

## RESEARCH ARTICLE

# Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes

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## Abstract

A previous analysis showed that *Gammaproteobacteria* could be the sole recoverable bacteria from surface-sterilized nodules of three wild species of *Hedysarum*. In this study we extended the analysis to eight Mediterranean native, uninoculated legumes never previously investigated regarding their root-nodule microsymbionts. The structural organization of the nodules was studied by light and electron microscopy, and their bacterial occupants were assessed by combined cultural and molecular approaches. On examination of 100 field-collected nodules, culturable isolates of rhizobia were hardly ever found, whereas over 24 other bacterial taxa were isolated from nodules. None of these nonrhizobial isolates could nodulate the original host when reinoculated in gnotobiotic culture. Despite the inability to culture rhizobial endosymbionts from within the nodules using standard culture media, a direct 16S rRNA gene PCR analysis revealed that most of these nodules contained rhizobia as the predominant population. The presence of nodular endophytes colocalized with rhizobia was verified by immunofluorescence microscopy of nodule sections using an *Enterobacter*-specific antibody. Hypotheses to explain the nonculturability of rhizobia are presented, and pertinent literature on legume endophytes is discussed.

## Introduction

The nitrogen-fixing symbiosis between plants of the family *Leguminosae* and prokaryotic partners is typically characterized by the formation of root or stem nodules that are induced and subsequently invaded by the specific microsymbionts. These include the well-known alphaproteobacterial group of *Rhizobiaceae* containing the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* (*Ensifer*), *Mesorhizobium*, *Azorhizobium*, and *Allorhizobium*, along with other taxa such as *Methylobacterium* (Sy *et al.*, 2001) and *Devosia* (Rivas *et al.*, 2002), and members of the *Betaproteobacteria* such as *Burkholderia* (Moulin *et al.*, 2001) and *Ralstonia* (Chen *et al.*, 2001). The support of microscopy to examine nodule symbioses has gained new importance in

light of these findings, and various studies have coupled the visual approach with the molecular characterization of symbionts (Chen *et al.*, 2005; Elliott *et al.*, 2007). The traditional strategy used to investigate nodule-associated microbial symbionts involves their isolation and cultivation from internal tissues of surface-sterilized nodules (Vincent, 1970). The description of symbiotic partnerships for the various legumes has therefore traditionally relied, as a starting point, on the culturability of the bacterial occupant within the nodule when streaked on yeast–mannitol-based agar plates. Despite the physiological transformation of vegetative bacteria into nondividing bacteroids, it is normally observed that rhizobia are regularly cultured from surface-disinfectected crushed nodules. This implies either that some vegetative rods (still confined or recently released from

infection threads) have not undergone the bacteroid conversion (Paau *et al.*, 1980; Timmers *et al.*, 2000), or that some bacteroids can be resuscitated back to culturable state, or both of these possibilities.

The vast majority of studies that have detected the taxa so far assigned have, however, dealt with cultivated species of legumes or with man-exploited natural plants. In contrast, little attention has been paid to the root-nodule symbionts of truly wild legumes, that is, those whose ecology is not directly affected by human action. As the *Leguminosae* family includes over 18 000 species (<http://www.ildis.org/Leguminosae/>), only a minor portion has been examined (mostly representing crops of agricultural interest), and knowledge of the biological diversity of interactions between legumes and microorganisms is still very limited. We previously addressed this issue by describing the unusual diversity of *Gammaproteobacteria* as the sole culturable nodule occupants within three wild *Hedysarum* species collected in various locations in Algeria (Benhizia *et al.*, 2004a, b). In those studies, rhizobia could not be cultured from any of the 52 nodules examined, leading to the hypothesis that some *Gammaproteobacteria* may represent an alternative endosymbiotic partner to rhizobia for these nodulated plants.

In addition to the theories and research on *Rhizobium*-legume interactions, a parallel and rarely converging field of knowledge is that of microbial endophytes. Evidence that the healthy plant interior can normally contain bacteria or fungi not necessarily related to a pathogenic context was first put forward by Perotti (1926), subsequently revisited by Old & Nicolson (1978), and is now well documented by many studies that have been reviewed over the years (Hallmann *et al.*, 1997; Sattelmacher, 2001). The majority of reports deal with culturable endophytes. Stems and roots of most plant species tested harbour a range of  $10^3$ – $10^6$  live internal bacteria per gram of fresh weight, whose roles are related to various interactive phenotypes. Legumes are in this respect no exception: Gagné *et al.* (1987) found bacteria in the xylem of alfalfa roots; and Sturz *et al.* (1998) found 22 species within red clover. Sturz *et al.* (1997) showed that red clover harbours rhizobia of different species (not limited to the endosymbiont, *Rhizobium leguminosarum* bv. *trifolii*) not just in nodules but systemic throughout the plant. Elvira-Recuenco & Van Vuurde (2000) found that *Pantoea agglomerans* and *Pseudomonas fluorescens* were the most common endophytes in various pea cultivars. Dong *et al.* (2003) experimentally created the conditions in which inoculated enterobacteria achieved internal invasion of *Medicago sativa* and *M. truncatula*, and Kuklinsky-Sobral *et al.* (2004, 2005) demonstrated the plant growth promoting rhizobacteria (PGPR) properties of soybean endophytes.

Nodules themselves can be colonized internally by several bacterial genera unrelated to rhizobial symbiotic nitrogen

fixation. Philipson & Blair (1957) found diverse species, including Gram-positive bacteria, in roots and nodules of healthy red and subterranean clover plants. Sturz *et al.* (1997) showed that rhizobia recovery from red clover nodule tissue could yield up to  $4.3 \times 10^9$  CFU g<sup>-1</sup> fresh weight, but that, at the same time,  $3.0 \times 10^5$  CFU g<sup>-1</sup> of nonrhizobial endophytes, belonging to 12 different species, could be cultured from the same nodules. *Agrobacterium* sp. has been reported in nodules of tropical legumes (De Lajudie *et al.*, 1999). In bean nodules, Mhamdi *et al.* (2005) found, along with *Rhizobium*, *Agrobacterium*-like bacteria, and proved that these could invade new nodules upon coinoculation with rhizobia and affect their nodulation performance (Mrabet *et al.*, 2006). *Actinobacteria* such as *Streptomyces lydicus* have been reported to colonize pea nodules (Tokala *et al.*, 2002). Furthermore, Bai *et al.* (2003) showed that *Bacillus subtilis* and *Bacillus thuringiensis* can naturally coinhabit soybean nodules along with *Bradyrhizobium japonicum*, and that these Gram-positive bacteria can enhance plant productivity in coinoculation experiments. A more recent report (Zakhia *et al.*, 2006) described the association of 14 bacterial genera with wild legume nodules in Tunisia.

In order to gain a better understanding of the incidence and diversity of natural legume–endophyte associations, we examined the microbial occupants inside nodules and other plant tissues of 11 wild legume species collected in Sardinia (Italy) and Algeria, using both the standard colony isolation method and a direct PCR amplification of prokaryotic DNA from nodules and other tissues. In parallel, microscopy-based approaches were undertaken to document the microbial colonization within these legume tissues.

## Materials and methods

### Plant collection and nodule examination

An extensive search was conducted to locate plants of interest in suitable biotopes within Sardinia during the springs of 2004 and 2005. The legumes sampled, the nearest urban settlement, the geographical coordinates of the sampling site, and the number of nodules collected and analysed were as follows: *Hedysarum spinosissimum* (Giòscari, 40°42'N, 8°33'E; and Castelsardo 40°54'N, 8°41'E, 15 nodules), *H. glomeratum* (Pimentel, 39°29'N, 9°04'E, and Segariu, 39°34'N, 8°57'E, 13 nodules), *Hippocratea unisiliquosa* (Castelsardo, 25 nodules), *Scorpiurus muricatus* (Castelsardo, 25 nodules), *Tetragonolobus purpureus* (Nurèci, 39°50'N, 9°01'E, 34 nodules), *Ornithopus compressus* (Bolòtana, 40°19'N, 8°57'E, 24 nodules), *Ornithopus pinnatus* (Bolòtana, 21 nodules), and *Psoralea bituminosa* (Castelsardo, five nodules). The collection and characterization of culturable bacteria (exclusively *Gammaproteobacteria*) from nodules of three Algerian species (*Hedysarum spinosissimum* ssp.

*capitatum*, *Hedysarum pallidum* and *Hedysarum cornoosum*) have been previously described (Benhizia *et al.*, 2004a, b). In the present work, these three African species (plus the recently found Algerian-endemic species *Hedysarum naudianum*; near Sétif, 36°12'N, 5°24'E, five nodules) have been further characterized by adding the direct-PCR bacterial identification and nodule microscopy approaches. According to the Italian botanical taxonomy (Pignatti, 1982), *H. glomeratum* (collected by us in Sardinia) is considered a synonym of *H. spinosissimum* ssp. *capitatum* (collected in Algeria). However, in view of several ecotypic differences and of the distance within the large range, we keep the distinction between the two species when describing the results.

### Bacterial strain isolation and culture conditions

Root segments bearing nodules from the plants were washed free of soil under running water, then encaged in a fine-mesh steel holder and surface-sterilized by immersion in 95% ethanol for 20 s followed by 5% sodium hypochlorite for 3 min, and finally washed seven times with sterile distilled water. As an alternative surface sterilization procedure, in place of the NaClO step, after the ethanol treatment, nodules were immersed in 0.1% HgCl<sub>2</sub> for 2 min, and the number of H<sub>2</sub>O washes was extended to 10. All treatments were performed under microbiologically controlled conditions. Tests to validate surface-sterilization of plant tissues were performed by touching the material several times on the surface of plate count agar (PCA, Difco) plates prior to isolation of the interior microbiota.

Surface-sterilized root nodules were transferred into empty sterile plastic dishes and cut in half with a flamed scalpel. One portion was processed for microscopy and the other was squashed in 50–150 µL of sterile physiological saline solution, the volume varying in proportion to the nodule size. About one-fifth of the resulting suspension volume was withdrawn for lysis and direct 16S rRNA gene PCR, with the aim of verifying the identity of the prevailing bacterial species by a culture-independent approach. The remaining portion from the squashed nodule suspension was streaked on yeast–mannitol agar (YMA) plates containing Congo-red (Vincent, 1970), on PCA, and on defined BIII-agar (Dazzo, 1982). As alternative rhizobial media with pH-buffering capability, YMA containing 4 g L<sup>-1</sup> calcium carbonate (Jordan, 1984) and medium I (Howieson *et al.*, 1988, also known as 1/2 Lupin agar) were also tested. The latter was modified using 0.17 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> and 0.13 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. Plates were incubated at room temperature for up to 2 weeks. All colonies obtained were purified and processed for DNA extraction.

The same protocols were used for the isolation of endophytic bacteria from preweighed and surface-sterilized portions of roots and stems.

### Plant cultivation and nodulation tests

Mature pods were collected from wild plants in their natural habitat and kept dry at room temperature until use. Seeds were removed manually from pods, surface-sterilized by immersion in 70% ethanol for 30 s followed by stirring in 0.1% HgCl<sub>2</sub> for 7 min, and rinsed in seven changes of sterile deionized water. Seeds were preimbibed for 3 h in the final wash. Dormancy was broken by mechanical scarification with autoclaved material as follows: seeds were transferred over a ribbed rubber sole fitted in a polypropylene box and gently streaked for 5 s with bodywork-grade medium-grain sandpaper. Alternatively, a vernalization treatment was applied by storing seeds at –20 °C for 3 days. Germination and concomitant verification of surface sterility were obtained by spreading seeds on YMA plates wetted with 10 drops of sterile water, and incubating inverted for 3 days in the dark. Germinated seedlings were transferred aseptically to sterilized plastic Leonard jars containing a water-washed, oven-dried, quartziferous sand–vermiculite 1/3 mixture, fed from the bottom with nitrogen-free Fähraeus solution. The rooting mixture was rehumidified with 1/10 vol of sterile nitrogen-free Fähraeus solution and autoclaved in plastic biohazard-type bags prior to transfer to the top portion of the Leonard jar assembly. Bacteria were inoculated by dispensing a 1-mL suspension of an overnight-grown liquid culture in yeast–mannitol broth (YMB) medium (c. 10<sup>8</sup> cells mL<sup>-1</sup>). Seeds were covered with a layer of autoclaved gravel, and the entire Leonard jar assemblies were transferred to a growth cabinet (Angelantoni, Sas. Massa Martana, Italy) programmed for a 16-h daylight photoperiod at 23 °C, night temperature of 18 °C, and 60% constant relative humidity. Plants were inspected as early as after 40 days for nodule formation and grown for up to 3 months. For nodulation by natural symbionts, uninoculated seedlings were transplanted to jars containing field-collected soil instead of the sand–vermiculite mix.

### Acetylene reduction activity (ARA) measurement

Nitrogenase activity was estimated by the acetylene reduction assay according to Somasegaran & Holben (1985) using a TRACE GC 2000 gas-chromatographer (Thermo Finnigan) equipped with a flame ionization detector.

### Nodule microscopy

#### Single-stain light microscopy and electron microscopy

Whole nodules and nodule halves obtained as described above were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) for 24 h at 4 °C and postfixed for 2 h at 4 °C

in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series, and then embedded in araldite resin. Thin sections (1  $\mu\text{m}$ ), obtained with a Reichert-Jung ultramicrotome, were stained with 1% toluidine blue for light microscopy. Ultra-thin sections (0.05  $\mu\text{m}$ ) were collected on copper grids, stained with uranyl acetate followed by lead citrate at room temperature, and then examined with a Hitachi 300 transmission electron microscope operating at 75 kV.

### Double-stain light microscopy

Nodules were washed in running tap water and fixed overnight in 1.5% glutaraldehyde in 200 mM phosphate buffer, pH 7.2. Samples were degassed for 5 min under vacuum and dehydrated in an ethanol series from 30% through 95%, then embedded in LR white resin. One- to 1.5- $\mu\text{m}$  sections were obtained using glass knives on a Reichert Om U3 ultramicrotome (C. Reichert Optische Werke, Vienna, Austria), and dried on a microscope slide by placing each section on a drop of 30% acetone in distilled water and transferring to a 60 °C hot plate dryer. Dry sections were stained by flooding with Astra-Blue (1% dissolved in 1% acetic acid) for 2 min, rinsed with distilled water, and double-stained with Basic Fuchsin (0.07% in H<sub>2</sub>O) for 2 min at the same temperature.

Sections were also stained on the hot plate with Aniline Blue Black (0.5% dissolved in 3.5% acetic acid) for 5 min, rinsed, and then stained with Basic Fuchsin (0.07% in H<sub>2</sub>O) at room temperature for 2 min (Kraus *et al.*, 1998). Slides were placed on top of a moistened paper tissue and covered with a Petri dish lid to prevent drying during staining. All of the above stains were purchased from Merck/BDH (Darmstadt, Germany). After staining, samples were rinsed in water, mounted with Eukitt medium (Sigma Chem. Corp, Saint Louis, MO), and examined by bright-field microscopy using a Leitz Dialux 22 microscope.

### Detection of *Enterobacter agglomerans* within nodules by immunofluorescence microscopy

Nodules were rinsed in water, dried on paper tissue, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2, and embedded overnight in paraffin (Paraplast Plus, Sigma). Sections (*c.* 7  $\mu\text{m}$  thick) were collected on microscope slides coated with poly-L-lysine, dried, treated with xylol (four washes), 1:1 xylol: absolute ethanol (EtOH), absolute EtOH, 95% EtOH, 90% EtOH, 70% EtOH, 50% EtOH; H<sub>2</sub>O; phosphate-buffered saline (PBS, Dazzo, 1982). Treated sections were soaked in 100 mM glycine (*p*-hydroxyphenylaminoacetic acid) for 1 h to cover the residual fixer response, treated with 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C to block nonspecific staining, incubated for 1 h at 37 °C

with the primary antibody (mouse IgG2a monoclonal antibody to *E. agglomerans* NCTC9381, Acris Antibodies GmbH, Hiddenhausen, Germany) diluted 1:20 in 1% BSA in PBS, washed in PBS four times, incubated for 1 h at 37 °C with the secondary antibody in 1% BSA in PBS (goat anti-mouse polyvalent immunoglobulins, fluorescein isothiocyanate-conjugated, Sigma Chemical Co.), washed in PBS four times, treated for 15 min in 0.1% Toluidine Blue to quench basal autofluorescence, washed extensively in PBS, and mounted in Mowiol with 1  $\mu\text{g mL}^{-1}$  4',6-diamidino-2-phenyl-indole. Samples were observed under an Olympus BX51 epifluorescence microscope equipped with a  $\times 100/1.35$  oil iris PlanApo objective. At least 1000 cells were scored for signals by each of two independent examiners. Only signals presenting rod-like shapes were considered. Selected images were acquired using a Magnafire camera (Optronics, Goleta, CA).

### Fluorescent stain microscopy

Nodules were fixed overnight using 4% paraformaldehyde in phosphate buffer, pH 7.4, and embedded in paraffin as described above. Four-micrometer-thick sections were mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma). The sections were deparaffinized by two extractions in xylene, followed by two extractions with absolute ethanol and then a single wash with 95% EtOH, 90% EtOH, 80% EtOH, 70% EtOH, 50% EtOH, and finally PBS. Endophytic bacteria were then stained with propidium iodide solution (1  $\mu\text{g mL}^{-1}$ ) containing antifade (23 mg mL<sup>-1</sup> DABCO, 80% glycerol, 2 mM Tris-HCl pH 8). Samples were examined under an Olympus BX51 epifluorescence microscope as described for immunofluorescence.

### DNA extraction

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50  $\mu\text{L}$  of lysis buffer [0.25% sodium dodecyl sulphate (SDS), 0.05M NaOH] in a 1.5-mL polypropylene tube, followed by stirring for 60 s on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min, and 10  $\mu\text{L}$  of the supernatant was mixed with 90  $\mu\text{L}$  of sterile water. Lysates were stored at 4 °C prior to PCR. For direct PCR analysis, nodules were squashed in 50  $\mu\text{L}$  of sterile water, and 10  $\mu\text{L}$  of the suspension was transferred to 50  $\mu\text{L}$  of lysis buffer and treated according to the protocol described above for DNA isolation.

### PCR amplification of the 16S rRNA gene and amplified ribosomal DNA restriction analysis (ARDRA)

One microliter of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the two 16S rRNA gene-targeted universal

bacterial primers 63F (5' CAGGCCTAACACATGCAAGTC) (Marchesi *et al.*, 1998) and 1389R (5' ACGGGCGGT-GTGTACAAG) (Osborn *et al.*, 2000) at 1  $\mu$ M each in a 25- $\mu$ L reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 4 min; and a final extension at 72 °C for 10 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 1  $\mu$ M of each primer, and 2.5 U Taq DNA polymerase, recombinant (Invitrogen Life Technologies). Amplicons were digested overnight at 37 °C upon mixing 5  $\mu$ L from the 25- $\mu$ L reaction volume with 1  $\mu$ L of CfoI enzyme (Pharmacia, Uppsala, Sweden) and 2  $\mu$ L of 10  $\times$  reaction buffer. Digested DNA was loaded on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed with a Kodak DC290 digital camera. Upon ARDRA analysis the isolates were sorted and selected for sequencing.

### Direct PCR from nodule extract

Ten microliters of the suspension resulting from squashing the nodule in 50–100  $\mu$ L of sterile physiological solution was mixed with 50  $\mu$ L of lysis buffer, and the same protocol as described above for DNA isolation was carried out.

### DNA sequencing

One microliter of the amplicon resulting from the above-described PCR amplification was mixed with 1  $\mu$ L containing 6.4 picomoles of the above-described forward primer 63F in a 0.2-mL polypropylene tube and then dried by incubating the open tube for 15 min at 65 °C in an I-Cycler thermal cycler. The template and primer mix was directly used for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analysed using CHROMAS 2.23 software (Technelysium Pty Ltd, Tewantin, Australia).

## Results

### Plant collection and nodule examination

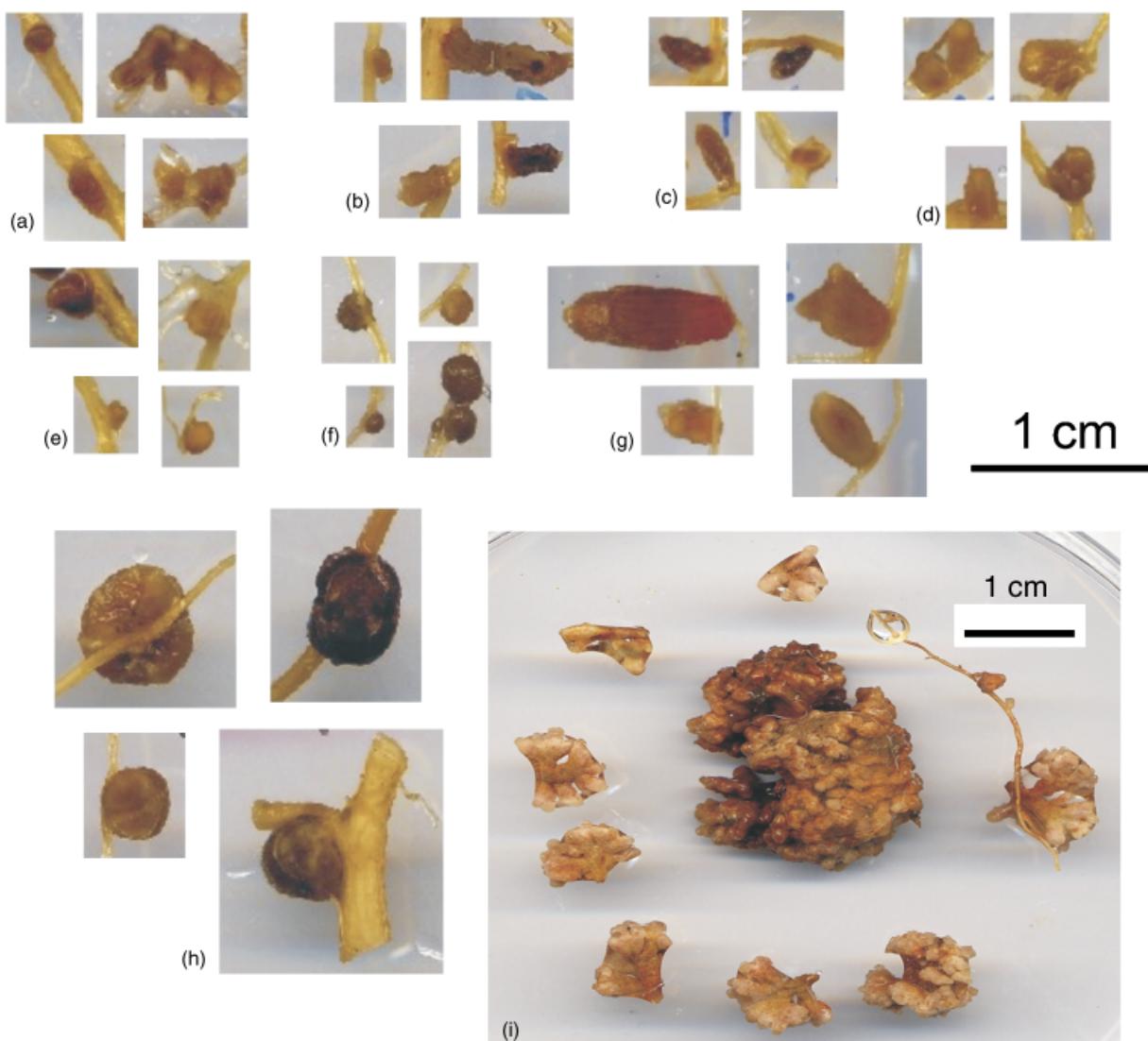
The root systems of all field-collected species bore tubercular structures that varied in number and shape (Fig. 1). Root nodules on *T. purpureus* and *P. bituminosa* were typically round and determinate, and very large in the case of the latter species, whereas the root nodules on the other legumes had elongated-indeterminate to irregular shapes. Multi-lobed nodules were present on roots of *H. spinosissimum* often varying in character according to the site of isolation.

Figure 1i depicts examples nodules for this case, all belonging to a vigorous plant specimen thriving in a sandy scree near Castelsardo and endowed with a single nodular cluster with a noticeable maximum diameter larger than 3.5 cm. In the more clayish soil of Giòscari, however, the same plant tended to form unilobed nodules.

### Nodule microscopy

A minimum of three nodules per plant species were inspected under optical as well as electron microscopy. Standard nodule histology techniques involving toluidine-stained thin sections and transmission electron microscopy (TEM) ultra-thin sections indicated that these root-borne hypertrophy structures from different host plants are consistent with the anatomy of genuine legume nodules, displaying an uninfected cortex, a peripheral vascular system, and a central tissue of host cells infected to various degrees with bacteria (Fig. 2a–h). TEM confirmed the presence of bacteria within the lumen of walled infection threads that crossed the walls of adjacent host cells, and provided evidence that infected host cells contained bacteroid-like cells of various sizes and shapes, including elongated rods (Fig. 3a and b), clubs (Fig. 3a and h), lobed cells (Fig. 3c–e and h), ellipsoids (Fig. 3b), coccobacilli (Fig. 3f), and spheres (Fig. 3g and h). Intracellular bacteria contained intracellular inclusions that were either electron-opaque (Fig. 3b) or electron-transparent (Fig. 3c and h), resembling polyphosphate or polyhydroxybutyrate storage granules, respectively.

We also tested two other novel double-staining procedures not previously used on root nodules (Aniline Blue Black/Basic Fuchsin and Astra Blue/Basic Fuchsin, Fig. 4a and b, respectively). Nodules present on 70-day-old plants of *H. spinosissimum* cultivated in a growth cabinet in Leonard jars filled with original soil from Giòscari were used. The double stains worked well by differentially staining structures such as cell walls, infection threads and bacteria. Basic Fuchsin stained the endosymbiotic bacteria pale red (Fig. 4b), whereas Astra Blue added contrast with blue staining of mucilages, including the lumen of infection threads and the pecto-cellulosic plant cell walls. Aniline Blue Black (Fig. 4a), a protein stain, was particularly suitable for staining bacteria within the plant cells whose cell walls were stained red by Basic Fuchsin. Compared with Toluidine Blue, these stains increased the contrast, providing more detail and improving the visualization of infection threads, which appear in colours different from those of the bacteroid tissue. Moreover, the physiological state of the plant nuclei appears to be proportional to the intensity of the blue colour upon Aniline Blue Black/Basic Fuchsin staining. Thus both procedures can be recommended in general histological studies of legume nodulation and other plant-microorganism interactions.

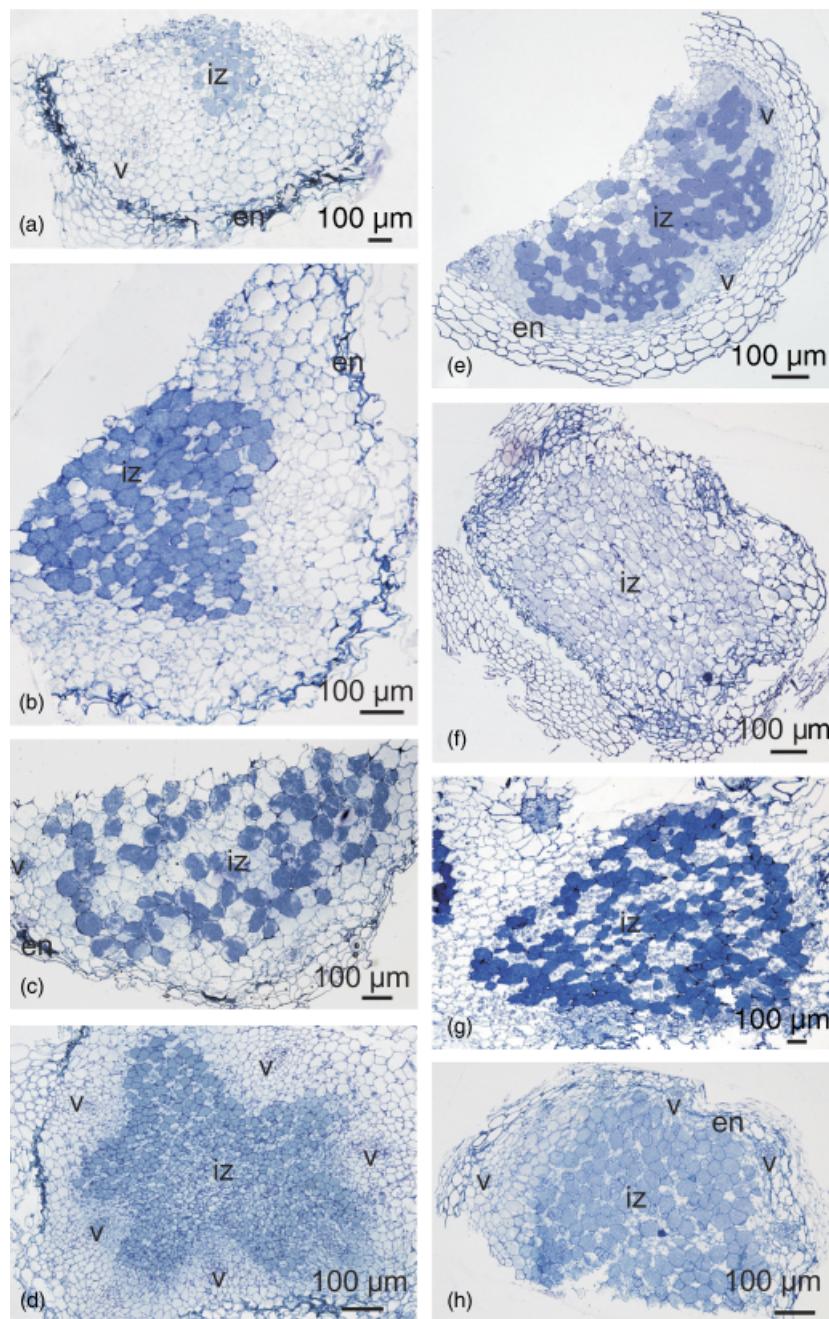


**Fig. 1.** Examples of excised root nodules of the various plants included in this study. (a) *Hedysarum glomeratum*; (b) *Hedysarum spinosissimum*; (c) *Hippocratea unisiliquosa*; (d) *Ornithopus compressus*; (e) *Ornithopus pinnatus*; (f) *Tetragonolobus purpureus*; (g) *Scorpiurus muricatus*; (h) *Psoralea bituminosa*. (i) Clustered root nodules of a healthy *Hedysarum spinosissimum* plant collected in Castelsardo.

### Bacteria isolation from nodules

In total, 79 nodules from the 2004 campaign and 25 nodules from the 2005 campaign were evaluated. The surface-sterilization treatment was generally efficient, as in most cases no colonies developed on the PCA plates upon which nodules were rolled prior to sectioning. In about 10% of the cases where surface sterilization was not achieved, the squashed nodule samples were not considered further. A total of 161 bacterial colonies were isolated from inside the surface-sterilized nodules from the eight plant species harvested in Sardinia and grouped by ARDRA. Partial sequencing (700–800 nucleotides) of the corresponding amplified

16S rRNA gene revealed at least 12 broad lineages, encompassing a diversity represented by several taxa as defined by GenBank database similarities. The ranked abundance of the various bacteria in the nodules is summarized in Table 1. Quite unexpectedly, rhizobial lineages were rarely found, amounting to only single-colony occurrences in four nodule squashes that also yielded various other taxa. The most common result (27%) from plating nodule squashes was no development of microbial growth on YMA, BIII or PCA plates. When growth did occur, fewer than ten colonies developed. This contrasts with typical results obtained using cultivated legumes, for which the rhizobial occupants rescued by such techniques normally form a profuse lawn along



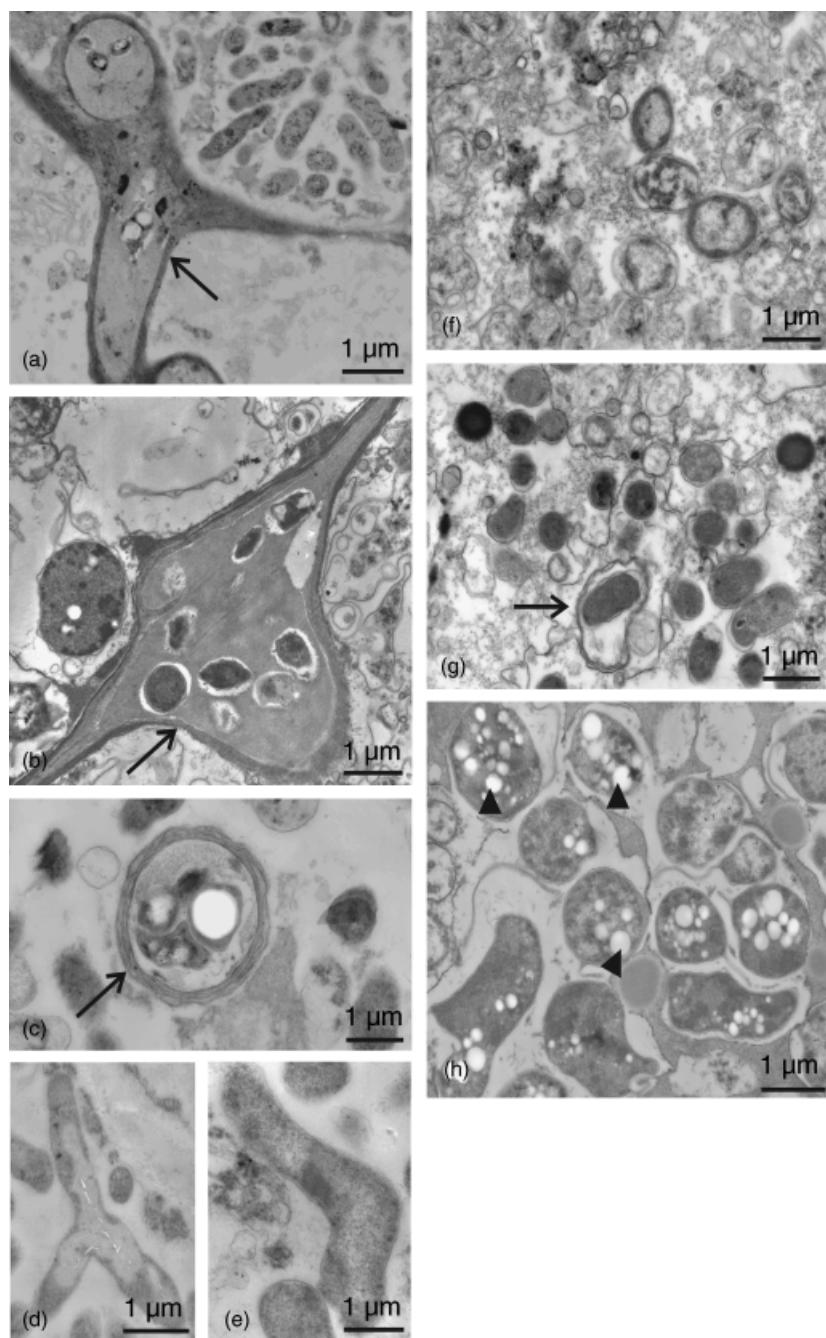
**Fig. 2.** Structure of the legume nodules. (a–h) Bright-field micrographs of sections stained with 1% Toluidine Blue. (a, d) *Ornithopus compressus*; (b) *Ornithopus pinnatus*; (c) *Tetragonolobus purpureus*; (e) *Hedysarum spinosissimum*; (f) *Hedysarum glomeratum*; (g) *Psoralea bituminosa*; (h) *Scorpiurus muricatus*. All examined nodules present a cortex containing vascular elements (v), an endodermis (en) and a central infected zone (iz). The reason why nodule halves rather than whole nodules were processed is that the complementary halves were used to isolate the hosted bacteria and enable a direct comparison between cultured taxa and microscopy.

most of the streak length on these plating media, indicating their abundance and culturability. In the present study, only nodules from *H. spinosissimum*, *T. purpureus*, and, from our previous Algerian campaign (Benzhizia *et al.*, 2004a, b), *H. spinosissimum* ssp. *capitatum*, *H. pallidum* and *H. carnosum* yielded a dense lawn of confluent bacterial colonies, but none of these were rhizobia. As controls in our experiments we often included nodules from the cultivated legume *Hedysarum coronarium*, which, by contrast, always yielded a fully culturable load of *Rhizobium sullae*, ruling out the

possibility that the results observed with the other legumes could have arisen from a general fault in the surface sterilization procedure.

#### Nodulation tests

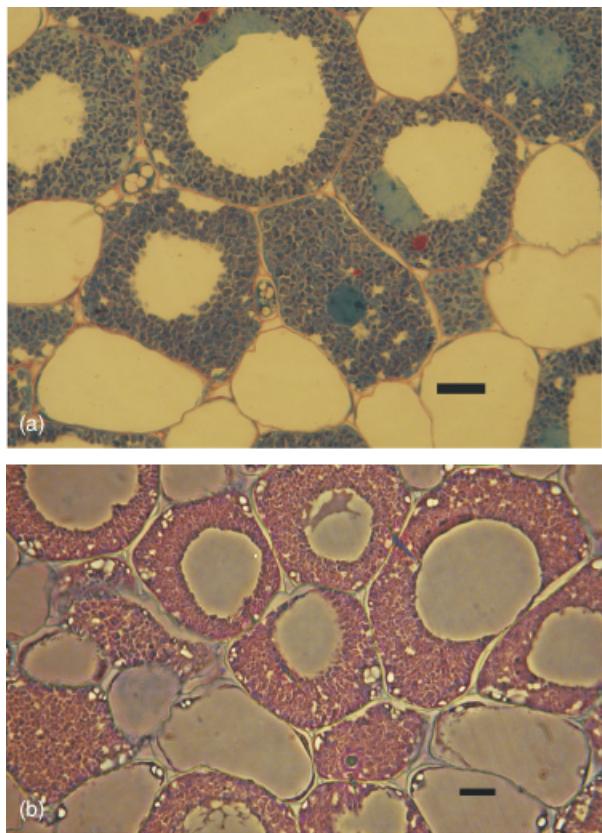
The absence of a consistent culturable rhizobial occupant revealed by the standard procedure used for the isolation of legume nodule symbionts confirmed our previous findings (Benzhizia *et al.*, 2004a, b). We next checked whether the



**Fig. 3.** Transmission electron micrographs of the infection zone in *Ornithopus compressus* and *Hedysarum spinosissimum* nodules. Arrows indicate (a) longitudinal- and (b, c) cross-sections of bacteria-containing infection threads (a, b) between and (c) inside a *Ornithopus compressus* host cell. (d, e) Endosymbiotic bacteroids of various shapes in *O. compressus* nodules. (f, g) *Hedysarum spinosissimum* micrographs from the same infected cell showing endosymbionts having different shapes and electron density. In (g) peri-bacteroid membranes surround the symbiotic bacteroids (arrow). (h) Rod-shaped bacteria containing granules of  $\beta$ -hydroxybutyrate (arrowheads).

various bacterial isolates could induce nodule formation on their hosts under gnotobiotic conditions. A series of nodule formation tests was performed in growth cabinets using sterilized sand/vermiculite in Leonard jars, with the cultivation period extended up to two months. Five plant species, propagated from surface-sterilized seeds, were tested, including all *Hedysarum* species used here and in our previous studies, plus *Ornithopus compressus*, which was tested for the first time. Tests were repeated four times in two laboratories

(Padova and Constantine). A total of 24 distinct purified isolates, encompassing all the diverse taxa in Table 1 and including 10 strains of *Gamma proteobacteria* from the previous study (Benhizia *et al.*, 2004a, b), were tested either alone or in mixed inocula containing up to 10 strains. These inocula included the strains whose 16S rRNA gene had high similarity to *Mesorhizobium* and *Rhizobium*. No nodules were produced in any of these gnotobiotic plant tests, including those inoculated with the above isolates related



**Fig. 4.** (a) Optical microscopy of Aniline Blue Black/Basic Fuchsin double-stained thin sections from a *Hedysarum glomeratum* nodule. Plant cell walls and bacterial infection threads (thick round cross-sections) stain red. Plant nuclei stain from dark to light blue in relation to cell physiological status. Residual plant cytoplasm stains very pale blue. Bacteroids filling partially or totally plant cells stain dark grey to brown. Vacuoles (large central cell lumens) and starch granules (clumped masses in small interstitial cells) remain white. Scale bar: 10 µm. (b) Optical microscopy of Basic Fuchsin/Astra Blue double-stained thin sections from a *H. glomeratum* nodule. Plant cell walls stain greenish blue, bacterial infection threads stain blue (pointed lozenge shape between two plant cells in the upper right of the image). Vegetative bacteria inside infection threads stain purple. Bacteroids stain pale fuchsia. Vacuoles do not stain, and starch granules appear refractile white. Scale bar: 10 µm.

to rhizobia. Occasionally, shovel-like swellings similar to those that develop in senna, *H. coronarium* (Squartini *et al.*, 1993), developed on *H. spinosissimum* roots, but these are known to be modified short lateral roots that form independently of bacteria (A. Squartini & F. Dazzo, unpublished data).

#### Direct 16S PCR from nodules

Because the negative nodulation results indicated that the various bacterial occupants did not induce the nodules from which they were isolated, we performed direct PCR from the squashed nodule samples, targetting 16S rRNA gene with

bacterial primers to test the hypothesis that the true rhizobial occupants may have lost their culturability within the nodules. The results (Table 2) clearly showed that, indeed, most of these nodules actually contain a dominant amount of rhizobial DNA, sufficient in most cases to overwhelm and outcompete in the PCR amplification the heterologous DNA of the other nonrhizobial occupants and produce a clean sequence chromatogram upon nucleotide sequencing of the amplicon. In essence, this approach is not intended to examine the diversity of bacteria in nodules but rather to reveal which is the most abundant species within them. The bacterial rRNA gene from some of the nodules gave a mixed but still readable sequence, with a major template series of peaks superimposed over a rather high background (data not shown), further confirming that rhizobia are dominant but not alone within the nodule. In the case of nodules of *H. spinosissimum*, *T. purpureus*, and the Algerian *Hedysarum*, for which the nonrhizobial occupants produced the most profuse growth on plates (rather than sparse colonies), the direct PCR also resulted in the dominant amplification of the gammaproteobacterial occupant. The only case for which the 16S rRNA gene sequence from the whole nodule squash matched the same taxon as the corresponding culturable isolate was the *Mesorhizobium* sporadically found in *T. purpureus*.

#### Cultivation of wild plant species from seed in their natural soil

The above studies were performed on flowering plants collected during April 2004 and 2005. To test whether the rhizobia eventually lose culturability within the nodules or during a possible late-seasonal physiological stress of the plants, a growth chamber test was set up using seedlings of *H. spinosissimum* and *H. glomeratum* derived from natural seeds collected in July–August. Seeds were surface-sterilized, germinated on PCA plates, and transplanted into Leonard jars filled with Sardinian soil from two sites where the two species naturally occur (one was a compact soil from Giòscari, and the other was a sandy soil from an erosive hill near the coastal city of Castelsardo). Plants were harvested as early as 40 days after germination (early three-leaf stage) in order to obtain young nodules induced and invaded by their natural microbial partners before exposure to the stress of mature plant senescence. Both hosts formed two to five nodules in both soils. Ten nodules were analysed, but even in this case the same situation as observed with all the field-collected plants was confirmed. That is, three nodule squashes did not yield colonies, and the other seven produced one to five colonies per nodule. The identity of these culturable taxa revealed 99% similarity to *Bacillus simplex* (DQ457600) from five nodules, 98% similarity to *Bacillus megaterium* (DQ457599) from two nodules, and one single

**Table 1.** Number of nodules in which members of the above phylogenetic groups (identified by sequence homology to database) were found in the various plants upon colony culturing and 16S rRNA gene sequencing

Host plant	<i>Hedysarum spinosissimum</i>	<i>Hedysarum glomeratum</i>	<i>Tetragonolobus purpureus</i>	<i>Hippocratea unisiliquosa</i>	<i>Scorpiurus muricatus</i>	<i>Psoralea bituminosa</i>	<i>Ornithopus compressus</i>	<i>Ornithopus pinnatus</i>	Total
No growth	0	3	0	9	5	4	1	2	24
<i>Enterobacteriaceae</i> * <sup>8</sup>	1	1	0	0	0	0	8	4	22
<i>Bacillus</i> <sup>†</sup>	2	2	1	5	0	0	0	1	13
<i>Pseudomonas</i> <sup>‡</sup>	6	0	0	0	0	0	6	0	12
<i>Curtobacterium</i> <sup>§</sup>	0	0	0	0	0	0	0	4	4
<i>Rhizobium</i> <sup>¶</sup>	2	0	0	0	0	0	0	1	3
<i>Staphylococcus</i> <sup>  </sup>	0	1	2	0	0	0	0	0	3
<i>Xanthomonas</i> <sup>**</sup>	2	0	0	0	0	0	0	0	2
<i>Microbacterium</i> <sup>††</sup>	0	0	0	0	0	0	0	1	1
<i>Arthrobacter</i> <sup>‡‡</sup>	0	0	0	0	0	1	0	0	1
<i>Mesorhizobium</i> <sup>§§</sup>	0	0	1	0	0	0	0	0	1
<i>Agrobacterium</i> <sup>¶¶</sup>	1	0	0	0	0	0	0	0	1
<i>Ralstonia</i> <sup>   </sup>	0	1	0	0	0	0	0	0	1
<i>Thiobacillus</i> <sup>***</sup>	0	0	0	1	0	0	0	0	1

The following numbered notations specify which taxa present in the GenBank database share the highest homologies with the sequences obtained. The accession number in parenthesis refers to the novel deposited sequences.

\*Including: *Enterobacter agglomerans* 97–100% (DQ457576), *Pantoea ananatis* 98% (DQ457592), *Erwinia persicina* 100% (DQ457577).

<sup>†</sup>Including: *Bacillus simplex* 100% (DQ457586), *Bacillus megaterium* 99% (DQ457585), *Paenibacillus* sp. 98% (DQ457593).

<sup>‡</sup>Including: *Pseudomonas* sp. 98–100% (DQ457590), *P. lini* 99% (DQ457578), *P. viridisflava* 100% (DQ457579).

<sup>§</sup>Including *Curtobacterium flaccumfaciens* 100% (DQ457589), *C. herbarum* 99% (DQ457575).

<sup>¶</sup>Including: *Rhizobium* sp. ORS1466 100% (DQ457594), ORS1465 96% (DQ457595).

<sup>||</sup>Including *Staphylococcus epidermidis* 100% (DQ457587), *S. pasteuri* 100% (DQ457583).

<sup>\*\*</sup>*Xanthomonas translucens* 100% (DQ457591).

<sup>††</sup>*Microbacterium* sp. 99% (DQ457581).

<sup>‡‡</sup>*Arthrobacter* sp. 99% (DQ457580).

<sup>§§</sup>*Mesorhizobium loti* 100% (DQ457584).

<sup>¶¶</sup>*Agrobacterium* sp. SDW052 99% (DQ457596).

<sup>|||</sup>*Ralstonia pickettii* 100% (DQ457588).

\*\*\**Thiobacillus* sp. 99% (DQ457582). The total number of nodules refers to the occurrence of each bacterial species and is therefore not coincident with the absolute number of nodules examined, as in some instances multiple microbial taxa were isolated from the same nodule. No remarkable differences in the occurrence of taxa were observed comparing the 2 years of isolation.

colony with 99% similarity to *Thiobacillus* sp. (DQ457598). Nevertheless, the direct PCR of bacterial 16S rRNA genes from the nodule squashes gave the same sequence with 99% identity to *Mesorhizobium* sp. strain H-4 (AF279889). In terms of endophyte dynamics, considering that, as in *H. coronarium*, nodules are first observed one month after seedling inoculation, the nodules examined in this case represent early phases of their ontogenesis and confirm that endophytic invasion of the nodules has already started at this stage.

Some of the *H. glomeratum* plants grown in soil from Giòscari were kept for up to three months in the growth cabinet. An analysis of seven nodules from these plants yielded culturable colonies with 100% similarity to *Staphylococcus aureus* (DQ457597), 100% to *Rothia mucillaginosa* (DQ457602), and, in the case of one nodule, an isolate (DQ457601) with 99% identity to *Mesorhizobium* sp. strain H-4 (AF279889), whose identity completely matches the one consistently obtained by direct PCR from nodule

squashes of this legume species, indicating that in this case a sporadic event of culturability of the rhizobial occupant was possible (the above GenBank accession numbers refer to the matching sequences). A subsequent nodulation test, this time under sterile conditions, on the same host plant *H. glomeratum* was run with this cultured strain, which proved able to reinvoke the abundant formation of nodules and sustain plant growth, as indicated by the green foliage and healthy aspect. However, attempts at reisolating the inoculated strain led again to the usual scenario: most nodules, from individual plants, did not yield any growth. Only from one nodule could we recover three single colonies, whose ARDRA profile confirmed the identity of the inoculated *Mesorhizobium*.

#### Acetylene reduction assays

The nitrogen-fixing functionality of nodules was tested by means of the acetylene reduction activity test. Nodules from

**Table 2.** Results of nucleotide sequencing by direct 16S rRNA gene PCR amplification from the nodule squash

Plant host	Number of nodules	GenBank code	Best-match homologies to GenBank taxa, found with direct 16S PCR of individual nodules
Sardinia, Italy			
<i>Hedysarum spinosissimum</i>	5	DQ457614	(Meso)rhizobium sp. ( <i>H. spinosissimum</i> ) H-4 (AF279889) 99%
	1	DQ457613	<i>Rhizobium</i> sp. (AY278884) 99%
	1	DQ457612	<i>Pantoea ananatis</i> (AY173021) 96%
<i>Hedysarum glomeratum</i>	2	DQ457611	(Meso)rhizobium sp. H-4 (AF279889) 99%
<i>Hippocrepis unisiliquosa</i>	2	DQ457615	(Meso)rhizobium sp. H-4 (AF279889) 100%
	2	—	not amplifiable
<i>Scorpiurus muricatus</i>	6	DQ457619	<i>Mesorhizobium</i> sp. ( <i>Argyrolobium uniflorum</i> ) (AY500256) 99%
<i>Tetragonolobus purpureus</i>	2	DQ457620	<i>Mesorhizobium</i> sp. ( <i>Argyrolobium uniflorum</i> ) (AY500256) 99%
	1	DQ457621	<i>Pseudomonas</i> sp. KD (AY456697) 98%
<i>Ornithopus compressus</i>	3	DQ457616	<i>Bradyrhizobium</i> sp. Spain 5 (AF461195) 100%
<i>Ornithopus pinnatus</i>	3	DQ457617	<i>Bradyrhizobium</i> sp. Spain 6 (AF461196) 98%
<i>Psoralea bituminosa</i>	2	DQ457618	<i>Mesorhizobium chacoense</i> (AJ278249) 98%
Algeria			
<i>Hedysarum</i> sp. ssp. <i>capitatum</i>	4	DQ457608	(Meso)rhizobium sp. ( <i>H. spinosissimum</i> ) H-4 (AF279889) 100%
	2	DQ457609	<i>Pseudomonas</i> sp. TM7.1 ( <i>Tuber magnatum</i> ) (DQ279324) 100%
	1	DQ457610	<i>Pantoea ananatis</i> (AY173021) 98%
<i>Hedysarum pallidum</i>	2	DQ457606	<i>Pantoea ananatis</i> (AY173021) 98%
	2*	—	Not amplifiable
	1	DQ457607	<i>Pseudomonas</i> sp. CLb01 (AY574283) 99%
<i>Hedysarum carnosum</i>	2	DQ457604	<i>Pantoea ananatis</i> (AY173021) 98%
	1	DQ457603	<i>Enterobacter hormaechei</i> (AJ853889) 98%
<i>Hedysarum naudinianum</i>	4	—	Not amplifiable
	1	DQ457605	<i>Pseudomonas</i> sp. CLb01 (AY574283) 99%

The number of nodules from which each given result was obtained is indicated, along with the GenBank accession number of a representative sequence from that group. The percentage identity with database sequences is indicated. \* Both nodules of *Hedysarum pallidum* from which no amplicons could be obtained are from plants collected in a mine site contaminated with high levels of antimony.

40-day-old plants grown in the growth chamber were assayed. Species tested included *H. glomeratum* grown in two types of soil (Giòscari or Castelsardo), *S. muricatus* grown in soil from Giòscari, and *H. glomeratum* grown in sterile conditions in vermiculite mix inoculated with the above-described single culturable strain of *Mesorhizobium*. As a control, *H. coronarium* of the same age inoculated in vermiculite with *R. sullae* type strain IS123 was used. Data indicated that the nodules from each of the combinations reduced acetylene to an extent not significantly different from that displayed by the sulla positive control. Values observed ranged between 2 and 10 nanomoles mg<sup>-1</sup> of nodule dry weight per hour. Attempts to cultivate rhizobia from other nodules from the same roots, not used for the ARA tests, yielded the usual nonculturability result. This analysis rules out the possibility that the nonrecoverable state of the rhizobia could correlate with a generally inefficient (fix-minus) phenotype of the nodules.

#### Variations tested in the isolation procedure

The hypothesis of a general problem resulting in too harsh a sterilization method was investigated. Using nodules either

collected in nature or developed in the growth cabinet, we tested various alternatives among the standard methods used for rhizobia (Vincent, 1970; Jordan, 1984; Somasegaran & Holben, 1985). However, using either hypochlorite or mercuric chloride-based procedures on nodules from *H. glomeratum* or *H. spinosissimum* did not alter the outcome. A lower concentration of NaClO (3%) was also tested, yielding the usual results. However, the same protocols used on positive-control nodules of sulla (*H. coronarium*) always enabled full recovery of *R. sullae* as ascertained by ARDRA and 16S rRNA gene sequencing. In light of possible osmotic damage or salinity impact we checked both saline solution and distilled water as alternatives for resuspending bacteria from nodule squashes, and, to dilute bacterial inhibitors that might be present, we performed serial dilutions prior to plating. None of these measures succeeded in solving the problem. We also considered the possible sensitivity to acidic pH for the rhizobia of our wild legumes, as indicated for certain strains of *Sinorhizobium meliloti* (Howieson *et al.*, 1988) and *Bradyrhizobia* from *Arachis* (Macciò *et al.*, 2002). In these species, colony development of rhizobia is halted by the acidification resulting from their own metabolism. The problem is often coupled with the need for calcium

(Howieson *et al.*, 1992), and we addressed it with media reported to circumvent the inhibition such as YMA containing calcium carbonate (Jordan, 1984) or media featuring the phosphate buffer (Howieson *et al.*, 1988; Nandasena *et al.*, 2001). However, none of these relieved the nonculturability phenomenon.

Exploring the possibility of a strict seasonal dependence of rhizobium viability, we collected *H. spinosissimum* in early February 2006 (in the Algerian site of Constantine), while still in its youngest recognizable stage, consisting of the newly emerged 3–4 cm tall plantlet displaying the first composite leaf and an average of two to three root nodules. The results (no culturable rhizobia) were no different from the ones observed in spring isolations. We again included, as a positive control, nodules from a spontaneous stand of *H. coronarium* collected on the same day in a nearby location, which produced, as expected, regularly growing streaks of *R. sullae*. Nevertheless, a possibility still exists that rhizobia from these hosts, for inherent physiological reasons, are particularly sensitive to all surface sterilization procedures so far used for the isolation of nodule symbionts.

### Presence of endophytic bacteria in other parts of the plants

In order to test whether internal colonization of the non-rhizobial species in these legumes is limited to nodules (as opposed to a systemic plant invasion), specimens of *H. spinosissimum*, *T. purpureus*, *P. bituminosa* and *S. muricatus* collected between April and May 2005 in two locations in Sardinia were examined for endophytic colonization in primary and secondary root segments and in

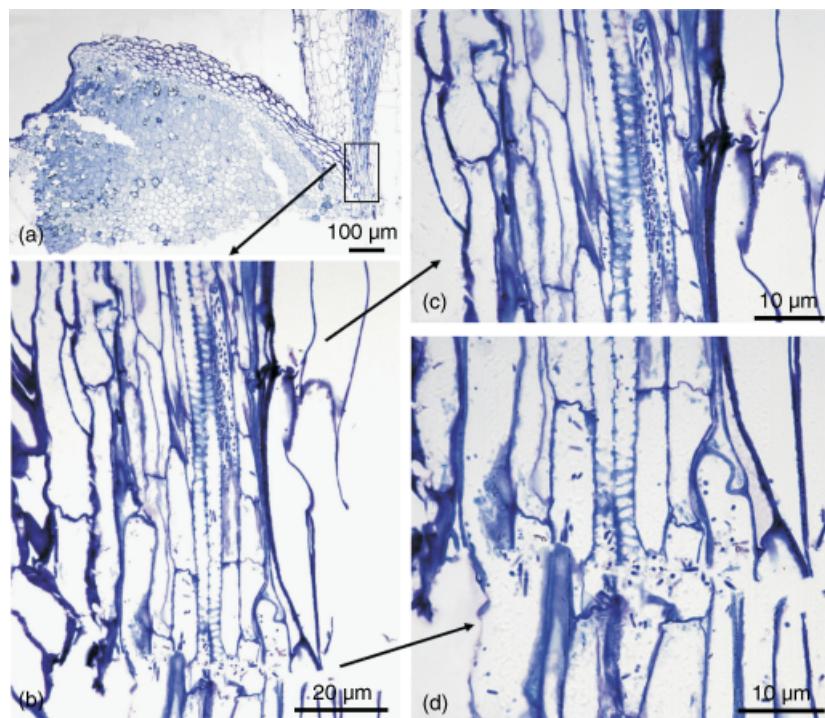
stems. For comparison, we examined the cultivated legume *sulla* (*H. coronarium*), whose nodules consistently yield their culturable, well-characterized rhizobial symbiont previously described by our group as the novel species *R. sullae* (Squartini *et al.*, 2002). Surface sterilization was confirmed by sterility control tests. The majority of plant species, including *sulla*, were found to contain an internal community of culturable bacteria within all parts examined. The number of CFUs on PCA plates (Table 3) reached values typically above  $10^5 \text{ g}^{-1}$  of root tissue, consistent with the numbers found by other authors for plant endophytism. The highest value was found in a primary root of *H. spinosissimum*, which yielded nearly  $10^7 \text{ CFU g}^{-1}$  of fresh weight. In our study, stems of the wild species generally harboured populations that were one to two orders of magnitude smaller than those found in the roots, with the exception of *H. coronarium*, the cultivated control, in whose stems nonrhizobial culturable endophyte densities were as high as in roots. Plants of *Scorpiurus muricatus* were the only case from which no culturable microbial community was recoverable on PCA plates. It should be recalled that nodules of this host were also often devoid of a culturable biota (Table 1).

Direct PCR was also performed on dominant bacterial 16S rRNA genes within macerated tissues of previously surface-sterilized root and stem segments. In about 75% of the cases (consistent with levels exceeding  $1 \times 10^4 \text{ CFU g}^{-1}$ ), the macerates produced a gel-visible amplicon from which a clean sequence could be obtained, enabling direct assay and determination of the dominant endophytes in these plant tissues. Such test revealed that macerates of *H. spinosissimum* and *H. coronarium* roots and *T. purpureus* stems

**Table 3.** Mean values of PCA-culturable colony forming units obtained from surface-sterilized portions of plants harvested in nature or germinated and grown in cabinet conditions

Plant and parts tested	Mean number of CFUs $\text{g}^{-1}$ fresh weight
Field-collected plants (late spring)	
<i>Hedysarum spinosissimum</i> , primary roots	$8.9 \times 10^4$ (Max. $1.1 \times 10^7$ )
<i>Hedysarum spinosissimum</i> , secondary roots	$4.3 \times 10^5$
<i>Hedysarum spinosissimum</i> , stems	$2.1 \times 10^3$
<i>Hedysarum coronarium</i> , primary roots	$2.5 \times 10^5$
<i>Hedysarum coronarium</i> , stems	$4.1 \times 10^5$
<i>Psoralea bituminosa</i> , primary roots	$3.6 \times 10^5$
<i>Psoralea bituminosa</i> , stems	$0.1 \times 10^2$
<i>Tetragonolobus purpureus</i> , primary roots	$5.0 \times 10^3$
<i>Tetragonolobus purpureus</i> , stems	$4.1 \times 10^2$
<i>Scorpiurus muricatus</i> , primary roots	0
<i>Scorpiurus muricatus</i> , stems	0
Plants grown from surface-sterilized seeds, transplanted in natural soil, in a growth chamber	
<i>Hedysarum spinosissimum</i> , 5-day-old seedlings roots (before transplant)	0
<i>Hedysarum spinosissimum</i> , 40-day-old primary roots	$0.3 \times 10^2$

Values are the average of three to six plant specimens per species. SDs (not shown) ranged in all cases within 10% of the mean value, with the exception of primary roots of *Hedysarum spinosissimum*, for which a single specimen with a particularly high value ( $1.1 \times 10^7$ ) was recorded.



**Fig. 5.** Toluidine-stained longitudinal sections from a nodule and root of *Hedysarum naudinianum*. (a) Sections were taken from the junction between a nodule and the primary root. (b) High magnification of the square in (a). (c, d) At higher magnification, bacteria-like rods are clearly visible in the vascular elements of the primary root.

contain a recurring endophyte having 98% similarity to *Pseudomonas* sp. K94.14 (AY456697).

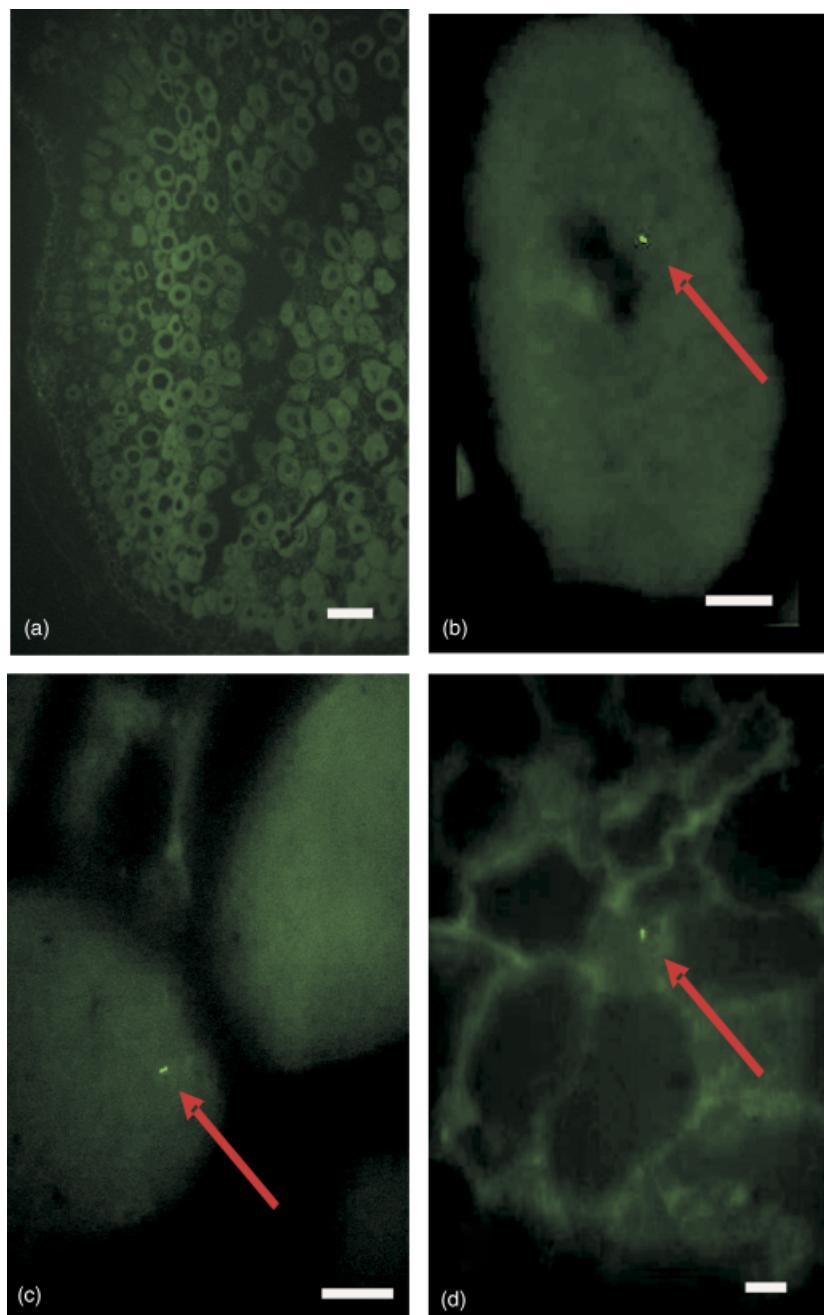
By varying the sampling date after planting in this same experiment, it was possible to ascertain how early the general endophytes establish in the plant and whether they are seed-borne. Roots of the young seedlings of *H. spinosissimum* were devoid of culturable endophytes, whereas surface-sterilized roots of 40-day-old plants harboured bacterial endophytes that were orders of magnitude lower than what we recorded at flowering stage under natural conditions (Table 3).

We also examined the relative abundance and location of endophytes *in situ* by plant microscopy. Figure 5, a toluidine-stained thin section, shows a junction between a nodule and the primary root of *H. naudinianum*, with short rod-shaped bacteria that gained access to the vascular tissue near the nodule. This suggests that an endophytic infection process is used to enter the plant, unlike that used by rhizobia. Stem invasion by bacteria in *H. spinosissimum* was investigated by epifluorescence microscopy upon staining thin sections with DNA-staining propidium-iodide, and indicated the presence of sparse bacteria in intracellular positions (data not shown). Finally, immunofluorescence microscopy was used to locate *Pantoea agglomerans* as the most abundant enterobacterial endophyte within *H. spinosissimum* nodules based on direct PCR results. Toluidine blue was used to quench root autofluorescence. One thousand plant nodule cells were examined. Of these, about 100 (10%) contained immunofluorescent-positive short rod-

shaped bacteria (Fig. 6). No immunofluorescent bacteria were found in c. 1500 plant cells examined in the negative-control plant sections. A nodule section of low magnification is presented in Fig. 6a, showing the typical central zone containing infected host cells with or without a vacuole in their centre. Higher magnification reveals plant cells containing isolated immunofluorescing rods (Fig. 6b–d). These results provide evidence that enterobacterial endophytes are not restricted to vascular bundles or outer cortical regions in legume nodules but can also occupy the effective bacteroid tissue.

## Discussion

The data presented here emphasize two important aspects of rhizobiology, namely that root nodules of some Mediterranean legumes harbour prevailingly nonculturable rhizobia, and that these same nodules are colonized internally by an array of culturable nonrhizobial endophytes. The microscopical analysis is supportive of this indication. Although the rhizobial endosymbiont exhibits various transition stages from vegetative to bacteroids, the striking variation in morphology of the bacteria within nodules is in line with the presence of other nonrhizobial taxa. Their occurrence is collectively supported by several lines of microscopical evidence. First, there appears to be more variation in bacterial morphotypes within a given host nodule cell than is typical for rhizobia (Fig. 3a–g and h). Second, the degree of electron opacity varied dramatically among some



**Fig. 6.** Antibody-mediated fluorescent microscopy immunolocalization of *Enterobacter* on thin sections of a *Hedysarum spinosissimum* nodule. (a) Low-magnification image of the nodule showing the background level of fluorescence. (b–d) Higher-magnification examples of single isolated fluorescent antigen-bearing vegetative rods (indicated by arrows) among dark masses of nonfluorescing bacteroids. Scale bars: (a) 0.1 mm; (b–d) 10  $\mu$ m.

intracellular bacteria within a given infected host cell, more so than the variation typically exhibited by rhizobia *in planta* (Fig. 3f and g). Third, peribacteroid membranes enclosed intracellular bacteria in some cases, whereas these signature ultrastructural features of the rhizobial endosymbiotic state were notably absent in other cases (compare, for example, Fig. 3g and h). Fourth, the immunolocalization with an *Enterobacter*-specific antibody points towards its coexistence in the bacteroid tissue.

By introducing a direct PCR analysis of nodule endophytes, the problem of nonculturability can be bypassed, and in most cases this approach reveals the putative rhizobial aetiological agent that resides in each nodule. Recovery of the rhizobial occupants to the culturable state could not be achieved under the conditions used. This phenomenon of nonculturable rhizobia within nodules appears to be commonplace, as we tested eleven legume species, including annuals and perennials, belonging to six genera distributed

in three tribes (*Hedysareae*, *Psoraleae* and *Loteae*). The condition appears widespread in both continents facing the Mediterranean Sea. Parallel studies of nodules from cultivated legumes in the same area, such as clover (Mateos *et al.*, 1992), pea (Corich *et al.*, 2001), and sulta (*H. coronarium*), routinely yielded fully culturable rhizobia, implying that physiological hindrances imposed by climatic or habitat factors could not explain why rhizobia lost their culturability within nodules of the legumes investigated in the present study. A question that arose was whether rhizobia could ever be cultured from nodules of these legumes. This was the case for *H. spinosissimum*, from which in Israel, Kishinevsky *et al.* (1996, 2003), using standard methods, isolated strains whose 16S rRNA gene sequence clusters in the *Mesorhizobium* branch with 99% identity to the sequence that we obtained by direct PCR from the unculturable occupant of nodules of the same species. To this we can add the single isolate from one of our *T. purpureus* nodules (Table 1). Thus, culturability appears to be an exception rather than the usual state. It should be noted, however, that the isolation of unexpected taxa from nodules can often be disregarded as the result of an inefficient sterilization procedure. While presenting preliminary results of these findings at a Nitrogen Fixation meeting (Benhizia *et al.*, 2004a) and shortly afterwards, we received three independent personal communications (Marta Laranjo, Therese Atallah, and Ines Soares) sharing the unpublished experience of having found (in Portugal, Lebanon and Uzbekistan, respectively) also *Enterobacteriaceae* or *Pseudomonadaceae* instead of rhizobia from nodules of several different wild legumes, including some of the ones investigated in our project. Further evidence of this phenomenon is the above-quoted report from Zakhia *et al.* (2006). Other studies also suggest the same pattern. Brundu *et al.* (2004) examined 15 wild species of *Medicago* in Sardinia and isolated 125 strains from nodules: only 29 were able to re-nodulate their host in gnotobiotic culture (as expected from a *Sinorhizobium* partner); the remaining 94 nodule isolates were saprophytes. Ben Romdhane *et al.* (2005) used a direct PCR protocol to study the symbionts of *Acacia tortilis* in Tunisia: 25.8% of their nodule-associated bacteria were not identified as rhizobia.

Our data showed that the diverse nodule endophytes are most often represented by a few CFUs, allowing identification of the unculturable rhizobium by direct nodule PCR. In nodules of some host species (*H. spinosissimum*, *T. purpureus*), however, the nonrhizobial occupant is sometimes abundant ( $\geq 5 \times 10^5$  CFU nodule $^{-1}$ ), yielding a dominant 16S rRNA gene sequence. In other cases, represented by four *Hedysarum* species examined in Algeria, the direct PCR analysis of nodules revealed nonrhizobial sequences or no amplifiable DNA, raising doubts about the presence of rhizobia at all. Interestingly, the culturable inhabitants of

nodules (*Pseudomonas*, *Enterobacter/Pantoea* and others) consistently produce a uniform lawn of growth instead of isolated colonies on plates. Our earlier hypothesis (Benhizia *et al.*, 2004b), that the gammaproteobacterial nodule occupants may represent the nitrogen-fixing symbiont replacing rhizobia for those legumes, seems unlikely in light of what we found here, implying that the rhizobia microsymbiont in nodules of Algerian *Hedysarum* species could be quantitatively overwhelmed and masked by endophytes in ways that prevent their detection even by PCR. The inability of pure cultures of the nonrhizobial occupants to nodulate the legume under microbiologically controlled conditions precludes the hypothesis of their involvement in that type of symbiosis. Moreover, dedicated microarray analysis using oligonucleotides for *nodC* and *nifH* genes (Bontemps *et al.*, 2005) on two gammaproteobacterial strains isolated from *H. pallidum* and *H. spinosissimum* (Benhizia *et al.*, 2004b) revealed no detection of these genetic determinants for nodulation or nitrogen fixation. However, the abundance of these bacterial endophytes within perfectly healthy plants suggests that other beneficial interactions may be operative. In retrospect, we can hypothesize that the different bacterial taxa found by Zakhia *et al.* (2006) in nodules of legumes, some of which had previously been reported to yield rhizobia (Zakhia *et al.*, 2004), might also represent endophytes growing from nodules whose rhizobia were not easy to culture as a result of the same phenomenon observed in this paper.

In addition to the early demonstration of endophytes within legume nodules, the main issue is the unexpected nonrecoverable state that affects rhizobia in these plants. Having observed the phenomenon also when reisolating from nodules originated in sterile vermiculite (after inoculating *H. glomeratum* seedlings with the only culturable strain that we obtained), it can be concluded that the phenomenon is caused by the plant itself and not by other possible environmental factors existing in the soils of origin. The acetylene reduction test on *H. glomeratum* and *S. muricatus* showed that nodules do possess nitrogenase activity comparable to that of sulta. Our current work aims to investigate the physiological status of the rhizobia within those nodules, using stains reporting membrane-integrity and respiratory activity to assess whether they are alive and metabolically active. If rhizobia loose viability in these legume root nodules, it would be difficult to explain their persistence in the soils at levels that do not limit legume nodulation, unless they could occupy other niches that permit their multiplication, for example as cereal root endophytes (Yanni *et al.*, 1997). On the other hand, there are reports of rhizobia such as *R. leguminosarum* (Alexander *et al.*, 1999) and *R. sullae* (Toffanin *et al.*, 2000) that enter the 'viable but not culturable' (VBNC) state, although the involvement of host legumes in this syndrome has not yet been established. It is possible that the diverse endophytes

coinhabiting the nodule may produce antagonistic compounds negatively affecting rhizobia, and/or may trigger a systemic host defence response resulting in the production of inhibitory compounds such as salicylic acid (De Meyer *et al.*, 1999) to which the rhizobial symbionts might have a differential sensitivity. Indeed, some nonpathogenic bacteria can themselves produce sufficient levels of salicylic acid to generate reactive oxygen species (De Meyer *et al.*, 1999; Audenaert *et al.*, 2002). We also plan to examine if these undomesticated legumes differ from those cultivated in agriculture in their production of metabolites that inhibit rhizobia *in planta* and/or when carried-over with rhizobia to culture on plating media. Investigating these aspects will provide a better insight into the microbial interactions occurring in native and introduced wild legume plants and will lead to a better understanding of their nitrogen-fixing symbioses.

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