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Endophyte Watching: combining molecular and microscopy approaches to isolate, identify, tag, and monitor fungi and bacteria inside plants.

Direttore della Scuola : Ch.mo Prof. Giuseppe Zanotti
Coordinatore d'indirizzo: Ch.mo Prof. Giorgio Valle
Supervisore : Ch.mo Prof. Andrea Squartini
Ch.mo Prof. Barbara Baldan

Dottorando : Alessandra Tondello

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Summary

This work aims at investigating plant-endophyte interactions under many different standpoints. We took into consideration bacterial as well as fungal microorganisms. Regarding the fungi, endophytic species occurring in orchid plants either as possible mycorrhizal partner as well as other internal colonizers were studied. We analyzed three different species of wild orchids found in the Euganean Hills area, North-Eastern Italy, namely *Orchis militaris*, *Spiranthes spiralis* and *Orchis purpurea*, which are mostly regarded as species in endangered status and for which little is known on the nature and presence of symbionts. An approach involving both molecular methods and microscopy techniques was used. Fungal isolation was performed from surface sterilized roots to obtain pure mycelia. A molecular approach allowed us to amplify the Internal transcribed spacer (ITS) region, starting both from root portions of the orchid plants and from pure cultured mycelia. This genetic region varies relatively little within species but dramatically between species and is easy to amplify because of its high copy number. In addition relatively few primers sets are needed due to the highly conserved SSU and LSU flanking regions. Different amplicons were obtained and analyzed from each species, at first upon their different ARDRA profiles obtained by enzymatic digestions. Representative cases were sequenced and results were examined by BLAST. Fungi of mycorrhizal nature and an additional series of endophytic ones, which are an important component of fungal biodiversity, were found. With different microscopy approaches (Fluorescence, Confocal and Transmission Electron Microscopy) we localized fungi within plant tissues and investigated their features. The hyphal septa found in *Orchis militaris* and *Spiranthes spiralis* samples were of basidiomycete type, which was confirmed by the results obtained from DNA extraction, ITS amplification and sequencing.

In parallel bacterial endophytes were considered, starting from a previous work in which the coexistence of rhizobia with diverse, endophytic bacterial taxa within nodules of wild legume plants had been demonstrated, using molecular and

microscopy-based approaches. In this work in order to co-localize the relevant endophytes inside plant tissues, different fluorescent proteins were used as markers for the different kinds of bacteria. Bacterial strains tagged with GFP were obtained using the pUTgfr2X plasmid, a delivery system for a mini-Tn5 trasposon, expressing kanamycin resistance and the GFP protein. While to obtain bacteria tagged with the *rf*p gene, a replacement of the *gfp* with a *rfp* gene was made starting from plasmid pRL765gfp, obtaining pRL765rfp. Both pRL765rfp and pUT gfp2X vectors were used to incorporate the GFP or RFP cassettes into the chromosome of *R. leguminosarum* bv. *trifolii*. In both cases plasmids were introduced by biparental mating. *Pseudomonas* sp. Hs1::gfp from a wild type *Pseudomonas* sp. isolated from wild legume nodules was obtained introducing pUTgfp2X by biparental mating. In parallel, to tag an *Enterobacter agglomerans* also isolated from legumes, pRL765rfp was introduced by electroporation. The four bacterial strains constructed were used to inoculate seedlings of *Trifolium repens* in nodulation tests. Tagged bacteria were localized on the surface and within plant tissues using Confocal microscopy.

Subsequently *Pseudomonas* sp. Hs1::gfp and *Enterobacter agglomerans* pRL765rfp were used as co-inoculant strains during nodulation tests performed with seeds of wild legume plants from Sardinia. Their ability to be true endophytes was investigated using jointly standard colony isolation methods and direct PCR amplification of prokaryotic DNA from nodules and other tissues. We found that *Pseudomonas* sp. Hs1::gfp, upon root inoculation was able to invade one of the wild species of legumes (*Tetragonolobus purpureus*) and be traslocated to its aerial portions.

Riassunto

Questo lavoro ha come obbiettivo, lo studio con diversi approcci delle interazioni tra piante ed endofiti, sono perciò stati esaminati sia microrganismi batterici che fungini.
Per quanto riguarda i microrganismi fungini, sono state studiate le specie endofitiche riscontrabili in piante di orchidea, sia come possibili partner micorrizici che come generici colonizzatori interni. Abbiamo analizzato tre diverse specie di orchidee selvatiche, considerate in pericolo, ritrovate nell'area dei Colli Euganei (*Orchis militaris, Spiranthes spiralis* e *Orchis purpurea*) per le quali poco si conosce riguardo alla presenza ed alla natura dei simbionti. Per questo studio è stato scelto un approccio che prevedeva l'utilizzo sia di metodologie molecolari che di tecniche di microscopia; inoltre per ottenere miceli fungini in coltura pura si è provveduto a isolarli dalle radici delle piante sterilizzate in superficie. L'approccio molecolare ci ha permesso di amplificare la regione ITS, partendo sia da miceli in coltura pura che direttamente da porzioni di radici. La regione ITS è una regione facilmente amplificabile con un numero relativamente esiguo di primers, per il suo alto numero di copie e per l'alta conservazione delle regioni che la fiancheggiano. Ulteriormente questa regione ci permette di ottenere interessanti informazioni, in quanto varia relativamente poco all'interno delle specie, ma molto tra specie diverse. Per ogni specie di orchidea analizzata sono stati ottenuti diversi ampliconi, che sono stati differenziati in base ai lori diversi profili ARDRA in seguito a digestione enzimatica. I casi più rappresentativi sono stati sequenziati e i risultati sono stati analizzati su piattaforma BLAST. Mediante analisi di omologie di sequenze sono stati identificati alcuni funghi di natura micorrizica e una serie di altri funghi endofitici, che risultano essere una componente importante della biodiversità fungina all'interno dei tessuti delle piante. Diversi tipi di microscopia: a fluorescenza, confocale ed elettronica a trasmissione sono stati usati per localizzare gli stessi funghi all'interno dei tessuti e analizzarne le caratteristiche. I setti (dolipori) ritrovati nelle ife fungine all' interno dei campioni di *Orchis militaris Spiranthes spiralis* erano caratteristici dei

basidiomiceti. Queste osservazioni hanno perciò permesso di confermare i risultai ottenuti dalle indagini molecolari.

Partendo da un precedente lavoro nel quale è stata dimostrata la coesistenza di rizobi e altri batteri endofitici all'interno di noduli di leguminose selvatiche, per questo progetto si sono considerati anche gli endofiti di tipo batterico. In particolare per localizzare gli endofiti all'interno dei tessuti delle piante si è deciso di utilizzare dei marker fluorescenti diversi per tipi differenti di batteri endofitici e rhizobi.

In alcuni ceppi batterici è stato inserito il gene codificante la proteina GFP usando un plasmide pUTgfr2X. Questo sistema trasporta un mini trasposone-Tn5, che esprime oltre alla proteina fluorescente anche la resistenza alla kanamicina. Per ottenere i ceppi marcati con RFP si è dovuto manipolare il plasmide pRL765gfp sostituendo il gene codificante la GFP con quello per la RFP, ricavando così un nuovo plasmide chiamato pRL765rfp. Per quanto riguarda i rizobi sia pRL765rfp che pUTgfp2X sono stati introdotti per coniugazione in *R. leguminosarum* bv. *trifolii*, così che i geni codificanti le proteine fluorescenti si integrassero nel cromosoma. Considerando invece le specie endofitiche diverse dai rizobi, un ceppo di *Pseudomonas* sp. Hs1::gfp è stato ottenuto introducendo per coniugazione in *Pseudomonas* sp. *wt* il plasmide pUTgfp2X. *Pseudomonas* sp. *wt* era stato precedentemente isolato da noduli di leguminose selvatiche così come *Enterobacter agglomerans*. Quest'ultimo ceppo (*Enterobacter agglomerans*) è stato marcato con la RFP introducendo il plasmide pRL765rfp per elettroporazione.

I quattro ceppi batterici ottenuti sono stati utilizzati per inoculare piantine di *Trifolium repens* nei test di nodulazione, utilizzando il microscopio confocale è stato possibile localizzare i batteri sulla superficie delle radici o all'interno delle stesse

Successivamente gli stessi endofiti marcati, utilizzati nei test di nodulazione su *T. repens* (*Pseudomonas* sp. Hs1::gfp e *Enterobacter agglomerans* pRL765rfp), sono stati co-inoculati in plantule di leguminose selvatiche della Sardegna per investigare la loro abilità di essere dei veri endofiti. Per questa ultima parte del lavoro si sono utilizzate comuni tecniche di isolamento e amplificazione del DNA dei procarioti

tramite PCR. *Pseudomonas* sp. Hs1::gfp in particolare è in grado di colonizzare una delle specie analizzate (*Tetragonolobus purpureus*) e di essere traslocato nelle parti aeree.

Introduction

The rhizosphere

The rhizosphere is the zone in which some of the most complex chemical, physical and biological interactions occur, between terrestrial plants and other organisms. This space can be defined as the narrow layer of soil in the vicinity of a plant, that is directly influenced by the roots (Hardoim *et al*. 2008). Rhizosphere represents a highly dynamic front for interactions between roots and pathogenic or beneficial soil microbes, invertebrates and root systems of competitors (Hirsch *et al*. 2003). All these relations may be classified as negative or positive associations; negative interactions include competition or parasitism among plants, pathogenesis by bacteria, fungi, and invertebrate herbivory, while positive ones include not only symbiotic associations with epiphytes and mycorrhizal fungi, but also root colonization by bacterial biocontrol agents and plant growth–promoting rhizobacteria (PGPR) (Fig.1). An important role in the establishment of both positive and negative relations is played by root exudates, which are transported across the cellular membrane and secreted into the surrounding rhizosphere. Plant products are also released from root border cells and root border-like cells, which separate from roots during their growth (Vicre *et al*. 2005). Root exudates are often divided into two classes of compounds: low-molecular weight and high-molecular weight exudates. Low-molecular weight compounds such as amino acids, organic acids, sugars, phenolics, and other secondary metabolites are considered to account for most of the diversity of root exudates. This class of compounds diffuses near the roots promoting symbiotic interactions and blocking growth of pathogens and competing plant roots. High-molecular weight exudates, such as mucilage (polysaccharides) and proteins, are less diverse but often compose a larger proportion of the root exudates by mass (Bais *et al*. 2006). Plant exudates clearly represent a significant carbon cost to the plant, but they have an active role in the regulation of symbiotic processes, and in the protection against deleterious bacteria. In addition plant exudates are often species-

specific. As an example, *Azospirillum* chemotaxis is induced by sugar, aminoacids and organic acids, but the degree of chemotactic response to each of those compounds differs among strains. Also rice exudates induce stronger chemotactic responses from endophytic bacteria than from non-growth promoting ones present in the rhizosphere (Compant *et al*.2005).

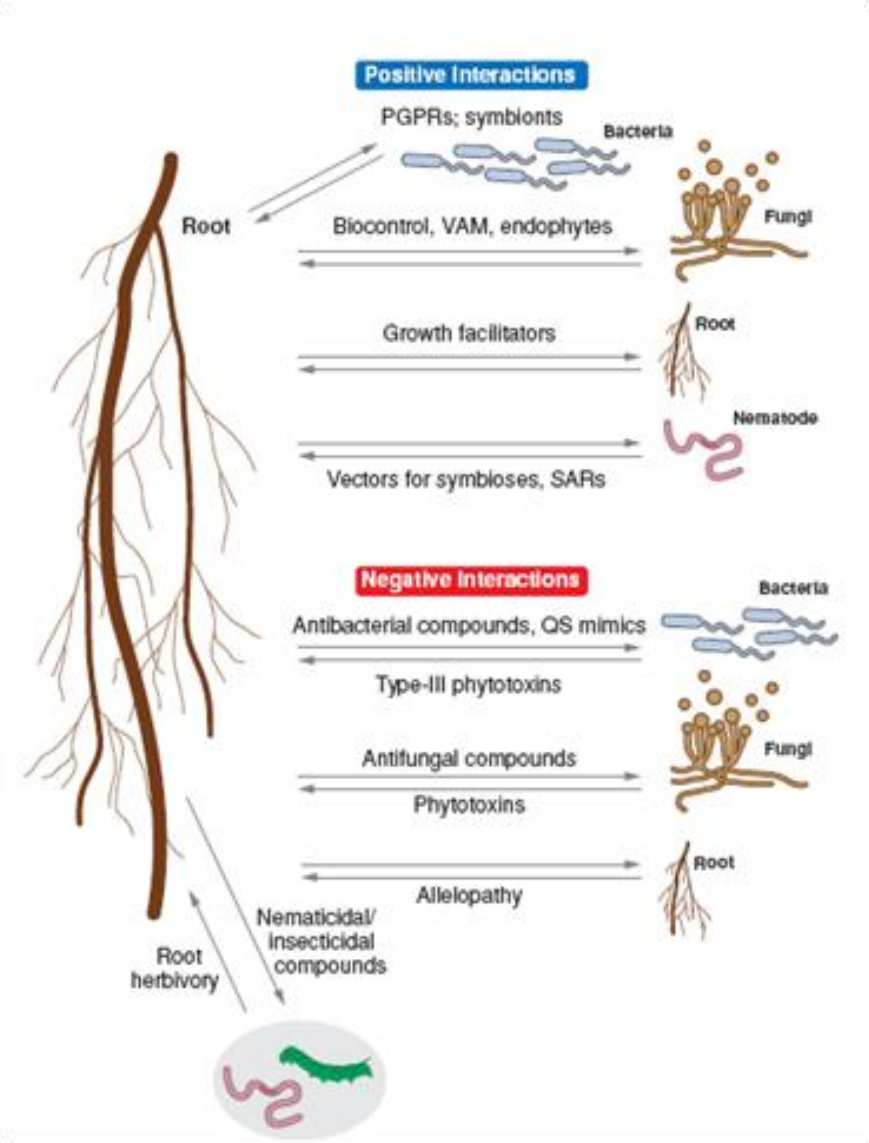

Fig. 1: schematic representation of possible rhizospheric interactions mediated by root exudates. From Bais *et al*. 2006

Therefore plants, like other organisms, live immersed in a thriving community of microbes and the diversity of microrganisms with which plants interact can bring diseases but also benefits. Plant-microbe interactions can positively influence plant growth through a variety of mechanisms, including fixation of atmospheric nitrogen by different classes of proteobacteria, increased biotic and abiotic stress tolerance imparted by the presence of endophytic microbes (Schardl *et al*. 2004), direct and indirect advantages conferred by plant growth–promoting rhizobacteria, by the production of phyto-hormones or by enhancing availability of minerals as the case of mycorrhizal fungi (Rosenblueth and Martìnez-Romero 2006). Bacteria in particular can also positively interact with plants by producing protective biofilms or antibiotics operating as biocontrols against potential pathogens, or by degrading plant- and microbe-produced compounds in the soil that would otherwise be allelopathic or even autotoxic.

Among the above described plant-microorganism relationships we focused the attention on endophytes. Bacteria as well as fungi can enter or reside in plants as endophytes without causing harm. Some plants could also be the unexpected reservoir of clinically relevant human and animal pathogens. As reviewed by Rosenblueth and Martìnez-Romero (2006), endophytes have been isolated from a large diversity of plants and in the same specimen they are not restricted to a single taxon but can encompass several genera and species. Endophytes normally occur at lower cell densities than external rhizospheric and pathogenic microbes. Endophytes can be defined as those microorganisms that colonize the internal tissues of plant showing no external signs of infection or negative effects on host and they could be better protected from biotic and abiotic stress, that normally condition rhizospheric populations (Ryan *et al*. 2007). Some criteria to recognize microorganisms as true endophytes exist, and require not only the isolation from surface-sterilized tissues, but also their visualization by microscopy inside plant tissues, and finally their capacity to re-infect disinfected seedlings (Rosenblueth and Martìnez-Romero 2006).

Fungal endophytes

Beneficial fungi that offer protection against pathogens can rapidly colonize the cortex of living plant roots without causing harm, but also some pathogenic or necrotrophic fungi, with a latent phase, can enter in the same root zone without causing diseases.

Endophytic fungi in particular are ubiquitous in plants and are the most likely source of establishment of new plant–fungus associations. Fungi that live within plant tissues can promote host growth (Cheplick *et al*., 1989), help mineral nutrition (Malinowski e Belesky 1999), and enhance resistance to pathogens (Sturz *et al*. 1999). Some endophytic fungi have been shown to protect plants from herbivores or to be responsible for the synthesis of novel secondary products (Stobel *et al.* 2004). *Streptomyces* sp. as an example, was shown to be widely distributed among wheat plants in the field and to have the ability to promote plant growth and to control a number of root-infective phytopathogenic fungi (Coombs and Franco 2003). *B. bassiana* was isolated from all opium poppy plants and it may have implications in the biological control of *T. papaveris*, including the possible systemic protection of the plant against this cynipid (Quesada-Moraga *et al*., 2006). Fungal endophytes benefit from occupying plants by gaining greater access to exudates, the first access to organic substrates after the death of the host and avoidance of competition, predation and parasitism from other soil organisms.

A particular group of endophytic fungi are the mycorrhizal ones, considered ancient plant partners widespread among plants, their benefits are well known and some plants, like orchids, are incapable to survive through their offspring stage without their mycorrhizal fungi. This particular association cannot be properly defined like an endophytism, because it represents an intimate association with a specialized interface, where exchange of materials occurs between living cells. To differentiate mycorrhizae from other fungal interactions Brundrett defines the former as a symbiotic association, between a fungus and a root of living plant, essential for one or both partners, that is primary responsible for nutrient transfer. Mycorrhizal fungi

attracted by exudates proliferate on the surface of plants, develop mechanisms for penetrating living plants without causing harm to their hosts, and the interaction occurs in a modified tissues where intimate contact results from synchronized plant-fungus development. Fungal hyphae interact with host plants, and at the other end with soil, so they can in principle form a connection network also between roots of different plants. Mycorrhizal fungi differ from other fungi primarily because they are dual soil-plant inhabitants that would have evolved to become efficient at growth and nutrient uptake in both soil and plants (Brundrett 2002). Mycorrhizal association are classified primarily by morphological features controlled by the host, as features controlled by the fungus are too highly variable. At least seven types of mycorrhizae have been recognized, although some are very similar to each other, for this reason some works, considering the plant's perspective, recognize only the three main structural lineages of mycorrhizae: Ectomycorrhizae (ECM), vescicular arbuscolar mycorrhizae (VAM) and orchid mycorrhizae (OM) (Imhof 2009, Brundrett 2004).

Ectomycorrhizae (ECM)

Ectomycorrhizal symbioses are important on a global scale because the dominant trees in most of the world's temperate and boreal forests and in large areas of tropical and subtropical forests are ectomycorrhized. ECM fungi are also important because they include edible fungi of high value, *Tuber sp* for example are ectomycorrhyzal ascomycete that produces subterranean ascomycota known as truffles (Pacioni *et al*. 2007). Ectomycorrhizal fungi are characterized by the presence of a hyphal mantle covering the root tip and of a Hartig net, that consists of labyrinthine hyphae surrounding the plant cells within the root cortex. Outside the root, the fungal mycelium forms an extensive network within the soil enhancing the capacity of the plant root system to absorb mineral elements and water. Fungi that form ectomycorrhizae do not belong to a monophyletic group. Over 5000 species of ectomycorrhizal fungi have been described, the symbionts span various phyla of fungi (Zygomycota, Ascomycota, and Basidiomycota), and occur in at least 15

families within the Basidiomycota and Ascomycota (Horton *et al*. 2001). Ectomycorrhizal fungi in addition need the presence of host plant to complete their life cycle. For a plant that grows in a habitat poor in nutrients the association with a large variety of fungal symbionts enhances its ability to uptake nutrients. These fungi in fact produce different enzymes that degrade organic molecules, obtaining organic N and P that is available to the plant. Related to ectomycorrhizae also the ecto-endomycorrhizae group exists, that comprises a limited number of fungal taxa. The difference from fungi classified as ectomycorrhizae is that in this case fungal hyphae can enter within the cells forming coils or haustorial structures.

Arbuscolar mycorrhizae (AM)

The development of the arbuscular mycorrhizal symbiosis, that is the most widespread type of fungal symbiosis, played a crucial role in the initial colonization of land by plants and in the evolution of the vascular species. Arbuscolar mycorrhizae are characterized by the formation of an unique symbiotic interface that serves to exchange nutrients and possibly signals. AM (previously referred to also as VAM for vescicular) can be definied as particular endomycorrhizae because the endophytic fungus undergoes complex morphogenesis inside the host root, whit development of intracellular structures. In the case of arbuscolar mycorrhizae the initial signal (strigolactone) of the plant is constitutively released, and induce the hyphal branching and metabolic activity of fungi. This molecule promotes fungal growth and increases their chance to encounter the plant roots When the mycelium contacts the host root surface it produces appressoria, multinucleate infection structures, that can be triggered by physical contact with isolated cell walls of epidermal root cells (Reinhardt 2007). This structure allows the colonization of root tissues forming inter- and intracellular hyphae, coils, highly branched arbuscoles and vesicles. Meristem and vascular tissues are resistant to mycorrhizal colonization, therefore only epidermis and cortical cells are colonized (Fig.2).

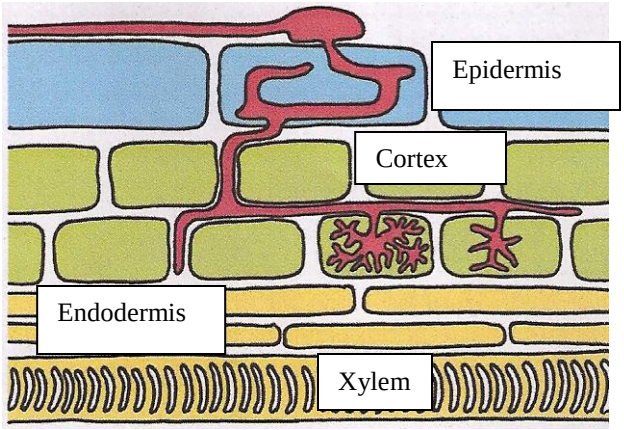

Fig. 2: colonization of root cortex by arbuscolar mycorrhizas. After the formation of appressoria an intracellular coil is formed in the first epidermal cell, then the fungus grows intercellularly to colonize the cortex, with the formation of arbuscular structures. Modified from Reinhardt 2007.

Colonization of the cortical cells induces a number of changes within cells, because fungal hyphae fill most of the space. Vacuole fragmentation, nuclear migration from the periphery to the centre, cytoskeleton rearrangements and plastid modifications are visible in the host cells that contain arbuscolar structures (Fig.3).

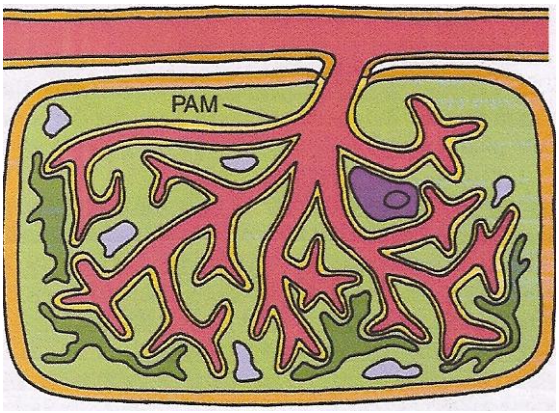

Fig. 3: development of the arbuscule within the host cell. During fungal colonization, the central vacuole becomes fragmented (BLUE), and the nucleus move to a more central position (PURPLE). The plastids form tubular structures that associate with the fine branches of the arbuscule. These remain surrounded by the periarbuscular membrane (PAM). Modified from Reinhardt 2007.

AM fungi have elaborated a specialized intracellular structure, for nutrient exchange with plants; it consists of highly ramified hyphae with very fine terminal tips, resulting in a surface-to-volume ratio that is greater than that of normal hyphae. VAM help plants to capture nutrients such as phosphorus and micronutrients from the soil, and absorb carbohydrates from host plant. The modern AM fungi are placed in the Zygomycetes order Glomales and in the absence of the host their growth is limited to a relatively short time (Bonfante, and Perotto 1995).

Orchid Mycorrhizae (OM)

The orchid mycorrhiza is the third distinct structural lineage of mycorrhizae. The evolution of orchid mycorrhizae is linked to extreme specialization, since orchid plants produce an abundant number of microscopic seeds, with limited storage materials, for dispersal into specialized habitats in different environments. These associations have hyphal coils in host cells with very few morphological signs, which renders hard to assess whether the fungi are specialized root inhabitants or plain invaders, in contrast with AM and ECM which display the host-fungus interface with highly specialized hyphae (Brundrett 2002). Orchid seeds are very small with minimal nutrient reserve, therefore upon germination, fungal hyphae promptly penetrate the cell walls of the orchid and form characteristic coils, called pelotons, within the cells. Growth of the fungus is restricted to cortical cells, probably by the deposition of phenolic compounds and the production of anti-fungal substances (Shimura *et al.,* 2007). Differently from other mycorrhizas pelotons are subsequently ‘digested’, and through this process the orchid is thought to receive the essential nutrients and carbon needed to grow. Nutrient exchange may also, or instead, occur across intact cell membranes prior to ‘digestion’ as in other intracellular mycorrhizas. The germinated seed develops into a mass of differentiated cells called protocorm, and remains in this form for a period that can extend up to several years, until leaves are produced. During this period of their life, many orchids are underground and rather than producing organic carbon through photosynthesis, they obtain all of their

energy from fungal pelotons. Therefore, before the production of leaves, all orchids go through a stage of their life-cycle in which they are mycoheterotrophs, rather than autotrophs. Most adult orchids have a poorly developed root system, therefore they retain their mycorrhizal partnerships because they are still heavily reliant on mycorrhizal fungi for mineral nutrition (Waterman and Bidartondo, 2008). In contrast to other mycorrhizal symbioses, it has long been thought that orchid mycorrhizal fungi receive few benefits from the interaction; in fact orchid mycorrhizae have historically been depicted as anomalous associations in which nutrient flux was plant-oriented (Leake 1994). However a recent study demonstrated bi-directional movement of carbon between adult *Goodyeara repens* and its fungal partner (Cameron *et al*., 2006, 2007). Orchidaceae have species with different levels of dependence on mycorrhizae, extending from fully auotrophic to fully- heterotrophyc associations. Generally all orchids need fungi to provide inorganic and organic nutrients for seed germination and early protocorm development. In addition in adult photosintetic orchids, N, P and water continue to flow from the fungal partner, but carbon exchange is essentially reversed with photosynthate providing incentive for continued fungal colonization (Dearnaley 2007). Most orchid mycorrizal fungi belong to the Rhizoctonia group, a diverse polyphyletic group including plant pathogens, endophytes, saprophytes and mycorrhizal fungi. There are also several exceptions as shown in both achlorophyllus and photosynthetically-active specimens of *Epipactis* which have a mycorrhizal interaction with fungi from the tree-ectomycorrhizal ascomycetes genus *Tuber* (Selosse *et al*. 2004) or *Neottia nidus-avis* which is specialized on fungi in the Sebacinaceae known to be ectomycorrhizal with trees (McKendrick *et al.*, 2002; Selosse *et al.*, 2002). *Corallorhiza maculata* and *C. mertensiana*, are instead specialized on ectomycorrhizal members of the Russulaceae (Taylor and Bruns, 1999; Taylor *et al*., 2004). In addition, other non-photosynthetic orchids specialize on free-living non-rhizoctonia fungi (Ogura-Tsujita and Yukawa, 2008). Some evidences indicate also that fungal partners may switch during the life of

the orchid, so that the fungal-orchid association appears sensitive to enviromental stimuli and can possibly adjust to favor survival of the plant partner.

The identification of orchid mycorrhizal fungi is a critical step in exploring the biology of this symbiosis, considering that fungal isolation from orchids is not always easy, isolation success in many orchid varies with season and in some cases simbionts are difficult or impossible to isolate. Electron microscopy examination of septal ultrastructures can not allow to recognize fungal species, but molecular methods based on fungal-specific PCR amplification of the nuclear ribosomial internal transcribed spacer (ITS) are helpful to overcome the problems associated with limited morphological variation and inefficient culturing (Taylor and McCormick 2008).

The Orchidaceae family

Orchidaceae are the most diverse of all angiosperm families, with estimates of 25000 species. Orchids comprise five subfamilies and approximatly 870 genera and are considered almost ubiquitous, occurring on all continents and even in some Antarctic islands (Chase *et al*., 2003). Particularly orchid-rich areas include the northern Andes of South America, Madagascar, Sumatra and Borneo for mostly epiphytic species, Indochina is rich in both epiphytic and ground species, and Southwestern Australia is a centre of ground orchid richness (Cribb *et al*., 2003). Orchidaceae, more than any other plant family, have a high proportion of threatened genera. The persistence of these plants is linked to abiotic and biotic factors that act in a linear sequence of interactions dependent on their level of criticality for growth, development and reproductive success. For example, for most ground orchids, the presence and vitality of mycorrhiza in soil around plants have a more immediate impact on plant persistence than other factors. The great taxonomic diversity of Orchidaceae is often attributed to their specialization to particular habitats, pollinators and mycorrhizal associations (Swarts and Dixon, 2009).

In this project endophytic species in orchids were investigated. We analyzed three different species of wild orchids found in the Euganean Hills area, North-Eastern

Italy: *Orchis militaris, Spiranthes spiralis* and *Orchis purpurea*, species mostly in endangered status and for which little is known on the nature and presence of symbionts (Fig. 4).

Orchis purpurea

Orchis purpurea perennates during the winter and its leaves appear above the ground in February. Plants have one to four basal leaves of elliptic-ovate to lanceolate shape, 2–5 cm wide and 6–20 cm long. Flowering takes place at the end of May. Flowering stalks vary in height between 25 and 60 cm and carry 10–50 bright white to purple-brown, self-compatible flowers. Seed capsules ripen by the end of June, and this is followed by dehiscence and seed dispersal in August (Jacquemyn *et al*., 2007).

Orchis militaris

Orchis militaris (L.) is a pseudobulbous orchid. Flowering stalk of purple colour is 20-60 cm height and carries 10-40 flowers. Basal leaves of ovate-lanceolate shape form a rosette. Flowering stage of this species starts from the end of April and finishes by July. This rare orchid species was found to colonize hills of ash and semicoke tailings from oil shale mines in north-eastern Estonia (Shefferson *et al*., 2008).

Spiranthes spiralis

Spiranthes spiralis (L.) is the latest-blooming native species of orchid in Italy, flower stalks and rosettes appear aboveground by the end of the summer (August–September) and the wintergreen rosettes die off in spring (May). The height of the flower stalks varies between 5 and 25 cm, white flowers are arranged in a spiral on

the upper half of the stalk. Flower stalks emerge aboveground some days or even some weeks earlier than the tiny rosettes. One plant may consist of one or a few rosettes, that normally have 2-6 leaves that are each up to 3-4 cm long and 0,5-1,5 cm wide. Emergence of rosettes and development of the flower stalk is at the expense of one or two small underground tubers (Willems 2000).

Fig. 4: ***Orchis militaris*** **(A),** ***Spiranthes spiralis*** **(B),** ***Orchis purpurea*** **(C).**

Bacterial endophytes

We previously defined endophytes as those microorganisms that can colonize internal plant tissues, we shall now consider in detail bacterial endophytes. The endophytic niche offers protection from the environment for those bacteria that can colonize plant tissues and establish within them, and plants can require the presence of associated bacteria for their growth and establishment in different ecosystems. Endophytic bacteria have been isolated from both monocotyledonous and dicotiledonous plants. Many evidences that the healthy plant interior can normally contain bacteria not necessarily related to a pathogenic context exist and a list of

bacterial endophytes and plant harbouring them has been reviewed by Rosenblueth and Martìnez-Romero (2006). A number of facultative endophytes have been reported from rice (Biswas *et al*., 2000, Chi *et al*., 2005), maize (Gutierrez-Zamora and Martìnez-Romero, 2001), pepper (Aravind *et al*., 2009), poplar trees (Taghavi *et al*., 2009); the endophytic bacterial communities inhabiting citrus plants were also studied and the most commonly isolated endophytic bacteria were *Methylobacterium* spp., *Curtobacterium flaccumfaciens*, *Pantoea agglomerans*, *Bacillus* spp., *Pseudomonas* spp., *Alcaligenes* spp. and *Enterobacter cloacae*, as an example for *Pantoea agglomerans* a number of interesting characteristics were suggested in addition to its potential as a bio-control agent (Andreote *et al*., 2008). Zinniel *et al*., (2002) isolated several hundred different endophytic colonizing bacterial strains from aerial tissues of four agronomic crop species and 27 prairie plant species and proposed that these microorganisms may be useful for biocontrol and other applications.

It appears that the bacteria best adapted for living inside plants are naturally selected, and also that some endophytes are more aggressive colonizer than others. A recent work revealed that both *Rhizobium* sp. and *Burkholderia* sp. can colonize the intercellular spaces in the root cortex of rice plants when inoculated separately, but the colonization process by gfp/gusA-tagged *Rhizobium* sp. was severely inhibited when co-inoculated with an equal number of wild type *Burkholderia* sp.; therefore *Burkholderia* resulted to be a more aggressive endophytic colonizer of rice than *Rhizobium*. (Singh *et al.*, 2009). Endophytes enter the plant tissue primarily through the root zone, however cotyledons and aerial portions of plants, such as flowers, stems and leaves may also be used for entry. Endophytes either become localized at the point of entry or are able to spread throughout the plant and such isolates can live within cells, in the intercellular spaces, or in the vascular system (Hallmann *et al*., 1997). Also seeds can carry different endophytes and by being seed-borne, endophytes assure their presence in new plants. In the root system some bacteria must find their way to penetrate through cracks formed at the emergence of lateral roots or

at the zone of elongation and differentiation of root (Sharma *et al*., 2005). In legume plants the nodule, the novel organs induced and occupied by soil bacteria collectively known as rhizobia, can be colonized internally by several bacterial genera unrelated to rhizobial symbiotic nitrogen-fixing bacteria. Reports provide information on isolates belonging to the genus *Bacillus* that were obtained from soybean root nodules (Bai *et al*., 2002). Also the collection and characterization of culturable Gammaproteobacteria (genera *Pseudomonas*, *Enterobacter*, *Klebsiella*) associated with peanut root nodules (Ibànez *et al*., 2009) or in nodules of wild legumes plants has been reported (Benhizia *et al*., 2004, Muresu *et al*., 2008)

The legume-Rhizobium interaction

We have already introduced the most widespread plant symbiosis that is between plants and mycorrhizal fungi, but a more specialized one is the symbiosis between plants and nitrogen-fixing bacteria, in this work we considered in particular the widely occurring legume-rhizobial symbiosis.

During legume-rhizobial interaction the bacteria invade the plant roots and the result of a molecular dialogue between the host plant and the bacteria unleashes nodule organogenesis (Oldroyd and Downie 2008). These special structures have been evolved by plants belonging to the Leguminosae family as a housing for nitrogen-fixing bacteria. The bacterial partner of this biological nitrogen fixation is restricted to a limited but diverse number of genera, that include both α-proteobacteria *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, and β-proteobacteria *Burkholderia*, and *Ralstonia* sp. (Moulin *et al*.,2001).

Nitrogen and Nitrogenase system

Nitrogen is a crucial component of amino acids and nucleotides, and therefore, is essential for life. It is often a limiting factor as it is only biologically accessible in the reduced state, in the form of ammonia or nitrate. The natural process taking care of

the reduction of atmospheric nitrogen is called biological nitrogen fixation and accounts for about a half of the annual amount of nitrogen fixed by both natural and human activities (Vitousek *et al*., 1997). Biological nitrogen fixation is restricted to prokaryotes that possess the Nitrogenase enzyme responsible for nitrogen reduction, which catalyzes the conversion of N_2 into a reduced form, which can then be used for growth by microorganisms and higher life forms. The nitrogenase system consists of two different enzymes which must function together in the nitrogen fixing process. One of the enzymes, the azoferredoxin is an iron-containing protein. The second enzyme, the molybdoferredoxin contains both iron and molybdenum. The two components combine and function together as a single system. This nitrogenase enzyme is also irreversibly inactivated by oxygen, therefore, biological nitrogen fixation requires anoxic or nearly anoxic conditions, this is one of the reasons for the development of the symbiotic nodules. In the symbiotic nitrogen-fixing organisms such as *Rhizobium*. Rhizobia induce nodules formation on plants of the *Leguminosae* family, which protect nitrogenase from oxygen. These tubercular structures contain oxygen-scavenging molecules such as leghaemoglobin, which confers a visible pink colour when the active nitrogen-fixing nodules of legume roots are cut open. Leghaemoglobin regulates the supply of oxygen to the nodule tissues (Brewin 2002).

Perception of Nod factors

Before the physical contact, the plant and the bacteria communicate through the exchange of signal molecules. The roots of the host plants secrete flavonoids and betaines that induce the expression of bacterial nodulation (*nod*) genes (Carlson *et al*.,1994**,** Long 1996). These genes encode for enzymes responsible for the production and the export of Nod factors, lipochitooligosaccharides, which trigger a number of early nodulation responses in the host plant. Nod factors consist of an oligomeric backbone of β-1,4-linked N-acetyl-D-glucosaminyl residues, N-acylated at the non-reducing end with species-/strain-specific substituents. Only particular types and mixtures of Nod factors allow a strain to nodulate a certain legume host, thus giving

rise to a first rhizobial determinant of host specificity (Spaink, 2000). Therefore variation in the amount and structures of Nod factors produced by a rhizobial species is a key factor determining its host range (Perret *et al*., 2000). Perception of these bacterial signalling factors by the plant triggers a series of morphological and physiological changes observable in the host plant early in the nodulation process, including root hair deformations, curling and branching, depolarization of the plasma membrane, generation of an oscillatory calcium signal (calcium spiking), remodeling of actin filaments near the root hair tip, induction of gene expression and the initiation of cell division in the root cortex, which establishes a meristem and nodule primordium (Gage 2004).

Attachment of bacteria to the roots and root hair deformation and curling

Attachment of rhizobia to host roots is supposedly the very early step required for infection and nodulation. A Ca^{2+}-bilding bacterial protein called rhicadhesin appears to be involved in bacterial attachment to legume root hairs. Legume lectins located at the root-hair tip would recognize and bind to specific carbohydrate structures that are present in the bacterial surface, so these molecules may also play an important role since they could serve as receptor for bacterial surface polysaccharides. Workum *et al.*, (1998) showed that exopolysaccharides could enhance bacterial binding to root hairs by inoculation of vetch roots with an exopolysaccharide-deficient *R. leguminosarum* bv. *viciae* mutant and observed a severely reduced number of infection sites. This first step of attachment is weak and reversible, a second binding step requires the synthesis of bacterial cellulose fibrils (Rodriguez-Navarro *et al*., 2007).

The root hairs mostly susceptible to rhizobial attachement and Nod factor action are those that have nearly finished growing. (root hair zone II, Fig.5).

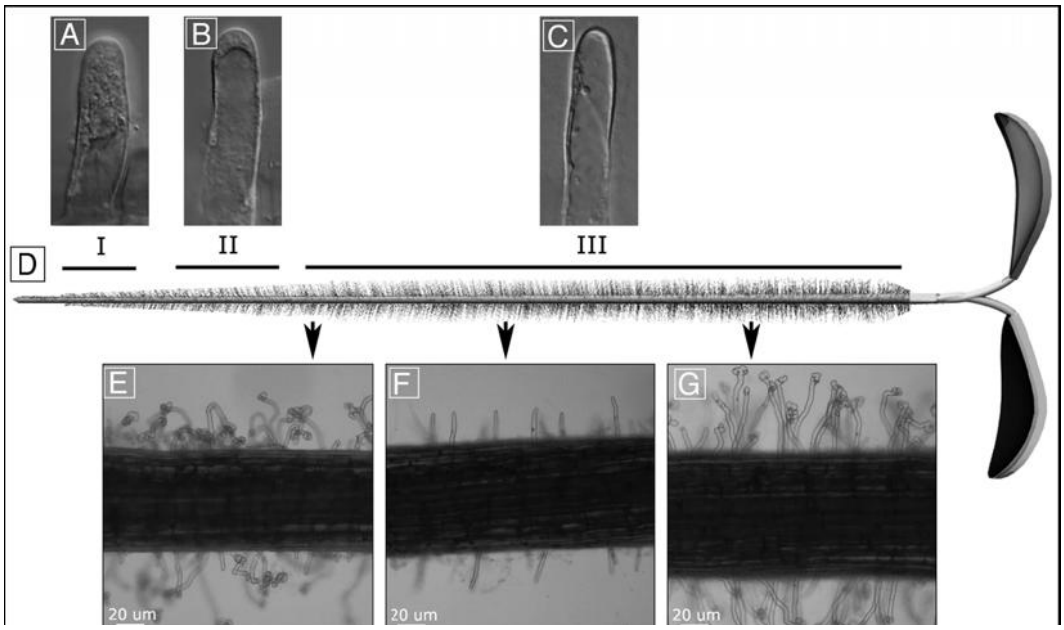

Fig. 5: Root hair morphology. (A, B, C) Typical root hairs from zones I, II, and III, respectively, of an uninoculated alfalfa plant. (D) Diagram of an alfalfa seedling, showing the locations of root hair zones I, II, and III. Modified from Gage, 2004.

Root hairs that have finished growing (root hair zone III) and root hairs that are actively growing with a strongly polarized internal organization (root hair zone I) are refractory to the deforming activity of Nod factors. Zone II root hairs are terminating growth and are different morphologically from actively growing root hairs in zone I. They do not display the large plug of cytoplasm below the root hair tip and a large vacuole is nearer to the tip. Nod factor-induced deformation of zone II root hairs begins with root hair tips swelling isodiametrically; this process is followed by the establishment of a new growing tip that resembles highly polarized, actively growing tips of zone I root hairs (de Ruijter *et al*., 1998, Heidstra *et al*., 1994, Miller *et al*., 1999, Sieber and Emons, 2000). It is interesting to consider why zone I and zone III cells do not deform in response to the addition of purified Nod factor. Zone III cells may be unable to deform because they have a secondary cell wall or because they no longer have the machinery in place to catalyze tip growth. Zone I cells may not

respond to Nod factor by deforming because they are already highly polarized and actively growing (Lhuissier *et al*., 2001). When added to the external medium, purified compatible Nod factors are sufficient to cause root hair deformation and branching, but they are not sufficient to cause the formation of tightly curled root hairs (shepherd's crooks) that are usually the sites of bacterial entry into plants. The responsiveness of root hairs to deform in the presence of Nod factors can also be modulated by plant hormones such as ethylene, which inhibits Nod factor signal transduction and can influence the degree of root hair deformation and the frequency of productive infections (Oldroyd *et al*., 2001). Thus, changes in ethylene levels, in ethylene signal transduction, or in other hormone signalling systems during root growth may explain the observed variability in root hair responsiveness to Nod factor (Gage, 2004).

In response to bacterial attachment, the root hairs curl around the bacteria and isolate them from the external environment. An infection site is formed within the root hair curl through local cell wall degradation. Robledo *et al*. (2008) in a recent study describe the purification, biochemical characterization, molecular genetic analysis, biological activity, and symbiotic function of a cell-bound bacterial cellulose (CelC2) enzyme from *Rhizobium leguminosarum* bv. *trifolii*, the clover-nodulating endosymbiont (Mateos *et al*., 1992) The purified enzyme can erode the noncrystalline tip of the white clover host root hair wall, making a localized hole of sufficient size to allow wild-type microsymbiont penetration. They also demonstrated that this CelC2 enzyme is not active on root hairs of the non-host legume alfalfa.

Infection Thread formation and progression

After cell wall degradation the bacteria gain access at the internal plant tissues through a tubular structure called Infection Thread. The start of growth of infection threads often coincides with an arrest in tip growth of the root hairs. The formation of the infection thread is the consequence of an initial plant plasma membrane invagination and growth down into the root hairs by the deposition of new membrane

and cell wall material. Within infection threads the bacteria are embedded in a matrix of glycoproteins from which they are protected by a layer of surface polysaccharides, and inside the thread they grow and divide, keeping the tubule filled with bacteria. In that way progression of the infection filament is probably driven by bacterial division and local cell wall and matrix deposition, the extending infection thread tip is connected to the nucleus of the root hair cell by thick and actively streaming columns of cytoplasm. Prior to the arrival of the tube of infection in the outer cortex cells, the cytoplasm of these cells redistributes into a column of cytoplasm, the pre-infection thread (PIT), which marks its way of growing (Kijne, 1992). Growing towards the root interior, the infection thread branches and enters the previously formed nodule primordium (Fig.6) (Timmers, 2008).

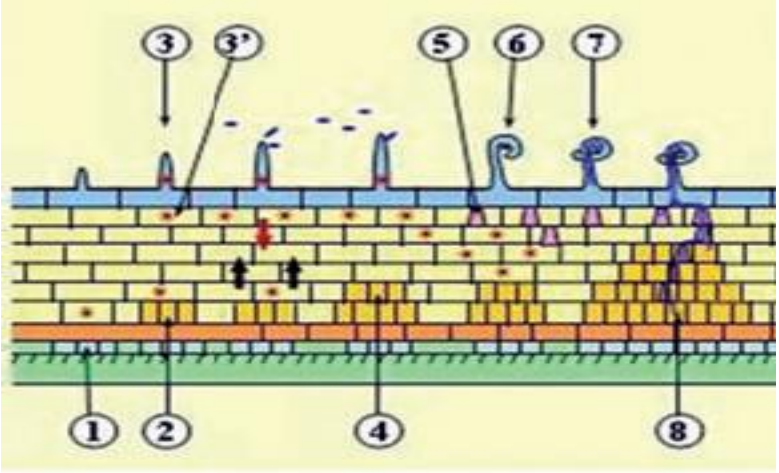

Fig.6: Schematic representation of the sequence of development during the setting up of the interaction between *Medicago* species and *Sinorhizobium meliloti*: (1) activation and cell division of the pericycle. (2) Activation and cell division of the internal cortex. (3) and (3') Activation of root hairs and external cortex. (4) Primordium formation (5) Formation of cytoplasmic bridges (PIT) (6) Deformation of root hairs. (7) Infection initiation in root hairs. (8) Progression of infection threads in the root cortex. Adapted and modified from Timmers, 2008.

Nodule primordia and determinate and indeterminate nodules

Concomitant with rhizobial infection of root hairs, Nod factors stimulate distant cells of the root pericycle layer to undergo cytoskeletal rearrangements and transient proliferation. Cortical cells near infection point and close to the protoxilem pole divide to establish the nodule primordium, a mass of rapidly proliferating cells (Crespi and Frugier, 2008). After infection threads have reached the nodule primordium, some non invaded cells organize the nodule meristem, which is necessary for nodule growth towards the root surface; bacteria are liberated from the network of the infection thread in the infection zone, which is present within central tissues of the nodule. Bacteria liberated into plant cells are called bacteroids. They remain surrounded by the peribacteroid membrane, forming the symbiosome. Depending on the nodule type, the bacteroids within the symbiosomes may divide once or a few times. The symbiosome either divides or does not, resulting in a symbiosome with a single or several bacteroids.,. Finally, the infected cells become completely filled with the symbiosomes (Timmers, 2008).

Legume nodules can be distinguished on morphological and anatomical criteria in determinate and indeterminate nodules. Indeterminate nodules are cylindrical and elongated due to a persistent apical meristem, and nodule organogenesis begins with cell divisions in the inner cortex and pericycle of the root, in these nodules different developmental zones can be distinguished. The determinate nodules, present in tropical legume plants originate from cell divisions in the outer cortex, do not have a persistent meristem and have a more spherical shape. In both cases the peripheral tissues of nodules surrounding the central tissues include the nodule parenchyma, a vascular system, the nodule endodermis and an outer cortex (Gualtieri and Bisseling 2000).

Different ways to infect legume plants

The best studied mode of infection of legumes is the one caused by root hairs deformation and infection thread growth, but an alternative mode of infection is the crack entry. During crack entry rhizobia invade the root interior trough natural wounds caused by splitting of the epidermis where young lateral roots or nodule primordia have been stimulated to develop and emerge. In addition two types of crack entry can be distinguished depending on the mode in which bacteria disseminate into the nodules. In the first case rhizobia directly invade some cortical cells and their dissemination takes place with the division of infected cells like the case of symbiosis between *Chamaecytisus proliferus* (tagasaste) and *Bradyrhizobium* sp. (Vega-Hernandez *et al*., 2001). The second method, observable for example in the aquatic legume *Neptunia*, involves the dissemination of bacteria in intercellular spaces followed by the formation of true tubular infection threads that penetrate nodule cells (Rivas *et al*., 2002) (Fig.7).

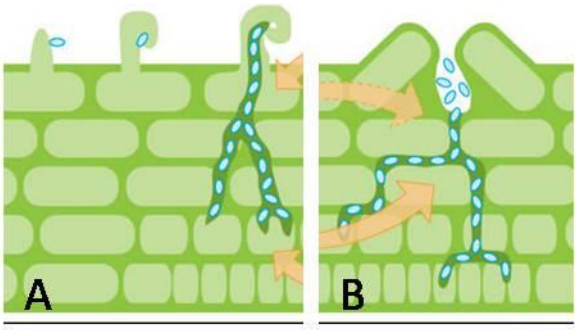

Fig. 7: Bacterial infection can occur either through root hairs (A) or crack entries (B). During root hair entry, epidermal responses are associated with nodulation (Nod) factor perception that leads to gene expression. During crack invasion the epidermis is breached and the bacteria gain direct access to cortical cells. Modified from Oldroyd 2008.

Studying Bacterial Endophytes

Endophytic bacteria have been studied mainly after culturing in laboratory media, but many bacteria are not prone to be cultured, as we observed in the case of nodules of Mediterranean legumes harbouring non-culturable rhizobia (Muresu *et al*. 2008). Using methods that do not require bacterial culturability and make use of the analysis of sequences from bacterial genes obtained from DNA extracted or amplified from internal plant tissues, a more complete view about endophytes has emerged. Molecular approaches for the isolation of bacterial endophytes and plant-associated bacteria have been reviewed by Franks *et al*. (2006). Microbial communities inhabiting stems, roots and tubers of various plants can be analized by 16S rRNA gene-based techniques such as terminal restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis as well as 16rRNA gene cloning and sequencing. The development of metagenomic techniques and new sequencer technologies promises to be very useful to investigate plant endophytes. Therefore studies that make use of both culture-based and culture-independent techniques can be particularly useful in the case of plant-endophytes interactions. Considering that the use of microscopy to visualize microorganisms inside plant tissues is also important to define a microorganism as a true endophyte, the use of tracking methods can facilitate the localization of bacteria in plant tissues. Autofluorescent protein methods are now a key tool for studing processes such as microbe-plant interactions. These techniques have been largely utilized to detect and enumerate microorganisms on plant surfaces (Tombolini *et al*., 1997) and within tissues (Gage *et al*, 1996; Germaine *et al*., 2004; Njoloma *et al*., 2005; Spinelli *et al*., 2005, Singh *et al*., 2009). GFP for example, but also other fluorescent proteins are useful biomarkers as they do not require any substrate or cofactor in order to fluoresce. In addition, different fluorescent protein can be used together to tag different endophytes and co-localize them in the same plant tissue. Confocal laser scanning microscopy in combination with fluorescent proteins can help to investigate plant-microbe interactions without

excessive manipulation of the samples, since this microscopy approach permits an optical sectioning of the samples.

Chapter I

Fungal Endophytes

Summary and scope I

The experimental trials described in this chapter aim at evaluating the presence and the taxonomical identity of fungal endophytes in roots of plants belonging to the Orchidacee family. Three different species, mostly in endangered status, of wild orchid plants were analyzed, and particular interest was given to the identification and localization of mycorrhizal fungi. The amplification of ITS region was chosen as the characterization method, whereas the use of different microscopy approaches permitted to evaluate fungal distribution within roots of orchid plants. In addition the TEM microscopy allowed to investigate structures of the hyphae found within plant tissues. This double approach allowed to confirm the results obtained by fungal DNA extraction, amplification and sequencing. We were also able to successfully culture some of the internal putative mycorrhizal symbionts that will allow testing their field-inoculation to plan novel fitness-enancing strategies for endangered orchids.

Materials and Methods I

Plant collection

Individuals of three wild orchid species, *Orchis purpurea* (Huds), *Orchis militaris* (L.), *Spiranthes spiralis* (L.) were collected at their flowering stage in the Euganean Hills area, North-Eastern Italy. In particular plants of *Orchis purpurea* species were collected in May 2007, *Orchis militaris* specimens were collected in mid May 2007 and all individuals of the threatened species *Spiranthes spiralis* were collected in September or October of two different years. All individuals were excavated with a clod of their surrounding soil and transferred to the laboratory. Root apparati were carefully cleaned from soil under running water and were sectioned to investigate the presence of fungal endophytes, in the entire root system. From each section we collected material for microscopy and molecular analysis. In addition, from *Spiranthes spiralis*, plant material was collected also for the isolation of fungal endophytes in plant culture.

Isolation of fungal endophytes

Fungal isolation was performed from surface sterilized roots. Sections of roots were immersed in 95% ethanol for 20 seconds followed by a treatment in 5% sodium hypochlorite for 3 minutes and finally washed 7 times with sterile distilled water. External portions of sections were excised under sterile conditions and each section was transferred into Petri dishes containing potato dextrose agar (PDA: potato starch 4g/l, dextrose 20 g/l, agar 15 g/l) or malt agar (MA: malt extract 30 g/l, agar 15 g/l). Petri dishes were incubated in the dark at 20°C to allow the development of hyphae.

Morphological observations

Sections of roots for fluorescence and confocal microscopy were stained in acridine orange 0.05% (Senthilkumar *et al*. 2000) for 10 min, rinsed with distilled water, and

placed on microscopy slides. These were visualized both under epifluorescence and confocal microscopes. For light and electron microscopy small pieces of roots or cultured mycelia were excised and fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9). Samples were post-fixed in 1% osmium tetroxide, dehydrated in a gradient series of ethanol and propylene oxide, and embedded in Epon-Araldite. Ultra-thin sections were observed, after uranyl acetate and lead citrate stainings, with a Hitachi H 300 EM operating at 75 kV. Mycelia grown in PDA or MA were stained with 0.005% DAPI solutions and observed under a fluorescence microscope.

DNA extraction and PCR amplification of ribosomal intergenic spacer

Root portions or mycelia were transferred into 1.5 ml polypropilene conical tubes, 50 µl of sterile TE buffer (Tris·HCl 10 mM, EDTA 1mM, pH 8) were added to the tubes. Samples were mashed by means of flame-sterilized forceps tips. Tubes were subsequently incubated for 30 sec in a microwave oven set at 700W. The procedure was repeated twice. Samples were allowed to cool at room temperature for 5 min and centrifuged for 10 sec to pellet plant debris. One µl of supernatant was withdrawn and used as template for the PCR reaction using the primer sets ITS1F-ITS4 or ITS1-ITS4 (ITS1: 5'TCCGTAGGTGAACCTGCGG3', ITS1 -F: 5'CTTGGTCATTTAGAGGAAGTAA3', ITS4: 5'TCCTCCGCTTATTGATATGC3') (White *et al*. 1990). One µl of the lysate containing the total DNA was treated in a PCR BioRad I-Cycler using primers at 1 µM each in a 25µl reaction volume, and adopting the following program: initial denaturation at 95 °C for 2 min; 30 cycles at 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 2.5 U Taq DNA Polymerase, recombinant (InVitrogen Life Technologies). Amplification products

were visualized by loading 5 µl from the PCR reaction on a 1.5% agarose gel in 0.5 x TBE buffer (Maniatis *et al*. 1982), run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital. In cases where multiple bands were observed each was separated by cutting the gel slice containing the band over the transilluminator and purifying DNA by means of a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified material was used as template for a further round of PCR amplification using the same condition and band purity was verified by electrophoresis as described above. In alternative to gel slicing and Qiaquick purification, the DNA band in the gel was simply touched by penetrating the agarose with a sterile plastic pipet tip to be subsequently dipped into the PCR reaction mix. Amplicons were digested with *Hinf*I restriction enzyme.

Ampicon Cloning

The purified ITS fragments were either sequenced directly or cloned in pGEM-T vectors (Promega), *E. coli* JM109 electrocompetent cells were transformed. After transformation some colonies were transferred in LB plate and bacterial cells lysed with 50 µl of lysis solution (0,25% SDS, 0,05M NaOH) at 95°Cfor 15 min. Plasmid inserts were amplified using the primers GEM-T1 (5'GCAGGCGGCCGCACTAGTGAT3') and GEM-T2 (5'CCGCCATGGCCGCGGGAT3').

DNA Sequencing

One µl of the solution resulting from the above described PCR amplification was mixed in 0.2 ml polypropylene tubes with one µl containing 6.4 picomoles of the forward primer used and dried by incubating the open tubes for 15 min at 65 °C in an I-Cycler thermal cycler. For the reverse stand a parallel reaction was performed separately using the reverse primer. 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 analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Tewantin Australia). Merging of the complementary strands was achieved by Lasergene software v.7.2 (DNASTAR Inc. Madison WI) and the similarities to database records were investigated online through the NCBI platform (http://www.ncbi.nlm.nih.gov) using the BLAST utility.

Results and Discussion I

Isolation of fungal endophytes from *Spiranthes spiralis*

Fungi that developed from the inner core of the plant roots were isolated. Fungal cultures grown in MA or PDA presented different morphology and different growth rates. It was important to observe that in most cases fungi isolated from tissue blocks demonstrate a rapid growth, while only in few cases there was a slower growth that can be consistent with fungi of mycorrhizal nature (Fig I 1).

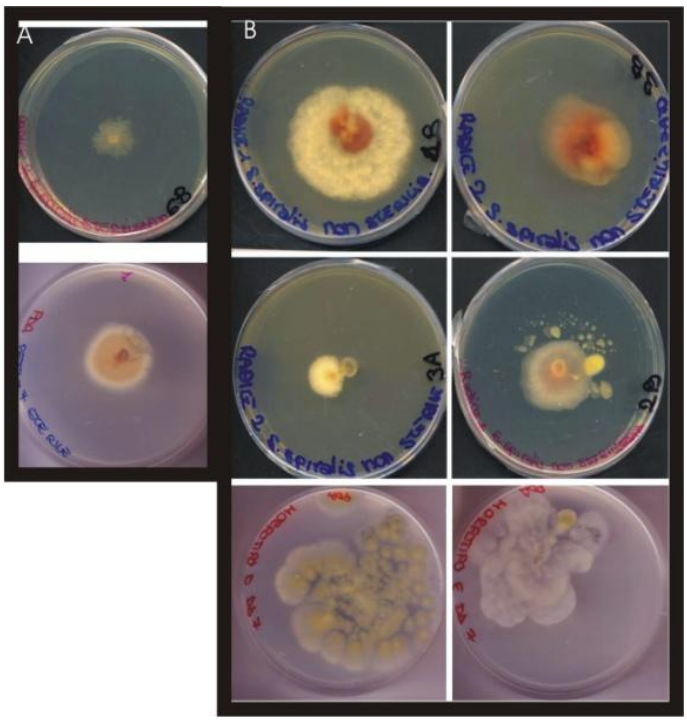

Fig.I 1: Colony morphology of some isolated fungi. Fungi with slower growth (A), and rapid growth (B)

Microscopy

To investigate the features of plant-fungus association we used different microscopy approaches. *Orchis purpurea* and *Orchis militaris* plants were investigated using fluorescence and bright field microscopy, while for *Spiranthes spiralis* we added also stereo and confocal microscopy. The investigation with stereo microscope was used to observe the anatomy of *Spiranthes spiralis* roots. Small underground tubers of *Spiranthes spiralis* plants appeared to be colonized by different kinds of fungal hyphae, with very diverse sizes (Fig.I 2 A). Images (Fig.I 2 B,C) show that both thick and thin hyphae seemed to enter into *Spiranthes spiralis* roots by structures that look like appressoria.

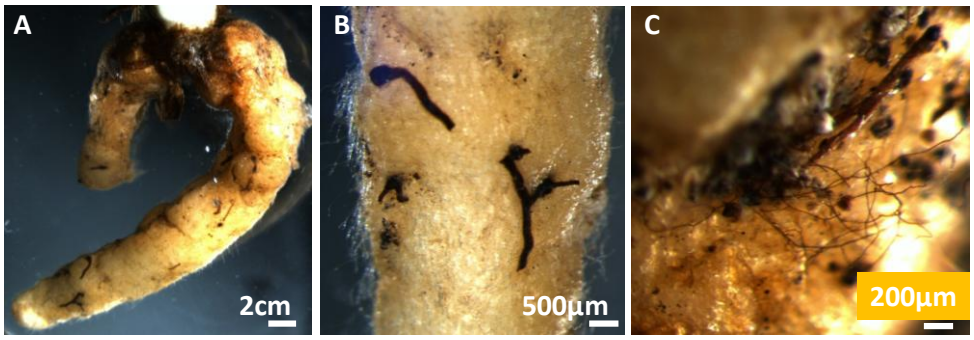

Fig.I 2: Underground tuber of *Spiranthes spiralis* colonized by fungal hyphae with different size (A); thick hyphae (B); thin hyphae (C)

Fluorescence microscopy performed on all species clearly showed evidence of internal root colonization (Fig.I 3 A, B, C). Blue light excitation of acridine orange-incubated sections revealed that the filling material stains accordingly, further supporting its fungal nature. Growth of fungus is restricted to cortical cells (Fig. I 3 B, C). Characteristic coils, called peletons were visible in the cells and in some cases transcellular hyphae were also observable (Fig.I 3 D, E). Confocal laser microscopy, performed on *Spiranthes spiralis* samples, helped to appreciate details of mycelial glomerular structures coiling inside cells, and hyphae passing through plants cells walls (Fig.I 3 F). Thin sections of root apparati, collected from *Orchis militaris* and

Spiranthes spiralis plants, observed with light microscopy confirmed an intracellular colonization (Fig.I 4 G, H, I, L). In the outer cortical cells under the root epidermis, hyphae occupied all the cell and were unclumped, freely running in all directions (Fig. I 4 I, L). Tranverse sections allowed to appreciate the occurrence of hyphae in the intercellular spacer between outer cortical cell (Fig.I 4 M). In the inner cortical cells, glomerular structures likely made of clumped thin hyphae were usually visible in the center of the cell. In most cases pelotons seemed to be digested by the orchid as described by Watermann and Bidartondo (2008) (Fig. I 4 H, I). Electron microscopy for *Orchis militaris* and *Spiranthes spiralis* was used to better investigate plant-endophytes interactions and fungal endophytes characteristics. This investigation confirmed the presence of fungal hyphae both inside plant cells (Fig.I 4 N) and in the intracellular spaces (Fig.I 4 O). In particular in the case of *Orchis militaris* we observed the presence in the same sample of hyphae having different sizes, shapes and cellular contents. Fungal cells appeared rich in electron transparent lipid bodies and they were surrounded by the host cell membrane (Fig.I 4 N). Pelotons of thin hyphae were well visible whit electron microscopy, they were surrounded by the host cell membrane, and often positioned near nuclei of plant cells (Fig.I 4 P, Q, R). In isolated hyphae it was possible to observe the presence of septa (Fig.I 4 N), while no septa types were recognizable in hyphae forming pelotons. *O. militaris* fungal cells were rich in electron-transparent lipid bodies, but other more electron-dense material was also visible (Fig.I 5 A, B). Hyphae appeared to be septate and constantly surrounded by the host membrane as usual in biotrophic interactions. The fungal septa both in *Orchis militaris* and *Spiranthes spiralis* samples were of basidiomycete type, they revealed the typical dolipore structure with entire parenthesomes and cell wall bulges at the edge of the pore (Fig.I 5 B,C,D). DAPI (4',6-diamidino-2-phenylindole) stain of the mycelium isolated from *Spiranthes spiralis* showed the presence of two distinct nuclei inside some fungal cells (Fig.I 5 E,F). In mycology this stage is known as di-karyon which normally follows plasmogamy and precedes meiosis.

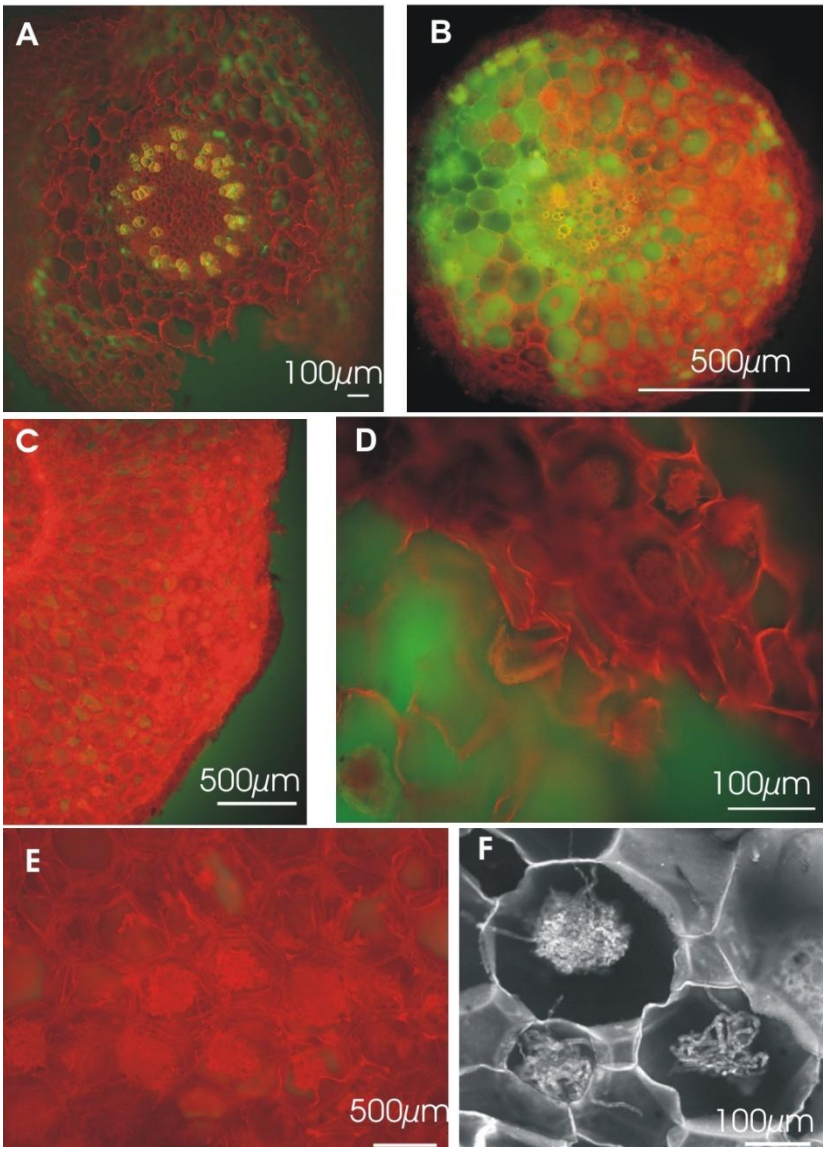

Fig.I 3: Root cross sections of *Orchis purpurea* (A), *Orchis militaris* (B) and *Spiranthes spiralis* (C). Fungal peloton staingin orange-red are visible in the cortex cells. Details of glomerular structure of the hyphae coils are shown in (D, E, F). Confocal microscopy on free hand sections of Spiranthes spiralis roots show intracellular colonization and fungal hyphae penetrating in cell walls of root cells (F).

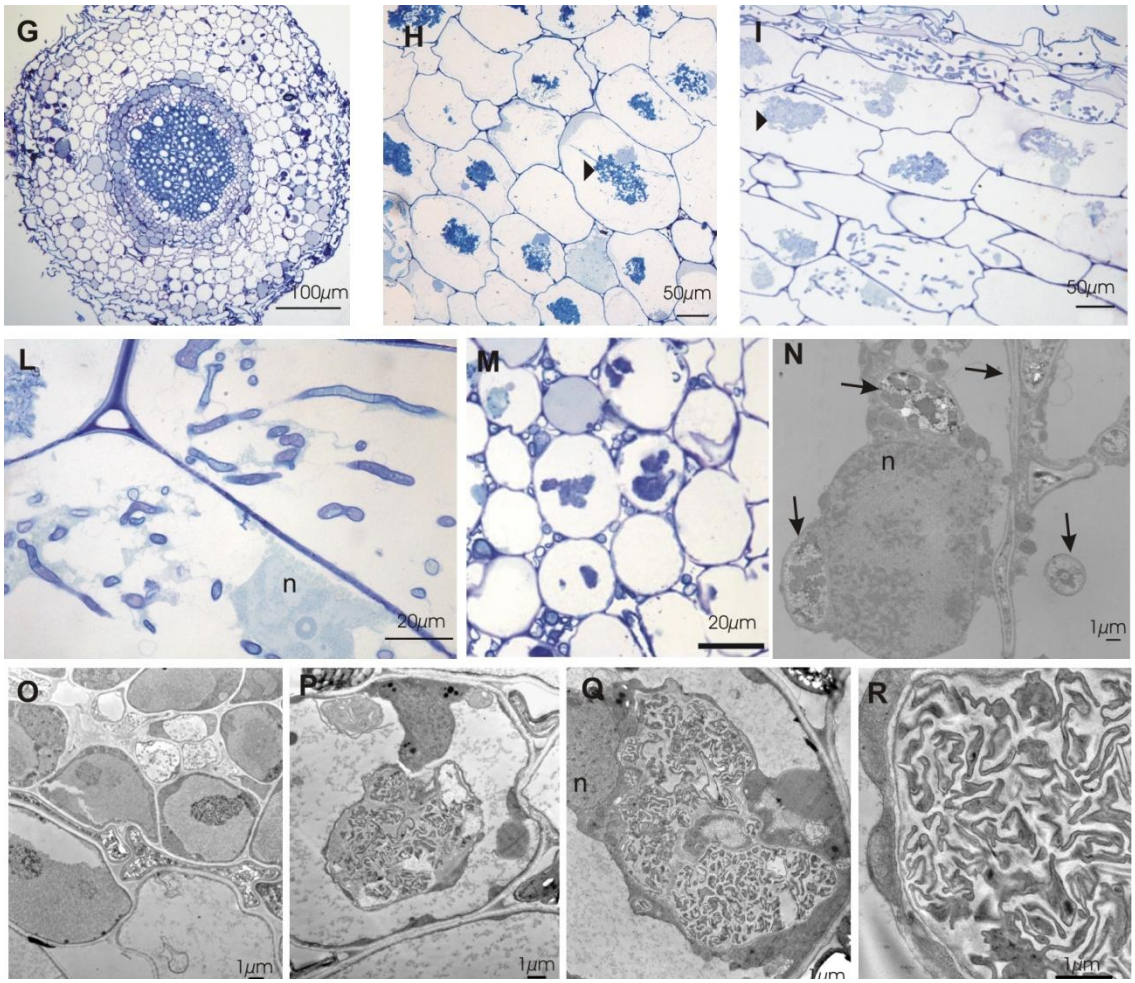

Fig.I 4: Light and electron microscopy on sections of *Orchis militaris* and *Spiranthes spiralis* roots. showing fungal colonization. Cross section showing cortical colonization (G); longitudinal section showing hyphae and glomerular structure in the inner and outer cortical cells (H,I). Outer cortical cells filled with unclumped hyphae (L); cross-sections of a root showing hyphae in the intercellular spaces between outer cortical cells (M); Transmission electron micrographs showing plant-endophytes interactions and fungal endophytes characteristics (N-R); hyphae of different size and shape inside host cells and in an intercellular space (N,O). Fungal peloton surrounded by the host cell membrane (P, Q, R).

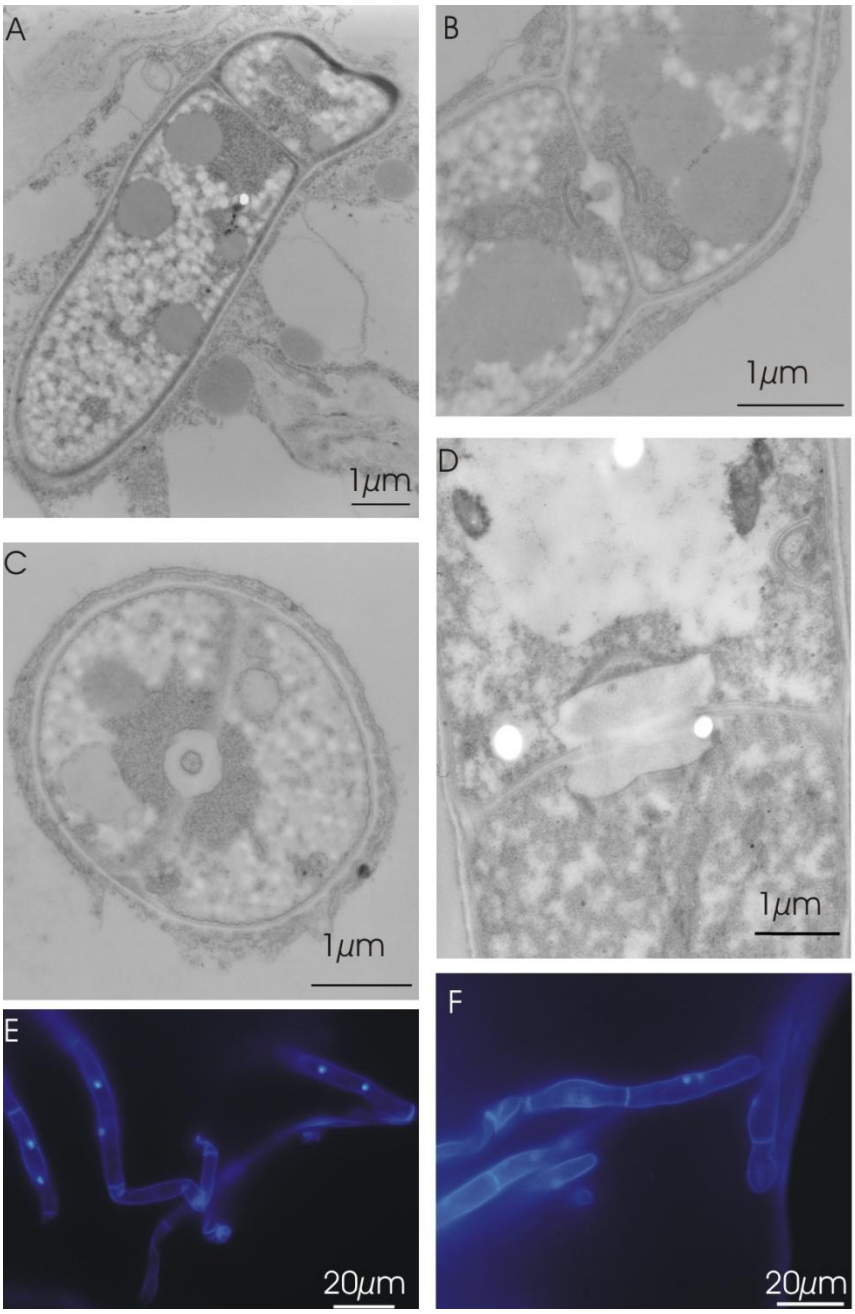

Fig.I 5: Anatomy and cytology of fungal hyphae found in *Orchis militaris* (A,B,C) and *Spiranthes spiralis* (D,E,F) . Transmission electron micrographs showing hyphae rich in lipid bodies into the cytoplasm (A, B, C, D); longitudinal and frontal section of dolipore septa with parenthesomes. DAPI-stained nuclei in one cell, two nuclei per cell (E, F).

Molecular analysis

The same number of specimens used for microscopy was subjected to molecular analysis. Fungal genomic DNA was extracted from root tissues and from fungal cultures. Selective PCR amplification using ITS1-ITS4, or ITS1F-ITS4 primers was carried out. The ITS region is easy to amplify because of its high copy number, in addition relatively few primers sets are needed due to the highly conserved SSU and LSU flanking regions (White *et al*. 1990). This region vary relatively little within species but dramatically between species. Amplification of the ITS region gave rise to amplicon electrophoretic profiles displaying bands of variable intensity running at position corresponding to 800-600 bp., as shown in fig.I 6. The primer pair ITS1F-ITS4 usually gave better results, with bands of higher intensity. The standard set of primers ITS1-ITS4 used by most labs to identify fungal isolates gave us less consistent amplification for all investigated species (Fig.I 6). The amplicons obtained were digested to identify different profiles. Representative cases were sequenced and results were examined by BLAST in the NCBI platform. Where mixed sequences were obtained PCR products were cloned in *E.coli* using pGEM-T vectors (Promega), following manufactures' instruction. Colonies were directly amplified using GEM-T1 and GEM-T2 primers, sequenced and also in this case results were examined with BLAST in the NCBI platform.

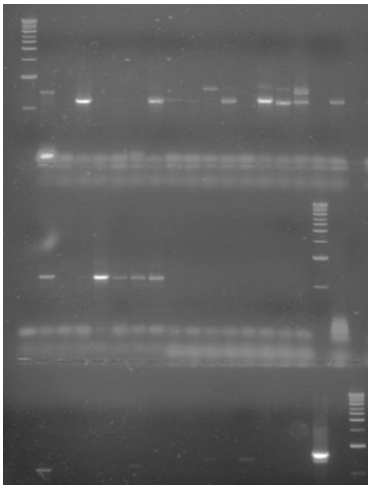

Fig.I 6: example of electrophoretogram with positive and negative cases

Amplicons from *O. purpurea* and *O. militaris* were obtained only from some of the tissues examinated as summarized in tab.I 1. For *O. purpurea* one band was found with high degrees of homology with *Davidiella tassiana* (GenBank code EF589865), a known endophytic species of fungus. Regarding *Orchis militaris* two bands identified as 4b1 and 4b2 were obtained. Band 4b1 (800 bp) (Genbank code EU490419) shared 99 % similarity with an uncultured fungus ascribed to the family Tulasnellaceae (Basidiomycota) found in roots of the same species *O. militaris* in Estonia (EU195344, Shefferson *et al*. 2008). The same level of similarity was shared with sequences from a series of uncultured fungi from the roots of *O. militaris* in Hungary (AM711604-AM711613, unpublished). Concerning band 4b2 (600 bp, Genbank code EU490420) we recorded a 99% identity with an uncultured ectomycorrhizal *Tetracladium* (mitosporic Ascomycota) found within *Salix herbacea* roots on a glacier of the Austrian alps (EU326166) and 98 % identity with an uncultured ectomycorrhizal ascomycete found in truffle grounds (AJ879646) (Vendramin et al., 2009).

Tab.I 1: Results of the ITS sequencing of *O. purpurea* and *O. militaris* endophitic fungi. The top-scoring similarities identified upon the bioinformatical BLAST analysis are reported along with their database record description and the percent homology with our query.

Orchis purpurea		
Op1	99%	*Davidiella tassiana* EF589865 Hidden diversity of endophytic fungi in an invasive plant
Orchis militaris		
4b1	100%	uncultured mycorrhizal fungus AM711604-AM711613 Orchid mycorrhizal fungal diversity of *Orchis militaris* habitats Unpublished
	99%	Uncultured Tulasnellaceae EU195344 Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills
4b2	98%	Uncultured Tetracladium EU326166 Mycobionts of Salix herbacea on a glacier forefront in the Austrian Alps.
	99%	Uncultured rhizosphere ascomycete AJ879646 Morphological and molecular typing of the below-ground fungal community in a natural *Tuber magnatum* truffle-ground

A high number of amplicons were obtained from *Spiranthes spiralis* samples, possibly due to more dense endophytic colonization but also to our higher sampling effort. From this species we also had the possibility to amplify DNA extracted from its cultured isolated fungi. BLAST results are summarized in tab.I 2 A and B. Different fungi have been found apparently associated with the same orchid plant, some with high degrees of homology with *Davidiella tassiana, Leptospheria sp., Alternaria tenuissima, Malasseziales*; while other fungal ITS regions showed of homology with *Ceratobasidium sp*. and *Rhizoctonia sp*. It is also possible to see that from plants of both the first and the second sampling we obtained amplicons with similarity around 90% with *Rhizoctonia sp*. (AJ318420-AJ318431) described as

mycorrhizal Rhizoctonia isolated from orchids in Singapore. Other amplicons gave similarity with *Vouchered mycorrhizae* (DQ028790) that is a mycorrhizal species found in Australian orchids. In *Spiranthes spiralis* plants in particular we could observe that in addition to fungi of ascertained mycorrhizal nature, an additional series of endophytic fungi, which are an important component of fungal biodiversity, was found.

Tab.I 2A: *Spiranthes spiralis* ITS sequncing result

***Spiranthes spiralis* 2006**		
SsE	100% 98%	*Leptosphaeria* sp. 3813 FN394721 Molecular diversity of fungal communities in agricultural soils and grassland from lower Austria Unpublished Uncultured *Leptosphaeriaceae* AJ879672 Morphological and molecular typing of the below-ground fungal community in a natural Tuber magnatum truffle-ground
SsF	99%	*Malassezia restricta* EU400587 Phylogenetic relationships of *Puccinia horiana* and other rust pathogens of *Chrysanthemum x morifolium* based on rDNA ITS sequence analysis
SsH	100% 100%	Uncultured *Davidiella* GU055694 Molecular diversity of fungal communities in agricultural soils and grassland from lower Austria Unpublished *Davidiella tassiana* anamorph: *Cladosporium herbarum* EU622923
SsI	87% 86%	*Ceratobasidium cornigerum* EU273525 *Rhizoctonia* sp. AJ318420 Molecular Phylogeny of Mycorrrhizal Rhizoctonia isolated from orchids in Singapore

Tab.I 3B: *Spiranthes spiralis* ITS sequncing result

***Spiranthes spiralis* 2007**		
Ss1	99%	*Alternaria sp*. FJ037742 Phylogenetic analysis of ITS DNA sequences of endophytic fungi in mangrove in China
Ss4	86%	*Monilina azalea* AB182266
6BITs	94%	*Vouchered mycorrhizae* (Ceratobasidium) DQ028790 Diversity of Mycorrhizal Fungi of Pterostylidinae (Orchidaceae) in Australia Unpublished
Fungo 2B	100%	*Fusarium oxysporum* strain EU429440 First report of *Fusarium oxysporum* on leek in Italy Uncultured fungus FN397369 As dominant ectomycorrhizal fungus, *Tuber melanosporum* affects fungal dynamics in truffle-grounds Unpublished
Fungo 3Achiaro	100%	Uncultured fungus FN391308 As dominant ectomycorrhizal fungus, *Tuber melanosporum* affects fungal dynamics in truffle-grounds Unpublished
Fungo 3Ascuro	100%	*Alternaria sp*. GQ169459
Fungo 3B rosa	98%	Uncultured fungus FN39736 As dominant ectomycorrhizal fungus, *Tuber melanosporum* affects fungal dynamics in truffle-grounds Unpublished
3Bgiallo	99%	*Fusarium solani* EU263916
Clone A	89%	Uncultured ectomycorrhiza (Ceratobasidiaceae) AY634129 Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees
Clone B	90%	*Rhizoctonia. sp.* AJ318431 Molecular Phylogeny of Mycorrrhizal Rhizoctonia isolated from orchids in Singapore

Molecular identification of fungi belonging to the Basidiomycota linked with microscopy observations, that revealed the presence of typical dolipore structure in fungal hyphea found inside root cells of *Orchis militaris* and *Spiranthes spiralis*, allowed us to investigate their putative endophytic fungi with mycorrhizal nature.

Conclusions I

Upon analyzing root samples from three species of wild orchid plants, different species of fungi have been found to live in internal association. In term of orchid-fungus relationships, apart from the bona fide mycorrhizal partner other root-associated non-mycorrhizal endophytes were recorded. Different taxa were found in the three diverse plant species.

Considering *Orchis purpurea* the only amplicon obtained showed homology with *Davidiella tassiana* a known endophytic fungus. This single amplification could be explained considering that isolation success in many orchid species varies with season and prior disturbance, and in some epiphytic orchids has been shown to decline within hours of collection of the sample (Suarez *et al*., 2006).

In the soldier orchid *Orchis militaris* one of the two amplicons obtained revealed homology with a fungus belonging to the Basidiomycota, found within roots of the same species of orchid but collected in Estonia. The presence of fungal hyphae compatible with Basidimycota inside plant root tissues was confirmed by microscopy observations, that allowed to visualize typical dolipore septa, characteristic of these fungi. The second amplicon revealed homology with an uncultured ectomycorrhizal ascomycete found in truffle grounds, but in this case no clear microscopical evidence of hyphae with Ascomycota characteristics in plant tissues could be obtained. However the presence of two or more fungi in association with the same plant is not unexpected, based on knowledge of endophytes in other plants. Their presence does not necessary prove that both play a trophic role of mycorrhizal nature, however it is not unusual to find diverse non-mycorrhizal fungal endophytes inhabiting the same orchid plant. A large amount of fungal endophytes was already found not only in terrestrial but also in epiphytic and lithophytic orchid roots (Bayman and Otero 2006).

A more diverse endophytic colonization was inferred in *Spiranthes spiralis* plants due to the high number of amplicons obtained from these samples. Amplicons

yielded similarity with fungi belonging both to the Basidiomycota and Ascomycota, and also for this orchid microscopy observations confirmed the presence of dolipore septa in fungal hyphae present inside plant cells or obtained in pure mycelia cultured from surface-sterilized root sections.

Therefore the results obtained, showed the presence of very diverse fungi related with at least two of the orchid species that we analyzed. These endophytes may have different effects on the host and enhance its survival (Yuan *et al.*, 2009).

Protection of orchid populations and orchid-associated fungi is important in maintaining global biodiversity. As photosynthetic orchids pass photosynthate back to their fungal partners, orchids and their associated fungi are contributors to the common mycelial network that appears to be a key to the integrity of terrestrial ecosystems (Sellosse *et al*., 2006). In order to plan actions for the conservation of this kind of endangered plant species, an integrated approach can be recommended. The ecological analysis can benefit from the microbial ecology-based perspective in trying to individuate possible limiting resources of biotic nature. The possibility that more than one fungal endophyte be required to fully sustain plant development, could provide novel insights in interpreting its environmental outcome. Future work will be devoted to assess which fungal symbionts do associate with other orchids of different abundance and status in the same area. In addition fungi in pure colture obtained from *Spiranthe spiralis* roots could be used for studies of germination in vitro of the same plant species or other orchid species to investigate the specificity of this particular interactions. In addition, the isolation and cultivation of the fungal endophytes will provide the possibility of in-situ land inoculation with the mycorrhizal symbionts. This practice will be tested in the pursuit of enhancing survival and fitness of these endangered orchid species.

Chapter II

Bacterial Endophytes

Summary and scope II

Studies on beneficial microbes interacting with plants and a comprehension of their functions within their hosts are important to address the ecological relevance of bacterial endophytes. Previous works (Benhizia *et al*., 2004, Muresu *et al*., 2008) demonstrated by molecular and microscopy-based approaches the coexistence of rhizobia with diverse endophytic bacteria taxa within root nodules of wild legume plants. In the present project the first aim was to explore plant-endophyte relationships by introducing different fluorescent proteins as markers for the different bacteria. To obtain marked derivatives, with a stable integration of the genes encoding for fluorescent proteins in the chromosome, delivery plasmids for a mini-Tn5 trasposon were used. One of the plasmids was purposely engineered to obtain a delivery system for the *rfp* gene. *Trifolium repens* was chosen as host for the first investigations and nodulation test were performed using two different techniques. This plant is easily cultivable in the laboratory under microbiologically-controlled conditions.

Tagged strains of endophytic origin, after clover, were tested also on Mediterranean wild legumes germinated from seeds collected in nature. The goal was to assess whether these species would be less selective than temperate agricultural crops and whether non-rhizobial endophytes would be more proficient to enter within their tissues.

During this project we also aimed at enhancing the culturability of rhizobia rescued from nodules of wild legume plants, upon addition of compounds protecting from reactive oxygen damage. This allowed to investigate the possibility that the non culturability of rhizobia observed in previous works, was due to damage caused by ROS. Subsequently rhizobia, isolated from two species of wild legume plants (*Hedysarum spinosissimum* and *Tetragonolobus purpureus*) and the tagged

endophytic strains obtained in the first part of the work were tested for their resistance to different concentrations of hydrogen peroxide.

Materials and methods II

Tagging bacterial strains

Bacterial strains and growth conditions

Wild type bacterial strains used in this study are listed in tab.II 1. *Rhizobium leguminosarum* bv. *trifolii* E11 was used as clover symbiont species and grown at 30°C in TY (5 g/l tryptone, 3 g/l yeast extract, 0.7 g/l $CaCl_2$, pH 6.8) or BIII media (0.23 g/l K_2HPO_4, 0.10 g/l $MgSO_4$ $7H_2O$, 1 ml/l oligo-elements solution, 1 ml/l vitamine solution, pH 6.8) (Dazzo, 1984). As bacteria prone to become endophytic we used some isolates from root nodules of Mediteranean wild legume species *Hedysarum spinossisimum* (Benhizia *et al*., 2004); *Pseudomonas* sp. Hs1 was grown at 30°C in King's B (20 g/l proteose peptone, 1.5 g/l ,$MgSO_4$ $7H_2O$ 1.5 g/l K_2HPO_4, 10 ml/l glycerol, pH 7.2) or NB media (0.3 g/l beef extract, 0.5 peptone, pH 7), *Enterobacter agglomerans* Hs6 was grown at 37°C in LB (10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl, pH 7).

Tab.II 1: Wild type bacterial strains used in this study

Strain	Reference
Rizobium leguminosarum bv. *trifolii* E11	(Yanni *et al.* 2001)
Pseudomonas sp. Hs1	(Benhizia *et al.* 2004)
Enterobacter agglomerans Hs6	(Benhizia *et al.* 2004)

E. coli strains containing different plasmids were grown at 37°C in LB supplemented with appropriate antibiotics (kanamycin 30µg/ml, ampicilin 50 µg/ml and streptomycin 50 µg/ml). In particular *E. coli* S17.1λpir was chosen as donor strains

for conjugations while *E. coli* cc118λpir and *E. coli* JM109 were chosen to host plasmids carrying *gfp* and *rfp* genes. Plasmids used in this study are listed in tab.II 2.

Tab.II 2: Plasmids used in this study

Plasmids	Relevant characteristic	Reference
pUTgfp 2X	pUTmini-Tn5::gfp	(Tombolini *et al.* 1997)
pRL76511p	pRL765::gfp	(Tombolini *et al.* 1997)
pRL765rfp	pRL765::rfp	This work

Construction of pRL756rfp delivery plasmid

Amplification of the red fluorescent protein gene

To express the *rfp* gene in the broad-host range vector its coding sequence was amplified from pRLT2-RFP plasmid (kind gift of Alex Costa, UniPD) by PCR using BD Advantage^{TM}2 PCR Kit (Clontech). Primers used were synthesized from SIGMA, and designed starting from m*rfp* gene (GenBank accession number EF362408, 651-1328) (Bischof *et al*., 2007). The forward primer correspond to 5'ACC CAT ATG AGG TCT TCC AAG AAT 3', the reverse one has the following sequence 5'GGC GGA TCC CTA AAG GAA CA 3' (melting temperature: forward: 64,5°C reverse: 67,0°C). *Nde*I and *Bam*HI sites were created respectively at the 5' and 3'ends of the amplicon. A Gradient PCR was performed (in thermal cycler *Eppendorf ep gradient s* using the following conditions: denaturation 95°C, 30 sec; annealing from 53.1°C to 60°C, 30 sec; elongation 68°C, 60 sec). PCR fragments were analysed by 1,5% agarose gel electrophoresis. To permit the following legation reaction amplicons were digested with *Nde*I and *Bam*HI restriction enzymes.

Plasmid DNA extraction, digestion and ligation

pRL76511p plasmid DNA was extracted from *E. coli* S17.1λpir using an Invitrogen plasmid extraction kit (Purelink™ Quick Plasmid Miniprep) following manual instructions. The gene *P11 gfp* was excised as *Nde*I-*Bam*HI fragment from pRL76511p plasmid, and fragments were analyzed by 1,2% agarose gel electrophoresis. PCR products after purification with QIAquick PCR Purification Kit (QIAgen), was ligated using T4 DNA ligase (Promega) into the digested pRL765 plasmid, under the control of the constitutive promoter psbA. The resulting plasmid was transformed into *E. coli* JM109 used as temporary host for cloning and subsequently in *E. coli* S17.1λpir, which is a replicon-mobilizer strain. Transformants showing a fluorescent phenotype were selected in LB media supplemented with kanamycin 30µg/ml.

Tagging of bacterial endophytic strains with fluorescent proteins

The insertion, into wild type bacterial strains, of genes encoding the fluorescent proteins was performed using bacterial transformation or conjugation as indicated below.

Tagging of *Rhizobium leguminosarum* E11 by biparental mating

Rhizobium leguminosarum E11 strain was marked either with green or red fluorescent proteins. pUTgfp2X or pRL765rfp plasmids were introduced by biparental mating using as donor strain *E. coli* S17.1λpir. An overnight culture of donor and a one day colture of the recipient were mixed in a 1:3 ratio. The conjugation mix was incubated for 48h on LB agar plates, then different dilution of the mix were spread on BIII + 10% sucrose, supplemented with kanamycin 60 µg/ml. Colonies showing green or red fluorescent phenotype were selected for the study.

Tagging of *Pseudomonas* sp. Hs1 by biparental mating

Pseudomonas sp. Hs1 resistant to chloramphenicol 15µg/ml originally isolated from internal tissues of a wild legume plant from Algeria (Benhizia *et al.* 2004) was chosen as recipient. The plasmid pUTgfp2X was introduced by biparental mating using as donor strain *E. coli* S17.1λpir. Overnight cultures of donor and recipient were mixed. The conjugation mix was incubated over night on LB agar plates and different dilutions of the mix were spread on King's B agar plates, containing cloramphenicol 15 µg/ml and kanamycin 30 µg/ml. Transformants showing green fluorescence under UV illumination were selected.

Electroporation of *Enterobacter agglomerans*

Enterobacter agglomerans strain Hs6 was tagged with the red fluorescent protein marker. Bacteria from the exponential growth phase cultures in test tubes were transferred on ice for 30min, washed four times with ice-cold sterile distilled water and resuspended at a concentration of 10^{10} cells ml^{-1} in 10% glycerol. Plasmid DNA (1 µl or 2 µl at a concentration of 0.5 µg/µl) was added to 80 µl of competent cells in a 2 mm electroporation cuvette. After 15 min of incubation on ice, the DNA was electroporated into the cells using an Bio-Rad Gene Pulser apparatus equipped with a pulse controller set a 2.5 kV; 25µF and 200Ω. Different volumes of culture (50-100µl) were plated on selective medium (LB containing kanamycin, 30 µg/ml).

The stability and the level of fluorescent protein expression was monitored by streaking out to single colony on appropriate agar plates without selective antibiotics for four successive times. Colonies and the cells were observed for their fluorescent phenotype under the fluorescence microscope LEICA DM500 equipped with filter for GFP.

Detection of fluorescent colonies in plant roots

To determine whether gfp-tagged bacteria could be properly detectable on plant roots a cell suspension was applied to sterile roots of *Trifolium repens*. Roots were

immersed in 95% ethanol for 20 seconds followed by 5% sodium hypochlorite for 3 minutes and finally washed 7 times with sterile distilled water. *Pseudomonas sp.* Hs1::gfp colonies were scraped off a King's B agar plate and resuspended in distilled water to a concentration of approximately 10^6 cell ml^{-1}; the bacterial suspension was applied on surface-sterilized clover roots. After 1h the roots were rinsed with distilled water and examined using Fluorescence and Laser Scanning Confocal Microscopes. The fluorescence microscope used to visualized the root surface was a Leica equipped with a mercury lamp and a 488nm filter. The Confocal Microscope was a Biorad equipped with two laser He/Ne and Ar.

The spatial relationships between rhizobia and non-rhizobial endophytes during colonization and infection of white clover were examined, and two procedures to perform nodulation test were used.

Trifolium repens nodulation tests

Nodulation tests in tubes

Seeds of white clover (*Trifolim repens*) were surface-sterilized by immersion in 70% ethanol for 30 sec, followed by stirring in 0.1% $HgCl_2$ for 7 min and rinsing in 7 changes of sterile deionized water. Seeds were pre-imbibed for 3 hours in the final wash. Germination and concomitant verification of surface sterility were obtained by spreading seeds on PCA plates wetted with 10 drops of sterile water. Plates were incubated inverted for three days in the dark. The primary root of clover seedling grew in the humid air space within the plate. Two germinated seeds were transferred aseptically to sterilized plastic tubes containing modified N-free Fahraeus agar medium (Dazzo 1982) (0,8% w/v agar). Each seedling was inoculated with 100µl of a suspension containing both the simbiont *Rhizobium leguminosarum* bv. *trifolii* and one of the two different fluorescent protein-tagged endophytes. Tubes were covered with black paper to shade roots, and were incubated in a growth chamber (Angelantoni, Sas. Massa Martana, Italy) programmed for a 16 h daylight at 23 °C, night temperature of 18 °C and 60% constant relative humidity.

Nodulation tests in plates

Trifolim repens seeds after surface sterilization performed as described above were germinated on N-free Fahraeus agar plates. Three seedlings were transferred in sterile conditions and laid flat on the surface of one new N-free Fahräeus agar plate. Roots were spot-inoculated with 20µl of inoculum suspension, containing both *Rhizobium leguminosarum* bv. *trifolii* and one the two types of endophytes all tagged with fluorescent proteins. A circular cover slip (12 mm diameter) was placed over each seedling root (Fig.II 1). Plates were incubated vertically in a growth chamber at the following conditions: 16/8h light/dark photoperiod and 22°/18°C thermoperiod, 70% relative humidity, for at least 14 days.

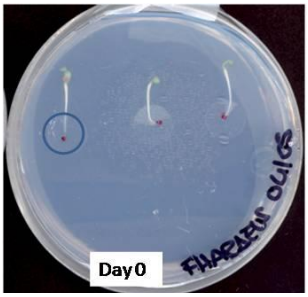

Fig.II 1:white clover seedlings on a Petri dish of N-free plant medium.

At various times, plants both from tubes and from plates were uprooted and examined by laser scanning confocal microscopy to follow the formation of infection thread at different time.

Bacterial inocula

To prepare the inoculum suspensions an overnight-grown liquid culture in BIII + 10% sucrose medium (approximately 10^6 cells/ml) of the simbiont *R.leguminosarum* and an overnight-grown liquid culture of each of the endophytic strains in the appropriate medium (approximately 10^6 cells/ml) were centrifugated and mixed in sterile Fahräeus medium. The correct volumes of these suspension were used as inoculum for each nodulation test (100µl of suspension in tube and 20 µl in plate). Different mixtures of bacterial strains were used to evaluate all the different possibilities of interaction between plant and endophytes.

Tab. II 3: inocula tested on white clover.

Test	Inoculum composition
Control	*R.l. bv trifolii* E11::gfp
Control	*R.l. bv trifolii* E11::rfp
Control	*Pseudomonas sp.*Hs1::gfp
Control	*E.agglomerans* Hs6 pRL765rfp
A	*R.l. bv trifolii* E11wt mixed with *Pseudomonas sp.*Hs1::gfp
B	*R.l. bv trifolii* E11wt mixed with *E.agglomerans* Hs6 pRL765rfp
C	*R.l. bv trifolii* E11::gfp mixed with *E.agglomerans* Hs6 pRL765rfp

Tab.II 4 Composition of modified Fahraeus solution. (components in g/l unless specified) (Dazzo,1982).

$CaCl_2$	0,1
$MgSO_4$ 7H2O	0,12
KH_2PO_4	0,10
$NaHPO_4$ 7H2O	0,15
Ferrous citrate	0,005
Trace element solution	0,1ml
Trace element stock solution contains: $MnCl_2$,$CuSO_4$,$ZnSo_4$,H_3BO_3 and Na_2MoO_4 all at 1g/l distilled water	

Confocal Laser Scanning Microscopy

At different time intervals, plants were uprooted from tubes or Petri dishes, gently washed with sterile modified Fahraeus solution; roots were cut into pieces in some cases containing also nodules and mounted on slide with water or sterile Fahräeus solution. Optical sections of roots were observed in a Biorad confocal microscope (University of Padova, Padova, Italy) or with a Zeiss LSM5 PASCAL confocal microscope (Michigan State University, East Lansing, Michigan, USA). Both confocal microscopes were equipped with two lasers He/Ne and Ar. 20X, 40X or 60X objectives were used for imaging. Usually, dual color images were acquired by sequentially scanning with optimal setting for GFP (excitation with the 488 nm Argon laser line, detection of emitted light between 505 and 530 nm) and mRFP (excitation with the 543 nm He/Ne laser line, detection of emitted light between 560 and 600 nm). Images were acquired as confocal z-series calibrating the number and the thickness of optical sections on each observed sample. In the case of Zeiss LSM5 PASCAL the projections of individual colour channels were merged with Zeiss LSM Data server software. For the Biorad confocal microscope Imaging software was used.

Wild legume plants

Bacterial isolation and culture conditions

Two species of wild legumes plants, *Hedysarum spinosissimum* and *Tetragonolobus purpureus*, were chosen as samples to bacterial isolation, in order to investigate the presence of non-culturable rhizobia within nodules. 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 seconds followed by a treatment with 5% sodium hypochlorite for 3 minutes; and finally washed 7 times with sterile distilled water. All treatments were performed under microbiologically controlled conditions. Tests to validate surface sterilization of plant tissues were performed by touching them several times on the surface of Plate Count Agar (PCA, Difco) plates prior to isolation of the interior microbiota. In tests aimed at increasing the fraction of culturable bacteria that could be isolated upon counteracting reactive oxygen species (ROS), a pool of surface-sterilized root nodules were transferred into empty sterile plastic tubes and squashed in 500 µl of PBS or 500 µl of PBS suplemented with ROS scavengers including: an antioxidant mix composed by Glutathione, Sodium Ascorbate and EDTA (1mM each) or an enzyme mix containing Catalase (20000 units/ml), Peroxidase (448 units/ml), and Superoxide dismutase (378 units/ml). The squashed nodules suspensions were streaked on yeast–mannitol agar Congo-red (YMA CR) plates. In alternative, nodules were squashed in plain PBS but plated on YMA plates whose top was previously spread with 100 µl of a stock of the antioxidant mix with each of the three components at 200 mM, or with 100 µl of the enzyme mix. Plates were incubated at room temperature for a period of over 10 days. Appearing colonies were counted daily and the different morphological types were characterized by DNA ARDRA fingerprints and sequencing.

DNA extraction from colonies

Bacterial cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 µl of lysis buffer (0.25% SDS, 0.05M NaOH) in an eppendorf tube, followed by stirring for 60 sec on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min and 10 µl of the supernatant were mixed with 90 µl of sterile water. Lysates were stored at 4 °C prior to PCR.

16S rDNA amplification and ARDRA analysis

One µl of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the two 16S rDNA-targeted universal bacterial primers 63F (5'CAGGCCTAACACATGCAAGTC) (Marchesi *et al.* 1998) and 1389R (5'ACGGGCGGTGTGTACAAG) (Osborn *et al*. 2000) at 1 µM each in a 25µl reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 sec, 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 $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 2.5 U Taq DNA Polymerase, recombinant (InVitrogen Life Technologies). Amplicons were digested overnight at 37 ° C upon mixing 5 µl from the 25 µl reaction volume with 1 µl of *Hinf6*I enzyme (Pharmacia, Uppsala, Sweden) and 2 µl of 10 x reaction buffer. The volume was brought to 20 µl with sterile distilled water. 5 µl of 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 by a Kodak DC290 digital camera.

DNA Sequencing

One µl of the amplicon resulting from the above described PCR amplification was mixed with 1 µl containing 6.4 picomoles of the above described forward primer 63F in a 0.2 ml polypropylene tube and then dried by incubating the tube open for 15 min at 65 °C in an ICycler thermal cycler. The template and primer mix was directly used

for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/AppliedBiosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.33 software (Technelysium Pty Ltd, Tewantin Australia).

H_2O_2 tolerance tests of the isolates

Isolates from nodules of *Hedysarum spinosissimum*, and *Tetragonolobus purpureus*, whose 16S rRNA gene had high similarity to *Rhizobium* and *Mesorhizobium*, and endophytes tagged with fluorescent proteins in this work (*Pseudomonas* sp.::gfp, *Enterobacter agglomerans* pRL765rfp) and wild type, were tested for their resistance to increasing concentrations of H_2O_2 (0, 10 µM, 100µM, 1mM, 10mM). For each bacterial strain a pre-inoculum suspension was prepared by resuspending a loopful of plate-grown colony in 5 ml of the appropriate growth media. TY was used for the growth of the four isolates from nodules of *Hedysarum spinosissimum*, and *Tetragonolobus purpureus*; LB was used for *Enterobacter agglomerans* wild type, while LB containing kanamycin 30 µg/ml for *Enterobacter agglomerans* pRL765rfp; NB was used to grow *Pseudomonas* sp., and NB containing kanamycin, 30 µg/ml for *Pseudomonas* sp.::gfp. The pre-inoculum suspension was entirely added to 200 ml of the appropriate liquid media, and the OD_{600} was checked in a spectrophotometer to ensure starting the growth curve from OD_{600}=0.05-0.1. The liquid colture was divided in falcon tubes (20ml of colture for each tube). Starting from a stock of H_2O_2 9,9M the appropriate volumes were added to each tube to obtain the five different concentrations of hydrogen peroxyde. The OD_{600} of each colture was recorded for at least 40 hours

Plant cultivation and nodulation tests

Mature pods were collected from five species of wild plants in their natural habitat and kept dry at room temperature until used. Seeds were removed manually from pods, surface-sterilized by immersion in 70% ethanol for 30 sec followed by stirring

in 0.1% $HgCl_2$ for 7 min, and rinsed in 7 changes of sterile deionized water. Seeds were pre-imbibed for 3 hours in the final wash. Dormancy was broken dipping seeds on boiling water for 30 sec. (Herranz *et al*., 1998) Germination and concomitant verification of surface sterility were obtained by spreading seeds on PCA plates wetted with 10 drops of sterile water, and incubated inverted until germination in the dark. Germinated seedlings were transferred aseptically to sterilized plastic Leonard jars (Fig.II 2) containing a water-washed, oven-dried, quartziferous sand/vermiculite 1/3 mixture, fed from the bottom with nitrogen-free Fahräeus solution (Vincent, 1970). The rooting mixture was re-humidified with 1/10 vol of sterile nitrogen-free Fahräeus 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 1 ml suspension of an overnight-grown liquid culture (approximately 10^8 cells/ml) of both wild type rhizobia species and endophytes tagged with fluorescent proteins. 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.

For nodulation by natural symbionts, uninoculated seedlings were transplanted to jars containing field-collected soil instead of the sand-vermiculite mix.

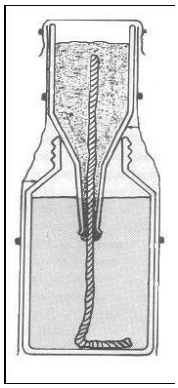

Fig.II 2:. Schematic representation of a Leonard jar system.

From these nodulation tests re-isolation of inoculated bacterial strains was attempted. Bacterial isolation was performed as described above starting from surface-sterilized root nodules, cylindrical portions (0.2 mm) of stems and portions of leaves. Each nodule and each portions of stems and leaves were transferred into empty sterile plastic tubes and squashed in 150 µl of NaCl. 30 µl of squashed suspensions were streaked on Petri plates. Growth media used for isolation were both YMA and also LB containing kanamycin 30 µg/ml and NB additionated with kanamycin 30 µg/ml and chloramphenicol 15 µg/ml to rescue possible co-inoculated endophytic *Pseudomonas* or *Enterobacter* tagged with plasmid-borne fluorescent protein markers. DNA was extracted from the colonies obtained and analyzed by ARDRA.

Results and Discussion II

Tagging bacterial strains

Plasmid manipulations

The pUTgfp2X plasmid (Fig.II 3) is a delivery system for a mini-Tn5 trasposon, expressing kanamycin resistance and GFP protein. This replicon acts as a suicide plasmid when transferred to bacteria lacking the λ pir system, as its origin of replication depends on the π protein that is produced in λ pir hosts. The transposable element is able to transpose from the delivery plasmid due to the trasposease activity encoded by *tnp* gene, which is present *in trans* outside the mini-Tn5 element (Elbeltagy *et al*. 2001). The Tn-5 transposon delivery system contains a PpsbA-RBS-gfp cassette. The *gfp* genes, plus the additional 35 bp region containing the RBS site are located downstream the constitutive psbA promoter. The GFP protein encoded by this plasmid is the red-shifted mutant P11 bearing an amino acid substitution at position 167; this leads to a shift of the maximum excitation wavelength of GFP from 396 to 471 nm without major changes in emission wavelength, 502 compared to 508 nm for wild-type GFP (Fig.II 6) (Heim *et al*., 1994)

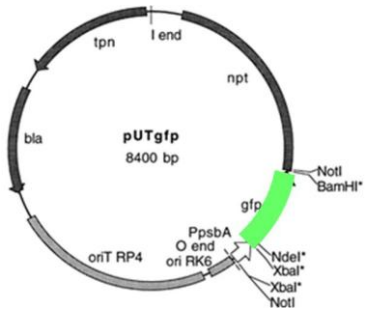

Fig.II 3: Structure of pUTgfp2x

The vector pRL76511p (Fig.II 4) is a delivery plasmid very similar to the pUTgfp ones, but *tnp* gene are present inside the mini-Tn5 element. (Tombolini *et al.* 1997). Both these plasmids were used successfully in other works to tag bacteria (Taghavi *et al*. 2009).

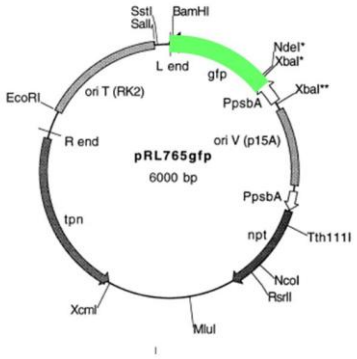

Fig.II 4: Structure of pRL76511p

In order to construct a rfp expression cassette that could be inserted in a variety of bacteria, we replaced the gene of the P11 GFP with the m*rfp* gene, which was derived from pRLT2-RFP plasmid (kind gift of Alex Costa, UniPD). The *gfp* gene was excised as *Nde*I-*Bam*HI fragment from pRL76511p plasmid. Three fragments were obtained with the restriction enzyme digestion (Figure II 5).

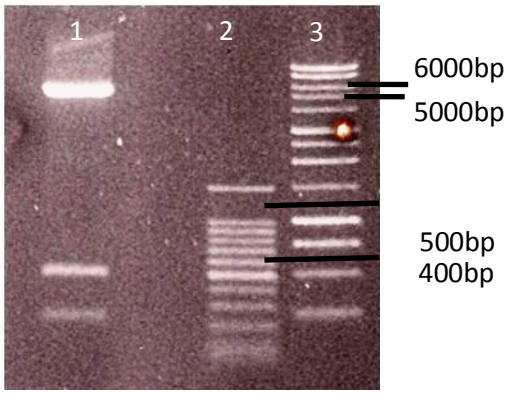

Fig.II 5: gel showing digested pRL76511p plasmid (lane 1), MW (lane 2 and 3)

In line 1 of the gel the first band is 5300bp long, the smaller two are 500bp and 200bp respectively, the sum of which gives an amplicon of 700bp corresponding to the *gfp* gene. The presence of two fragments is caused by the existence of a recognition site for *Nde*I (5' CA-TATG 3', 3'GTAT-AC 5') in position 255bp (Fig.II 6) within the *gfp* gene.

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5'Atg agt aaa gga gaa gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggt
  M   S  K    G   E   E   L   F   T   G   V   V    P  I   L   V   E   L   D   G
 Gat gtt aat ggg cac aaa ttt tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga
  D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D   A   T   Y   G
 Aaa ctt acc ctt aaa ttt att tgc act act gga aaa cta cct gtt cca tgg cca aca ctt
  K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P   W   P   T   L
 Gtc act act ttc tct tat ggt gtt caa tgc ttt tca aga tac cca gat cat atg aaa cag
  V   T   T   F   S   Y   G   V   Q   C   F   S   R   Y   P   D   H   M   K   Q
 Cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa aga act ata ttt ttc
  H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R   T   I   F   F
 Aaa gat gac ggg aac tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt
  K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G   D   T   L   V
 Aat aga atc gag tta aaa ggt att gat ttt aaa gaa gat gga aac att ctt gga cac aaa
  N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I   L   G   H   K
 Ttg gaa tac aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat gga
  L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D    K  Q   K   N   G
 Atc aaa gtt aac ttc aaa att aga cac aac att gaa gat gga agc gtt caa cta gca gac
  I   K   V   N   F   K   I   R   H   N   I   E   D   G   S   V   Q   L   A   D
 Cat tat caa caa aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac
  H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P   D   N   H   Y
 Ctg tcc aca caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt
  L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D   H   M   V   L
Ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta tac aaa taa 3'
 L   E   F   V    T   A   A   G   I   T   H   G   M   D   E   L   Y   K   -
```

Fig.II 6: P11 gfp gene sequence; Ile 167-Thr modification in green, recognition site for *Nde*I in red

The coding region of *rfp* was amplified from pRLT2-RFP plasmid by polymerase chain reaction. The BD Advantage 2 Polymerase Mix used for this PCR is comprised of BD TITANIUM™ Taq DNA Polymerase, a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus BD TaqStart™ Antibody to provide automatic hot-start PCR. Primers were designed upon the database available sequence of the *rfp* gene taking care to add *Nde*I and *BamH*I sites at the 5' and 3' respectively, and ensuring that there was sufficient length near the end of the primers for the enzyme to efficiently bind and cut (Fig.II 7).

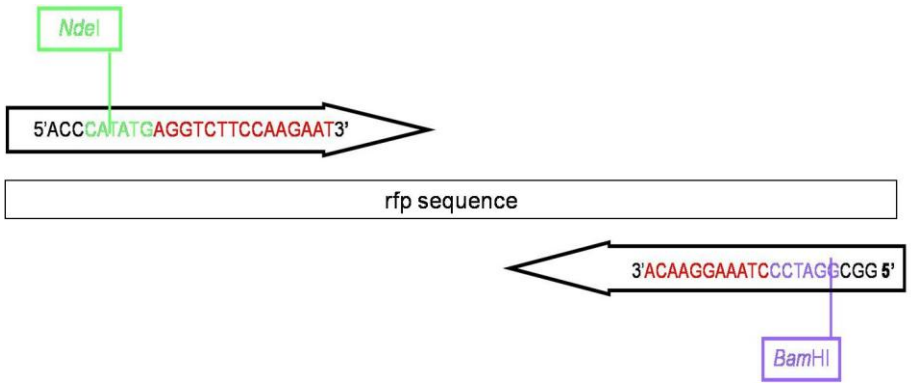

Fig.II 7: scheme of primers designed

As can be seen from the electrophoretic analysis on agarose gel of the PCR products, the expected band of 700bp was obtained (Fig.II 8) .

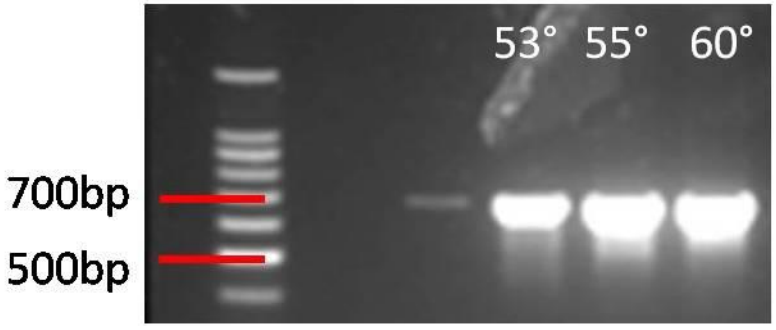

Fig.II 8: Electrophoretic analysis on agarose gel of PCR products showing amplicons corresponding to the expected size of the *rfp* gene obtained from all temperatures tested during gradient PCR.

All temperatures tested during the gradient PCR gave good results. We obtained amplicons with *Nde*I and *Bam*HI sites at the 5' and 3' respectively which could be conveniently cloned in the vector with ends obtained by digestion with the same restriction enzymes. In order to ligate the amplicons with the digested vector T4 DNA ligase (Promega) was used.

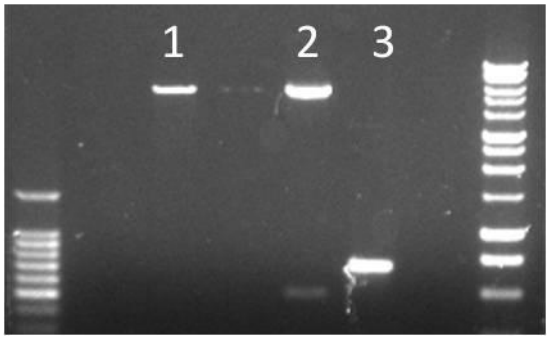

Fig.II 9: electrophoretogram showing (1) digest of the pRL76511p plasmid without *gfp* gene linearized and purified from a gel, (2) digested pRL76511p showing the gfp band, (3) the rfp band amplicon obtained.

Two different ligase reactions were performed, one using digested vector after extraction from a agarose gel (Fig.II 9, lane 1), the second one using just the whole digested vector excising the *gfp* gene to be replaced by the *rfp* amplicon (Fig.II 9, lane 2). Both ligation products were transformed into *E. coli* JM109 used as temporary host to be subsequently introduced into the mobilizer strain *E. coli* S17.1λpir. Only the electroporation from the second ligation reaction gave transformants. Reasons could be due to the limiting amount of vector DNA recovered from the gel. Transformants showing red fluorescent phenotype were grown on selective media. Colonies and bacterial cells were detected initially with fluorescence microscope using an 540nm excitation filter. Upon completion of this procedure plasmid pRL765rfp was therefore obtained, which is based on a mini-Tn5 trasposon and containing the PpsbA-RBS-rfp cassette, encoding a rfp gene with maximum excitation wavelength of 584 nm and emission wavelength of 607 nm (Campbell *et al.,* 2002). *E. coli* harbouring multiple copies of pRL765rfp plasmid expressed a

strong red fluorescence visible both in colonies and as single cell resolution (Fig.II 10). Interestingly old colonies of rfp-tagged bacteria acquired a red phenotype detecteble also under visible light (Fig.II 11).

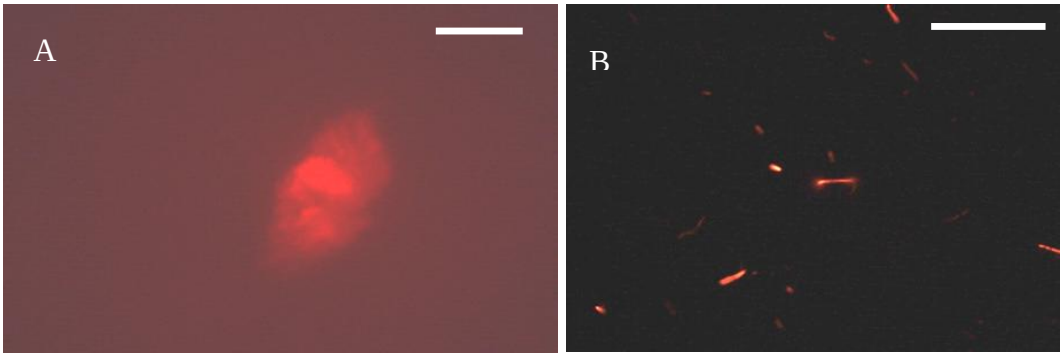

Fig.II 10: images show *E. coli* JM109 expressing the *rfp* reporter gene. (A) fluorescent microcolony and in (B) individual fluorescent cells are visible

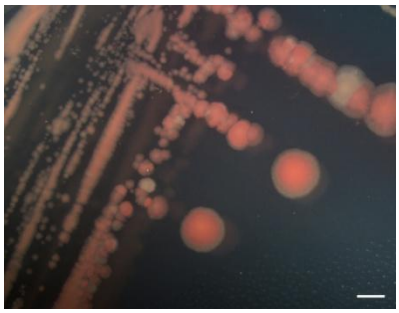

Fig.II 11: old colonies of rfp tagged *E.coli* JM109 showing a red phenotype visible also without the appropriate excitation.

Tagging of environmental bacterial strains with fluorescent proteins

The pUTgfp2X and the pRL765rfp plasmids were used to tag endophytic strains. The methods utilized to introduce constructs and the corresponding tagged bacteria obtained are summarized in tab.II 5.

Tab.II 5: tagged bacterial strains

Wild type strains	plasmids	Construct introduction method	Tagged strain designation
Rhizobium leguminosarum bv.trifolii E11	pUTgfp2x	Conjugation	E11:gfp
Rhizobium leguminosarum bv.trifolii E11	pRL765rfp	Conjugation	E11::rfp
Pseudomonas sp. Hs1	pUTgfp2X	Conjugation	Hs1::gfp
Enterobacter agglomerans Hs6	pRL765rfp	Transformation	Hs6 pRL765rfp

Both the pRL765rfp and pUT gfp2X vectors were used for incorporation of the *gfp* and *rfp* cassette into the chromosome of *R. leguminosarum* bv. *trifolii*. In both cases plasmids were introduced by biparental mating using as donor strain *E. Coli* S17.1λpir. Colonies with fluorescent phenotype (+) were selected on BIII with 10 % sucrose supplemented with kanamycin 60 µg/ml. Such defined medium (Dazzo, 1984) is supposedly more selective than other media for *Rhizobium* cultivation; while with the addition of sucrose we prevented the growth of donor *E coli* strains. *Pseudomonas* sp. Hs1::gfp from wild type *Pseudomonas* sp. was obtained introducing pUTgfp2X by biparental mating using as donor strain *E. coli* S17.1λpir. To tag *Enterobacter agglomerans* pRL765rfp was introduced by electroporation.

pUT gfp2X is a suicide plasmid, when transferred into bacterial strain enable to produce π protein for RK6 origin of replication the transposable element is able to transpose from the delivery plasmid. This ability is due to the trasposease activity encoded by *tnp* gene which is present in trans outside the mini-Tn5 element. The result is a stable gfp integration into the chromosome of the host endophytes *R. leguminosarum* bv. *trifolii* and *Pseudomonas* sp. The pRL756rfp delivery plasmid is similar to pUTgfp ones, but the *tnp* gene is contained inside the mini-Tn5 element, and its integration into the chromosome of *R. leguminosarum* bv. *trifolii* is prone to possible further transposition. Instead for *Enterobacter agglomerans,* which like *E. coli* is in the family Enterobacteriaceae, the vector persists as plasmid. Both the

pRL765rfp and pUT gfp2X delivery system gave successful incorporation of genes encoding for fluorescent proteins, into the chromosome of simbiont *R.leguminosarum* bv. *trifolii*, and *gfp* into *Pseudomonas* sp.

E. coli cells harboring multiple copies of *gfp* or *rfp* gene were found to express strong, green or red fluorescence clearly detectable by fluorescence or confocal microscopy (Fig.II 11B), while for E11::gfp, E11::rfp and Hs1::gfp fluorescence phenotypes observed were much weaker (Fig.II 12 A,B,C), however the cells were enough visible by fluorescence or confocal microscopy.

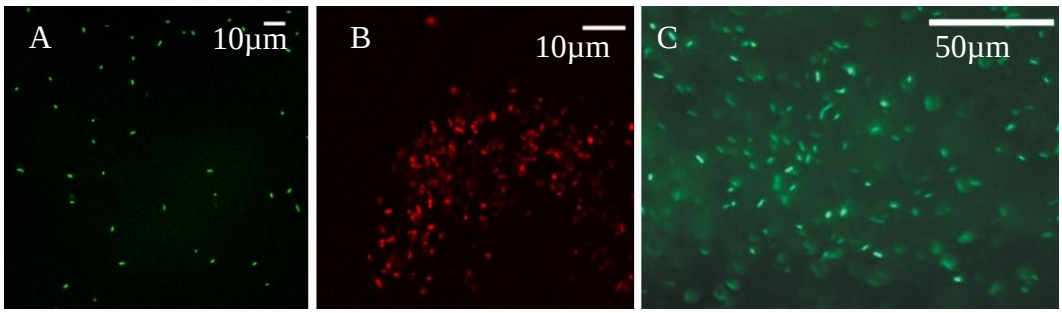

Fig.II 12: fluorescence or confocal microscopy images showing bacterial cells tagged with fluorescent proteins; E11::gfp (A), E11::rfp (B), Hs1::gfp (C).

Some preliminary tests were performed to determine if gfp tagged bacteria could be really seen on plant roots. A cell suspension containing *Pseudomonas sp.* Hs1::gfp was applied to sterile roots of *Trifolium repens*. As first trial root surface was examined under the fluorescence microscope. In figure II 13 a root hairs is visible A fluorescence, B phase-contrast and C the merge. Problems encountered during the observation, due to a strong auto-fluorescence of the plant tissues, are clearly visible, in particular in A the green fluorescence of root hair make impossible to observe gfp tagged bacteria, which are better visible in the space around the root in panel B. However we were able to localize gfp-tagged bacteria, if root hairs were very thin, like in figure II 13 D ,E ,F in which auto-fluorescence due to the plant tissues is caused by the thicker root (D). No bacterial cells were recorded directly on the root tip, where root-associated population tend to concentrate (Espinosa-Urgel 2004).

Assemblages of cell expressing GFP could be easily visualized on root surface of *Trifolium repens* in figure II 13 G (arrow). Extensive plant cellular debris caused by root cutting were shown in phase-contrast micrographs (Fig.II 13); their presence made difficult the visualization of bacterial cells. Considering all these problems we also tested the Laser Scanning Confocal Microscope equipped with two lasers, Ar and HeNe, to visualize plant tissues and bacteria in two different channels. Figure II 14 A and D show plant tissues, B and E show the presence of gfp-tagged bacteria near root hairs and C and F are a merge of the first and the second images in which false colors were given, green for gfp tagged bacteria and red for plant tissues. Figure II 14 shows just root hairs, while plant root would be visible in a deeper focal plan. The use of the Confocal Microscope and appropriate filters helped us to minimize the problem of plant auto-fluorescence, for these reasons we decided to use confocal techniques for our following observations.

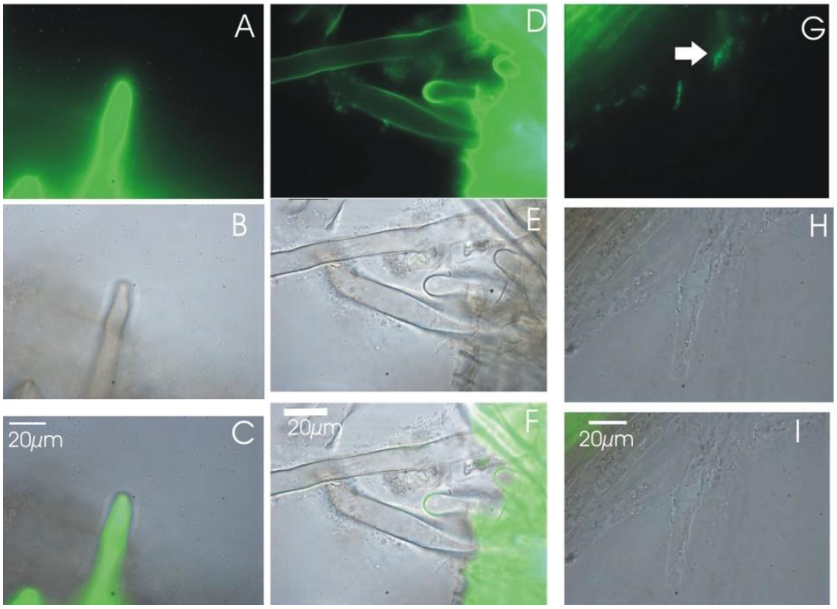

Fig.II 13: Use of GFP to detect *Pseudomonas sp*. Hs1::gfp in white clover roots with fluorescence microscopy. Fluorescent images (A,D,G), Phase-contrast image (B,E,H), and their merge (C,F,I). Auto fluorescence of the root is clearly visible in panel A and D, Fluorescent assembling of bacteria is observable near root hairs in G (arrow).

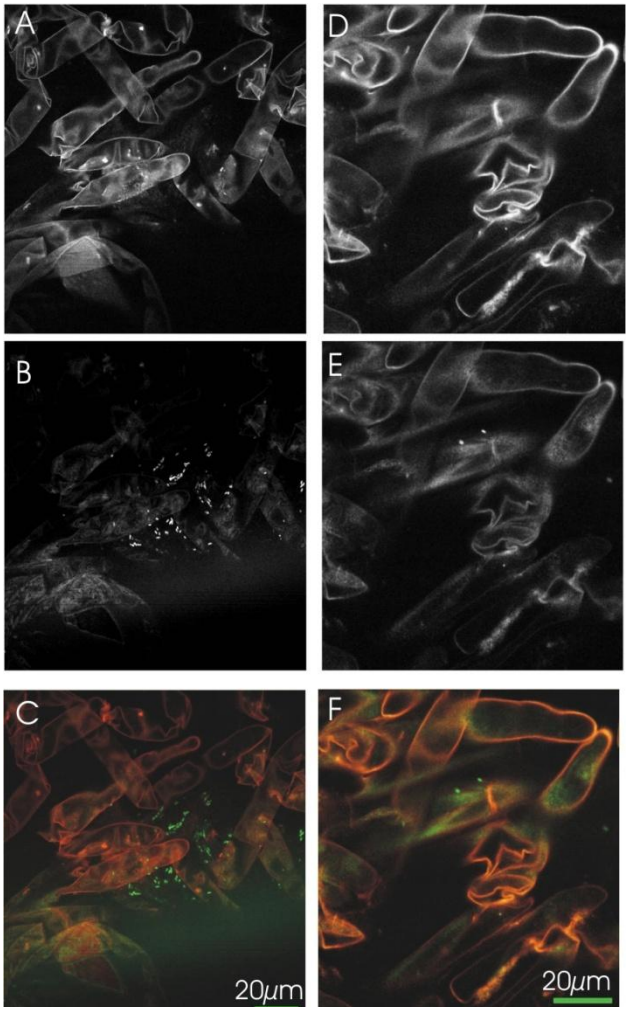

Fig.II 14: Confocal microscopy on white clover roots. With red channel plant tissues are visible A, D. GFP-tagged bacteria near root are observable with green channel B, E, in the merge both plant structures and bacterial cells are visible.

Trifolium repens nodulation tests

To investigate plant-endophyte spatial relationships, including the co-localization of endophytes (*Pseudomonas* sp., *Enterobacter agglomerans*) and rhizobia (*Rhizobium leguminosarum* bv. *trifolii*) within the same plant root, we constructed bacterial strains that constitutively expressed Fluorescent Proteins (FP) and used these to inoculate *T. repens* plants. In this project two procedures for nodulation tests were used, both involved aseptical germination and growth of seedlings in sterile conditions, followed by inoculation with a bacterial suspension containing both rhizobia and non-rhizobial strains. *T. repens* was chosen because is easily cultivable in Fahraeus agar medium, enabling to follow the different stages of the nodulation process under microbiologically-controlled conditions. Sterile plastic tubes were used for the first nodulation method; this allowed to follow the formation of nodules for at least 1 month. 100µl of inoculum suspension were dispensed exactly on each seedlings' root, which entered deeper in the N-free Fahraeus agar medium (0,8% w/v agar) during the nodulation process. In the second artificial method seedlings were laid flat in Petri plates, roots were spot-inoculated with 20µl of bacterial suspension and a round coverslip was placed over each inoculum point. The space, between the agar surface and the coverslip, should be filled with the suspension without trapping air bubbles that could affect the nodulation test. This second method enabled to follow the nodulation of *Trifolium repens* for no longer than 15 days, but knowing exactly the inoculation point we were able to find easily infection threads. In addition we could observe the development of root nodules day by day.

During all these trials we tested different mixtures of bacterial strains. First we used an inoculum containing *R.leguminosarum* E11 wild type mixed with one of the two non-rhizobial endophytes tagged with fluorescent proteins: E11 wt mixed with Hs6 pRL765rfp or E11wt mixed with Hs1::gfp (Fig.II 15, 16). We observed the presence of tagged endophytes on the surface of white clover roots, but we could not visualize formation and development of infection treads, since in this test the rhizobia responsible of this process were not tagged. We confirmed that *Enterobacter*

agglomerans Hs6 pRL765rfp, in which the vector for rfp gene persists as plasmid with a high copy number, maintained its fluorescent phenotype also in the absence of selection, as previously showed during its cultivation in LB agar plates without selective antibiotics. Figure II 15 shows an example of a nodulation trial inoculated with E11wt and Hs6 pRL765rfp, roots were observed with confocal microscopy 40 days after the inoculation. Images C and F come from the merge of the images obtained with the observation of the samples with the red channel (Fig.II 15 A, D) and in bright field (Fig.II 15 B, E). Red cells were visible around root hairs (Fig.II 15 C) and in the crevices between cells (Fig.II 15 F arrow), which is one of the points where normally root-associated population tend to concentrate (Espinosa-Urgel, 2004).

When we observed samples inoculated with a mixed bacterial population of E11wt and Hs1::gfp it became easy to visualize green bacterial cells, correspondent to Hs1::gfp cells. *Pseudomonas* sp was tagged using the pUTgfp2X delivery plasmid, that produces a stable integration into the chromosome of this strain of two copies of *gfp* gene. The presence of two genes encoding for GFP enhanced the fluorescent phenotype of the bacterial cells. Also in these tests we observed bacterial cells near root hairs (Fig.II 16 A), and the presence of micro-colonies attached to root hairs (Fig.II 16 C,D) and between root cell walls (Fig.II 16 B). An example in figure II 16 shows that we visualised Hs1::gfp cells associated with root hairs during the early stage of infection process, in particular during root hair deformation (Fig.II 16 E,F,G,H). Confocal microscope observation of GFP cells using excitation with the 488 nm Argon laser line, allowed us to visualize not only tagged endophytes but also plant tissues, however it was still possible to well distinguish bacterial cells. This double visualization was not an hindrance as it helped to better investigate the plant-endophyte spatial relationships using only one excitation setting.

Test tubes inoculated with E11::gfp mixed with Hs6 pRL765rfp gave us the possibility to investigate not only the plant-endophytes display, but also the interactions between rhizobia and non-rhizobial endophytes. The presence of both

green and red cells was well observable on the surface of roots (Fig.II 17 D), between root hairs (Fig.II 17 C) and on their tip (Fig.II 17 E). Micro-colonies formed with both E11::gfp and Hs6 pRL765rfp were visualized on root hairs tip (Fig.II 17 F,G,H). Upon observing nodules emerged from *T. repens* roots nine days after inoculation, we found large numbers of red bacterial cells covering their surfaces (Fig.II 17, I), as if bacteria were seeking a way of entry in plant tissues. Optical sections of the same nodules showed infection threads filled only with *R. leguminosarum* E11 gfp tagged, no red cells were visible in intracellular spaces or within infection threads (Fig.II 17, L).

When observing white clover plants grown in the test tube we were able to identify and to section with optical method only few infection threads. Figure II 18 shows examples of these results. Bacteria attached to the root hair tip induced the typical root hair deformation and a red cell is visible in the pocket of the curled root hair tip in figure II 18 A. Green fluorescent rhizobial cells were detected inside intracellular infection threads during primary infection of host root hairs, therefore we could easily follow their formation (Fig.II 18 B,C), the elongation through root hairs (Fig.II 18 F,G) and their progression in the root cortex (Fig.II 18 D,E). Just in few cases (Fig.II 18 A,D) we appreciated the presence of *E. agglomerans* Hs6 pRL765rfp in the upper part of infection threads in development.

Regarding the nodulation method performed in plates, very similar results were obtained. From the observation of root nodules emerged from *T. repens* roots, 14 days after inoculation with a bacterial suspension containing *R. leguminosarum* E11 gfp tagged and *E. agglomerans* Hs6 pRL765rfp, we could appreciate the presence of micro-colonies on the surface of nodules and also at the point of emergence of the nodule from root (Fig.II 19 A,B,C,D). From these tests we performed hand sections of the nodular structures, which allowed us a deeper observation of their internal features with confocal microscopy. Thick networks of intracellular infection threads were easily detected by virtue of the green fluorescent rhizobial cells present inside (Fig.II 19 E, F, G). Neither in these trials red fluorescent bacterial cells (Hs6

pRL765rfp) were found inside root nodule tissues. In seedlings grown in plates, vertically incubated, roots during elongation did not enter the agar medium, and knowing exactly the point of inoculation, it was simple to follow the formation and elongation of many infection threads (Fig.II 20 A, B, C, D and Fig.II 21 A, B,C). At least 15 infection threads were identified, in some cases we detected very long structures, which progressed very deep into the root (Fig.II 20 C,D), in other cases we appreciated the presence of two or more infection threads in the same portion of observed root as visible in the two optical sections of the same field (Fig.II 20 E,F). An interesting result is that *Enterobacter agglomerans* rfp-tagged cells were detected in early pre-penetration structures of the infection process, above all in the pocket of the overlap of the markedly curled deformation at the root hair tip, but not within intracellular infection threads (Fig.II 21 A,B,C).

It is important to observe that the gfp-tagged construct of *Rhizobium leguminosarum* bv. *trifolii* used in this study retained its infection and nodulation abilities notwithstanding the insertion of the miniTn5-Km-gfp. This is confirmed by control experiments in which plants inoculated only with *R. leguminosarum* E11 tagged with red or green protein, regularly developed nodules devoid of endophytes. Seedlings inoculated only with one of the two non-rhizobial species tagged with fluorescent protein did not develop nodules and showed presence of bacterial cells only on the surface of roots.

Both the two artificial techniques used to study the spatial relationships of rhizobia and non-rhizobial endophytes during colonization and infection of white clover, allowed us to visualized endophytes at single cell resolution on the surface and in the internal tissues of the plant.

It can be remarked that the two endophytes tested had originally been isolated from nodules of Mediterranean wild legumes within the genus *Hedysarum*. We hereby observe that these bacteria are apparently not prone to be internalized in *Trifolium repens*, a species from temperate regions which features a strict constraint in terms of host specificity. The two tagged strains tested although colonizing the external

portion of the roots seemed to be denied entry in white clover. This prompted a subsequent series of tests, described in the following part of the present work, in which we tested the hypothesis that Mediterranean wild legumes would be less selective than temperate agricultural crops and non-rhizobial endophytes would be more proficient to co-infect them along with rhizobia in mixed inoculation tests.

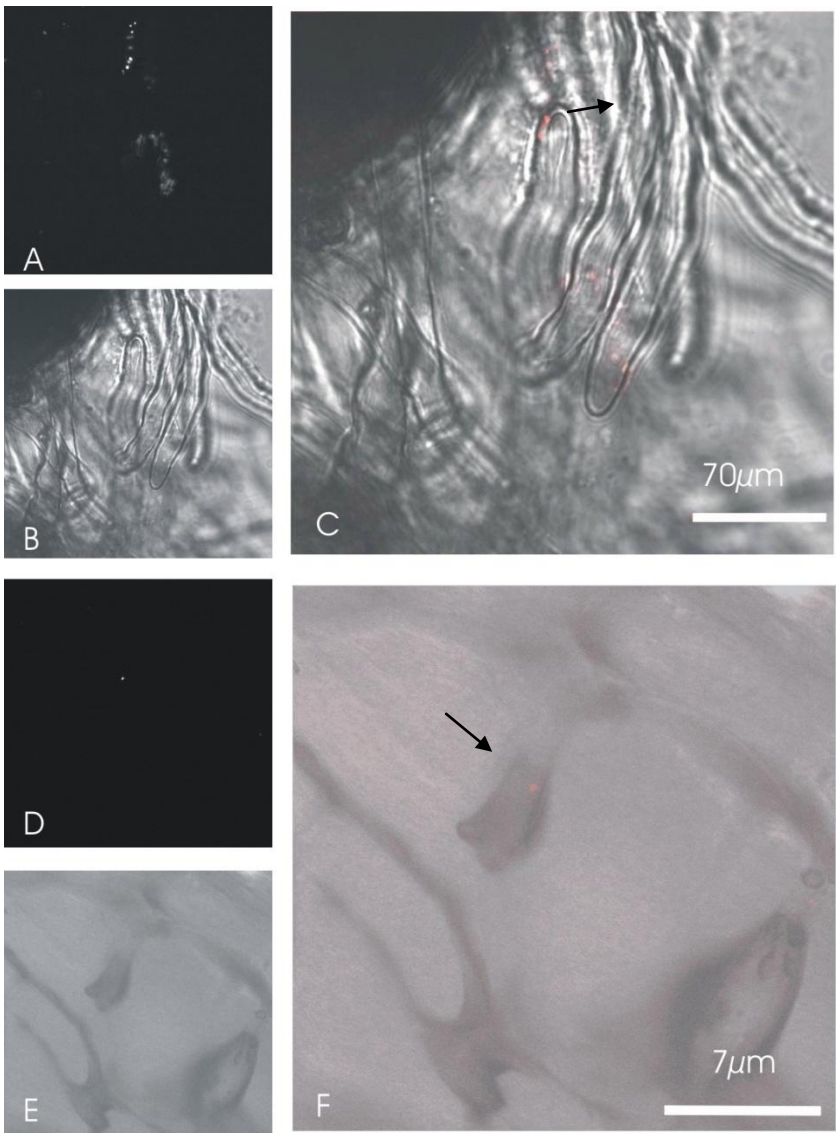

Fig.II 15: Visualization of *Enterobacter agglomerans* Hs6 pRL765rfp on roots of *Trifolium repens*. Nodulation test performed in tubes. (C) Confocal reconstruction of red cells near root hairs obtained by merging red channel (A) and bright-field image (B). (F) Image showing the presence of a red cell in the crevice between cells. (D) Red channel of F, (E) bright-field image.

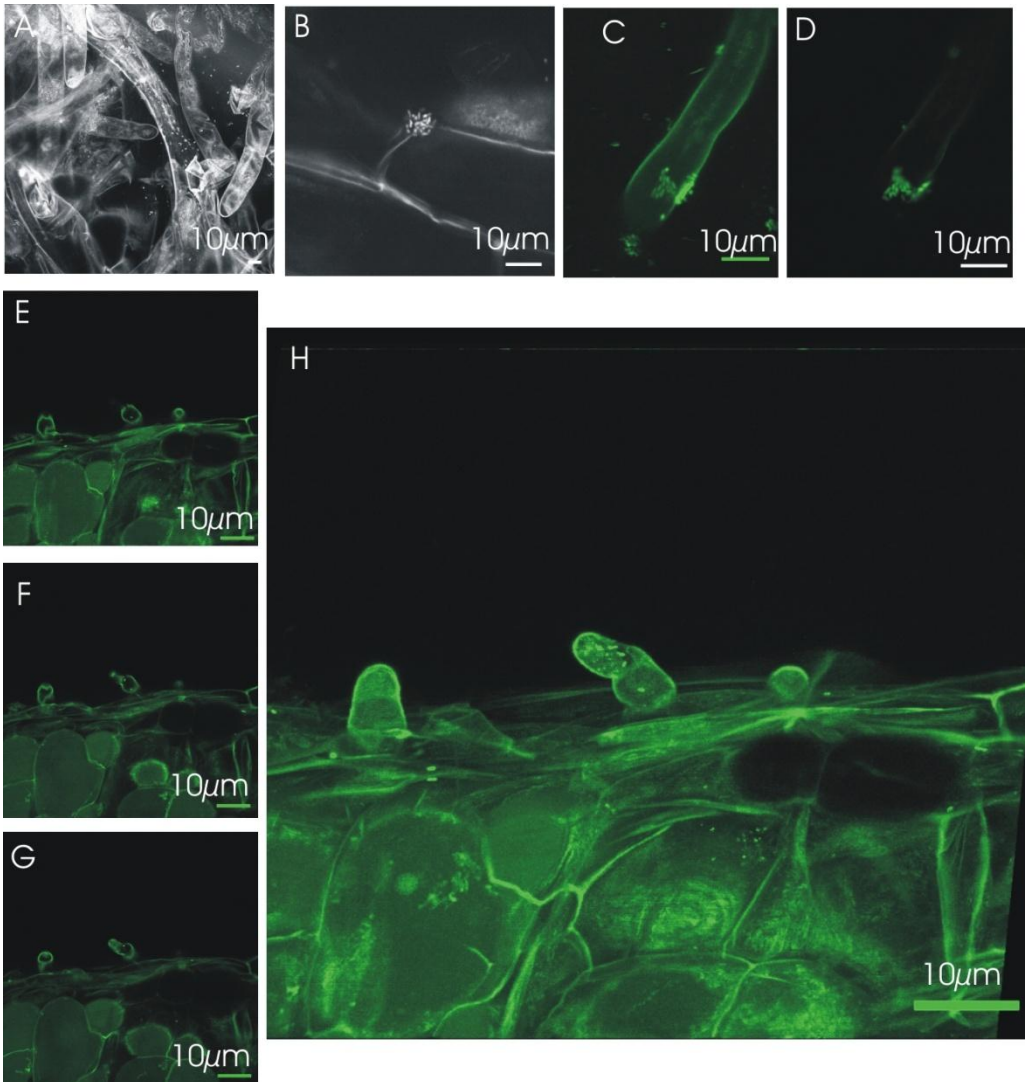

Fig.II 16:Confocal laser scanning micrographs of *Pseudomonas* sp. gfp tagged colonizing roots of white clover grown in tubes. (A) observation with the green channel of green bacterial cells between root hairs, plant tissues gave some autofluorescence. (B) presence of micro-colonies between root cell walls (C,D) Two optical sections of micro-colonies attached to root hairs. (E,F,G) Confocal optical sections of Hs1::gfp cells associated with root hairs during root hairs deformation. (H) Confocal reconstruction of optical sections E, F, G of a root hair deformation observed with green channel.

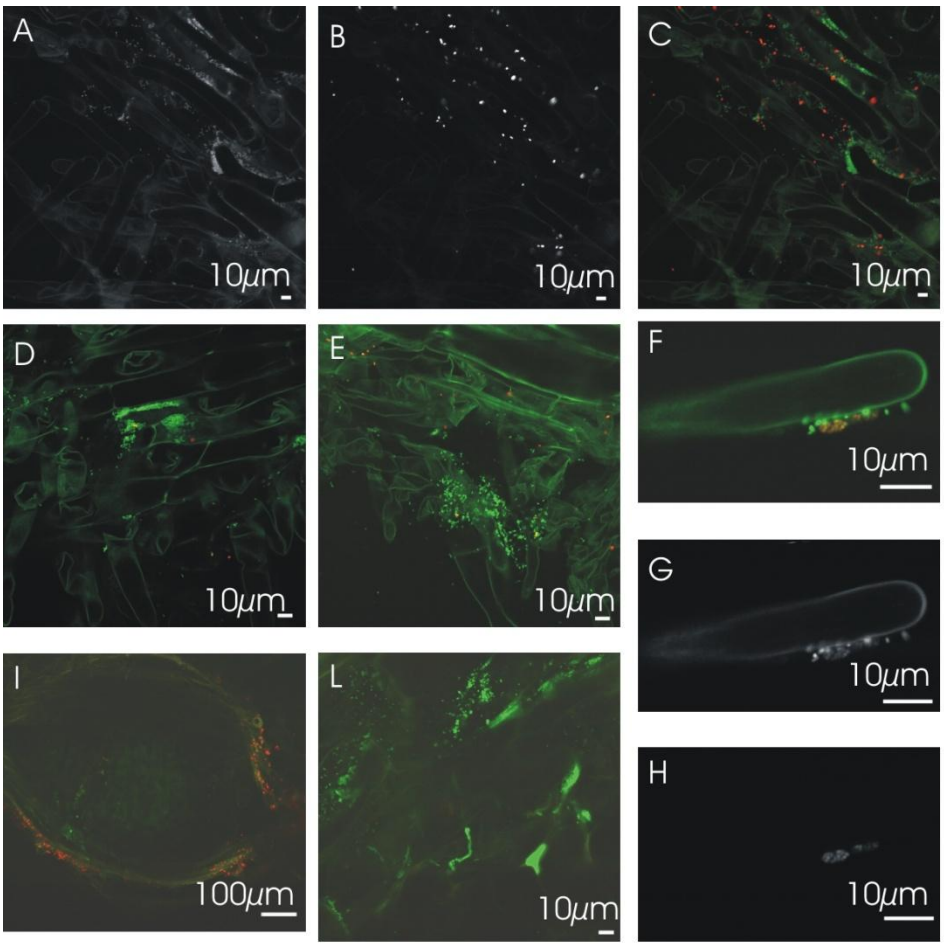

Fig.II 17: Confocal laser scanning micrographs of roots of white clover grown in tubes inoculated with E11::gfp mixed with Hs6 pRL765rfp. Presence of both green (A) and red cells (B) was well observable on surface of roots, A merging of green and red channels is visible in (C).Confocal reconstruction of mixed bacterial population between root hairs (D) and on their tips (E). Micro-colonies formed by both E11::gfp and Hs6 pRL765rfp were visualized on merged image of root hair tip (F), acquisition in green channel of image F is visible in G while the red one is visible in H. Nodule emerged from *T. repens* roots nine days after inoculation covered by many red bacterial cell (I). Optical section on nodule showing infection threads filled only with *R. leguminosarum* E11 gfp-tagged (L).

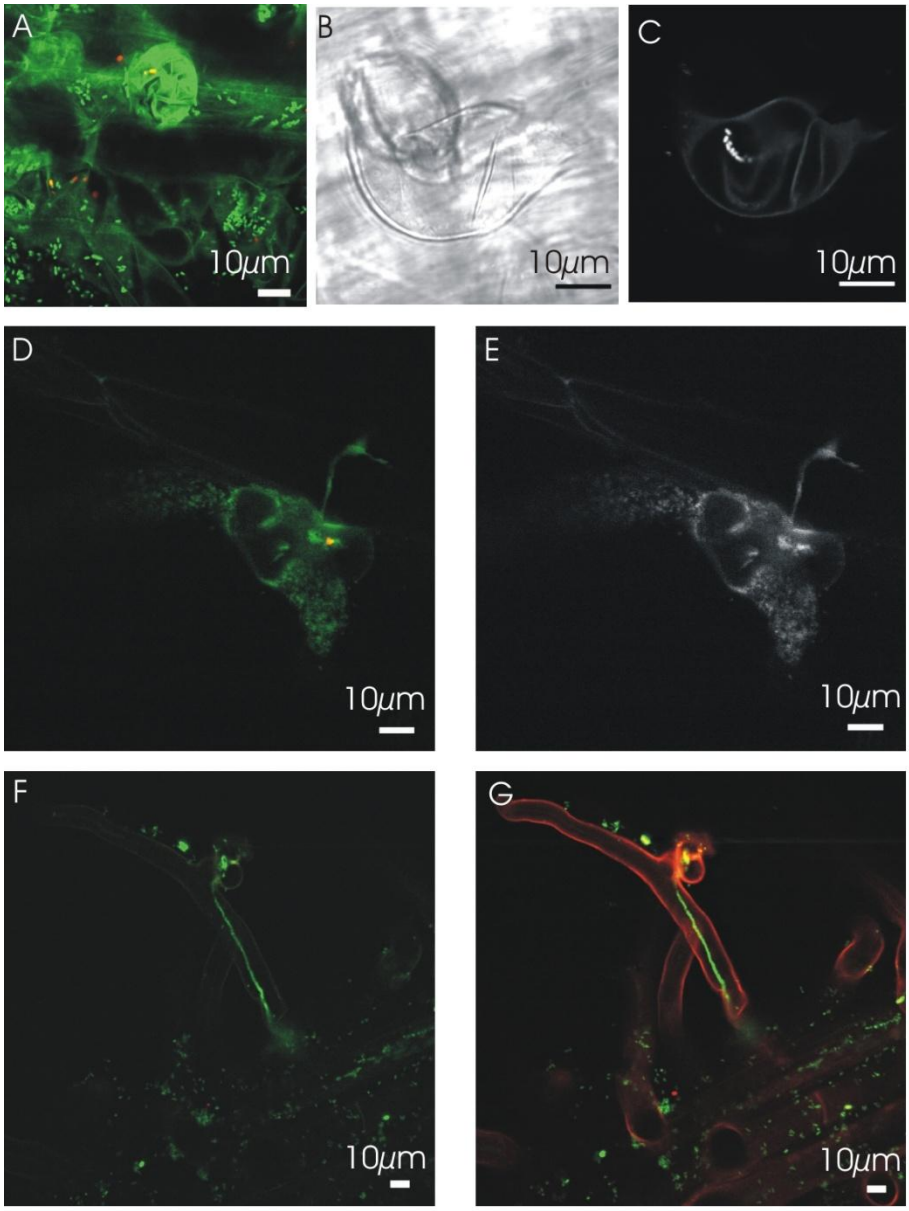

Fig.II 18: Infection threads in *T. repens* roots. Confocal reconstruction showing bacteria cells attached to the root curled hairs tip a red cells is visible in the pocket formed by root hair tip (A). Visualization of an infection threads in bright field (B) and with laser excitation, green cells are visible within IT (C). Confocal image of a curled root hairs and an infection thread growing in the root cortex, *E. agglomerans* Hs6 pRL765rfp in the upper part of the filament (D), in (E) the sole green channel observation is shown. Confocal reconstruction of elongation of an infection thread in the root hairs (F), panel (G) shows the same image of F but without cutting the λ correspondent to plant tissues autofluorescenc

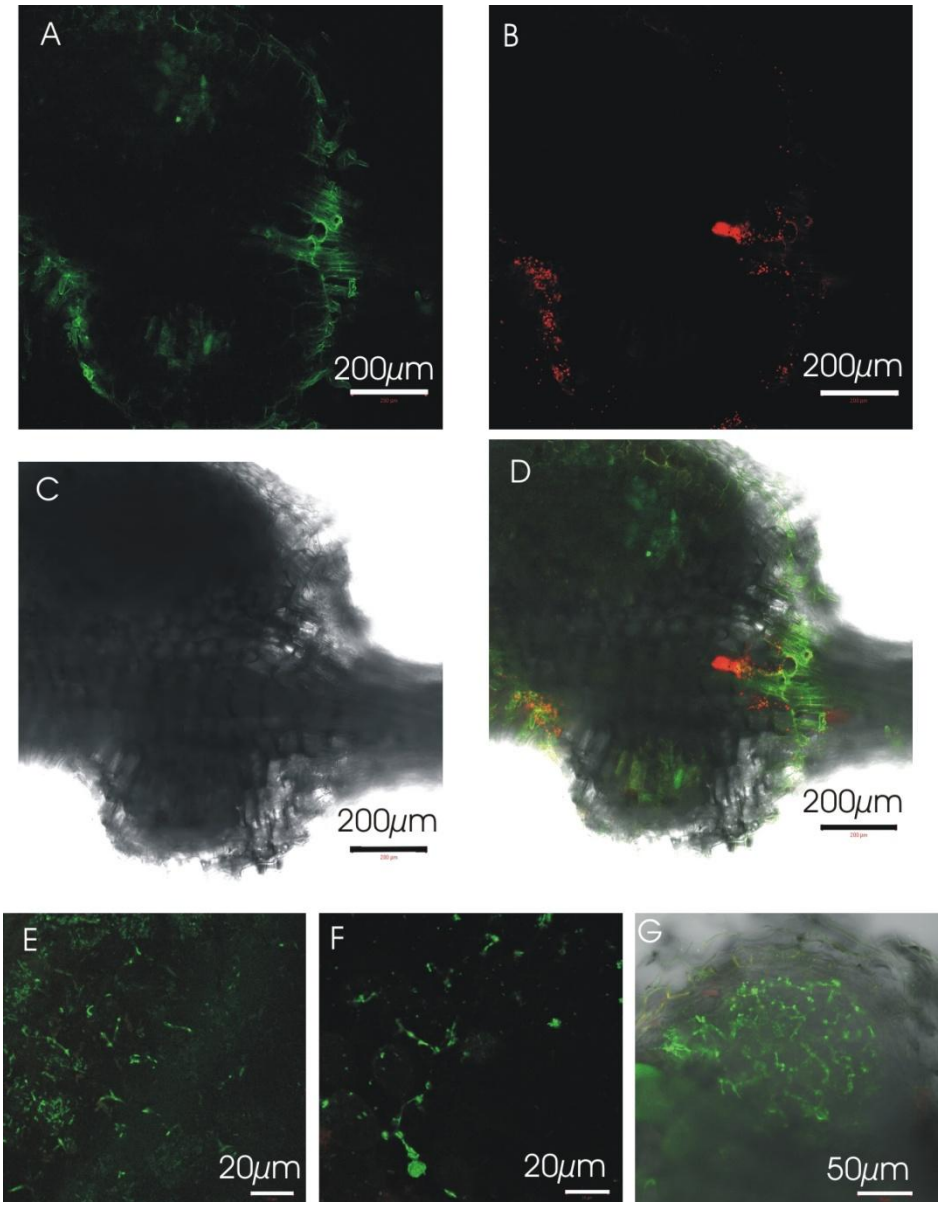

Fig.II 19: Nodules emerged from *T. repens* roots inoculated with *R. leguminosarum* E11 gfp tagged and *E. agglomerans* Hs6 pRL765rfp. Combined fluorescence and bright-field images showing micro-colonies on the surface of nodule (D), green channel (A), red channel (B), bright-field (C) of D panel. Optical sections of root nodules (E,F,G), thick networks of intracellular infection threads are visible.

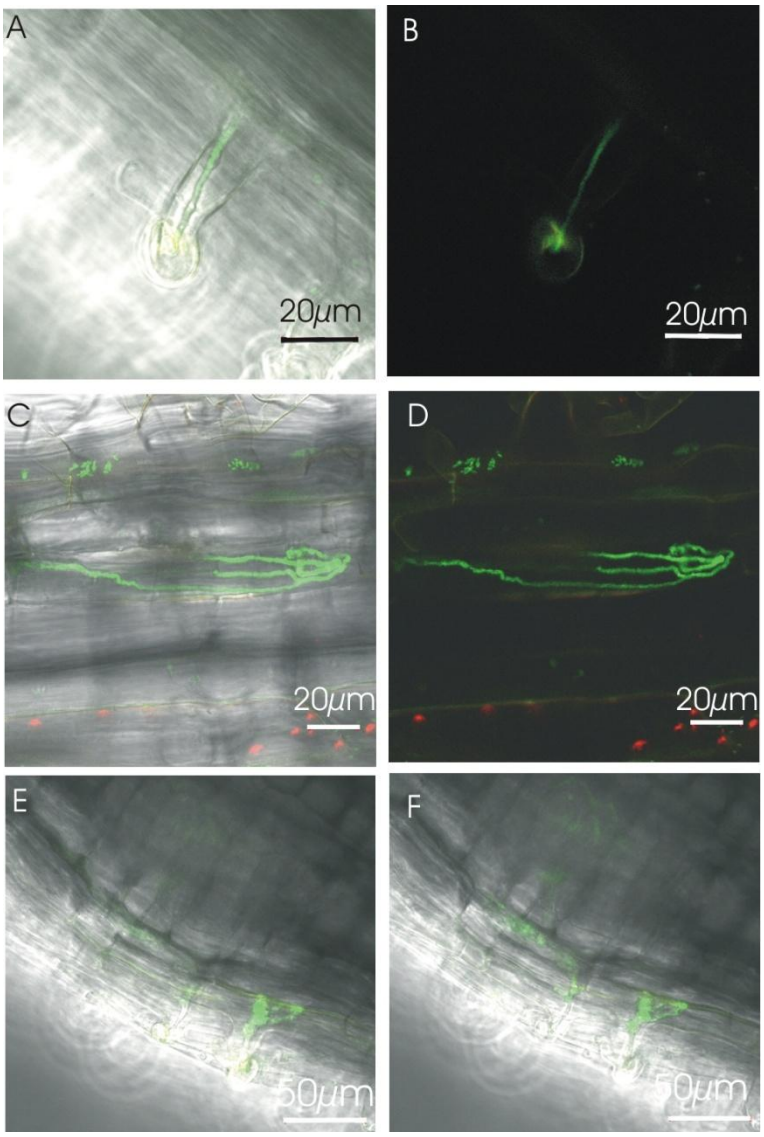

Fig.II 20: root hairs curling and progression of infection threads towards the root inner cortex. (A, C, E, F) Combined fluorescence and bright-field images of infection threads elongation along root hairs and cotex. Panels E and F shows two consecutive sequences of the same field.

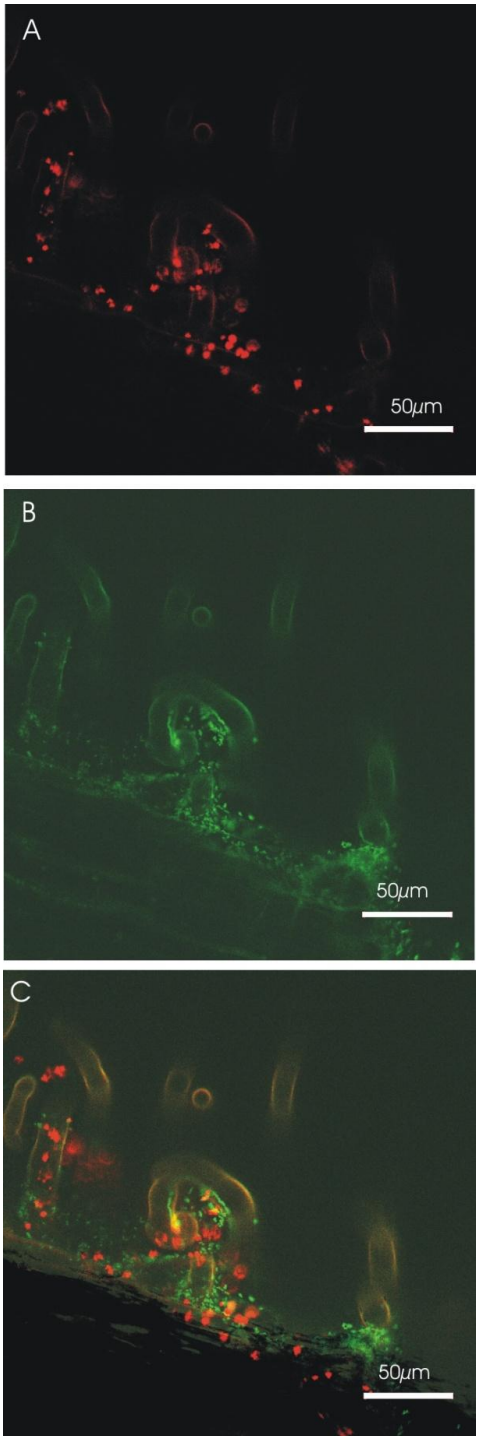

Fig.II 21: root hairs deformation during early pre-penetration. (A) red channel, (B) green channel, (C) merge of the firs and the second image. *Enterobacter agglomerans* rfp tagged cells were detected in the pocket of curled deformation at the root hair tip, but not within intracellular infection threads. Several other green and red cells are visible on root surface.

Wild legume plants

Plants collection, nodules processing and bacteria isolation from nodules

Wild legume species examined, *Tetragonolobus purpureus* and *Hedysarum spinosissimim*, were collected in Sardinia at Piscinas and Gioscari respectively, during spring 2009 (Fig.II 22). The root systems of field-collected species bore tubercular structures that varied in number and shape. Root nodules on *T. purpureus* were typically round and determinate and they were elevate in number, whereas the roots of *H. spinosissimum* presented only few multi-lobed nodules.

Fig.II 22: *Tetragonolobus purpureus* (A) and *Hedysarum spinosissimim* (B).

After surface sterilization a pool of nodules sampled from different individuals of the each species was squashed in the presence of the two kinds of mixes containing ROS scavenging systems; the resulting suspensions were streaked on different YMA plates as described in the scheme (Fig.II 23). Considering results obtained in a previous work, in which we showed the consistently unculturable state of rhizobial endosymbionts from within the nodules of eleven wild legume species of Sardinia (Muresu *et al*. 2008), in the present experiment we aimed at increasing the number of culturable rhizobia with the addition of systems protecting from possible damage from reactive oxygen.

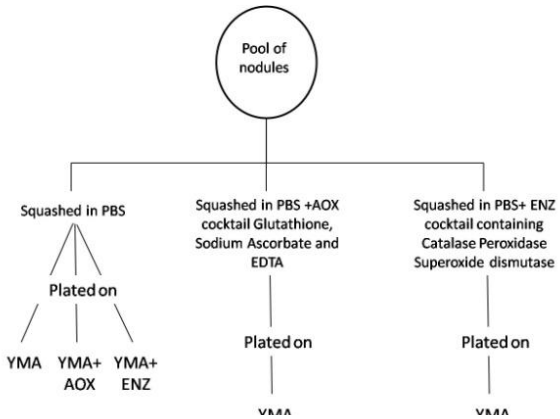

Fig. II 23: procedure used for nodules processing.

Colonies, with rhizobial morphology, grown on the different kinds on plates were counted. Both for *Tetragonolobus purpureus* and *Hedysarum spinosissimim* the CFU/ml obtained in each kind of plates was plotted in relation with their time of appearance expressed in hours from plating. For *T. purpureus* (Fig.II 24 A) the scavenging systems do not appear to enhance the culturability of rhizobia from root nodules. Only after 100h a large number of small mucoid colonies arose, but this growth was not linked with the presence of scavenging systems in the squashed solution or on plates, as the PBS control gave the same result. The colonies kinetics would comply to relatively slow-growing bacterial species. On the contrary, in the case of *H. spinosissimim* large numbers of mucoid colonies appeared after 48 hours in YMA plates streaked with the squashed nodules suspensions containing the anti-oxydant (AOX) mix, and a lower but still higher than control number of colonies, with a different morphology, appeared on plates streaked with nodules suspensions containing the enzyme (ENZ) mix. According to these results, the presence of the AOX cocktail in the squashing buffer seems to promote the rescue of nodule endosymbionts, while a certain effect on different ones is exerted by the presence of enzymatic protecting agents.

To test if grown colonies were true rhizobia, representative cases from each morphology and plate type were purified, processed for DNA extraction, and their 16S rRNA genes were amplified. Prior to sequencing the amplicons obtained they were grouped by ARDRA comparing the results with reference rhizobial strains. The results of an NCBI database BLAST analysis of the sequences obtained are shown in Tab.II 6. Two isolates from *T. purpureus* and two from *H. spinosissimim* for which 16S rRNA gene proved high similarity to *Rhizobium* or *Mesorizobium* were chosen to verify their ability to re-induce nodules formation in wild legume plants.

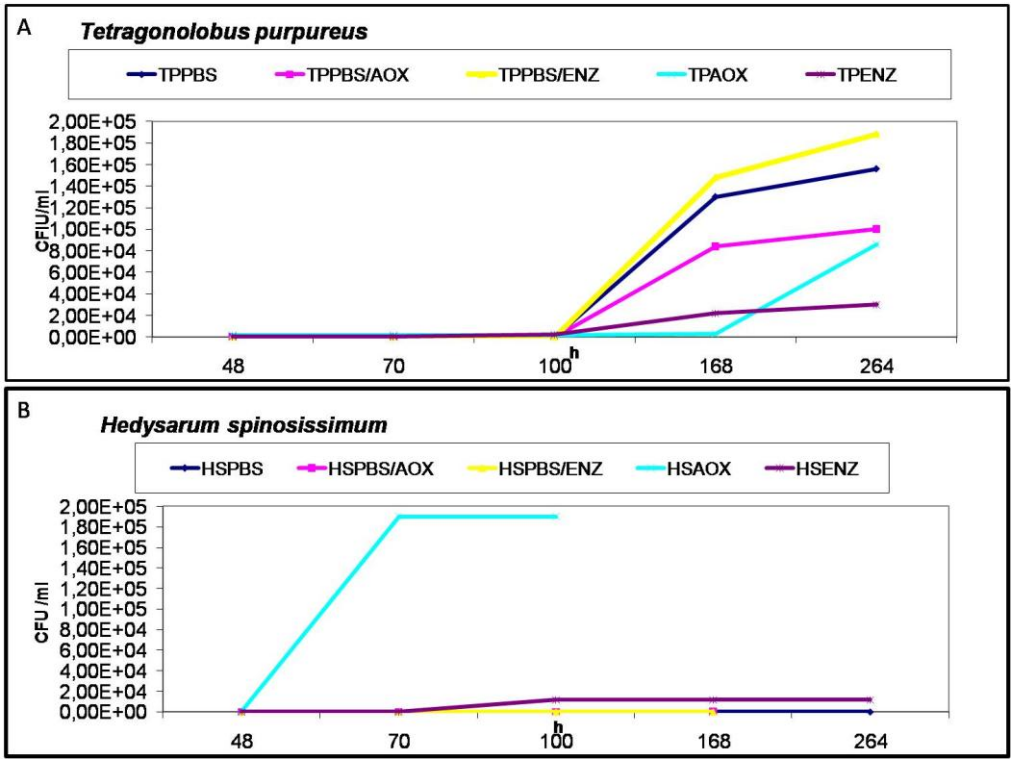

Fig.II 24: Plate counts vs. time in response to the presence of ROS scavenging systems.in the nodule squashing buffer. Plate Codes include plant species name (HS= *Hedysarum spinosissimum* TP= *Tetragonolobus purpureus*; Squash buffer (PBS=phosphate buffer saline control, AOX= chemical antioxydant mix, ENZ= enzymatic mix). Plates were made of plain YMA or of YMA plus one of the two types of mixes (AOX or ENZ).

Tab.II 6: strains isolated from wild legume plants.

Strain designation	Top scoring database similarities of 16S rDNA
Tp AOX 3	*Rhizobium* sp. 94% (EF540493)
Tp AOX 13	*Mesorhizobium* sp. 96% (AY225398)
Hs AOX 8	*Rhizobium* sp. 97% (Y10176) *Phyllobacterium* sp. 98% (FJ263028)
Hs ENZ 9	*Rhizobium* sp. 96% (FJ648703)

Resistance to H_2O_2

Upon the results obtained from isolation of bacteria in presence of scavenging systems, bacterial strains Tp AOX 13 and Tp AOX 3, Hs AOX 8, Hs ENZ 9 were tested for their resistance to different concentrations of H_2O_2. The same experiment was performed with two known endophytic bacteria (*Pseudomonas* sp. and *Enterobacter agglomerans*), testing both their wild types and the derivatives tagged with fluorescent proteins described in previously. OD_{600} of bacterial liquid coltures, in the presence of different concentrations of hydrogen peroxyde, was measured until stationary phase. As can be observed from the plots in figure II 25, both TP AOX 13 and TP AOX 3 were sensitive at concentrations of 1mM and 10mM of H_2O_2, while strains that came from *H. spinosissimum* were sensitive only to 10mM of H_2O_2. Regarding the growth of the non-rhizobial endophytes in the presence of H_2O_2, both for wild type and tagged strains no turbidity appeared in the presence of the highest concentration (10mM) of hydrogen peroxide, with the exeception of *Pseudomonas* sp.::gfp which, unlike its wild type, was resistant also to the highest concentration tested. This is an interesting finding that can be interpreted in light of other works in literature that point out how fluorescent proteins as gfp and rfp can act as efficient oxygen radical quenchers (Palmer *et al*., 2009). A study of Bou-Abdallah *et al*., (2006) showed that GFP can quench O_2. The mechanism of quenching appears to involve weak SOD-like activity at low O_2 concentrations while at higher

concentrations quenching still occurs but at the expense of structural changes in the protein. The observation that there is no loss of activity, or fluorescence, when GFP is exposed to low fluxes of O_2 radicals, is important because low fluxes of O_2 are present in most organisms under normal conditions. The fact that our rfp tagged *E. agglomerans* was not resistant to the high concentration of H_2O_2, as the gfp-tagged *Pseudomonas*, could be due to the fact that the RFP encoded by the m*rfp* gene is a monomeric form of the protein.

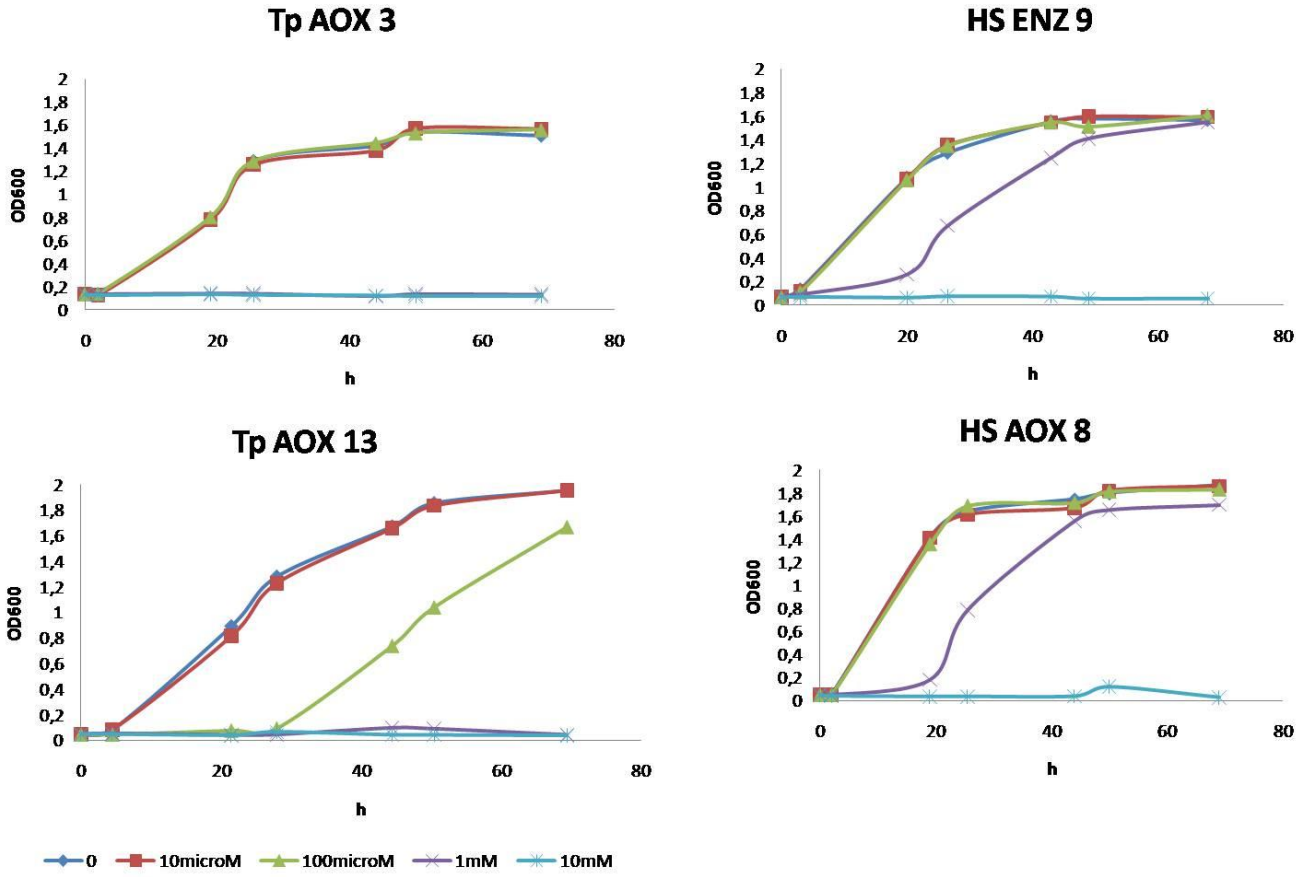

Fig. II 25: growth kinetics of the different isolates in the presence of a series of H_2O_2 concentration

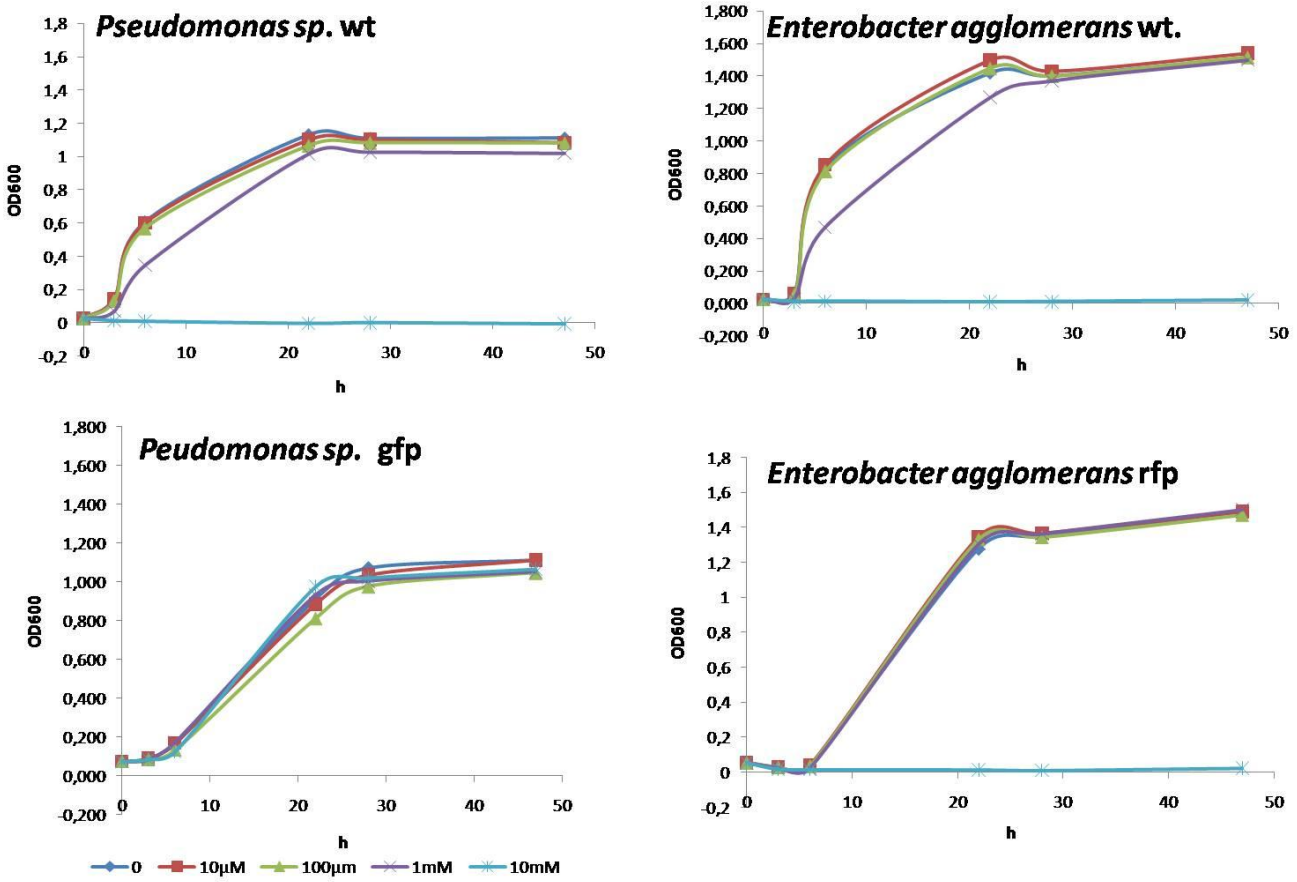

Fig.II 25: growth kinetics of the different isolates in the presence of a series of H_2O_2 concentrations

Nodulation tests

Next we checked whether the four different bacterial isolates could re-induce nodule formation on their hosts under gnotobiotic conditions. In addition, by including in the inoculums suspension *Pseudomonas* sp.::gfp and *E. agglomerans* pRL765rfp we investigated plant-endophyte relationships testing the effect of co-inoculation in terms of possible facilitated invasion of the legume by these non-rhizobial bacteria. A series of nodulation tests were performed in the growth cabinet using sterilized sand/vermiculite in Leonard jars and extending the cultivation for up to two months (Fig.II 26). Six plant species, propagated from surface-sterilized seeds were tested: *Hedysarum spinosissimim, Tetragonolobus purpureus, Ornithopus compressus, Ornithopus sativus, Lotus ornithopodioides, Hedysarum coronarium* (sulla). The four isolates obtained from the previous experiment were tested in mixed inocula containing also tagged endophytes. *Hedysarum coronarium* was used as a control and its inoculua suspensions contained the natural symbiont *Rhizobium sullae* mixed with tagged endophytes. All species tested in sand/vermiculite, excluding *T. purpureus* for which not enough germinating seeds were available, were also investigated in Leonard jars containing soil from Sardinia, intended as a source of natural legume-nodulating bacteria. The inocula of these last series of tests contained only *Pseudomonas* sp.::gfp and *E. agglomerans* pRL765rfp. Not all species survived these in-vitro tests, only *T. purpureus, H. coronarium* and *O. compressus* grown in sterilized sand/vermiculite and *H. spinosissimim* grown in soil endured the assay and grew well. The other plant species did not survive as presumably they did not find in the inocula the appropriate simbiont to form N-fixing nodules.

Fig.II 26: Example of nodulation test in Leonard jars. Plants were cultivated in sterile quartziferous sand-vermiculite and inoculated with Tp AOX 3, Tp AOX 13, Hs AOX 9, Hs ENZ 8 mixed with tagged endophytes

T. purpureus was the species that gave the highest number of nodules (hundreds per plant) with a round shape like determinate nodules. They presented structures that seemed vascular elements on the surface (Fig.II 27 A), these did not appear on nodules recovered from roots of *T. purpureus* plants grown in field. *H. spinosissimim* and *H. coronarium* gave nodules with irregular shape (Fig.II 27 B,C), and an high number of shovel-like swellings similar to those that develop in *sulla*, *H. coronarium* (Squartini et al., 1993), were seen on *H. spinosissimum* roots (Fig.II 27). *O. compressus* did not appear in healthy conditions and its roots presented only 5 very small nodules.

Fig.II 27: Examples of root nodules of the different plants included in this study. (A) *T. purpureus*, (B) *H. spinosissimim*, (C) *H. coronarium*.

We attempted bacterial isolation from nodules, stems and leaves of plants that survived and formed root tubercular structures. The surface-sterilization treatment was generally efficient, since in most cases, no colonies developed on PCA plates upon which nodules and areal parts were rolled prior to sectioning. The most common result from plating squashes suspensions was no development of microbial growth on LB Km and NB Km plates (used to rescue the two tagged endophytes). No growth at all occurred from stems. Growth occurred by plating nodule suspensions in PCA although less than ten colonies developed. And very few colonies (1 to 3) grew on YMA. This, while reaffirming our previously reported non-culturability of rhizobia from wild Mediterranean legumes, contrasts with typical results using cultivated legumes, where the rhizobial occupants rescued by such techniques normally form a profuse lawn along most of the streak length on these plating media, indicating their abundance and culturability. Only with *T. purpureus* this kind of result was obtained from one of the processed nodules, from which a lawn of confluent mucoid colonies was obtained. Interestingly it was possible to re-isolate colonies with green fluorescing phenotype and antibiotic resistance markers from surface-sterilized leaves of this host, 10 colonies grew on LB (Km 30 µg/ml) plates and 19 on NB (Km 30 µg/ml, Cm 15 µg/ml) agar plates. These were considered bona-fide colonies of *Pseudomonas* sp.::gfp which was present in the inoculum. To confirm this hypothesis these colonies were purified, processed for DNA extraction, and their 16S rRNA genes were amplified. We grouped them by ARDRA and we compared the restriction profiles with that one obtained by a pure colony of *Pseudomonas* sp.::gfp, confirming that colonies isolated from leaves of *T. purpureus* were *Pseudomonas* sp.::gfp. This indicates that such endophyte, upon root inoculation was able to invade the plant and be traslocated to its aerial portions. The strain is the one displaying increased levels of H_2O_2 tolerance due to its gfp, which can have conferred a fitness enhancing property during the route of internal plant colonization.

Conclusions II

One of the most useful aspects of the Fluorescent Proteins for biological studies is that they can be monitored in living cells, with minimal disruption of the system. In order to investigate plant-endophyte spatial relationships novel plasmids were constructed for the delivery of a red fluorescent protein tag in gram negative bacteria (pRL765rfp). pUTgfp2X was used as delivery system for the gfp gene. Derivatives of *Rhizobium*, *Pseudomonas* and *Enterobacter* were constructed with different fluorescent markers suitable for their tracking inside plants. The use of confocal laser scanning microscopy associated with the use of tagged bacterial strains, proved to be a useful tool for the fine-scale locatization of endophytic bacteria on and within plant roots.

At first we can conclude that both the gfp- and the rfp-tagged constructs of *Rhizobium leguminosarum* bv. *trifolii* used in this study retained the ability to infect and nodulate their host plants after the insertion of the marker gene and the antibiotic resistance. Fluorescent rhizobial cells could be easily detected inside intracellular infection threads during primary infection of *Trifolium repens* root hairs and on root surface also several days after inoculation.

The two tagged strains of endophytic origin (*Enterobacter agglomerans* pRL765rfp and *Pseudomonas* sp.::gfp) maintained as well their fluorescent phenotype during nodulation tests and in the absence of selection.

In particular rfp-tagged *Enterobacter agglomerans* could be detected on the surface of *Trifolium repens* roots and in early pre-penetration structures of the infection process. Thus in this plant species, the non-rhizobial endophyte appears to accompany its co-inoculated rhizobial counterpart up to the shepherd crook deformation event (Hac phenotype), but does not enter infection threads along with rhizobia during incipient penetration and growth within infection threads in primary host infection. Therefore endophytes originally isolated from African legumes were found incapable of passing the infection thread stage when inoculated in non-host crop legumes of temperate ranges as white clover. In contrast, when tested in other Mediterranean wild legumes upon co-inoculation with rhizobia they were able to invade the plant and to efficiently reach its upper

parts to be eventually reisolated from the interior of surface-sterilized leaves. In fact using standard isolation methods we could identify colonies of *Pseudomonas* sp.::gfp from leaves of *Tetragonolobus purpureus* after nodulation tests performed under gnotobiotic conditions with diverse species of wild legume plants. Further uses of dual fluorescence markers will allow to co-localize different bacterial taxa within plant tissues and to better understand the mode(s) of entry of endophytes into root nodules. This will open the way to different innovative applications in the field of symbiosis, biocontrol and other endophytic plant-microbe interactions.

In addition we observed that endophytic strains harbouring the introduced green fluorescent protein marker acquire substantially higher levels of resistance to hydrogen peroxide. This trait can open the perspective use of GFP also as a fitness-enhancing phenotype for bacteria targeted to colonize host plants displaying high production of reactive oxygen species.

In addition we obtained results suggesting that the culturability of rhizobial species from nodules of *Hedysarum spinossisimum* can be enhanced by the use of systems protecting from possible damage from reactive oxygen in squashed nodules suspension, during the procedures for bacterial isolation from nodules.

All these evidences concur to our increased understanding of the details underlying the interaction between plants and bacteria within the boundaries of endophytism and in its connections with nitrogen fixing symbioses.

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Appendix 1

Muresu R., Polone E., Sulas L., Baldan B., Tondello A., Delogu G., Cappuccinelli P., Alberghini S., Benhizia Y., Benhizia H., Benguedouar A., Mori B., Calamassi R., Dazzo F. B. and Squartini A.

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

RESEARCH ARTICLE

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

Rosella Muresu[1], Elisa Polone[2], Leonardo Sulas[1], Barbara Baldan[3], Alessandra Tondello[3], Giuseppe Delogu[4], Piero Cappuccinelli[4], Sara Alberghini[2], Yacine Benhizia[5], Hayet Benhizia[6], Ammar Benguedouar[5], Bruno Mori[7], Roberto Calamassi[7], Frank B. Dazzo[8] & Andrea Squartini[2]

[1]C.N.R., Sezione di Sassari, c/o Dipartimento di Scienze Agronomiche e Genetica Vegetale Agraria, Università degli Studi di Sassari, Sassari, Italy; [2]Dipartimento di Biotecnologie Agrarie, Università di Padova, Padova, Italy; [3]Dipartimento di Biologia, Università di Padova, Padova, Italy; [4]Dipartimento di Scienze Biomediche, Sassari, Italy; [5]Laboratoire des Biotechnologies, Département des Sciences de la Nature et de la Vie, Université Mentouri, Constantine, Algeria; [6]Laboratoire de Génétique, Biochimie et Biotechnologies Végétales, Département des Sciences de la Nature et de la Vie, Université Mentouri, Constantine, Algeria; [7]Dipartimento di Biologia Vegetale, Università di Firenze, Firenze, Italy; and [8]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA

Correspondence: Andrea Squartini, Dipartimento di Biotecnologie Agrarie, Università di Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy. Tel.: +390498272923; fax: +390498272929; e-mail: squart@unipd.it

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rhizobia; endophytes; wild legumes; nonculturable.

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-disinfected 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 4.3×10^9 CFU g^{-1} fresh weight, but that, at the same time, 3.0×10^5 CFU g^{-1} 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 Segarìu, 39°34′N, 8°57′E, 13 nodules), *Hippocrepis 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 carnosum*) 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 naudinianum*; near Sètif, 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% $HgCl_2$ for 2 min, and the number of H_2O 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-1 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^{-1} K_2HPO_4 and 0.13 g L^{-1} KH_2PO_4. 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% $HgCl_2$ 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.* 10^8 cells mL^{-1}). 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 μm), obtained with a Reichert–Jung ultramicrotome, were stained with 1% toluidine blue for light microscopy. Ultra-thin sections (0.05 μm) 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-μm 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_2O) 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_2O) 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 μm 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_2O; phosphate-buffered saline (PBS, Dazzo, 1982). Treated sections were soaked in 100 mM glycin (*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 μg mL^{-1} 4′,6-diamidino-2-phenyl-indole. Samples were observed under an Olympus BX51 epifluorescence microscope equipped with a × 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 μg mL^{-1}) containing antifade (23 mg mL^{-1} 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 μL of lysis buffer [0.25% sodium dodecyl sulphate (SDS), 0.05M NaOH] in a 1.5-mL polypropilene 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 μL of the supernatant was mixed with 90 μL of sterile water. Lysates were stored at 4 °C prior to PCR. For direct PCR analysis, nodules were squashed in 50 μL of sterile water, and 10 μL of the suspension was transferred to 50 μL 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′ACGGGCGGTGTGTACAAG) (Osborn *et al.*, 2000) at 1 μM each in a 25-μL 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 $MgCl_2$, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer, and 2.5 U Taq DNA polymerase, recombinant (Invitrogen Life Technologies). Amplicons were digested overnight at 37 °C upon mixing 5 μL from the 25-μL reaction volume with 1 μL of CfoI enzyme (Pharmacia, Uppsala, Sweden) and 2 μL of 10 × 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 μL of sterile physiological solution was mixed with 50 μL 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 μL 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. Multilobed 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.

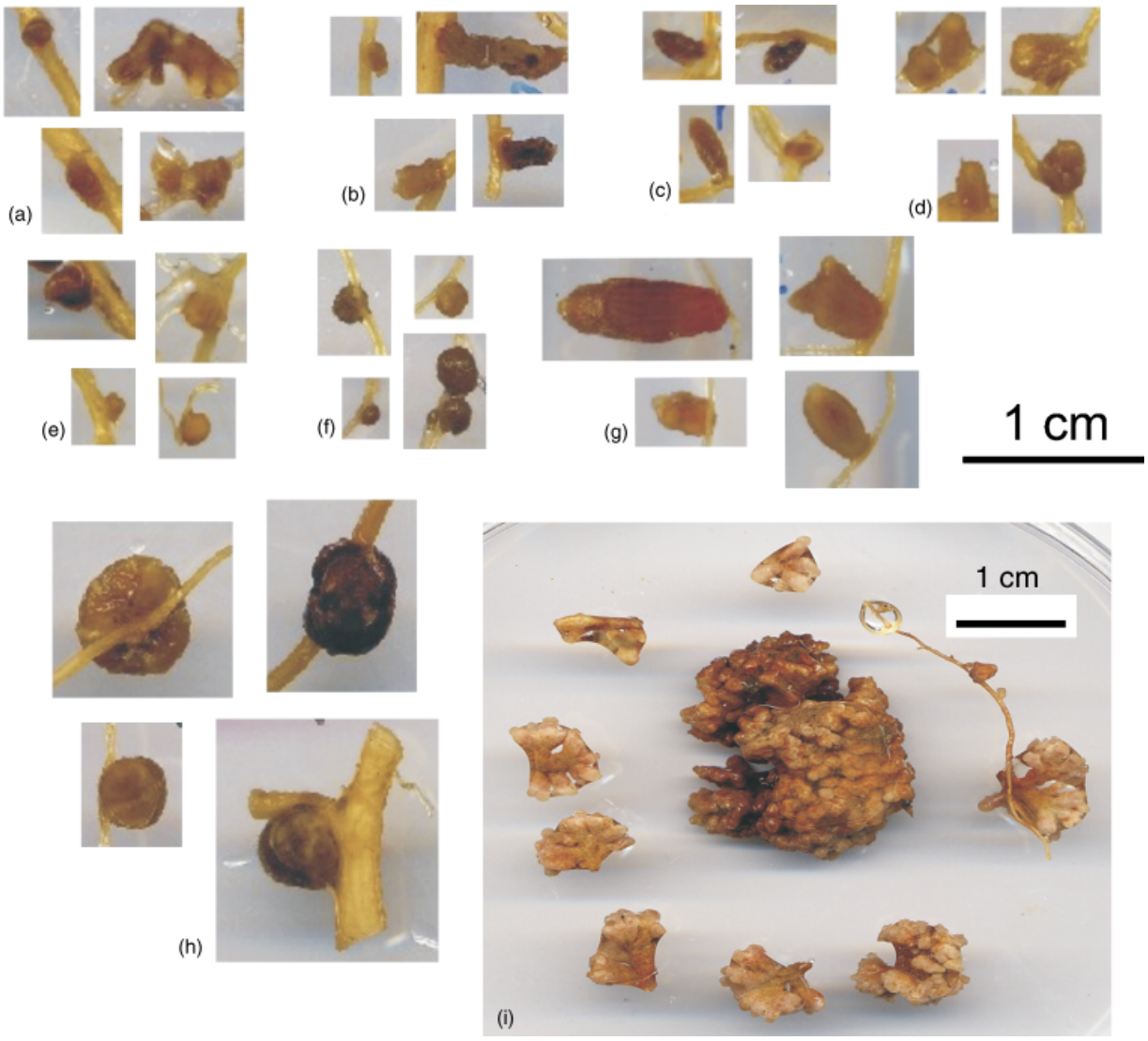

Fig. 1. Examples of excised root nodules of the various plants included in this study. (a) *Hedysarum glomeratum*; (b) *Hedysarum spinosissimum*; (c) *Hippocrepis unisiliquosa*; (d) *Ornithopus compressus*; (e) *Ornithopus pinnatus*; (f) *Tetragonolobus purpureus*; (g) *Scorpiurus muricatus*; (h) *Psoralea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

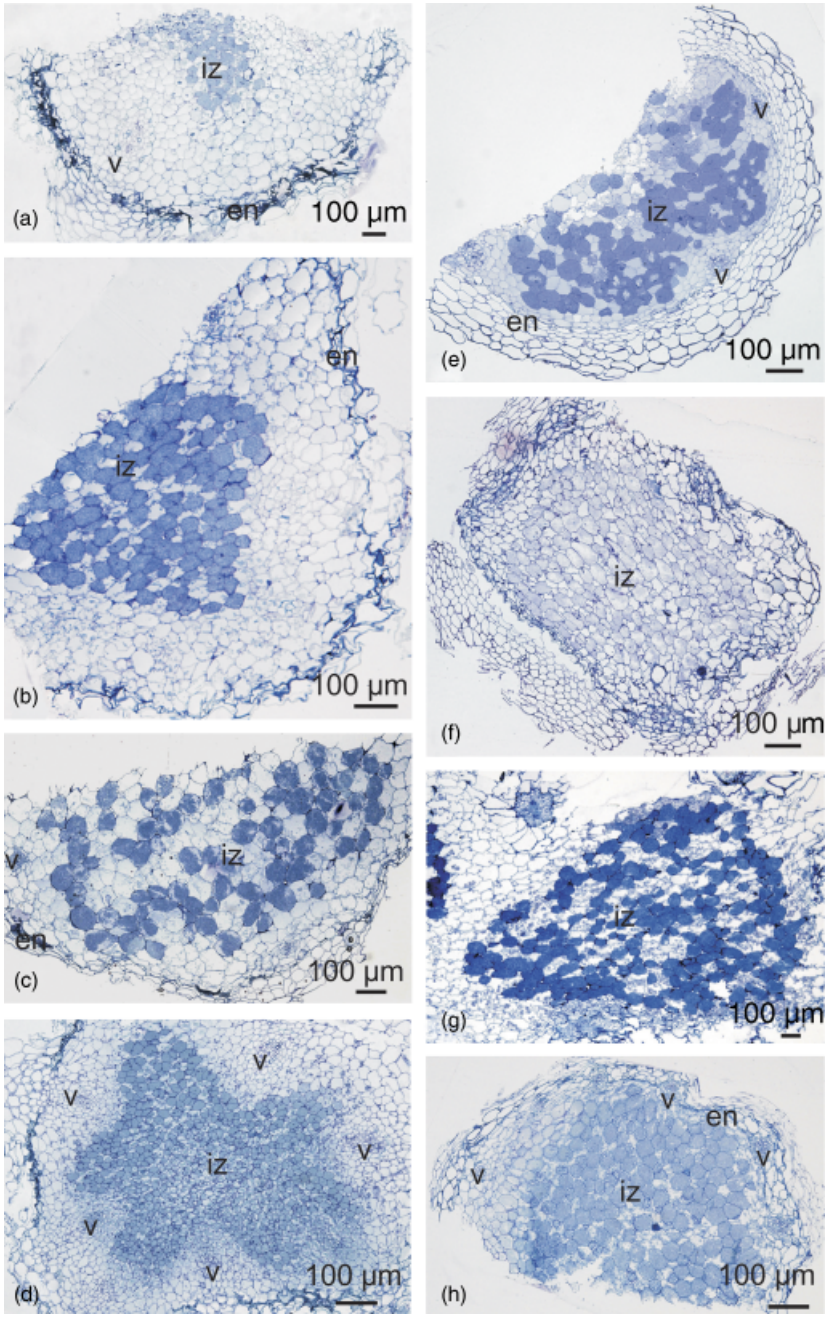

Fig. 2. Structure of the legume nodules. (a–h) Bright-field micrographs of sections stained with 1% Toluidine Blue. (a, d) *Ornithopus compressus*; (b) *Ornithopus pinnatus*; (c) *Tetragonolobus purpureus*; (e) *Hedysarum spinosissimum*; (f) *Hedysarum glomeratum*; (g) *Psoralea bituminosa*; (h) *Scorpiurus 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 (Benhizia *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 (Benhizia *et al.*, 2004a, b). We next checked whether the

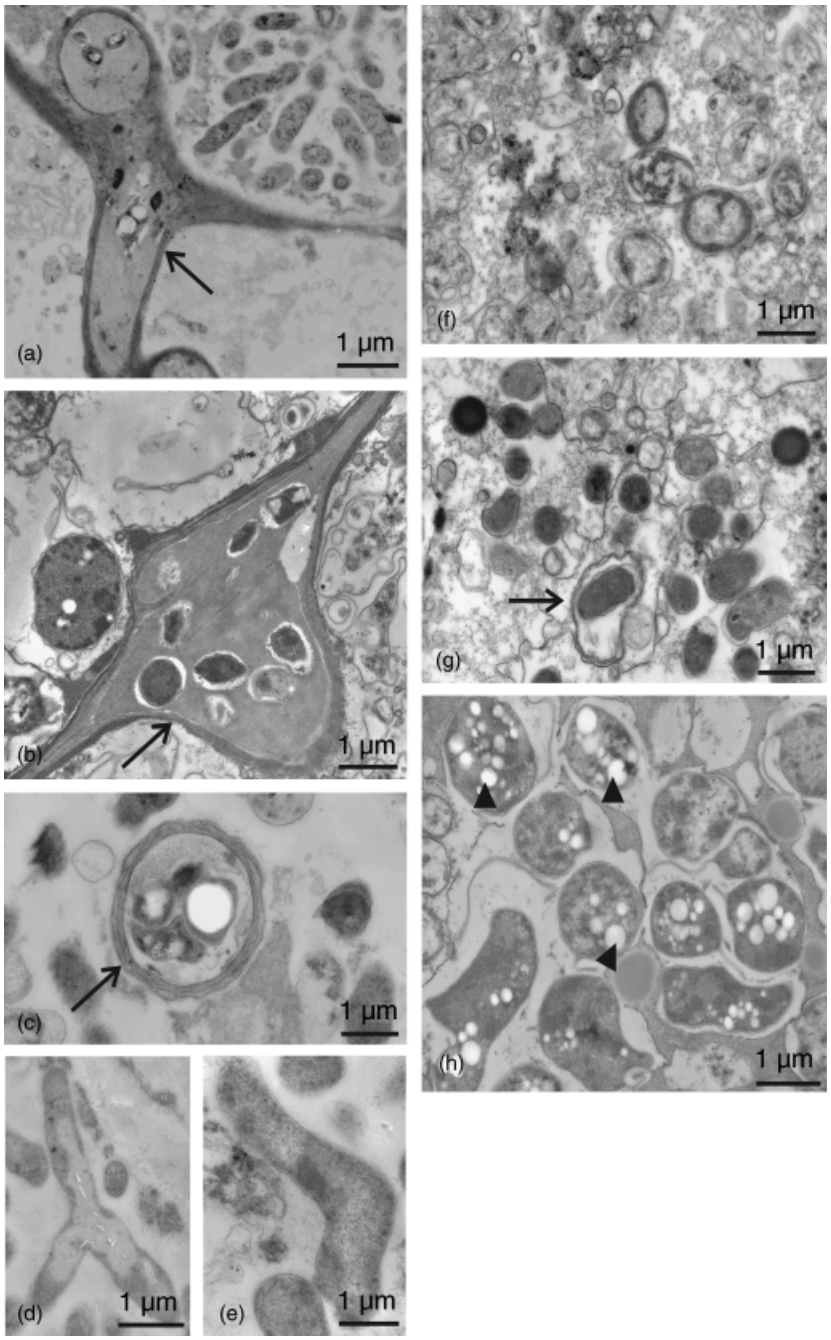

Fig. 3. Transmission electron micrographs of the infection zone in *Ornithopus compressus* and *Hedysarum spinosissimum* nodules. Arrows indicate (a) longitudinal- and (b, c) cross-sections of bacteria-containing infection threads (a, b) between and (c) inside a *Ornithopus compressus* host cell. (d, 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 electrondensity. In (g) peri-bacteroid membranes surround the symbiotic bacteroids (arrow). (h) Rod-shaped bacteria containing granules of β-hydroxybutyrate (arrowheads).

various bacterial isolates could induce nodule formation on their hosts under gnotobiotic conditions. A series of nodulation 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 *Gammaproteobacteria* 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

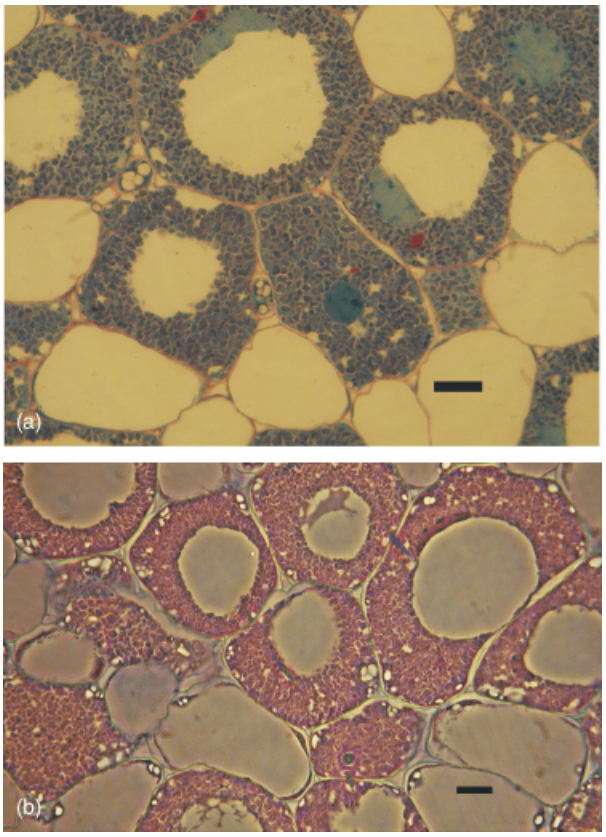

Fig. 4. (a) Optical microscopy of Aniline Blue Black/Basic Fuchsin double-stained thin sections from a *Hedysarum glomeratum* nodule. Plant cell walls and bacterial infection threads (thick round cross-sections) stain red. Plant nuclei stain from dark to light blue in relation to cell physiological status. 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 sulla, *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	*Hedysarum spinosissimum*	*Hedysarum glomeratum*	*Tetragonolobus purpureus*	*Hippocrepis unisiliquosa*	*Scorpiurus muricatus*	*Psoralea bituminosa*	*Ornithopus compressus*	*Ornithopus pinnatus*	Total
No growth	0	3	0	9	5	4	1	2	24
Enterobacteriaceae[*]	8	1	1	0	0	0	8	4	22
Bacillus[†]	2	2	2	1	5	0	0	1	13
Pseudomonas[‡]	6	0	0	0	0	0	6	0	12
Curtobacterium[§]	0	0	0	0	0	0	0	4	4
Rhizobium[¶]	2	0	0	0	0	0	0	1	3
Staphylococcus[‖]	0	1	2	0	0	0	0	0	3
Xanthomonas[**]	2	0	0	0	0	0	0	0	2
Microbacterium[††]	0	0	0	0	0	0	0	1	1
Arthrobacter[‡‡]	0	0	0	0	0	1	0	0	1
Mesorhizobium[§§]	0	0	1	0	0	0	0	0	1
Agrobacterium[¶¶]	1	0	0	0	0	0	0	0	1
Ralstonia[‖‖]	0	1	0	0	0	0	0	0	1
Thiobacillus[***]	0	0	0	0	1	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).

[†]Including: *Bacillus simplex* 100% (DQ457586), *Bacillus megaterium* 99% (DQ457585), *Paenibacillus* sp. 98% (DQ457593).

[‡]Including: *Pseudomonas* sp. 98–100% (DQ457590), *P. lini* 99% (DQ457578), *P. viridiflava* 100% (DQ457579).

[§]Including *Curtobacterium flaccumfaciens* 100% (DQ457589), *C. herbarum* 99% (DQ457575).

[¶]Including: *Rhizobium* sp. ORS1466 100% (DQ457594), ORS1465 96% (DQ457595).

[‖]Including *Staphylococcus epidermidis* 100% (DQ457587), *S. pasteuri* 100% (DQ457583).

[**]*Xanthomonas translucens* 100% (DQ457591).

[††]*Microbacterium* sp. 99% (DQ457581).

[‡‡]*Arthrobacter* sp. 99% (DQ457580).

[§§]*Mesorhizobium loti* 100% (DQ457584).

[¶¶]*Agrobacterium* sp. SDW052 99% (DQ457596).

[‖‖]*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 reinduce 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			
Hedysarum spinosissimum	5	DQ457614	*(Meso)rhizobium* sp. (*H. spinosissimum*) H-4 (AF279889) 99%
	1	DQ457613	*Rhizobium* sp. (AY278884) 99%
	1	DQ457612	*Pantoea ananatis* (AY173021) 96%
Hedysarum glomeratum	2	DQ457611	*(Meso)rhizobium* sp. H-4 (AF279889) 99%
Hippocrepis unisiliquosa	2	DQ457615	*(Meso)rhizobium* sp. H-4 (AF279889) 100%
	2	–	not amplifiable
Scorpiurus muricatus	6	DQ457619	*Mesorhizobium* sp. *(Argyrolobium uniflorum)* (AY500256) 99%
Tetragonolobus purpureus	2	DQ457620	*Mesorhizobium* sp. *(Argyrolobium uniflorum)* (AY500256) 99%
	1	DQ457621	*Pseudomonas* sp. KD (AY456697) 98%
Ornithopus compressus	3	DQ457616	*Bradyrhizobium* sp. Spain 5 (AF461195) 100%
Ornithopus pinnatus	3	DQ457617	*Bradyrhizobium* sp. Spain 6 (AF461196) 98%
Psoralea bituminosa	2	DQ457618	*Mesorhizobium chacoense* (AJ278249) 98%
Algeria			
Hedysarum sp. ssp. *capitatum*	4	DQ457608	*(Meso)rhizobium* sp. (*H. spinosissimum*) H-4 (AF279889) 100%
	2	DQ457609	*Pseudomonas* sp. TM7.1 (*Tuber magnatum*) (DQ279324) 100%
	1	DQ457610	*Pantoea ananatis* (AY173021) 98%
Hedysarum pallidum	2	DQ457606	*Pantoea ananatis* (AY173021) 98%
	2*	–	Not amplifiable
	1	DQ457607	*Pseudomonas* sp. CLb01 (AY574283) 99%
Hedysarum carnosum	2	DQ457604	*Pantoea ananatis* (AY173021) 98%
	1	DQ457603	*Enterobacter hormaechei* (AJ853889) 98%
Hedysarum naudinianum	4	–	Not amplifiable
	1	DQ457605	*Pseudomonas* 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^{-1} 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 nonrhizobial 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 $10^5\,g^{-1}$ 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 $10^7\,CFU\,g^{-1}$ 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 $1 \times 10^4\,CFU\,g^{-1}$), 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 g^{-1} fresh weight
Field-collected plants (late spring)	
Hedysarum spinosissimum, primary roots	8.9×10^4 (Max. 1.1×10^7)
Hedysarum spinosissimum, secondary roots	4.3×10^5
Hedysarum spinosissimum, stems	2.1×10^3
Hedysarum coronarium, primary roots	2.5×10^5
Hedysarum coronarium, stems	4.1×10^5
Psoralea bituminosa, primary roots	3.6×10^5
Psoralea bituminosa, stems	0.1×10^2
Tetragonolobus purpureus, primary roots	5.0×10^3
Tetragonolobus purpureus, stems	4.1×10^2
Scorpiurus muricatus, primary roots	0
Scorpiurus muricatus, stems	0
Plants grown from surface-sterilized seeds, transplanted in natural soil, in a growth chamber	
Hedysarum spinosissimum, 5-day-old seedlings roots (before transplant)	0
Hedysarum spinosissimum, 40-day-old primary roots	0.3×10^2

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 (1.1×10^7) was recorded.

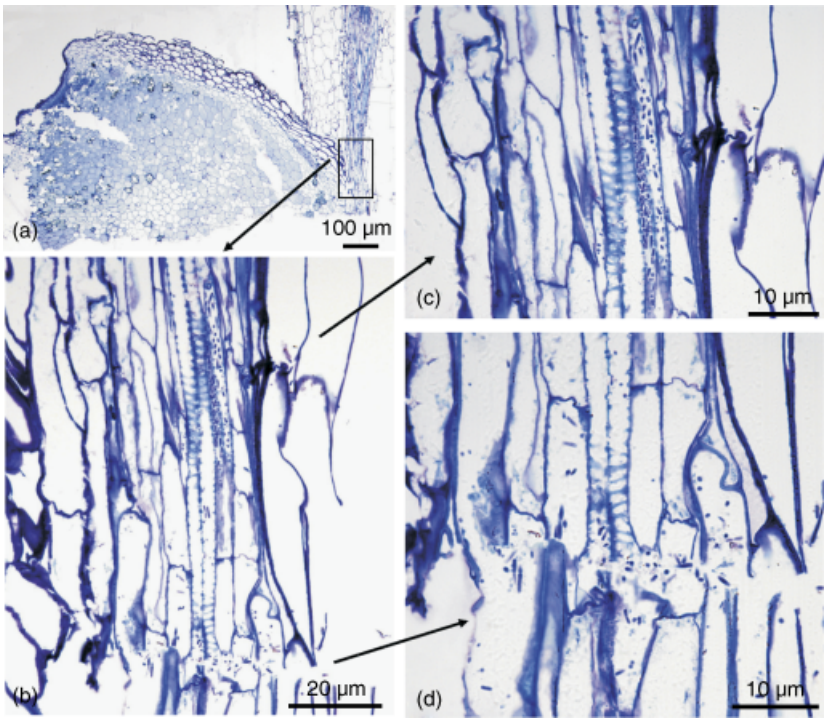

Fig. 5. Toluidine-stained longitudinal sections from a nodule and root of *Hedysarum naunidianum*. (a) Sections were taken from the junction between a nodule and the primary root. (b) High magnification of the square in (a). (c, d) At higher magnification, bacteria-like rods are clearly visible in the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

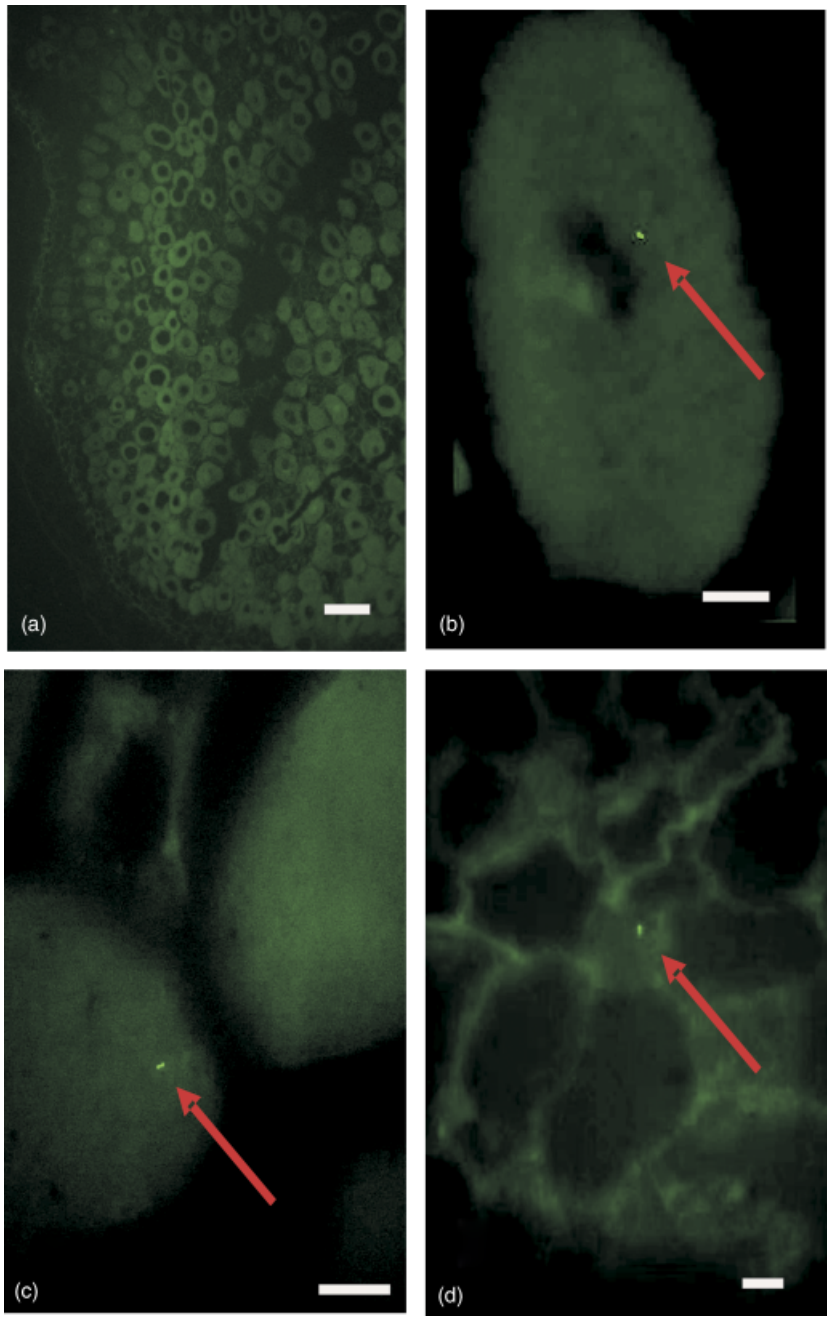

Fig. 6. Antibody-mediated fluorescent microscopy immunolocalization of *Enterobacter* on thin sections of a *Hedysarum spinosissimum* nodule. (a) Low-magnification image of the nodule showing the background level of fluorescence. (b–d) Higher-magnification examples of single isolated fluorescent antigen-bearing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

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 sulla (*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 that 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 sulla. 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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Appendix 2

Tondello A., Vendramin E., Villani M., Baldan B. and Squartini A.

Analysis, determination and cultivation of endophytic fungi associated with the orchid *Spiranthes spiralis*

Analysis, determination and cultivation of endophytic fungi associated with the orchid *Spiranthes spiralis*

Alessandra TONDELLO[1,2], Elena VENDRAMIN[2], Mariacristina VILLANI[1], Barbara BALDAN[1], Andrea SQUARTINI[2]*

[2] Dipartimento di Biologia,Università degli Studi di Padova, Italy.
[2] Dipartimento di Biotecnologie Agrarie, Università degli Studi di Padova, Italy

Mycorrhizas can sustain plants in most habitats through mutualistic interactions with fungi. The presence of these in internalized location can be due to true symbiotic relationships or to different levels of endophytism. In the case of orchids the fungal assistance is crucial throughout the seedling stage and only established plants begin to return organic carbon to the symbiont. Fungi from orchids are often difficult to isolate and their cultivation depends on seasonal and physiological conditions. The submediterranean species *Spiranthes spiralis* (L.) Chevall. is the latest-blooming native orchid in western Europe. Its rosettes appear in summer to die off in the following spring, while stalks flower in September (Willems and Dorland, 2000). The putative mycorrhizal fungi were isolated in culture from the surface-sterilized *Spiranthes spiralis* roots and their taxonomical identity was assessed by molecular techniques upon amplification of diagnostic ribosomal DNA regions. In parallel, DNA was isolated directly from the root tissues for comparison. Selective PCR amplification using ITS1-ITS4, ITS1F-ITS4 primers was carried out. Additionally, using fluorescence and confocal microscopy on acridine orange-stained freehand sections we observed a diffuse cortical colonization by intracellular hyphae. Their further ultrastructural details were resolved by electron microscopy.

Keywords: *Spiranthes spiralis*, orchid mycorrhiza, fungal endophytes.

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***Corresponding author:** squart@unipd.it

Appendix 3

Tondello A., Baldan B., and Squartini A.

Co-localizing symbiont and endophytic bacteria in legumes by tagging with different fluorescent proteins

Co-localizing symbiont and endophytic bacteria in legumes by tagging with different fluorescent proteins

Alessandra TONDELLO[1,2], Barbara BALDAN[1], Andrea SQUARTINI[2]*

[1] Dipartimento di Biologia, Università degli Studi di Padova, Italy
[2] Dipartimento di Biotecnologie Agrarie, Università degli Studi di Padova, Italy

Bacterial endophytes can promote plant growth and yield, suppress pathogens, solubilize nutrients or contribute to nitrogen uptake in plants. The use of fluorescent reporters is nowadays a key tool for studying microbe-plant interactions. In a previous work we assessed the presence of different endophytes in addition to rhizobia inside root nodules of wild legume plants and showed that rhizobia share nodules with a variety of different co-infecting taxa (Muresu *et al.*, 2008). In the present report we explore the plant-endophyte relationships attempting the co-localization of endophytes (*Pseudomonas* sp., *Enterobacter agglomerans*) and rhizobia (*Rhizobium leguminosarum* bv. *trifolii*) by the introduction of different fluorescent proteins as bacterial markers for the different kinds of bacteria. This would help their localization throughout the plant and in particular the distinction of rhizobia from other endophytes co-infecting root nodules. Green fluorescent protein (GFP) and red fluorescent protein (RFP) markers were integrated into the bacterial chromosome. For the marked constructs, a suicide plasmid, carrying the gfp gene in a transposable element was mobilized into the endophytic species. To obtain rfp-tagged bacteria, a replacement of *gfp* with *rfp* was made starting from the pRL765gfp plasmid. The use of dual fluorescence markers will allow to co-localize different bacterial taxa within plant tissues and will enable to plan different innovative applications in the field of symbiosis, biocontrol and other endophytic plant-microbe interactions.

Keywords: endophytes,confocal fluorescence microscopy.

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***Corresponding author:** squart@unipd.it

Appendix 4

Vendramin E., Gastaldo A., Tondello A., Baldan B., Villani M.,
and Squartini A.

Identification of two fungal endophytes associated with the endangered orchid *Orchis militaris* L.

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Identification of Two Fungal Endophytes Associated with the Endangered Orchid *Orchis militaris* L.

Vendramin, Elena[1], Andrea Gastaldo[1], Alessandra Tondello[1,2], Barbara Baldan[2], Mariacristina Villani[2], and Andrea Squartini[1]*

[1]*Dipartimento di Biotecnologie Agrarie, Università di Padova, viale dell'Università 16, 35020 Legnaro, Padova, Italy*
[2]*Dipartimento di Biologia, Università di Padova, via U. Bassi 58/B, 35131 Padova, Italy*

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A survey of the endangered orchid *Orchis militaris* populations was carried out in north-eastern Italy. The occurrence of fungal root endophytes was investigated by light and electron microscopies and molecular techniques. Two main sites of presence were individuated in the Euganean Hills, differing as to the percentage of flowering individuals and of capsules completing maturity. Fluorescence microscopy revealed an intracellular cortical colonization by hyphal pelotons. Two ITS PCR products co-amplified. Sequencing revealed for the former an identity and a high similarity (99%) with a Tulasnellaceae (Basidiomycota) fungus found within tissues of the same host in independent studies in Hungary and Estonia, suggesting an interesting case of tight specificity throughout the Eurosiberian home range. The second amplicon had 99% similarity with *Tetracladium* species (Ascomycota) recently demonstrated as potential endophytes. TEM revealed two different hyphal structures. Double fungal colonization appears to occur in *Orchis militaris* and the possible requirement of a specific fungal partner throws light on the causes of this plant's rarity and threatened status.

Keywords: *Orchis militaris*, endangered taxa, Euganean hills, mycorrhizal symbionts

The majority of vascular plant species engage in mutualistic interactions of trophic nature with defined taxa of fungi. The resulting association is broadly referred to under the collective term of mycorrhiza [7]. Recently reviewed [3], this definition encompasses a number of morphofunctional types involving different plant families and corresponding fungal groups with variable degrees of specificity. Among these types, the orchid mycorrhiza is a category with important peculiarities, as the fungi often sustain the life of these small-seeded plants through the delicate offspring stage and begin receiving carbon only once the plant is established [17, 28]. Some orchid species however remain achlorophyllous throughout their adult phase and persistently exploit their mycorrhizal fungus without a mutualistic return of organic carbon [29]. The biology of orchid mycorrhiza has been thoroughly reviewed [5, 21]. Different methods have been used to investigate these interactions; microscopy has been for a long time the primary approach [9]. The use of fluorescent stains has helped visualizing root invasion in optical microscopy [20, 22]. The advent of biomolecular techniques has allowed to verify the fungal identity with high degree of accuracy [8, 13, 15]. These studies, based on sequence comparison of the ribosomal operon region, have pointed out that the traditional association of fungi of the Rhizoctonia group (phylum Basidiomycota; subphylum Agaricomycotina) as the typical orchid symbionts [28] has several exceptions. Selosse *et al*. [24] showed that both achlorophyllous and photosynthetically active specimens of *Epipactis* are in mycorrhizal interaction with truffles (i.e., ascomycetes of the genus *Tuber*). Girlanda *et al.* [10] found that the Mediterranean orchid *Limodorum abortivum*, another member of the Neottiae tribe, was predominantly associated with basidiomycetes belonging to the *Russula* genus. In the present investigation, we focused our attention on the soldier's orchid *Orchis militaris*, an endangered Eurosiberian species whose occurrence into the Mediterranean regions is rare and localized [6]. The plant is also recorded as sporadic in Britain and confined to a few sites in Sussex, Kent, and Buckinghamshire [4]. Among the reasons that might have led to its threatened status, the presence and diffusion of specific fungal symbionts can be one of the possible limiting factors. In the northern side of its host range, this plant's fungal associations have been investigated in Estonian mine soils [27]. As pointed out by a number of evidences, the issue that mostly determines the distribution range and the persistence of plant species, once climatic requirements are met, is the below-ground interaction with

**Corresponding author*
Phone: +390498272923; Fax: +390498272929;
E-mail: squart@unipd.it

specific microorganisms [14, 30]. Accordingly, plants ending up in red lists may be suffering a limitation imposed by their microbiota population dynamics, whose status is in turn affected by environmental pollution, soil management practices, and land usage in general. In this study, we aimed at verifying whether *O. militaris* was endowed with a mycorrhizal colonization in the natural habitats under study, and, if that were the case, which was the identity of its associated fungi. In the first instance, we performed a botanical survey on this rare plant distribution in an area located in north-eastern Italy, which we coupled to the analysis of its root associations, using both microscopy- and DNA-based approaches.

Material and Methods

Plant Distribution in the Study Area

The occurrence of *O. militaris* in the area of Euganean Hills was surveyed and a census of its populations and their productivity was made by recording the following: number of specimens, number of fruits per specimen, and number of pods reaching maturity per specimen. Descriptive statistics was computed (mean, median, standard deviation) and differences among sites were evaluated by the Kruskal–Wallis one-way analysis of variance by ranks test.

Plant Collection, Root Tissue Processing, and Epifluorescence Microscopy

Orchis militaris whole specimens were collected at the flowering stage in mid-May on Mt. Lozzo, in the Euganean Hills, north-eastern Italy (45°17'47"N; 11°37'12"E). Plants were excavated with a clod of their surrounding soil and transferred to the laboratory. Root apparati were carefully cleaned from the soil under running water. Free-hand sections of both roots and tubers were obtained. Cylindrical portions were transferred into 1.5-ml polypropylene conical tubes to be used for DNA extraction and stored at −20°C. Free-hand cross-sections for light and fluorescence microscopies were stained in acridine orange 0.05% [26] for 10 min, rinsed with distilled water, and placed on microscopy slides. These were visualized under an Olympus BX60, equipped for epifluorescence with a mercury lamp. Digital images were acquired by an Olympus Camedia C3040 camera.

Light and Electron Microscopies

For light and electron microscopies, small pieces of roots were excised and fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9). Samples were post fixed in 1% osmium tetroxide, dehydrated in a gradient series of ethanol and propylene oxide, and embedded in Epon-Araldite. Semi-thin sections (1 µm) were stained with 1% Toluidine Blue. Ultrathin sections were observed, after uranyl acetate and lead citrate staining, with a Hitachi H 300 EM operating at 75 kV.

DNA Extraction and PCR Amplification of Ribosomal Intergenic Spacer

Samples obtained as described above were thawed, and 50 µl of sterile TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8) was added to the tubes, and root portions were mashed by means of flame-sterilized forceps tips. The tubes were subsequently incubated for 30 s in a microwave oven set at 700 W. The procedure was repeated twice. Samples were allowed to cool at room temperature for 5 min and centrifuged for 10 s to pellet plant debris. One µl of supernatant was withdrawn and used as template for the PCR reaction. Primers used included ITS1, ITS1F, and ITS4 [32]. The following primer pair combinations were tested: ITS1–ITS4 and ITS1F–ITS4.

One µl of the lysate containing the total DNA was treated in a PCR BioRad I-Cycler using primers at 1 µM each in a 25-µl reaction volume, and adopting the following program: initial denaturation at 95°C for 2 min; 30 cycles at 94°C for 1 min, 45°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1 µM of each primer, and 2.5 U *Taq* DNA Polymerase, recombinant (InVitrogen Life Technologies). Amplification products were visualized by loading 5 µl from the PCR reaction on a 1.5% agarose gel in 0.5× TBE buffer [18], run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera. In cases where multiple bands were observed, each was separated by cutting the gel slice containing each band over the transilluminator and purifying the DNA by means of a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified material was used as template for a further round of PCR amplification using the same condition, and band purity was verified by electrophoresis as described above. For an alternative to gel slicing and Qiaquick purification, the DNA band in the gel was simply touched by penetrating the agarose with a sterile plastic pipet tip to be subsequently dipped into the PCR reaction mix.

DNA Sequencing

One µl of the solution resulting from the above-described PCR amplification was mixed with 1 µl containing 6.4 picomoles of the forward primer, used in 0.2-ml polypropylene tubes, and then dried by incubating the open tubes for 15 min at 65°C in an I-Cycler thermal cycler. A parallel reaction was performed separately using the reverse primer. The template and primer mix was directly used for dideoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Tewantin, Australia). Merging of the complementary strands was achieved by Lasergene software v.7.2 (DNASTAR Inc., Madison, WI, U.S.A.) and the similarities to database records were investigated online through the NCBI platform (http://www.ncbi.nlm.nih.gov) using the BLAST utility. A neighbor-joining tree against selected database sequences was constructed using the software Mega v. 4 [16].

Results

Plant Occurrence and Population Data

From the two sites investigated, 44 individuals were found on Mt. Cero and 101 on Mt. Lozzo. Of these, 48% and 65% had flowers, respectively, and 32% and 56 % bore fruits. The latter site appeared to offer more favorable conditions,

sustaining a larger population that performed better in terms of both flowering and fructification. Descriptive statistical analyses further underlined the higher productivity of the Mt. Lozzo site; the right tail of the bell-shaped distribution of the flower number contained a conspicuous number of individuals bearing a high number of flowers per plant (32–34), whereas on Mt. Cero the maximum value of flowers recorded per plant was 24. The minimum numbers observed instead were 14 and 2 flowers per plant, respectively. The Kruskal–Wallis test indicated a statistically significant difference between the two stations in terms of number of flowers per specimen but not for the number of pods reaching maturity. As regards habitat, *O. militaris* appeared to prefer the ecotonal border between thermophilic oak woods and arid meadows.

Microscopic Approaches Reveal Different Fungal Structures Colonizing *O. militaris* Roots

Six plant specimens, three from each of the two sites, were dissected and analyzed. Microscopy clearly showed evidences of internal root colonization in samples from both sites. The brightfield image (Fig. 1a) showed how a large crown of the cross-section (mostly visible on the right side) was cluttered by cell-filling pelotons within the cortex. Blue light excitation of acridine orange incubated sections revealed that the filling material stained accordingly, further supporting its fungal nature. Fungal mycelium appeared confined to the cortex and only marginally approaching the central stele in which the yellow-staining vascular bundle was visible (Fig. 1b). Details of mycelial glomerular structures coiling inside cells are visible in Fig. 1c. As regards other portions of the root apparatus, as the storage tubers were stained with the same procedure and proved devoid of mycelia, cells were filled only with nonstaining amyloplasts (data not shown). Light microscopy on thin sections, obtained from tissues close to the root portions used for molecular typing, confirmed an intracellular colonization with fungal hyphae. In the outer cortical cells under the root epidermis, hyphae occupied all the cell and were unclumped, freely running in all directions (Fig. 2a, 2e, and 2b). In the inner cortical cells, glomerular structures made of thin clumped hyphae were usually visible in the center of the cell (Fig. 2a and 2c). Transverse sections allowed to appreciate a massive occurrence of hyphae in the intercellular spaces between outer cortical cells (Fig. 2d). They appeared to be of different morphology, suggesting the occurrence of at least two fungi. This was confirmed by the electron microscopy: intercellular spaces harbored hyphae having rather different sizes, shapes, and cellular contents (Fig. 2f). In the same zone, the hyphae into the cytoplasm of the cortical cells presented septa that separated fungal cells rich in electron transparent lipid bodies (Fig. 2g, 2h, and 2i). The host cell membrane always surrounded the hyphae and a thin layer of electron-dense interfacial material was observed between the latter and the host cell membrane (Fig. 2g, 2h, and 2i, arrows). The septa in these hyphae are of basidiomycetous type; in Fig. 2g (longitudinal view) and Fig. 2i (front view), the presence of entire parenthesomes (arrowheads) and cell wall bulges at the edge of the pore clearly revealed the typical dolipore structure. By electron microscopy, it was not possible to define which fungal type the clumped hyphae, building pelotons in the inner cortical cells, belonged to. Pelotons, surrounded by host cell membrane, were constituted by randomly distributed very thin hyphae, and no septa types were recognizable (Fig. 2e). Some fungal hyphae appeared collapsed (Fig. 2f).

PCR Amplification of Fungal ITS Region

The same number of specimens used for microscopy was subjected to molecular analyses. Extraction of cell content from root tissues and polymerase chain reaction amplification consistently gave rise to amplicon electrophoretic profiles displaying two bands of variable reciprocal intensity running at positions corresponding to 800 and 600 bp (hereafter designated band 4b1 and 4b2, respectively). Each of the two resolved bands was purified by extraction from the gel and reamplification under the same conditions. Amplicon purity was ascertained again by electrophoresis and DNA sequencing was carried out. Results were the following: band 4b1 (800 bp) (GenBank code EU490419)

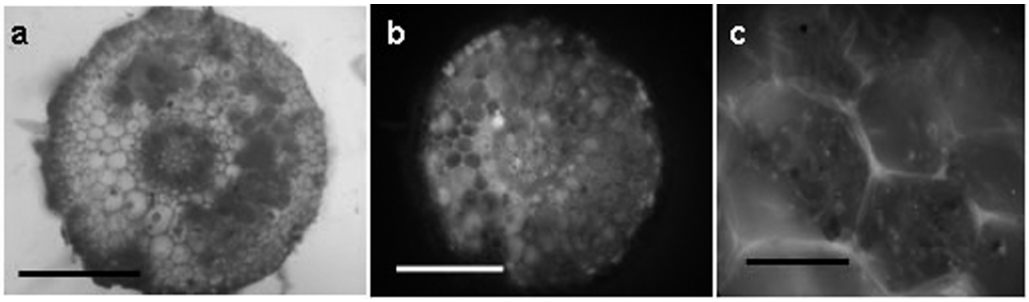

Fig. 1. Root cross-sections of *Orchis militaris* stained with acridine orange.
(**a**) Brightfield observation; (**b** and **c**) fluorescence images obtained using a WIB filter. Fungal pelotons staining orange-red are visible in over half of the cortex cells. Details on the glomerular structure of the hyphal coils are shown in *c*. Scale bars: 500 μm (**a**, **b**), 50 μm (**c**).

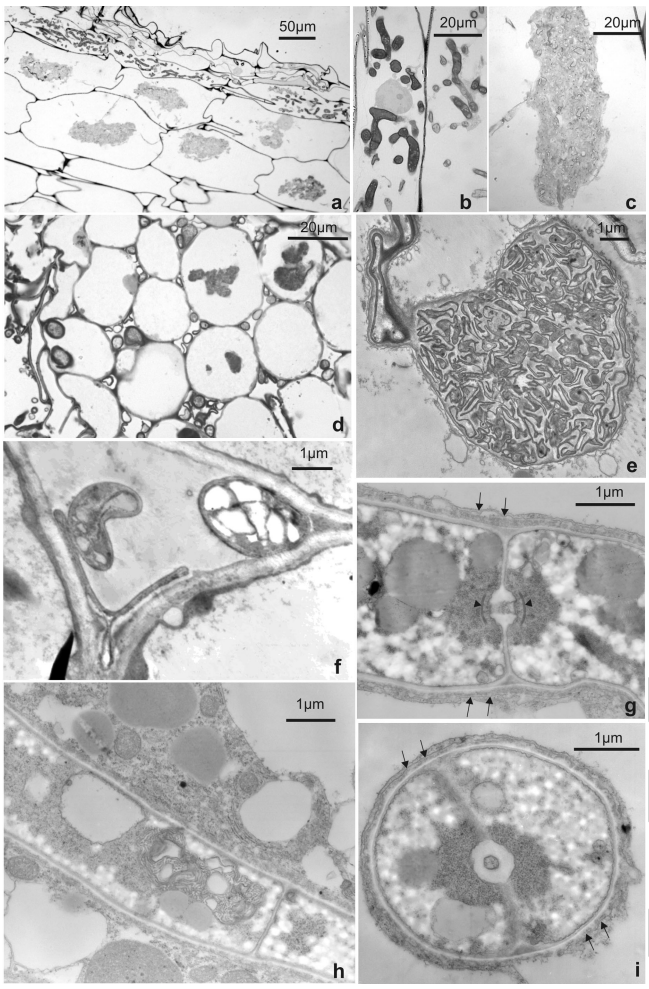

Fig. 2. Light and electron microscopies on sections of *O. militaris* roots, showing fungal colonization.
(**a**) Longitudinal section showing hyphae in the inner and outer cortical cells. (**b**) Outer cortical cells filled with unclumped hyphae; (**c**) inner cortical cell showing glomerular structures made by thin clumped hyphae; (**d**) cross-sections of a root showing hyphae in the intercellular spaces between outer cortical cells. (**e–i**) Transmission electron micrographs showing a fungal peloton surrounded by the host cell membrane (**e**); hyphae of different size and shape in an intercellular space (**f**); in the outer cortical cells, hyphae are rich in lipid bodies into the cytoplasm (**h**). Longitudinal (**g**) and frontal (**i**) sections of a dolipore septum with entire parenthesomes (**g**, arrowheads) and cell wall bulges; arrows in *g* and in *i* indicate the host cell membrane around fungal hyphae.

shared 100% similarity with sequences from a series of uncultured fungi from the roots of the same species of *O. militaris* in Hungary (AM711604–AM711613, unpublished), and 99% similarity with an uncultured fungus ascribed to the family Tulasnellaceae (Basidiomycota) found again in roots of *O. militaris* in Estonia (EU195344) [27]. Aside from this set of very related database entries, the further nearest similarities were separated by a substantial gap, scoring not more than 84% with isolates of *Epulorhiza* sp., values in the range between 81% and 86% with uncultured Tulasnellaceae, and 81% with a sequence (AJ549121) from

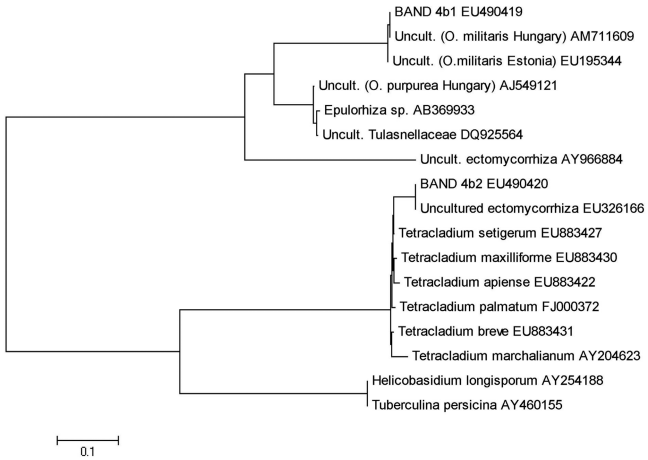

Fig. 3. Neighbor-joining clustering alignment tree showing the phylogenetic relationships between the two amplicon sequences obtained from *O. militaris* roots (bands 4b1 and 4b2) and selected database relatives.
GenBank accession codes are indicated. The optimal tree with the sum of branch length=2.01056074 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

an uncultured fungal symbiont of *Orchis purpurea*. Lower colinearities were shared with ectomycorrhizal basidiomycetes (AY966884), *Helicobasidium longisporum* (AY254188), and *Tuberculina persicina* (AY460155).

As regards band 4b2 (600 bp, GenBank code EU490420), we recorded a 99% identity with an uncultured ectomycorrhizal *Tetracladium* (mitosporic Ascomycota) found within *Salix herbacea* roots on a glacier of the Austrian alps (EU326166), 98% identity with an uncultured ectomycorrhizal ascomycete found in truffle grounds (AJ879646), and values in the 96–97% range with a series of different *Tetracladium* species including *Tetracladium maxilliforme* from mycorrhizal root tips of *Pinus sylvestris* (DQ068996). An identity of 96% was recorded with an uncultured ectomycorrhizal ascomycete isolate from the orchid *Cephalanthera damasonium* (AY833029). A neighbor-joining cladogram including some of the nearest database relatives of the two sequences was elaborated and is shown in Fig. 3.

Discussion

Two different fungal taxa, both with affinities to known plant associative fungi, have been found within the roots of *Orchis militaris*. The first belongs to the phylum Basidiomycota, and the second to the Ascomycota. Only the former is therefore within the rank that includes the *Rhizoctonia sensu lato* group, traditionally indicated as the fungal symbionts of orchids. For this fungus, indicated

by DNA homology as a *bona fide* member of the Tulasnellaceae family, a marked instance of host specificity arises from database matches. Indeed, the only cases of significant homology (99%, which is within the species boundary) pertain to isolates from roots of *Orchis militaris* collected in two geographical locations (Estonia and Hungary) very distant from the one of the present work. It is interesting to note the extent of fungal species consistency across the boundaries of this Eurosiberian plant range, as well as the restriction of this fungus association to *O. militaris*, as far as present databases allow to assess. This observation indicates how a possible constraint of a specific mycorrhizal partner could be operative for *O. militaris*. According to the literature [5], specificity for fungi by orchids can lead to higher rates of seed germination and a more efficient physiological association. Orchids undergoing prolonged dormancy periods, or those that are confined to shady habitats, are postulated to have a higher dependency on fungal carbon than evergreen or annually flowering plants, or those growing in exposed habitats [10]. As a consequence, the former can have an advantage from an efficient specific association. Fungal specificity and orchid rarity may also be correlated when the fungal partner is itself rare or distributed patchily in the environment. The consistency of identity at the species level (99%) with isolates from the same plant collected in Italy, Hungary, and Estonia suggests that this species, which is particularly threatened and rare in the southern part of its home range, could have a particularly tight host-specificity constraint. Many examples in the literature indicate that orchids and fungi associate through compatibility barriers whose nature is not entirely known. A narrow level of specificity in an orchid is envisaged as a possible reason for its rarity and vulnerability, just as a narrow food preference would in an animal species [21].

The second fungal species that co-amplified is reminiscent of the findings commented by Selosse *et al*. [23] on the unexpected but arising occurrence, as plant endophytes, of the so-called Ingoldian fungi (aquatic hyphomycetes whose teleomorphs are classified within the Ascomycota). *Tetracladium* is one of the most recurring cases for these species that appear to spend part of their cycle as water fungi, but have the capability of entering plants, including orchids. It has been postulated that these asexual aquatic hyphomycetes could exploit two niches in their lifestyle, moving from the water into plants, wherein endophytism could possibly allow the onset of their sexual stages [23]. *Tetracladium* sequences have been found also in roots of the orchid *Cephalanthera longifolia* [1]. *Tetracladium*-related sequences were also found in a survey of ectomycorrhizal fungal communities in stands of *Tuber magnatum* [19].

Among the novel aspects of the present work stands the fact that two fungi have been found to be associated with the same orchid host plant. Such dual infection of the same roots used to be considered an infrequent finding in orchid–fungi interactions [15]. The two phylogenetically distant taxa appear to share closely spaced tissues, as their amplicon bands arose within the same PCR reaction. The intensity of their amplification bands was about equal, indicating similar biomass and template abundance with no strong dominance of one of the two within the plant tissues. The presence of two fungi in association with the same plant does not necessarily prove that both play a trophic role of mycorrhizal nature. One likely interpretation is that the first could be the custom mycorrhizal partner, whereas the second could be an endophyte whose possible beneficial (or merely commensal) role remains to be established. Occurrence of endophytic microfungi in plant roots and stems, including orchids, is reported [3], but an unsolved issue standing out in these kind of studies is indeed the difficult distinction between orchid mychorrhizal and orchid endophytic fungi [2]; the former being ascertained mutualists and the latter simply microorganisms growing inside plant tissues without causing symptoms. Possibilities exist that, in case of dual presence, one of the fungi could be in mycoparasitic relation towards the other [31]. There is also a possibility that additional, yet to be detected, species could also be present and that the interactive picture could be even more complex.

Using the microscopy approach as complement to molecular-based indications, we can visually confirm the location of least one of the two kinds of fungi, as dolipore-bearing septa, characteristic of Basidiomycota were clearly evidenced. The possible co-presence of the Ascomycota in the root could nevertheless be consistent with the two different hyphal types observed in intercellular spaces, as judged by their size and cellular content. In addition, it can be underlined that the abundant central pelotons and coils cannot be tributed univocally to any of the two taxonomical divisions owing to the degenerate state of these hyphae, which is typically reported to occur in orchid mycorrhizae [12, 15, 24]. These could therefore either belong to the second taxon, indicated by the molecular analyses, or represent a differentiated stage of the first one.

In terms of plant fitness, the site on Mt. Lozzo appears to offer remarkably better conditions, yielding a more than double population. In both sites, however, the number of maturing pods was equal to about 50% of the flowers. The plant does not thrive inside woods either, where it is supposedly limited by low light, nor in the dry open fields, where it endures competition for nitrogen and water from better-adapted vegetation. It appears to strictly depend on the ecotonal transition zone. A moderate human or animal disturbance (hay reaping, pasture, recreational activities) appears to be of help, as it limits forest spread and reduces the vigor of competing meadow species. The presence of rocky outcrops and calcareous gravel from sedimentary depositions is also important, as it originates bare ground

spaces that are particularly suitable for *O. militaris* growth. The discussed results, constituting the first report on the possibility of a double myceliar interaction with roots of *Orchis militaris,* enable to cast some further light on the biology of this endangered species and to speculate on the reasons of its rarity in a considerable part of the home range. Studies in the literature have examined habitats and conditions limiting the diffusion of related orchids such as *Orchis simia* [33], a population of which, originating from a single individual, was followed in The Netherlands for several years. Juveniles would appear only four years after the mother plant had flowered and the overall spread of the species was slow, amounting to a total of 65 individuals in 10 years. Mycorrhizae are regarded as a critical issue in overall terrestrial plant survival. To some authors, they could be envisaged as the key of the plants' success in land colonization [14]. Considering the dependence of orchids on these interactions in overcoming the offspring stage, and having ascertained the presence of mycorrhizal taxa in *O. militaris*, we could hypothesize that their availability through soil could be a factor limiting this *Orchis* establishment and diffusion. The need for two different fungi, if both were critically involved in mutualistic interactions, could further reduce the environmental chances for *Orchis militaris*, contributing to explain its critical status. The requirement could also be reciprocal, in that the distribution and persistence of the fungi themselves could require the plant presence; it has long since been reported that orchid mycorrhizal fungi are hardly ever isolated from soil far from their hosts [11], although orchid seed baiting methods have in part counteracted such a view.

An additional factor that is to be considered in interpreting a plant species distribution is the possibility of being connected through a myceliar net to one or more plants. Such phenomena, defined under the term of Common Mycorrhizal Network, are earning an increasing awareness by the scientific community. An important consequence of the hyphal web is the possible transfer of nutrients among plants. In this respect, even green chlorophyllous orchids have been shown to obtain organic carbon from other plants *via* the fungus to an extent reaching values up to 85% [25]. Concerning such possibilities, it is to be reported that *O. militaris* stems were consistently more numerous when found near woody shrubs of *Cotynus coggygria* or *Viburnum lantana* on the Mt. Cero site. The second location examined, Mt. Lozzo, where *O. militaris* numbers were more than double, is also more densely covered by bushy vegetation and profuse groups of the orchid individuals were typically spotted near *C. coggygria*, *Ostria carpinifolia*, *Quercus pubescens*, or *Rubus ulmifolium*.

The observations presented suggest that, to plan actions for the conservation of this kind of endangered plant species, an integrated approach has to be recommended. The ecological analysis can benefit from the microbial ecology-based perspective in trying to individuate possible limiting resources of biotic nature. The possibility that more than one fungal endophyte is required to fully sustain plant development could provide novel insights in interpreting its environmental outcome. Future work will be devoted to assess which fungal symbionts do associate with other orchids of different abundance and status in the same area. In addition, attempts to isolate and cultivate the fungal endophytes will be carried out to provide the possibility of *in-situ* land inoculation with the mycorrhizal symbionts. This practice will be tested in the pursuit of enhancing the survival and fitness of these endangered orchid species.

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