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Movement of Rhizobia Inside Tobacco and Lifestyle Alternation from Endophytes to Free-Living Rhizobia on Leaves

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Rhizobia are well-known for their ability to infect and nodulate legume roots, forming a nitrogen-fixing symbiosis of agricultural importance. In addition, recent studies have shown that rhizobia can colonize roots and aerial plant tissues of rice as a model plant of the *Graminaceae* family. Here we show that rhizobia can invade tobacco, a model plant belonging to the *Solanaceae* family. Inoculation of seedling roots with five GFP-tagged rhizobial species followed by microscopy and viable plating analyses indicated their colonization of the surface and interior of the whole vegetative plant. Blockage of ascending epiphytic migration by coating the hypocotyls with Vaseline showed that the endophytic rhizobia can exit the leaf interior through stomata and colonize the external phyllosphere habitat. These studies indicate rhizobia can colonize both below- and above-ground tissues of tobacco using a dynamic invasion process that involves both epiphytic and endophytic lifestyles.

Keywords: Alternative lifestyle, endophytic and epiphytic rhizobia, tobacco, GFP tagging, rhizobial movement.

Rhizobium can form symbiotic nitrogen-fixing nodules on legume roots and provide these plant hosts with fixed nitrogen, enabling them to grow productively in nitrogen-limited agricultural soils. The amount of symbiotic nitrogen fixation accounts for most of the world's land-based biological nitrogen fixation and contributes significantly to global grain production [2]. Therefore, the *Rhizobium*-legume symbiosis is the most thoroughly studied plant-microbe interaction at the molecular level [11, 20, 31, 33].

Recently, much attention has been devoted to studies on the beneficial association of rhizobia and cereals, since these bacteria were found to be natural endophytes of important cereal crops and promoted their growth with an increase in grain yield at harvest while reducing their dependence on chemical fertilizer inputs, independent of root nodulation and biological N_2-fixation [34, 35]. Rhizobia are now known to develop endophytic associations with roots of domesticated and wild rice [3, 4, 6, 25, 34, 35], maize [13], wheat [22, 29], barley [22], canola [16, 22, 24], lettuce [24], and *Arabidopsis thaliana* [30]. Therefore, this beneficial endophytic association of rhizobia with various types of plants (and the consequential promotion of plant growth that ensues) heightens its interest and potential value as a broad host-range biofertilizer for sustainable agriculture to produce the world's most important crops.

Originally, it was demonstrated that the association of rhizobia with cereals was mainly located in root interior tissues including intercellular spaces, cortical parenchyma and the vascular system such as xylem vessels [25, 27]. More recently, it has been shown that rhizobia inoculated into the rice rhizosphere not only infect and colonize its root interior, but also conduct an ascending endophytic migration within roots into leaf sheaths and leaves where they develop dense local endophytic populations [7]. Thus, this endophytic *Rhizobium*-cereal association is far more inclusive, invasive and dynamic than previously thought,

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including dissemination in both below-ground and above-ground tissues and enhancement of growth physiology by several rhizobial species.

The finding of rhizobial migration within the rice tissue interior prompted us to investigate whether rhizobia can also infect tobacco and similarly show their disseminating migration ability like they do in rice. The results of this study show that the rhizobia can also infect tobacco roots and ascend to aerial plant tissues via two routes, demonstrating alternating lifestyles involving epiphytic - endophytic - epiphytic colonization cycles in association with this Solanaceous plant.

Materials and Methods

Rhizobia, plasmid, and plant

Five *Rhizobium* strains and one plasmid were used in this study (Table 1). The plasmid vector pHC60 [6] encodes for tetracycline resistance and contains the *gfp* gene that is constitutively expressed from a constitutive *lacZ* promoter without the required expression of *lacZ*. This vector contains a stability region so that expression of its *gfp* is more stably maintained within bacterial cells in the absence of selective pressure [6]. The half-life of the GFP protein is approximately one day [32] and so green fluorescent cells are considered to be metabolically active *in situ* and can be observed for a long time due to the *gfp*-gene constitutively expressed. For construction of the GFP-tagged strains, the pHC60 vector was transferred to the wild type rhizobia species using the triparental mating method [9].

Seeds of tobacco (*Nicotiana tabacum* L.) Honghuadajinyuan and four legume species (*Medicago sativa* L., *Sesbania rostrata, Astragalus sinicus* L. and *Pisum sativum* L.) were obtained from the Institute of Botany, Chinese Academy of Sciences, Beijing, China. These legumes were used to identify whether the GFP-tagged rhizobial strains of *Sinorhizobium meliloti* 1021 and USDA 1002, *Azorhizobium canlinodans* ORS 571, *Mesorhizobium huakui* 93, *Rhizobium leguminosarum* USDA 2370 can nodulate their respective host legume.

Tobacco Seed Treatment and Plant Growth Under Gnotobiotic and Greenhouse Conditions

Tobacco seeds were treated with 95% ethanol for 5 min, washed 3 times with sterile water, then with 1% $AgNO_3$ for 3–5 min, and finally washed 3 times with sterile water. Seeds thus treated were placed on LB plates in the dark for 3 days at 28°C to verify that they were surface-sterilized. Afterwards, they were germinated on wet Whatman # 1 filter paper in Petri dishes for 2–3 weeks to obtain axenic seedlings with two cotyledons and longer hypocotyls with sufficient length to be easily used for the Vaseline- coating hypocotyl experiment (below in detail). Then they were transferred directly into bottles which were 8 cm in diameter, 15 cm in height and contained 200 cm^3 of sterilized vermiculite plus 120 ml of half-strength MS medium [23] without sucrose. Each bottle contained ten or more seedlings. The seedlings in 18 bottles were inoculated with GFP-tagged rhizobia. Several days later, 9 bottles as three replicates of each treatment were used for examination of inoculated GFP-tagged rhizobia by confocal laser scanning microscopy, and 9 others also as three replicates were used for viable plating experiments. The seedlings in the remaining bottles were uninoculated negative controls. The bottled seedling cultures after inoculation were covered with sterile, adhesive transparent paper with many small holes (Zhentai, Beijing, China) and incubated in a growth chamber programmed with a 14-h photoperiod and 28/25°C day/night cycle for seedling growth.

Table 1. Plasmids and bacterial strains used in this study.

Plasmid and Rhizobial strains	Characteristics and antibiotic amount used	Source
pHC60	a broad host range plasmid with *gfp*, Tc^r (10 μg/ml)	City University of New York (reference 6)
Sinorhizobium meliloti 1021	Sm^r (50 μg/ml)	Inst of Plant Physiology, Shanghai, China
Azorhizobium caulinodans ORS 571	Amp^r (100 μg/ml) Cb^r (500 μg/ml)	Academy of Agriculture, Beijing, China
Sinorhizobium meliloti USDA 1002	Km^r (50 μg/ml)	Chinese University of Agriculture, Beijing, China
Rhizobium leguminosarum USDA 2370	Sm^r (50 μg/ml)	Chinese University of Agriculture, Beijing, China
Mesorhizobium huakuii 93	Sm^r (10 μg/ml)	Nanjing University of Agriculture, China
Sinorhizobium meliloti 1021 (*gfp*)	Sm^r (50 μg/ml) Tc^r (10 μg/ml)	City University of New York
Azorhizobium caulinodans ORS 571(*gfp*)	Amp^r (100 μg/ml) Cb^r (500 μg/ml) Tc^r (10 μg/ml)	This study
Sinorhizobium meliloti USDA 1002 (*gfp*)	Km^r (50 μg/ml) Tc^r (10 μg/ml)	This study
Rhizobium leguminosarum USDA 2370 (*gfp*)	Sm^r (50 μg/ml) Tc^r (10 μg/ml)	This study
Mesorhizobium huakui 93 (*gfp*)	Sm^r (10 μg/ml) Tc^r (10 μg/ml)	This study
E. coli DH5α (*gfp*)	Tc^r (10 μg/ml)	This study

Sm^r, streptomycin resistance; Amp^r, ampicilin resistance; Cb^r, carbenicillin; Km^r, kanamycin; Tc^r, tetracycline resistance.

Other axenic seedlings were planted in individual pots containing 4 liters of sterilized vermiculite and sand (1:1) in order to compare the difference from those grown in bottles in growth chamber. After inoculation of GFP-tagged rhizobia, they were grown in greenhouse programmed with natural light photoperiod of 10-14 h, temperature 25–30°C day/20–25°C night cycle.

Rhizobia Inoculation

To prepare the inoculum, the GFP-tagged rhizobia were cultured at 28°C for 48 h in TY medium [1] containing 5 g tryptone, 3 g yeast extract and 0.88 g $CaCl_2 \cdot 2H_2O$ per liter with antibiotics mentioned in Table 1. Cultured cells were harvested at 2,180 g, washed twice in PBS buffer (pH 7.4), and re-suspended in the same buffer to 10^8 cells/ml ($OD_{600\,nm}$=0.8) [19]. After the axenic seedlings were grown in bottles for a 3 days recovery, 5 ml inocula of the rhizobial suspension (5×10^8/ml) were delivered carefully to the roots by introducing the pipette tip below the vermiculite-sand surface while avoiding contamination of the above-ground epidermal surface. Plants were inoculated with 25 ml of the rhizobial suspension one week after transplantation into pots.

Microscopic Examination

Tissues of roots, stems, and leaves were excised from the tobacco plants after they were removed from the bottles at 3, 7, 10, 14, 21, 28, 35, 42, 49, and 70 days after inoculation (DAI). For microscopy, fresh stem and leaf tissue segments were fixed for 30 min in 0.5% glutaraldehyde in 200 mM phosphate buffer (pH 7.2) to intensify their red autofluorescence without affecting the green fluorescence of the GFP protein [17, 21], followed by rinsing with sterile water. Freehand longitudinal and cross-sections were made with a razor blade that was washed between cuttings with sterile water and 70% ethanol and wiped with sterile absorbent paper to avoid cross-contamination of tissues during excision and sectioning. Tissue sections were rinsed with sterile water, mounted in 0.2% agar in 0.2 M phosphate buffer (pH 7.2) on slides and examined using a Bio-Rad MRC 1024 laser scanning confocal microscope with 488 nm and 568 nm bandpass filters to excite Gfp and capture the green fluorescence from GFP-tagged bacteria and the red autofluorescence from host tissue, respectively. A Nikon E800 scanner and digital camera were used to acquire confocal images of GFP-labeled bacteria and host cells in optisections positioned at the cut surface and others located approximately 20 μm beneath the surface of the tissue sections. These images were then merged into loss-less montage composite images using Confocal Assistant Software Ver. 4.02 (Todd Clerke Brelje, URL ftp://ftp.genetics.bio-rad.com/Public/confocal/cas). The local abundance and distribution of green fluorescent bacteria within leaf cross-sectioned tissue were measured using Center for Microbial Ecology Image Analysis System software (CMEIAS,) [8]. For calculating cell abundance, each bacterium was represented by a projected area of 2 adjacent pixels, equivalent to 1.92 μm^2.

Vaseline-Coating Hypocotyl Experiment

The hypocotyl region between roots and two cotyledons of each tobacco seedling was carefully coated with autoclaved viscous Vaseline by using a sterilized Chinese brush pen. Then 10 hypocotyl-coated seedlings were transplanted into one bottle as a replicate. After three days of recovery in a growth chamber, they were inoculated with GFP-tagged rhizobia and incubated further. All together there were three replicates. At the same time, the plants with Vaseline-coated hypocotyls were also transplanted in pots and grown in greenhouse. Other seedlings without Vaseline coating were inoculated with GFP-tagged rhizobia were grown at the same condition as those mentioned above and used as negative controls.

Viable Plate Counting of Endophytic Rhizobia Populations Within the Tissue Interior

The tobacco roots from gnotobiotic seedlings were carefully removed from each bottle, excised into roots, stems and leaves, washed with sterile water, blotted dry, and weighed. Then, each of them was surface-sterilized by vortexing for 1 min in PBS containing 1% bleach, 0.1% SDS, and 0.2% Tween 20 [10]. After surface-sterilization, they were rinsed 4 times with sterile water, placed on TY agar plates for 1 h, and then removed. These plates developed no colonies when incubated for 2 days at 28°C, verifying that the excised roots, stems and leaves were surface-sterilized by this protocol. To enumerate the viable, endophytic rhizobia, the excised roots, stems and leaves were macerated with a sterile mortar and pestle, diluted in PBS containing 20% glycerol, and spread on TY agar plates supplemented with tetracycline (10 μg/ml) and the other appropriate antibiotic(s) for each test strain (Table 1).

To enumerate the viable populations of endophytic rhizobia inside tobacco grown in the pots in greenhouse, the roots, stems and leaves were collected and processed as described above. This plating experiment was replicated three times, each with 30 plants.

To determine whether the legume root-nodulation characteristics were affected by passage of the GFP-tagged rhizobial inoculum through the tobacco plants, isolates recovered from the tobacco plating experiments were tested for nodulation ability on their respective legume host (*M. sativa* L. for *S. meliloti* 1021 and USDA1002, *S. rostrata* for *A. caulinodans* ORS571, *A. sinicus* L. for *M. huakui* 93, and *P. sativum* L. for *R. leguminosarum* bv. viciae USDA 2370). Legume seeds were surface-sterilized with 70% ethanol for 10 min, washed 3 times with sterile water, then with 0.1% $HgCl_2$ for 10 min and washed 3 times with sterile water. Axenic legume seedlings were transferred to sterile tubes (4 cm diameter×30 cm length) containing 100 cm^3 of vermiculite and 20–30 ml of Fähraeus nitrogen-free nutrient medium [12], then inoculated with 1 ml containing 10^6 cells of GFP-tagged rhizobia inoculum and cultured in the growth chamber. At 30 DAI, the root nodules were removed, sliced in half and examined by confocal microscopy to check for green fluorescence at the cut face of sections.

RESULTS

Infection, Colonization, and Dissemination of Endophytic Rhizobia in the Interiors of Tobacco Roots, Stems, and Leaves

Examination of tobacco seedlings inoculated with *S. meliloti* 1021 at 7 DAI showed green fluorescent bacterial cells colonized epiphytically on the root epidermis, including within some lysed root hairs (figure not shown). At 10 DAI, fluorescent bacterial cells colonized around lateral root junctions and between displaced root epidermal cells where they had gained entry into the root cortex and propagated to higher populations in intercellular spaces,

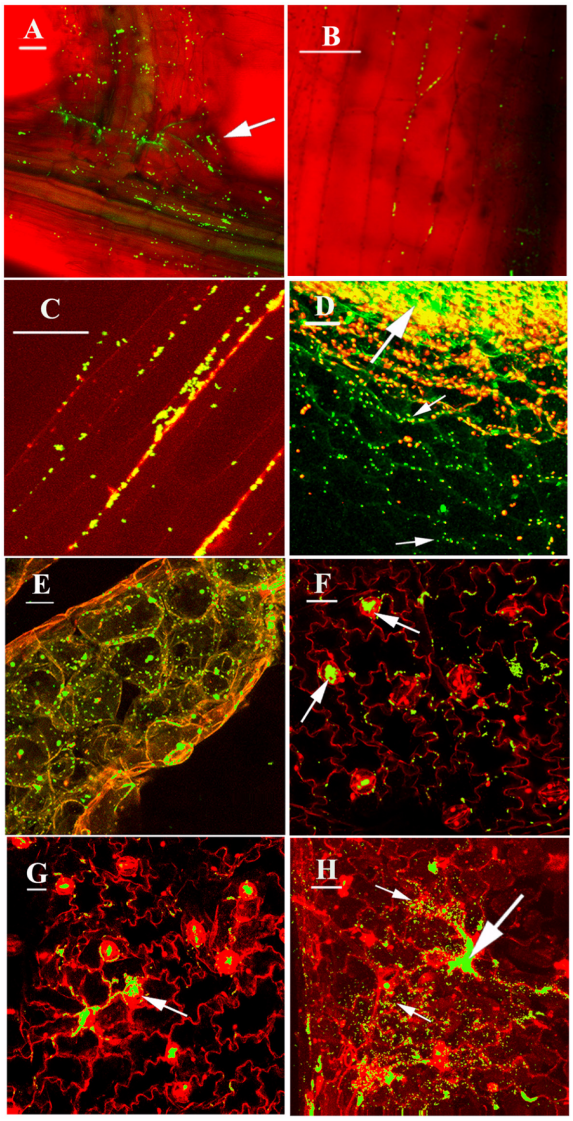

Fig. 1. Confocal laser scanning micrographs of endophytic, GFP-tagged cells of *Sinorhizobium meliloti* 1021 colonized within healthy, below- and above-ground tobacco tissues.
A, rhizobia in tobacco roots harvested at 10 DAI, showing colonization and infection at lateral root (arrow) emergence. This image was prepared from a confocal optisection located 24 μm beneath the epidermal root surface. **B**, longitudinal section of stem tissue harvested at 21 DAI, showing random, discontinuous rows of green fluorescent bacteria in intercellular spaces of the cortex near the vascular system. **C**, longitudinal section of stem tissue harvested at 28 DAI, showing that fluorescent bacteria were more extensively colonized in intercellular spaces. **D**, transverse section of stem tissue harvested at 28 DAI, showing that more green fluorescent bacteria situating in intercellular spaces of stem cortex (small arrows), large arrow indicating xylem vessel of stem vascular system. **E**, longitudinal section of the first leaf tissue at 35 DAI, showing green fluorescent bacteria colonizing leaf mesophyll tissue. **F**, leaf epidermis at 35 DAI, showing dense local populations of fluorescent rhizobia in stomata (arrows) and regularly dispersed rhizobia in intercellular spaces of abnormal polygon epidermal cells. **G**, the leaf epidermis at 42 DAI, showing numerous fluorescent rhizobia colonizing the stomata, and spreading out (arrow). **H**, the leaf epidermis at 49 DAI, showing the numerous fluorescent rhizobia partially covering the leaf surface around the stomata at random (small arrows) when they were spreading out (large arrow). Bar scale equals 50 μm.

resulting in various sized aggregates of green fluorescent bacteria distributed within the root interior (Fig. 1A) .

Longitudinal stem sections of seedlings harvested at 21 DAI had random discontinuous rows of GFP-tagged rhizobial cells located within cortical intercellular spaces near the vascular system (Fig. 1B). At 28 DAI, green fluorescent bacteria had colonized the intercellular spaces of the cortex more extensively (Fig. 1C). Transverse sections of stem contained rhizobia located in intercellular spaces of cortex (Fig. 1D), consistent with their distribution in longitudinal sections of the stem. At 21 DAI, some fluorescent cells of rhizobia were randomly dispersed inside mesophyll tissue of the first tobacco leaf (figure not shown). However, at 35 DAI numerous rhizobia had colonized the first leaf within intercellular spaces and mesophyll cells (Fig. 1E). CMEIAS digital image analysis of Fig. 1E indicated that at least 1,441 (Š 25%) of the 5,741 green fluorescent bacteria present in the leaf cross-section were located within abodes confined by the mesophyll cell walls, whereas the remainders were located within intercellular spaces. Confocal microscopy indicated that 20–50% of the inoculated samples were colonized internally by GFP-tagged rhizobia by 20–30 DAI, and 100% of the plant samples were colonized endophytically by these bacteria within 35 days and later.

Lifestyle Alteration of Rhizobia from Endophyte to Epiphyte

Other first leaf samples harvested at 35 DAI contained numerous fluorescent rhizobia forming various sized aggregates within stomata and on intercelluar spaces of epidermal cells with abnormal polygon form in morphology (Fig. 1F). *In situ* CMEIAS image analysis of the first leaf epidermis at 35 DAI indicated that the individual aggregates of green-fluorescent rhizobia ranged in size between 17 to 7,274 cells covering 9.1% of the phylloplane surface. The spatial densities of fluorescent bacteria (cells/mm^2) on the first leaf epidermis were 14,399 at 35 DAI (Fig. 1F), 25,145 at 42 DAI (Fig. 1G), and 38,316 at 45 DAI (Fig. 1H), and the largest microcolony aggregates sampled at these later times were 3.11-fold and 5.25-fold higher than the largest microcolony found at 35 DAI. These local increases in population density *in situ* suggest that the endophytic rhizobia eventually exit the leaf interior through stoma cavities and then grow into microcolonies on the phylloplane epidermal surface (Fig. 1G and 1H). Finally, the endophytic rhizobia exited through stomata on the second and third leaves of the inoculated tobacco plants and populated their leaf epidermal surfaces (figures not shown).

Blocking Epiphytic Rhizobial Ascending Migration Along the Stem Surface by Vaseline-Coating the Hypocotyl

In order to verify that the epiphytic GFP-tagged rhizobia on leaf surface (Fig. 1H) really come from within the leaf

interior, a special method of Vaseline-coating hypocotyl was utilized to block epiphytic rhizobia from ascending along the stem surface while still simultaneously allowing their endophytic migration up within internal tissues. Following inoculation of hypocotyl surfaces on axenic seedlings, green-fluorescent rhizobia could be found on stem and leaf surfaces by confocal microscopy and cultured on TY agar if the plants were left uncovered, but these methods did not detect green fluorescent bacteria on these aerial epidermal surfaces if a ring of vaseline were applied above the hypocotyl position when inoculated, indicating that this method blocked the rhizobial epiphytic ascending migration. Under these same conditions, endophytic green fluorescent bacteria could be detected from cut surfaces of interior stem and leaf tissues, indicating that the Vaseline coating did not prevent endophytic colonization of these tissues. Similar results were obtained using tobacco plants grown in pots in the greenhouse without gnotobiotic conditions.

Enumeration of Culturable Endophytic Rhizobia Within Tobacco Tissues and Nodulation of Isolates from Tissues on Legume Roots

In order to verify that the rhizobial endophytes in root interior could migrate up from inoculated tobacco roots to their aerial plant parts (like in rice [7]), we examined rhizobial populations within the below-ground and above-ground tissues. Three different plating experiments verified this endophytic colonization strategy of disseminating migration from primary host root infection to aerial plant parts. In the first experiment, enumeration of GFP-tagged *Sinorhizobium meliloti* 1021 inoculated into the rhizosphere of gnotobiotically-grown tobacco plants indicated a transient burst of endophytic population growth followed by maintenance of persistent or slightly declining populations within roots, stems and leaves (Fig. 2).

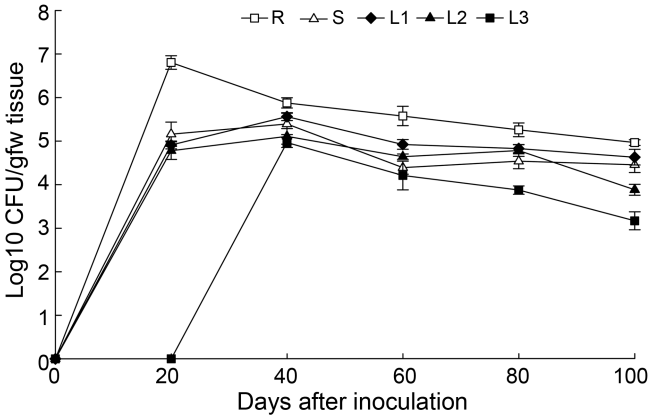

Fig. 2. Population dynamics of GFP-tagged *Sinorhizobium meliloti* 1021 in various tobacco tissues after inoculation of roots and growth in enclosed gnotobiotic culture.
Tissue samples were surface-sterilized; roots (R), stem (S), the first leaf (L1), the second leaf (L2). Data points and bars are means and standard errors of the mean from three replicates at each sampling time.

The second experiment extended these results indicating that all test strains endophytically colonized tobacco roots, stems and leaves, with *Sinorhizobium meliloti* 1021, *Azorhizobium caulinodans* ORS 571 and *Mesorhizobium huakui* 93 achieving higher endophytic populations than did *Rhizobium leguminosarum* USDA 2370 and *Sinorhizobium meliloti* USDA 1002 (Fig. 3). Interestingly, *A. caulinodans* ORS571 and *M. huakuii* 93 developed higher culturable endophytic populations in aerial plant parts than in the roots. Thus, the degree to which rhizobia establish endophytic populations within tobacco varies among different species. This is different from endophytic populations of these rhizobial species in rice [7].

The third plating experiment compared the persistence of viable populations of endophytic *Sinorhizobium meliloti* 1021 within roots, stems and the first, second and third leaf of tobacco in enclosed gnotobiotic bottle culture versus in open pots in the greenhouse. The results (Fig. 4) indicated that the rhizobial endophytic populations were 1-3 orders of magnitude higher when cultured in enclosed gnotobiotic culture than in open pots, except for the first leaf, in which the population was at the same magnitude level. This difference is likely to be due to the higher humidity and temperature of the enclosed gnotobiotic culture method.

For above each plating experiment, all ten randomly picked colonies were able to nodulate their host corresponding, specific legume host under gnotobiotic conditions, and longitudinal sections of nodules showed green fluorescent

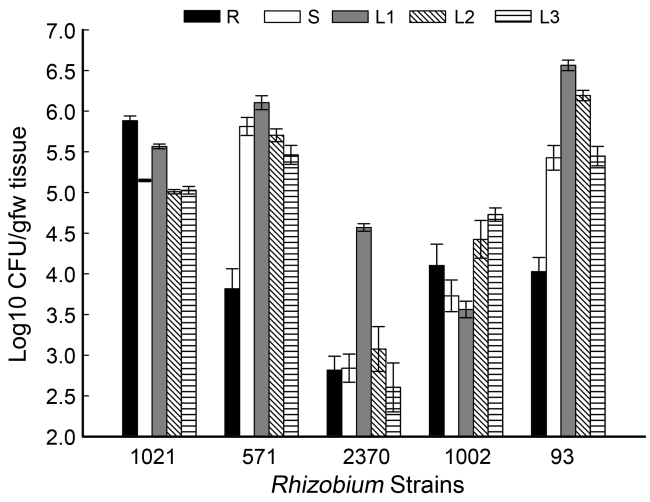

Fig. 3. Culturable population densities of GFP-tagged *S. meliloti* 1021, *Azorhizobium caulinodans* ORS 571, *Rhizobium leguminosarum* USDA 2370, *S. meliloti* 1002 and *Mesorhizobium huakuii* 93 within surface-sterilized tissue samples of roots (R), stems (S), 1st leaf (L1), 2nd leaf (L2), and 3rd leaf (L3) of tobacco plants grown under gnotobiotic condition and harvested at 40 DAI.
Data reported are the means ± standard errors of the means from three tissue sample replicates plated on TY media.

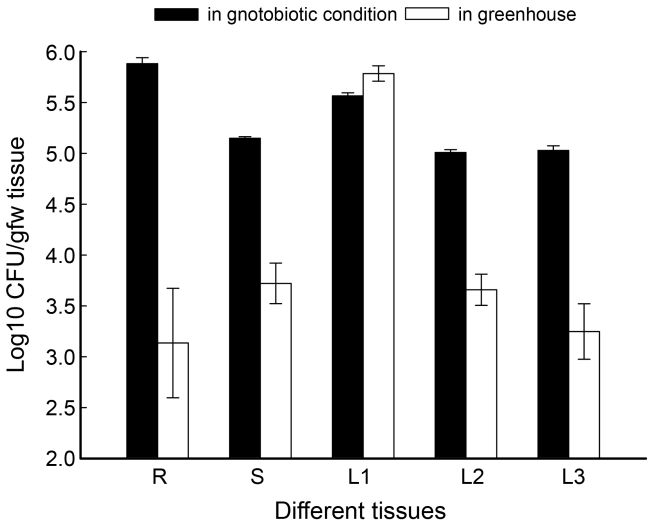

Fig. 4. Culturable population densities of GFP-tagged *S. meliloti* 1021 within tissue samples of roots (R), stems (S), 1st leaf (L1), 2nd leaf (L2), and 3rd leaf (L3) of tobacco plants grown in growth chamber under gnotobiotic condition and in open potted soil plus vermiculite (1:1 ratio) in greenhouse and harvested at 40 DAI. Data reported are the means ± standard errors of the means from three tissue sample replicates plated on TY media.

bacteria, confirming that the endophytic rhizobia in tobacco are the same inoculated strains of GFP-tagged rhizobia.

Discussion

Direct confocal microscopy reconfirmed that rhizobia can endophytically colonize tobacco roots and also migrate from within its root interior up to above-ground aerial parts of its stems and leaves like it does in rice [7]. Further experiments using Vaseline coating of the hypocotyls to experimentally block epiphytic ascending migration indicated both epiphytic and endophytic ascending migration of rhizobia on tobacco. In contrast, only endophytic ascending migration was found in rice, possibly because its leaf sheath surface was not favorable for epiphytic migration. An additional important finding in this study was that the endophytic rhizobia could exit internal tobacco leaf tissues through the stromata and then disperse and actively colonize the phylloplane leaf surface. Thus, rhizobia can significantly alter their ecological niche by displaying a dynamic lifestyle, starting with free-living persistent bacteria in soil, then as rhizoplane epiphytes, followed by endophytic colonization within below-ground and above-ground plant tissues, and finally as phylloplane epiphytes. How great it is!

These results using rhizobia are consistent with earlier studies of Hallmann *et al*. [15] and Rahme *et al*. [26] who reported that bacterial endophytes could enter leaf stomata if they disseminated on plant surfaces, and by Gyaneshwar *et al.* [14] and James *et al.* [18] who found that *Herbaspirillum seropedicae* and *Gluconacetobacter diazotrophicus* clustered around stomata leaf cavities during their colonization of rice and sugarcane. Future experiments are required to resolve the morphological status of the leaf mesophyll cells containing these endophytic GFP-tagged rhizobia and whether their presence promotes tobacco plant growth.

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