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From Roots to Leaves: The Capacity of *Micromonospora* to Colonize Different Legume Tissues

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ABSTRACT

An important number of *Micromonospora* strains have been reported from nitrogen-fixing root nodules of legume and actinorhizal plants. However, the question of whether this bacterium can also be found in other parts of these plants remains unanswered. More than 150 strains were recovered from different *Lupinus angustifolius* and *Pisum sativum* tissues, including leaves, stems, roots, and nodules. In all, 97% of the isolates were identified by 16S ribosomal RNA gene sequence in the target genus and were associated with 27 different *Micromonospora* spp. Plant-polymer-degrading enzymes are suspected to play a role in the colonization of plants. To this end, bacterial enzymatic activity assays for amylases, cellulases, chitinases, pectinases, and xylanases were determined. All strains produced xylanases and pectinases, while

98.6, 98, and 94.6% of them produced amylases, cellulases, and chitinases, respectively. The most productive strains included seven isolates from *P. sativum* and one from *L. angustifolius*. Strain *Micromonospora lupini* ML01-*gfp* was used to determine its capacity to reach and colonize different plant organs using *P. sativum* as the plant model. Stem and leaf samples were monitored by optical and fluorescence microscopy to locate the tagged strain. These results strongly suggest that *Micromonospora* is able not only to infect nitrogen-fixing nodules but also to reach other parts of the host plant, especially the leaves.

Keywords: ecology, endophytes, microbiome, microorganism, plants, rhizosphere and phyllosphere

Providing food for a global population that is estimated to reach 9 billion by 2050 will require that agricultural yields increase by 70 to 100% (FAO 2009; Liu et al. 2017; Reid and Greene 2012). One proposal to increase crop productivity is the use of microbial endophytes as biofertilizers. Endophytes are microorganisms that live, at least part of their lives, inside healthy plant tissues, in many cases providing a benefit to its host (Hardoim et al. 2008; Rosenblueth and Martínez-Romero 2006; Sturz and Nowak 2000). Indeed, plants are thought to rely on their microbial partners to thrive in adverse environmental conditions, which are increasing because of climate change (Liu et al. 2017; Redman et al. 2011).

Many bacteria-plant interactions begin in the rhizosphere (Bulgarelli et al. 2012, 2013; Kent and Triplett 2002). Plants release significant amounts of photosynthates or exudates from their roots, which influence microbial communities and act as attracting signals (Bulgarelli et al. 2013). Furthermore, some of these bacteria also colonize the rhizoplane and even reach internal plant tissues (Hardoim et al. 2008; Harrison and Griffin 2020; Haruna et al. 2018). Bacterial density and diversity in the different compartments or plant organs vary, being highest in the rhizospheric environment and lowest in the plant's stems and leaves (Bais et al. 2006; Benizri et al. 2001; Bulgarelli et al. 2013; Hallmann 2001). It is suggested that combinations of root exudates that act as attractants, the ability to bind to the roots, and overcoming the plant's immune system have a decisive impact on the ability of a soil bacterium to reach internal plant tissues (Bulgarelli et al. 2013; Liu et al. 2017; Reinhold-Hurek et al. 2015). Although species diversity in the different plant parts varies and depends, in part, on the capacity of a bacterium to overcome the different plant barriers, there is evidence that some overlap exists (Bai et al. 2015; Bodenhausen et al. 2013; Bulgarelli et al. 2014). Thus, it is expected that some of the soil bacteria that interact with plants may be found in all or some of the plant's internal organs. Learning about the composition of the bacterial communities between the different plant parts (e.g., leaves, roots, or shoots) is essential for the development of biofertilizers in agriculture (Liu et al. 2017).

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There is now good evidence that endophytic bacteria secrete plant cell-wall-degrading enzymes that include cellulases, xylanases, and pectinases, among others, to access plant tissues (Compant et al. 2010; James et al. 2002; Kandel et al. 2017; Lodewyckx et al. 2002; Reinhold-Hurek et al. 2006; Sessitsch et al. 2012). On the other hand, many bacterial endophytes contain a very low amount of these enzymes (Mastrorunzio et al. 2008; Trujillo et al. 2014). Therefore, our understanding of the distribution and role of these proteins across endophytic bacteria is far from complete. Several studies have shown that *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* appear to be the dominant groups in plant-associated microbiomes (Bulgarelli et al. 2012; de Oliveira Costa et al. 2012; Haruna et al. 2018; Kandel et al. 2017; Liu et al. 2017). *Micromonospora* is a soil, Gram-positive, filamentous actinobacterium that, in the last two decades, has been frequently isolated from the rhizosphere and roots and, mainly, from nitrogen-fixing nodules of legume and actinorhizal plants (Carro et al. 2012, 2013; Garcia et al. 2010; Genilloud 2015; Trujillo et al. 2006, 2007, 2010, 2015; Valdés et al. 2005). The presence of *Micromonospora* within the internal tissues of several legume nodules was confirmed by fluorescent tagging and immunoelectron microscopy (Benito et al. 2017; Trujillo et al. 2010). Several studies have also demonstrated that micromonosporae strains degrade various plant polymers and have an important role in organic material turnover (de Menezes et al. 2008, 2012; Ichiwaki et al. 2017). However, except for cellulose, enzymatic profiles have been studied in a low number of strains. Interestingly, the genome of the model endophytic strain *Micromonospora lupini* Lupac 08, isolated from *Lupinus angustifolius* (Trujillo et al. 2007), contained a high number of genes encoding for enzymes involved in plant-polymer degradation (Trujillo et al. 2014). Similarly, Carro et al. (2018) showed that most *Micromonospora* strains isolated from diverse environments and representing 40 species also contained several of these genes. More recently, it was reported that some of these genes were overexpressed when *Micromonospora* was grown in the presence of root exudates (Benito 2020). The ability of *Micromonospora* to infect and colonize different legume species and function as a potential plant-growth-promoting bacterium is relevant because this microbe enhances symbiosis without interfering with the host and its nodule-inducing and nitrogen-fixing microbes (Benito et al. 2017; Martínez-Hidalgo et al. 2015; Trujillo et al. 2014).

The present study was designed to investigate whether *Micromonospora* was able to colonize upper plant tissues in addition to nitrogen-fixing nodules. The capacity of the strains to produce amylases, cellulases, chitinases, pectinases, and xylanases and their distribution across the plant part organs was also investigated. Finally, green fluorescent protein (*gfp*)-tagged *Micromonospora* ML01-*gfp* was used to follow its translocation from the roots to upper parts of the plant. More than 80 strains were isolated from plant tissues other than nitrogen-fixing nodules and most of them produced important amounts of several hydrolytic enzymes. In addition, strain *Micromonospora* ML01-*gfp* was found in different parts of the host plant, especially in the leaves. These results further support the idea that *Micromonospora* stands out as a good candidate to enhance plant growth and contribute to a more sustainable agriculture.

MATERIALS AND METHODS

Strain isolation from different plant tissues. In all, 14 plants of the legumes *Pisum sativum* and *L. angustifolius* (7 each) were collected in Salamanca (40°57'24" N, 5°39'31" W) and Cabrerizos (40°58'38" N, 5°35'48" W), Spain, respectively. Leaves, roots, and stems were washed with running tap water to remove soil and

rinsed in sterile distilled water. The different plant tissues (roots, stems, and leaves) were surface sterilized by immersing in ethanol (70% v v⁻¹) for 1 min, transferred to 3.5% (wt v⁻¹) sodium hypochlorite solution for 2 min (5 min for roots), and rinsed five times with sterile distilled water. Nodules were sterilized in HgCl₂ (2.5% wt v⁻¹) for 2 min and rinsed five times with sterile distilled water. Samples were crushed with a sterile homogenizing pestle and the resulting slurry plated onto solid yeast extract mannitol (YMA) (Vincent 1970) and yeast extract-humic acid (yeast extract at 0.1% wt v⁻¹, humic acid at 0.02% wt v⁻¹, and agar at 1.8% wt v⁻¹) agars. Plates were incubated at 28°C for 3 to 4 weeks, with monitorization of growth every week. *Micromonospora*-like colonies were picked from the isolation plates and subcultured onto ISP 2 agar (Shirling and Gottlieb 1966). Pure cultures were kept at -80°C in 20% (v v⁻¹) glycerol suspensions for long-time preservation and at 4°C on plates for maintenance.

DNA extraction and strain identification. Extraction of genomic DNA for 16S ribosomal RNA (rRNA) gene sequence identification was carried out as previously described using the REExtract-N-Amp Plant Sigma Kit (Trujillo et al. 2010). A multiple sequence alignment of 16S rRNA genes was performed using MUSCLE v3.8.31 (Edgar 2004) with default options. A phylogenetic tree was built using FastTree v2.1.8 (Price et al. 2010) with options -nt (nucleotide) and -gtr (general time reversible). The tree and associated metadata (host species, plant identification number, and tissue location) were visualized using iTOL v4 (Letunic and Bork 2019). The 16S rRNA gene alignment was used to compute the genetic distances between all pairs of isolates using snp-dists (<https://github.com/tseemann/snp-dists>).

BOX-PCR profiles. BOX profiles were used to determine identical genotypes isolated from the different plant tissues. BOX-PCR fingerprint profiles were generated following the methodology described by Trujillo et al. (2010). Briefly, the REExtract-N-Amp Plant Sigma Kit was used for PCR amplification (20 µl per reaction) following the manufacturer's recommendations. The parameters used were 7 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C; followed by a final extension at 72°C for 10 min. PCR products were electrophoresed at 75V for 2 h on a 2% agarose gel in freshly prepared 1× Tris-borate-EDTA buffer.

Production of hydrolytic enzymes. The ability of the strains to produce plant cell wall hydrolytic enzymes was examined. Enzymatic activity assays for amylases, cellulases, pectinases, and xylanases were carried out on M3 agar (Rowbotham and Cross 1977) supplemented with the corresponding substrate in the following manner: carboxymethylcellulose (CMC) (0.5% wt v⁻¹), starch (0.5% wt v⁻¹), xylan (0.5% wt v⁻¹), and pectin (0.5% wt v⁻¹). Strains were grown on ISP 2 agar for 5 to 7 days, bacterial suspensions (10⁹ CFU ml⁻¹) were prepared in saline solution (0.85% wt v⁻¹), and 20 µl was inoculated on the different tests and incubated at 28°C. Results were recorded at 4, 7, and 14 days after inoculation. CMC and xylan plates were stained with Congo red (Mateos et al. 1992), while starch and pectin plates were flooded with lugol (Yamamoto 1988). Chitinase activity was determined according to the protocol previously described (Jagmann et al. 2010). Agar plates supplemented with purified chitin (1% wt v⁻¹) were inoculated with 20-µl bacterial suspensions (10⁹ CFU ml⁻¹). Activity was detected as clearing zones after 14 to 45 days of incubation at 28°C. All tests were done in triplicate. The enzymatic activity indices were calculated based on the ratio of the hydrolysis halo and the colony diameter (Hankin and Anagnostakis 1977; Huang et al. 2012).

Plant colonization studies. *Seed germination and inoculation assays.* *L. albus* and *P. sativum* seed were germinated axenically following the procedure previously described (Benito et al. 2017).

After germination (approximately 3 to 5 days), seedlings were transferred to pots containing sterile vermiculite and watered with nitrogen-free nutrient solution as needed. All seedlings were kept in a plant growth chamber with mixed incandescent and fluorescent lighting programmed for a 16-h photoperiod, with a constant temperature of 21 to 22°C and 50 to 60% relative humidity. Upon the appearance of the first leaves, the plants were inoculated with the appropriate bacterial suspensions (10^8 CFU ml⁻¹) in the following manner: (i) plants inoculated with *gfp*-tagged *M. lupini* (strain ML01-*gfp*); (ii) plants coinoculated with *M. lupini* ML01-*gfp* and the appropriate nitrogen-fixing strain, *Bradyrhizobium* sp. CAR08 for *Lupinus* plants and *Rhizobium pisi* DSM 301321^T for *Pisum* plants; (iii) plants inoculated with the corresponding nitrogen fixer; and (iv) noninoculated plants. In all cases, 12 plants per treatment were used. Nutrient solution was supplemented with nitrogen for treatments i and iv.

Localization of *Micromonospora* in *Pisum* leaves. *Pisum* leaves were prepared using a Scotch tape method, as described by Lawrence et al. (2018). A thin layer of clear nail varnish was applied to an epidermal section of the adaxial (upper) side of the leaf and, after drying, clear Scotch tape was placed on top of this section, lightly pressed, and gently peeled. Fluorescence microscopy (Nikon Eclipse 80i model) was used to locate *gfp*-tagged *Micromonospora* ML01-*gfp* in the leaf cells using standard filter settings (excitation at 488 nm and emission at 515 to 560 nm). The first observation was made 7 days after inoculation and monitored every 4 days until the plants were fully developed. Noninoculated tissues were used to detect sample autofluorescence.

Reisolation of *Micromonospora* ML01-*gfp* from plant tissues. *Lupinus* and *Pisum* plants (five each) were harvested after 8 and 4 weeks, respectively. Roots, nodules, leaves, and stem tissues from the different inoculation treatments (see inoculation experiments) were randomly selected and surface sterilized as described above. Individual processing of the different tissues was carried out and reisolation of *Micromonospora* ML01-*gfp* was done on YMA agar plates supplemented with apramycin (25 µg ml⁻¹). Identification of the isolated colonies was carried out by 16S rRNA gene amplification and fluorescence expression.

Detection of *Micromonospora* in plant tissues by *gyrB* amplification. Chickpea and lupine samples (roots, nodules, stems, and leaves) were randomly collected from the different inoculation treatments (see above) and were used to extract DNA and amplify a partial fragment of *Micromonospora* ML01-*gfp gyrB* using the REDExtract-N-Amp Plant kit. Amplification and sequencing of the *gyrB* gene was done as previously described (Garcia et al. 2010).

Statistical analyses. All statistical analyses were carried out using IBM SPSS statistics, Stata, and R software. Data for enzymatic activities were standardized using Z scores and compared to determine the most productive strains. Also, to test whether there were significant enzyme degradation differences between the two plant species sampled and the isolation tissues, a linear regression model was applied. Statistical differences of the genetic distances in different plants were computed using the Wilcoxon test.

RESULTS

Recovery of *Micromonospora* from different plant tissues. Colonies with a typical *Micromonospora*-like morphology appeared on the isolation plates after 15 days of incubation, becoming perfectly visible after 4 weeks. In total, 151 isolates were obtained: 44 strains were recovered from *Lupinus* tissues and 107 from *Pisum* tissues (Table 1). In *Lupinus* plants, *Micromonospora*-like colonies were recovered from all tissues sampled but the number of isolates varied greatly. Leaves yielded the largest number of isolates ($n = 21$);

in contrast, only 1 strain was recovered from stems. In *Pisum* plants, the strains with *Micromonospora* morphology were only isolated from leaves and nodules, with 53 and 54 isolates, respectively. Most *Micromonospora*-like strains isolated from roots and nodules from both plants were isolated on YMA agar. However, the strains recovered from leaves were isolated on YMA and humic acid agar media. The absence of microbial colonies on control plates confirmed that isolates obtained were recovered from internal plant tissues.

Strain identification and BOX-PCR fingerprinting. 16S rRNA gene sequencing confirmed that 147 of the 151 selected strains belonged to the target genus, *Micromonospora*. A list of the strains, their closest species, and identity percentages is given in Supplementary Table S1. The remaining four isolates—PSH10, PSH12, PSH33 and PSH39—isolated from *Pisum* leaves showed similarity values (99.0 to 99.8%) with *Pseudonocardia soli*, *Micrococcus aloeverae*, *Streptomyces alboniger*, and *Nocardiopsis umidischolae*, respectively. Subsequent analyses excluded these strains, because they did not belong to the target genus. A maximum-likelihood phylogenetic tree was built using the 16S rRNA gene sequences to obtain an overview of the genetic diversity of the *Micromonospora* strains isolated in this study (Fig. 1A). In all, 72 isolates were related to the species *Micromonospora noduli* and *M. saelicesensis* (38 and 34, respectively), representing 49% of the total strains, and these were recovered from multiple tissues of all plants (Fig. 1B). This was followed by *M. vinacea* (10 isolates, 6.8%), *M. echinospora* (8, 5.4%), and *M. zamorensis* and *M. palomenae* (7 each, 4.8%). Twenty-one additional species were identified but none presented an overall prevalence >3%. Species distribution by plant and organ type are provided in Supplementary Fig. S1.

Notably, for the two most abundant species, *M. noduli* and *M. saelicesensis*, variability in the branch lengths suggests the existence of genetic diversity as opposed to pure clonality. In addition, *M. saelicesensis* showed clustering of isolates by host species and plant tissues, with most strains recovered from *L. angustifolius* and leaves (Fig. 1A).

Pairwise genetic distances between the 16S rRNA gene sequences for all of the isolates corresponding to *M. noduli* and *M. saelicesensis* were also computed. The distribution of the single-nucleotide polymorphism revealed that isolates recovered from the same plant were more similar than those from different ones (Fig. 2A). The presence of bacterial clones in the same plant was further confirmed using a molecular typing approach. BOX-PCR profiles of selected isolates revealed that several strains recovered from different tissues within the same plant potentially represented clones (Supplementary Fig. S2). Using the genetic pairwise genetic distances of the 16S rRNA genes, we identified that isolates from nodules in the same plant were more similar than isolates from

TABLE 1
Number of *Micromonospora* isolates obtained from the different plant species and tissue types

Plant organ	Number of strains from	
	<i>Lupinus</i>	<i>Pisum</i>
Nodules	12	54
Stems	1	0
Roots	10	0
Leaves	21	49
Total isolates	44	103

Fig. 1. A, Maximum-likelihood phylogenetic tree of *Micromonospora* spp. isolates. External rings indicate host species (*Pisum sativum*, green and *Lupinus angustifolius*, purple), the plant identifier, and tissue (leaf, stem, root, or nodule). Clades corresponding with the two most abundant species (*Micromonospora saelicesensis* and *M. noduli*) are colored in red and blue, respectively. **B**, Proportion of total isolates recovered from each host species, plant, and tissue that correspond with *M. saelicesensis* (red) or *M. noduli* (blue). H = leaf (hoja in Spanish), N = nodule, and R = root.

Fig. 2. **A**, Distribution of the pairwise genetic distances (number of single-nucleotide polymorphisms [SNPs]) computed on the 16S ribosomal RNA gene sequence for *Micromonospora saelicesensis* and *M. noduli* that were recovered from the same plants (green) or different plants (yellow). **B**, Genetic distances computed for isolates recovered from leaves, nodules, and different tissues from the same or different plants. NS, not significant; *, significant differences $P < 0.01$.

nodules in different plants (Wilcoxon tests, $P < 0.01$) (Fig. 2B). This difference was also significant for *M. saelicesensis* isolates recovered from leaves (Wilcoxon test, $P < 0.01$). However, a comparison of isolates from nodules and leaves from the same plant did not show statistically significant differences with comparisons between isolates from different plants (Fig. 2B).

Production of hydrolytic enzymes. All strains produced xylanases and pectinases. Cellulases and amylases were recorded for 98 and 98.6% of the isolates, respectively. Chitinase production was detected in 94.6% of the strains, but only after 14 to 45 days of incubation.

Overall Z scores of the 44 strains isolated from the *L. angustifolius* (Fig. 3A) and the 103 isolates recovered from *Pisum sativum* (Fig. 3B) were graphically represented. These results showed that the most productive strains (with significant results) included seven isolates from *P. sativum* and one from *L. angustifolius*. Isolate PSH36 stood out as the most productive (P value < 0.01) with 9.28 deviations above the mean, with pectinases, cellulases, and amylases showing more than 2 deviations. On the other hand, the production of xylanases and chitinases was also higher than the mean but with values less than one deviation. Strains LAN08, PSH03, PSH17, and PSH16 had the next best enzymatic production values (P value < 0.05), followed by PSH21, PSH20, and PSN23 (P value < 0.10) (Fig. 3A and B). Conversely, strains PSH04 and PSN16 showed the lowest capacity to produce these enzymes (P value < 0.01). Specifically, strain PSH04 showed 7.24 deviations under the mean, with individual enzyme production values 2 deviations below. Strains PSN06 and LAR02 (P value < 0.05) and LAN01, LAH20, LAH15, and LAH21 (P value < 0.10) showed even lower values (Fig. 3A and B).

Next, a linear regression model was used to determine significant differences in enzyme production in relation to the host plant or tissue type, with a confidence interval of 95%. When the host plants were compared, the strains recovered from *P. sativum* showed higher productivity for xylanases, chitinases, and pectinases, being especially high for the latter one. Cellulase production, on the other hand, was significantly lower (Fig. 3C).

In the case of tissue types, enzyme production activities were unrelated. Root isolates showed significant production for amylases and chitinases while cellulase production for the leaf strains was significantly higher than that of the root and nodule isolates. The strain isolated from the stem sample was removed from this analysis because it was not representative.

Localization of *Micromonospora* spp. in different host tissues. Previously, we demonstrated that *gfp*-tagged *Micromonospora* ML01-*gfp* colonizes the roots and internal nodular tissues when coinoculated with an appropriate nitrogen-fixing strain on *Medicago* and *Trifolium* spp. (Benito et al. 2017). In this work, we used the same strain to determine its potential capacity to reach and colonize other plant organs using *P. sativum* as the plant model. Stem and leaf samples were monitored by optical and fluorescence microscopy to locate *M. lupini* ML01-*gfp*.

Leaves from different zones of the plant were randomly selected to localize strain ML01-*gfp* (Supplementary Fig. S3). The first evidence of the presence of *Micromonospora* in the foliar tissues was observed 11 days post inoculation (dpi) within the stomata; however, *Micromonospora* cells were not easily visible (zone 1) until 16 dpi. After this time, *Micromonospora* cells were readily visible inside stomata and in the surrounding leaf cells, especially after 20 dpi (Fig. 4A to D). It is interesting to note that *Micromonospora* was detected in three of the four zones sampled (Supplementary Fig. S4) however, localization of the bacterium in zones 2 and 3 was only achieved after 20 and 24 dpi, respectively. A detail of *Micromonospora* ML01-*gfp* hyphae congregating near the leaf

stomata is shown in Figure 4B and C. Localization of strain ML01-*gfp* in stem tissues was not possible due to the difficulty in processing these samples.

Reisolation of *Micromonospora* from *Lupinus* and *Pisum* tissues. To confirm the hypothesis that *Micromonospora* also colonizes different parts of the host plant (not only nodular and root tissues), as supported by the isolation experiments, surface-sterilized *Lupinus* roots, nodules, stems, and leaves were used to reisolate *M. lupini* ML01-*gfp*. Eight weeks after inoculation, *Micromonospora* ML01-*gfp* was recovered from nodules and leaves of the coinoculated plants (treatment ii, *Bradyrhizobium* sp. CAR08 + ML01-*gfp*). Specifically, from the eight leaves analyzed, *Micromonospora* colonies were recovered from three randomly chosen leaves, each containing four, five, and four isolates, respectively.

Reisolation of *M. lupini* ML01-*gfp* was also carried out from *Pisum* plant tissues 4 weeks after inoculation. The bacterium was recovered from surface-sterilized roots, nodules, stems, and leaves of coinoculated plants. Specifically, *Micromonospora* isolates were recovered from six leaves, three stems, six roots, and eight nodules from 10 samples processed from each tissue type.

All isolates recovered expressed the GFP protein when observed under a fluorescence microscope. In addition, 16S rRNA gene sequencing confirmed the identity of *M. lupini* ML01-*gfp*. Taken together, these results confirmed that the strain originally inoculated corresponded to the one recovered.

Detection of *Micromonospora* in plant tissues by *gyrB* amplification. *gyrB* gene amplicons of the expected size (548 bp) were obtained from the DNA samples of coinoculated *Lupinus* (60 dpi) and *Pisum* (30 dpi) plant samples (Supplementary Fig. S4). BLAST comparison of the sequenced PCR products confirmed that all fragments corresponded to the partial *gyrB* gene of *M. lupini* ML01-*gfp*, with a similarity of 99 to 100%.

DISCUSSION

Endophytic actinobacteria, including species of *Micromonospora*, have been isolated from stems, roots, and leaves of various plants included in the families Acanthaceae, Amaryllidaceae, Cruciferae, Cyperaceae, Gramineae, Meliaceae, Poaceae, Rubiaceae, and Zingiberaceae, among others (Kirby and Meyers 2010; Kizuka et al. 2002; Kuncharoen et al. 2018, 2019; Taechowisan et al. 2003; Okazaki 1995). However, most of these isolations have been regarded as casual, due to the low numbers of strains recovered. In contrast, many *Micromonospora* strains have been reported from nitrogen-fixing nodules of legumes and actinorhizal plants (Carro et al. 2012, 2013; Riesco et al. 2018; Trujillo et al. 2010, 2015), demonstrating that these organs are a natural habitat of this bacterium. In this work, we showed that *Micromonospora* can also be isolated from surface-sterilized roots, stems, and leaves of *L. angustifolius* and *P. sativum*. The distribution of *Micromonospora* spp. was not homogeneous and varied at both the tissue type and plant species level. The highest number of isolates was obtained from nodules and leaves of both plants, while roots and stems yielded lower numbers. In the case of stems, only one strain was isolated. The low presence of endophytes in stems has been explained by some authors as a transitional passage from the soil and roots to the aerial parts of the plant (Chi et al. 2005; Compant et al. 2005; James et al. 2002; Liu et al. 2017; Monnerat et al. 2009).

The 16S rRNA gene sequence analysis revealed that 97% of the strains isolated belonged to the target genus *Micromonospora* and were highly diverse, with 27 different species potentially identified, and the strains recovered from the *P. sativum* samples showing the highest diversity. This study also pointed out that many isolates had similarity values $>99\%$ with the species *M. noduli* and

Fig. 3. Accumulated Z scores for hydrolytic enzymes assayed and graphic representation of the linear regression model. Overall distribution of Z scores for the **A**, *Lupinus angustifolius* and **B**, *Pisum sativum* isolates (*, **, and *** indicate P value < 0.1, 0.05, and 0.01, respectively). **C**, Comparison of bacterial enzymatic production based on their plant and tissue origin; *L. angustifolius* and nodules were used as plant or tissue reference (ref = 0), respectively (confidence interval 95%).

M. saelicesensis, as reported in other studies (Carro et al. 2013; Riesco et al. 2018; Trujillo et al. 2015). Interestingly, the highest species diversity was found on the leaves of both plant species, again with *M. noduli* and *M. saelicesensis* being the most frequently isolated (Supplementary Fig. S1).

Although the 16S rRNA gene has limitations in resolving phylogenetic relationships at the species level, the phylogenetic analysis based on this marker provided an overview of the species abundance and distribution of the new isolates. The fact that >47% of the study strains were recovered in the cluster *M. noduli* and *M. saelicesensis* confirms previous observations and strongly indicates that these are the most abundant or prevalent species. Furthermore, it was also noted that these species are found distributed in different tissue types. These results also highlighted the possibility that several of the strains isolated may represent new species and should be further characterized.

BOX-PCR fingerprints were used to identify potential clones present in different tissues of the same plant. The identification of the same strain in different parts of a plant strongly suggests that, similar to other endophytic bacteria, genus *Micromonospora* is also capable of moving through the plant and colonizing different plant tissues (e.g., nodules and leaves), as was the case for strains PSN07 and PSH32. Movement of bacteria along their host plants to reach different tissues (e.g., roots to leaves) has been reported for bacteria such as *Bacillus thuringiensis* in cotton (Monnerat et al. 2009), *Rhizobium* spp. in rice (Chi et al. 2005), or *Burkholderia* spp. in grapevines (Compant et al. 2005). The current data, however, do not provide any information about the mechanism used by *Micromonospora* to move upward, assuming the strains originated from the rhizosphere.

More than 90% of the *Micromonospora* strains produced at least one or more of the hydrolytic enzymes assayed, indicating that these traits are a common feature of these endophytes. Indeed, the genome sequence of several of these isolates (PSN01, PSN13, and LAH08) revealed that genes encoding for several plant-polymer-degrading hydrolases were detected in high numbers (Riesco 2020). Plant-pathogenic bacteria and fungi are known to access internal plant tissues by actively degrading plant cell wall compounds (Kubicek et al. 2014; Pérez-Donoso et al. 2010; Thapa et al. 2017).

In the case of beneficial bacteria, plant-polymer-degrading enzymes are thought to be involved in internal tissue colonization without causing damage to the host plant (Carro and Menéndez 2020; Compant et al. 2005; Reinhold-Hurek et al. 2006). The production of hydrolytic enzymes, especially cellulases, appears to be a common feature in the genus *Micromonospora* (Carro et al. 2018; Trujillo et al. 2014), and these molecules are known to be involved in recycling of particulate organic matter in terrestrial and freshwater environments (de Menezes et al. 2012). The production of several hydrolytic enzymes (e.g., cellulases, pectinases, xylanases, and so on) by most of the endophytic *Micromonospora* strains isolated strongly suggests that these compounds have an important role in the colonization process as reported for other bacteria, including *Azoarcus*, *Burkholderia*, and *Rhizobium* spp. (Compant et al. 2005; Reinhold-Hurek et al. 2006; Robledo et al. 2008). Furthermore, recent transcriptomic analyses of several endophytic *Micromonospora* strains isolated from nodular tissues strongly suggests that several glucanase and pectinase lyases may play an essential role in establishing infection and colonization of the host plant (Benito, 2020). It has been proposed that rhizobia induce a pectate lyase in *Lotus japonicus*, the host legume, which is required for infection by the bacterium (Xie et al. 2012). In a similar way, *Micromonospora* may contribute by producing pectinases to facilitate infection by *Rhizobium* spp. Thus, pectinases also appear to be strong candidates to help the bacterium penetrate the plant tissues (Hugouvieux-Cotte-Pattat et al. 2014; Kandel et al. 2017; Mhlango et al. 2018). In the case of chitinases, these may confer protection to the plant by inhibiting fungal pathogens, or as signal molecules to elicit the host's defense mechanism (Carro and Menéndez, 2020).

The isolation of *Micromonospora* strains from different tissues of the host plants (*Lupinus* and *Pisum* spp.) suggests that this bacterium has the capacity to infect tissues other than nodules. The localization of fluorescent *Micromonospora* ML01-*gfp* in different parts of the plant tissues screened strongly suggests a dynamic infection process that allows the bacterium to migrate endophytically upward, moving from the roots into the stems and leaves, as reported for other bacteria (Chi et al. 2005; Monnerat et al. 2009). The use of specific primers to detect a partial *gyrB* gene fragment of *Micromonospora* ML01-*gfp* provided additional evidence that the bacterium moved throughout the plant and infected other tissues. The recovery of strain ML01-*gfp* from randomly chosen surface-sterilized tissues is another proof that *Micromonospora* has the capacity to infect and colonize different parts of *Lupinus* and *Pisum* plants. Overall, the present findings indicate the complex relationship of plant-microbe interactions. Understanding these interactions is essential to designing new strategies for a more productive and sustainable agriculture.

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Fig. 4. Localization of *Micromonospora lupini* ML01-*gfp* in *Pisum* leaves captured after 24 days postinoculation. **A**, *M. lupini* ML01-*gfp* expressing fluorescence; **B** and **C**, fluorescence micrographs of *Micromonospora* ML01-*gfp* located on open stomatal pores; and **D**, ML01-*gfp* hyphae entering an open stomata pore. Bar = 10 μ m.

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