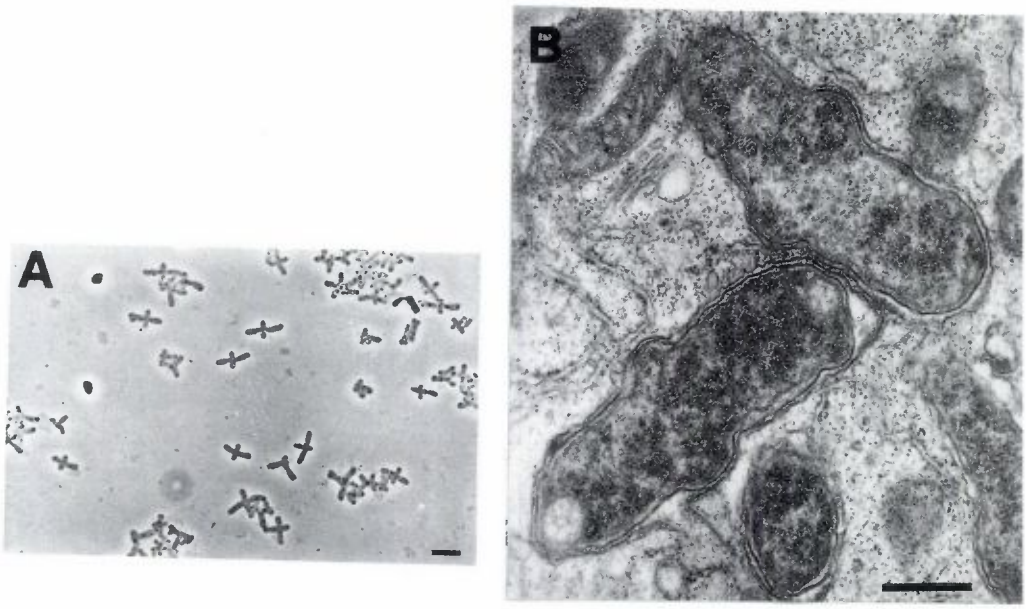

Figure 10. Bacteroids of *R. 'hedysari'* strain CC1335 in nodules of *H. coronarium*. (A) Phase contrast micrograph of a nodule squash. Note the frequent crucifix shape of the enlarged bacteroid. (B) Transmission electron micrographs of nodule symbiosomes containing endosymbiotic bacteroids. Note the continuity of the bacteroid outer membrane just beneath the closely associated peribacteroid membrane surrounded by the peribacteroid membrane. Bar scales are 5 μm in (A), 0.5 μm in (B).

Rhizobium 'hedysari' bacteroids have a unique cross shaped morphology

A large proportion of differentiated bacteroids in squash mounts of *H. coronarium* nodules have two small perpendicular branches, approximately one-third to one-half the distance into their longest dimension, resembling a crucifix (Fig. 10A). TEM of symbiosomes inside a host nodule cells (Fig. 10B) confirmed this cruciform morphology of mature bacteroids.

Our results show some of the particular aspects of this relatively unexplored, drought-tolerant bacterial-plant symbiosis and represent the basis for undertaking the characterization of *Rhizobium 'hedysari'* at genetic level. Experiments aimed at obtaining phylogenetic data to allow proper taxonomical description of the *Rhizobium* symbiont are in progress.

Acknowledgements

We wish to thank Francisco J. Ollero for providing strain IS123, Joann Palma and Stuart Pankratz for assistance with preparing specimens for microscopy, Roberto Frizzerin for technical assistance, Anita Toffanin for stimulating discussions, and Guy Orgambide for help during the nodulation test. Research was supported in part by M.A.F., special grant "P.F. Biotecnologie Avanzate". Part of this work was presented at the 9th International Congress on Nitrogen Fixation, Cancun, Mexico, 1992.

REFERENCES

Ballatore, G.P. 1963. La Coltivazione della Sulla. *L'Informatore Agrario* **52:** 5–15.

Beringer, J.E. 1974. R-factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **84:** 188–198.

Cabrera, E. Villa A. and Ruiz-Argueso, T. 1979. Diversidad y caracterizaciòn de la flora nativa de rizobios que nodulan en zulla (*Hedysarum coronarium*). In: *VII Congreso Nacional de Microbiologia.* Cadiz, (Abstr. Vol.), p. 294.

Casella, S., Gault, R.R., Reynolds, K.C., Dyson, J.R., and Brockwell, J. 1984. Nodulation studies on legumes exotic to Australia: *Hedysarum coronarium*. *FEMS Microbiol. Lett.* **22:** 37–45.

Dazzo, F. 1984. Leguminous root nodules. In: *Experimental Microbiol. Ecology.* R. Burns and J. Slater, eds. Blackwell Scientific Publishers, Oxford, pp. 431–446.

Dazzo, F. and Petersen, M. 1989. Applications of computer-assisted image analysis for microscopic studies for the *Rhizobium*-legume symbiosis. *Symbiosis* **7:** 193–210.

Espuny, M.R., Ollero, F.J., Bellogin, R.A., Ruiz-Sainz, J.E., and Perez-Silva, L. 1987. Transfer of *R. leguminosarum* bv. *trifolii* symbiotic plasmid pRtr5a to a strain of *Rhizobium sp.* that nodulates on *Hedysarum coronarium*. *J. Appl. Bacteriol.* **63:** 13–20.

Glatzle, A., Schulte-Batenbrock, T., and Brockwell, J. 1986. Symbiotic incompatibility between two forage species of *Hedysarum* grown in Morocco and their homologous rhizobia. *FEMS Microbiol. Lett.* **37:** 39–43.

Lupi, F., Casella, S., Toffanin, A., and Squartini, A. 1988. Introduction to *Rhizobium 'hedysari'* in alkaline clay-loam soil by different inoculation techniques. *Arid Soil Res. Rehabil.* **2:** 19–28.

Napoli, C., Dazzo, F., and Hubbell, D. 1975. Production of cellulose microfibrils by *Rhizobium*. *Appl. Microbiol.* **30:** 123–131.

Nuti, M.P. and Casella, S. 1989. Advances in the utilization of rhizobia in arid environments. *Arid Soil Res. Rehabil.* **3:** 243–258.

Ollero, F.J., Espuny, M.R., and Bellogin, R.A. 1989. Mobilization of symbiotic plasmid from a strain of *Rhizobium sp. (Hedysarum coronarium). System. Appl. Microbiol.* **11:** 217–222.

Rae, A., Bonfante-Fasolo, P., and Brewin, N. 1992. Structure and growth of infection threads in the legume symbiosis with *Rhizobium leguminosarum*. *The Plant Journal* **2**: 385–395.

Sarno, R. and Stringi, L. 1981. Sulla (*Hedysarum coronarium* L.). In: *Coltivazioni Erbacee*. R. Baldoni and L. Giardini, eds. Patron, Bologna, pp. 897–907.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root Nodule Bacteria*. I.B.P. Hanbook no. 15, Blackwell Scientific Publications, Oxford.