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COMPARATIVE PROTEOMIC AND TRANSCRIPTOMIC PROFILING OF *Micromonospora* STRAINS ASSOCIATED WITH LEGUMES

Memoria presentada por **Patricia Benito Santano** para optar al Grado de Doctor ("Mención Internacional") por la Universidad de Salamanca

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AUTORIZAN:

La presentación y defensa de la Tesis Doctoral titulada "**Comparative proteomic and transcriptomic profiling of *Micromonospora* strains associated with legumes**", elaborada por Dña. **Paricia Benito Santano** y realizada bajo nuestra dirección en el Departamento de Microbiología y Genética de la Universidad de Salamanca, para la obtención del grado de Doctor ("Mención Internacional").

Y para que así conste, extendemos la presente autorización en Salamanca, a 20 de enero de 2020.

Esta tesis está dedicada a todas las personas que han estado junto a mi en este largo camino, que me han dado la oportunidad de trabajar en lo que quiero, me han ayudado, apoyado y creído en mi en todo momento, incluso cuando los experimentos no salían como esperaba.

A todos vosotros gracias, sin vuestro incansable apoyo nunca lo habría conseguido.

"*We know better the mechanics of celestial bodies than the functioning of the soil below our feet*"

"S*abemos mejor la mecánica de los cuerpos celestes que del suelo que pisamos*"

Leonardo Da Vinci

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Abbreviations

µg: Micrograms

µl: Microliters

µM: Micromolar

1D: One-dimensional

2D: Two-dimensional

A: Amps

ABC: ATPbinding cassette

ACC: 1-aminocyclopropane-1-carboxylate

bp: Base pair

BZ: Bacterioid zone

C: Cortex

cDNA: Complementary DNA

CFU: Colony-forming unit

CLSM: Confocal laser scanning microscopy

CMC: Carboxymethylcellulose

COG: Clusters of orthologous groups

Ct: Threshold cycle

CV: Coefficient of variation

DEGs: Differentially expressed genes

DEPC: Diethyl pyrocarbonate

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dpi: Days post inoculation

EDTA: Ethylenediaminetetraacetic acid

FCH: Fold change

FDR: False discovery rate

GAE: Expressed genes in absence of exudates

GFP: Green fluorescent protein

GPE: Expressed genes in presence of root exudates

HA: Yeast extract/humic acid

$HgCl_2$: Mercury (II) chloride

IAA: 3-Indole acetic acid

IEF: Isoelectric focusing

ISP 2: Yeast extract/malt extract agar

KEGG: Kyoto Encyclopedia of Genes and Genomes

LB: Luria-Broth

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

mCherry: Red fluorescent protein

MEGA: Molecular evolutionary genetic analysis

mg: Milligrams

ml: Milliliters

mm: Millimeters

NaCl: Sodium chloride

NGS: Normal goat serum

nm: Nanometers

PB: Phosphate buffer

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PGP: Plant growth promotion

PGPB: Plant growth-promoting bacteria

RLE: Relative log expression

RNA: Ribonucleic acid

mRNA: Messenger RNA

ncRNA: noncoding RNA

RNase: Ribonuclease

RNA-Seq: RNA Sequencing

rpm: rounds per minute

rRNA: Ribosomal ribonucleic acid

RT-PCR: Real-Time PCR

SDS: Sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEPs: Significant expressed proteins

TBS: Tris-buffered saline

TCEP: Tris (2-carboxyethyl) phosphine hydrochloride

TEM: Transmission electron microscopy

TY: Tryptone yeast extract

v/v: Volume by volume

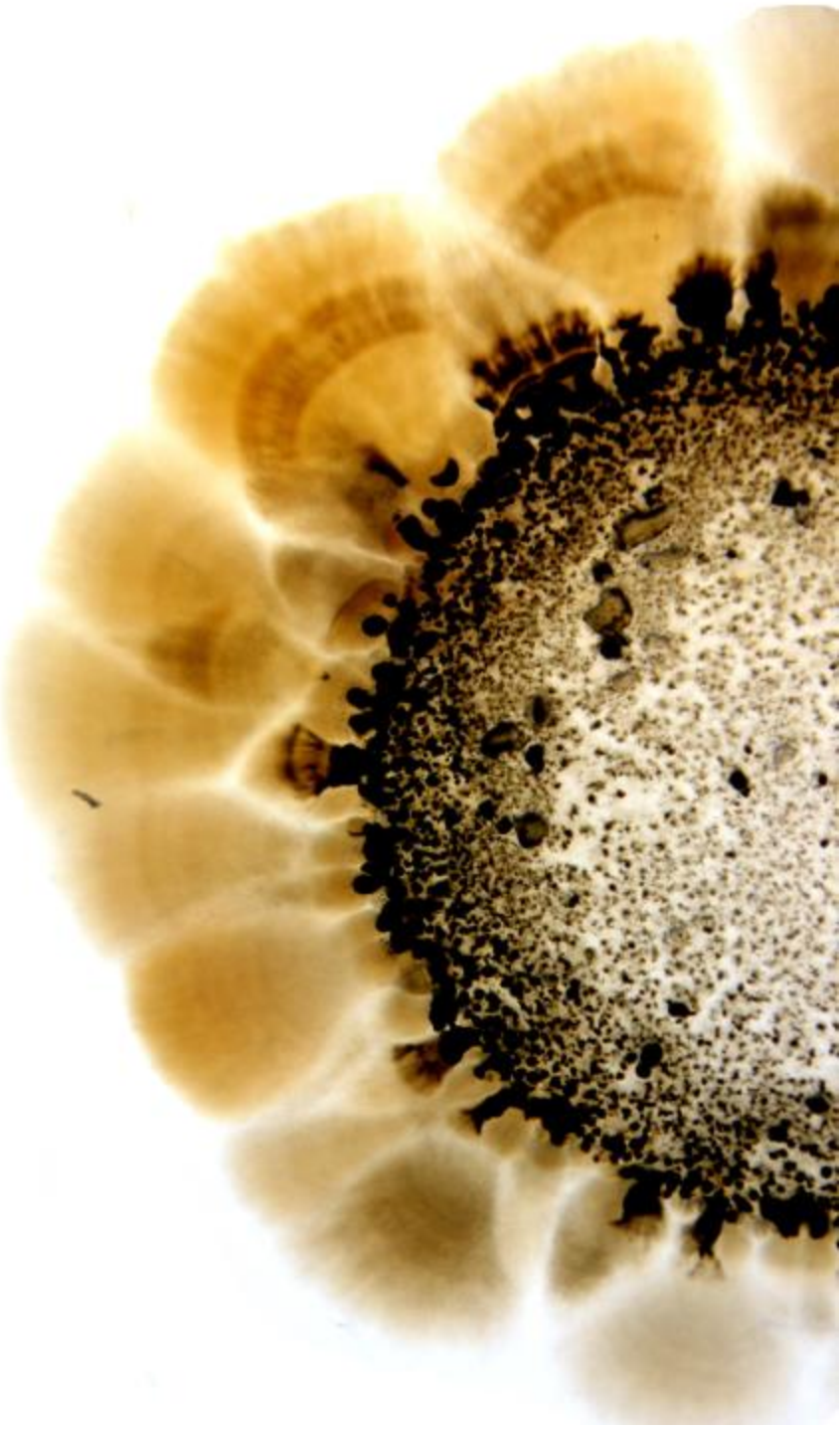
V: Volts

w/v: Weight by volume

YMA: Yeast extract mannitol

µm: Micrometers

GENERAL INTRODUCTION



1. General introduction

1.1 Legumes

Legumes are a family of the order Fabales that are represented by trees, shrubs or herbs, which can be biennials or perennial. It is a family of cosmopolitan distribution with approximately 730 genera and about 19,400 species, being the third largest family of angiosperms (Mabberley, 1997; Lewis et al., 2003). Two distinctive characteristics of this group are the production of a fruit wrapped in a pod and the shape of its flowers, often papilionaceous. They are distributed throughout the world, although they are more frequent in tropical and subtropical regions and less abundant or absent in arctic and alpine regions and the understory of cool temperate forests (Rundel, 1989). Legumes are the second most important family of agricultural crop species after grasses and its production is mostly intended for livestock feeding and human consumption, being one of the most consumed foods in the world after cereals. This is because legumes are a great nutrient source given their high protein and mineral (iron and zinc) content, as well as their amounts of fiber vitamins and other bioactive molecules (Amarowicz and Pegg 2008; Gętek et al., 2014). Spain is the second country in the European Union (behind Turkey) that produces more tons of legumes per year and the first European country where more legumes are consumed (MAPA, data 2018). The autonomous community with more land area dedicated to the legume cultivation is Castilla y León, in which 154,009 ha (18% of the national total) are dedicated to grain legumes and 190,768 ha for forage legumes. Also, this autonomous community is the second largest producer of legumes (86,042 t) behind Castilla-La Mancha (159,871 t) (MAPA, data 2018) (**Figure 1**).

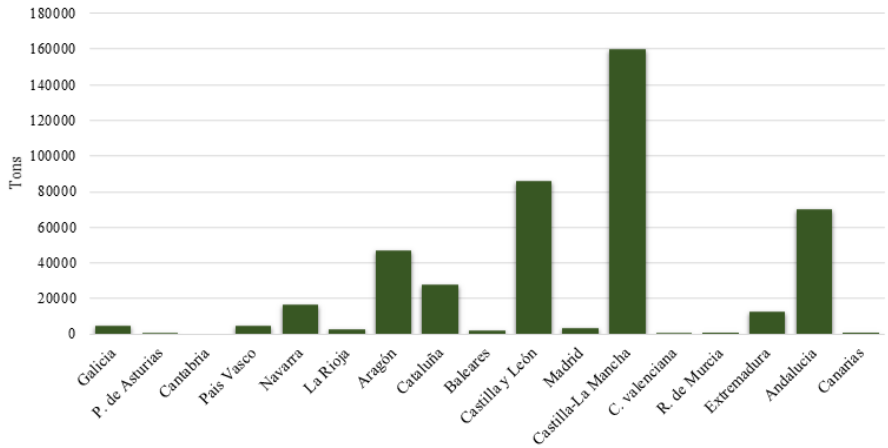


Figure 1. Legume production in Spain

Legumes are not only important at the nutritional level but also at the ecological level because they play an important role in the terrestrial nitrogen cycle (Sprent, 2001), establishing symbiotic relationships with nitrogen-fixing bacteria. In addition, this family of plants reduce soil erosion, have low dependence on industrial fertilizers, reduce greenhouse gas emissions and are a large carbon reservoir (Millennium Ecosystem Assessment, 2005).

1.2 *Pisum sativum* (common pea)

Pisum (common name pea) is a genus of the family Fabaceae, native to northeast Africa (Mediterranean basin) and southwest Asia. Three pea species are currently known: Common pea (*Pisum sativum*), tawny pea (*Pisum fulvum*) and abyssinian pea (*Pisum abyssinicum*). The common pea (*P. sativum*), also known as the garden or field pea, is an annual dicot species that belongs to the legume family (**Figure 2**). It is an important crop with various uses such as vegetable, grain, feed and fodder crop. (Zohary and Hopf, 2000). Domesticated about 11,000 years ago, pea is the most widely grown grain legume in Europe and the fourth worldwide and represents a versatile and inexpensive protein source for human and animal feeding. Dried peas are the legumes more produced in Spain, surpassing the cultivation of beans, chickpeas and lentils, three of the most consumed legumes in this country. Most of dried pea production is destined for animal feed (MAPA, data 2018).

Figure 2. *Pisum sativum* plants. (**a,b**) flowers; (**c**) peapod; (**d**) seeds.

Pea is grown mainly in the wet seasons such as autumn or spring because the seed germination begins with the water capture. The crop in other seasons of the year only produces a discontinuous germination and low crop productivity. In the Mediterranean region, it is grown in spring, gathering the seeds in summer. This crop demands high exposure to sunlight and does not tolerate acid soils, with pH values below 6.5 (Hernández et al., 2001).

1.3 *Lupinus* (lupine)

The genus *Lupinus* belongs to the legume family Fabaceae and includes more than 200 species across the world. About 90% of this genus is originated in North and South America and the remainder of the species are found in the Mediterranean region and northern Africa. Most of the economically important species come from the Mediterranean region. Three species of the genus *Lupinus* native to the Mediterranean region are grown around the world. They are the white lupin (*Lupinus albus*), narrow-leafed lupin or blue lupin (*Lupinus angustifolius*) and yellow lupin (*Lupinus luteus*).

Most lupine species have seeds with a good content of proteins (around 30-50%), carbohydrates, fiber and minerals (mainly cobalt, phosphorus and potassium). However, all species contain toxic and bitter alkaloids (especially spartein, lupinin, lupinic acid and lupanin), which are not suitable for human or animal feed. For this reason, since the 1930s varieties without alkaloids have been obtained (Castroviejo and Pascual, 1999). In addition to its nutritional importance, *Lupinus* has a great ecological importance. This genus not only establishes symbiotic relationships with nitrogen-fixing bacteria but is a valuable group of legumes for sustainable phosphorus management. In soils with low assimilable phosphorus, lupin forms specialized cluster root structures and/or release phosphorus-mobilizing carboxylates that convert this mineral into a soluble form (Lambers et al., 2012).

Lupinus albus (**Figure 3 a,c**) is one of the 200 lupin species and is original from South-Eastern Europe and Western Asia. It is a winter-growing legume that can be found in the wild on disturbed and poor soils where competition from other species is reduced (Clark, 2014). White lupine has a higher protein content (around 46%) than narrow-leafed lupin. In Spain and Portugal, the cultivation of this variety has been traditionally developed for human consumption of seeds as well as stock feed, fodder and green manure (Jansen, 2006). As previously mentioned, lupines are rich in alkaloids, in the case of white lupine the seeds must

be detoxified by soaking before cooking and consumption. Currently, there are modern sweet varieties without alkaloids which do not require detoxification (Clark, 2014).

Blue lupin (*L. angustifolius)* (**Figure 3 b,d**) is one of the most abundant lupines in the Iberian Peninsula. It is a legume perfectly adapted to a large number of soils and environmental conditions, being present in virtually all provinces. It usually grows in abandoned crops, wastelands, roadsides, thickets and, like weeds, in cereal and olive groves (Castroviejo and Pascual, 1999). However, blue lupin seeds sometimes cannot be used for animal or human consumption due to its high content of alkaloids. Nonetheless, some varieties such as "sweet" cultivars can be used as a protein source in animal feeding and forage. Blue lupin stubbles are used for forage and silage, and for late winter and early spring grazing. Bitter cultivars are grown mainly to improve the soil quality due to their contribution to the amount of available nitrogen and phosphorus. (Castroviejo and Pascual, 1999).

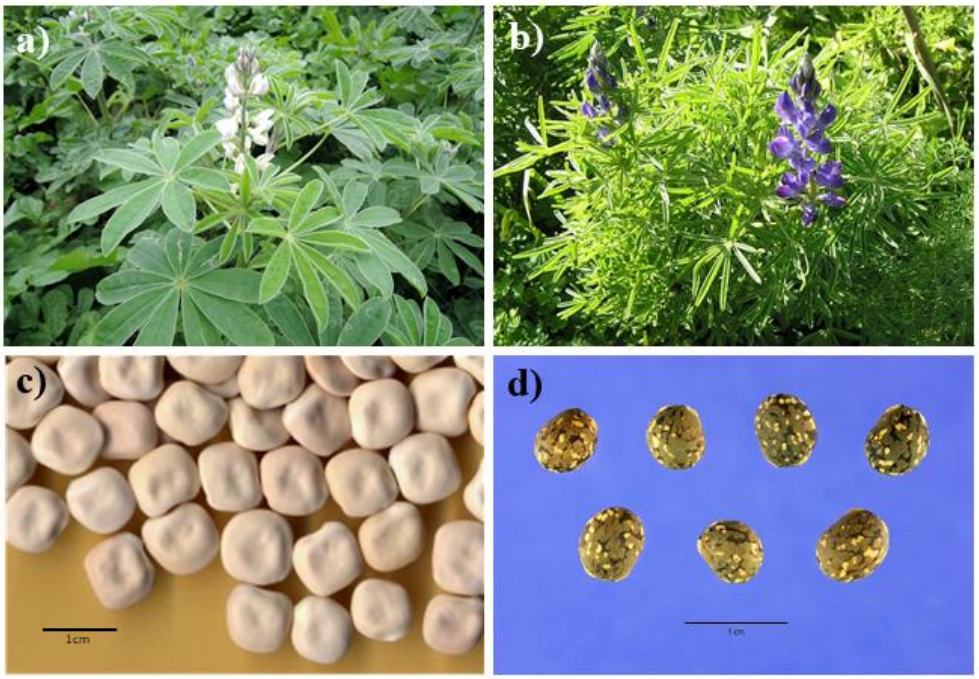


Figure 3. Lupinus plants. (**a**) *Lupinus albus*; (**b**) *Lupinus angustifolius*; (**c**) *L. albus* seeds; (**d**) *L. angustifolius* seeds.

1.4 Plant-bacteria interactions and their influence on the plant development

Microbial communities that live in the soil can establish different interactions with the surrounding plants (Schenk et al., 2012). These interactions may have a neutral, beneficial, or detrimental effect on the plant, depending on the type of microorganism and physiological state of the host. Microorganisms that interact with plants may be present in the immediate vicinity of the roots (rhizosphere), on the root surface (rhizoplane) or inside the internal tissues (endosphere) (Bulgarelli et al., 2013; Reinhold-Hurek et al., 2015). When we think about plant-bacteria interactions or microbial infection to plants, symptoms of diseases, detrimental effects and reduction in vigor, yield and quality of crops come to our mind. However, this is not true in the case of endophytic microorganisms, only a minor fraction of them may also cause diseases (Andreote et al., 2014). Most of endophytes that colonize the internal plant tissues do not cause symptoms or disease to their host, but can help in their proper development (Wani et al., 2015). Actually, all plant species can require the presence of associated bacteria for their growth and establishment in different ecosystems (Ortíz-Castro et al., 2009).

Bacterial root colonization usually begins with the recognition of specific compounds present in the root exudates (De Weert et al., 2002). The root exudate composition can be determined by plant genotype, cultivar, growth stage, physiological status, microbial abundance and diversity, biotic and abiotic stress and environmental (soil) conditions (Hesse et al., 2003; Malinowski and Belesky 2006; Haichar et al., 2008; Singh et al., 2011; Qawasmeh et al., 2012). Differences in root exudate composition may influence the bacterial communities and their colonization process (Lugtenberg et al., 2001). Some compounds may have negative, attractive and/or repulsive effects for certain microorganisms, which can not only influence microbial diversity but also its gene expression (Bais et al., 2006). The microorganisms from the rhizosphere attracted by exudates can colonize both the rhizoplane and the internal root tissues. However, not all microorganisms present in the rhizosphere and/or rhizoplane are able to colonize the plant's endosphere, since they must possess key genetic machinery to colonize and persist in it (Hardoim et al., 2008). The internal root tissue invasion can happen through the sites of lateral root emergence, root cracks, tips and intercellular spaces in the root and/or pathogen- or predation-induced wounds (**Figure 4**) (Reinhold-Hurek and Hurek, 1998; Böhm al., 2007; Chi et al., 2005). In addition, these endophytic microorganisms can be equipped with lipopolysaccharides, flagella, pili and cell-wall degrading enzymes that facilitate their entry into the root (Duijff et al., 1997; Dörr et al., 1998; Böhm et al., 2007).

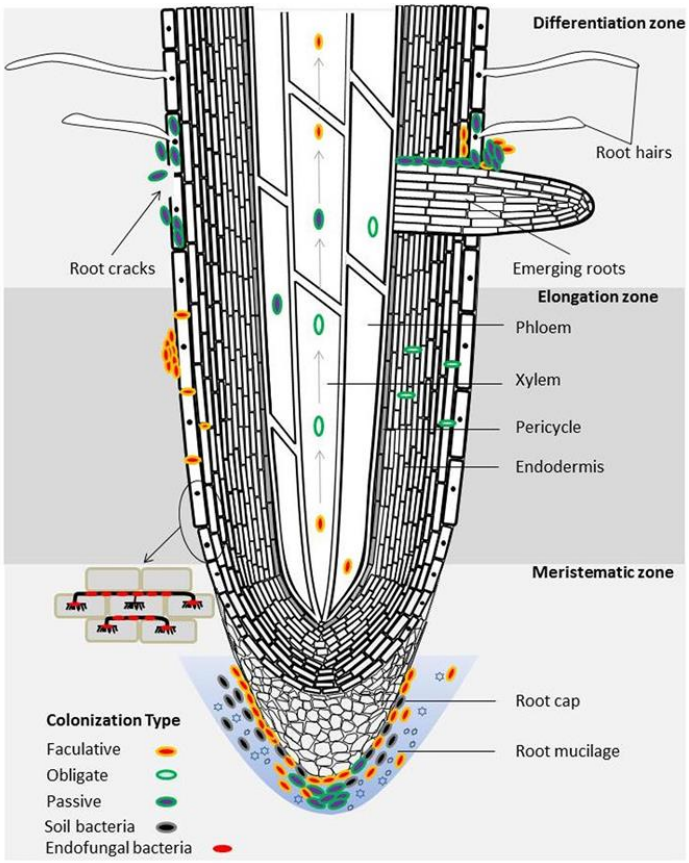


Figure 4. Microbial root colonization (Liu et al., 2017)

Bacterial endophytes can be classified as obligate or facultative according to their life strategies. Obligate endophytes are strictly dependent on the host plant for their growth and survival and are transmitted to other plants through plant-plant contact or plant-insect-plant transmission. In the case of facultative endophytes, they can temporary live inside plants and after, in other habitats (Hardoim et al., 2008). However, independent of their life cycle many endophytes have shown plant growth-promoting (PGP) effects (Hallmann, 2001, Compant et al., 2005a, Compant et al., 2008, Sessitsch et al., 2004, Hallmann and Berg, 2007). Plant growth-promoting bacteria (PGPB) are generally defined as a heterogeneous bacterial group which can live in rhizosphere, rhizoplane and endosphere of plants and can facilitate plant growth (Lugtenberg and Kamilova, 2009). The presence of these bacteria can increase agricultural production by accelerating the seed germination, promoting plant establishment under adverse conditions, improving plant growth or preventing pathogen infections (Hurek et al., 2002; Ryan et al., 2008). PGPB can stimulate and improve plant development through direct and indirect mechanisms. The direct mechanisms are related to compounds that can help plant growth. Among the direct mechanisms, the production of phytohormones (indoleacetic acid (IAA), gibberellic acid, zeatin, cytokinins and ethylene), nitrogen fixation,

ethylene modulation (1-aminocyclopropane-1-carboxylic acid (ACC) deaminase), volatile organic compounds production, iron acquisition (siderophores) and phosphate solubilization stand out. On the other hand, indirect mechanisms minimize and protect the plant against deleterious effects of biotic and abiotic stresses. This protection can be produced by competition for nutrients, production of molecular inhibitory substances (ammonia, cyanogens, sulfides, aldehydes, alcohols, and ketones), production of cell-wall degrading enzymes (cellulases and chitinases) and biocidal secondary metabolites (antibiotics) (Ryu et al., 2003; El-Tarabily and Sivasithamparam, 2006; Glick, 2012; Bouizgarne, 2013; Dey et al., 2014). Other indirect factors are associated with plant response reactions. Plants are equipped with several types of defense responses: induced systemic resistance (ISR) and systemic acquired resistance (SAR) against biotic factors and primed by the influence of beneficial microbes and pathogens respectively. Induced systemic tolerance (IST) is usually associated with abiotic stresses (Schuhegger et al., 2006; van Loon, 2007; Choudhary and Johri, 2009; Yang et al., 2009).

Among the great microbial diversity present in the soil with PGP properties, Gram-negative bacteria have been the best studied, being Bacteroidetes and Proteobacteria the most prominent phyla. However, many Gram-positive bacteria included in the phyla Firmicutes and Actinobacteria are also excellent plant growth promoters, in addition to being involved in biocontrol and bioremediation processes. The most commonly species studied are *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Gluconacetobacter*, *Enterobacter*, *Serratia*, *Paenibacillus*, *Streptomyces* and *Frankia* (Francis et al., 2010; Bhattacharyya and Jha 2012). In the case of *Frankia*, it was the first actinobacterial genus isolated from inside plant (Callaham et al., 1978). *Frankia* is a nitrogen-fixing actinobacterium, which establishes a symbiotic relationship with actinorhizal plants belonging to eight families (*Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae, and Rosaceae*) (Wall, 2000). This actinobacterial genus, like rhizobia, induces specific root organs called nodules where the bacteria are able to fix atmospheric nitrogen (Diagne et al., 2013; Ngom et al., 2016), but also, it can exert beneficial effects on the plant development by production of phytohormones, siderophores, enzymes involved in phosphate solubilization and antibiotics, among others. Among the PGP actinobacteria, the *Streptomyces, Micromonospora* and *Microbacterium* genera also stand out (Sathya et al., 2017). Unfortunately, our knowledge about the relationship between plants and Actinobacteria is still poor. Above all, in the case of molecular interactions and genetic

changes that occur both in the plant and in the bacteria, limited data are currently available on it.

1.5 *Micromonospora,* an unexpected endophyte of nitrogen-fixing nodules

Micromonospora is a bacterial genus belonging to phylum *Actinobacteria*. It was first described in 1923 by Ørskov (Ørskov, 1923) and it is currently composed of 101 species with valid names (October, 2019 http://www.bacterio.net/micromonospora.html) (Parte, 2014). The type species of the genus is *Micromonospora chalcea*, which was reclassified from its original name, "*Strepthotrix chalcea*" (Foulerton, 1905). The species of the genus *Micromonospora* are Gram-positive, aerobic and their genomes are usually large (6.1-7.3 Mb) and have a high content of guanine plus cytosine (72-74%) (Genilloud, 2015; Trujillo et al., 2014a). *Micromonospora* species are chemo-organotrophic and mesophilic with optimum temperature ranges between 20 ºC and 40 ºC, but not above 50 ºC. They tolerate basic pH 8.0-10.5, but they do not grow below pH 5.0 or above pH 10.5. In addition, they are able to grow on concentrations of 1.5 to 5% of sodium chloride, but not greater than 6% (w/v) (Genilloud, 2015).

Micromonospora colonies generally have a raised and folded appearance on agar media (**Figure 5 a**). Most of them are pigmented and can show a wide range of colors from orange, yellow, red and brown, to blue, greenish blue and even purple (**Figure 5 b**). In many old cultures change to brown, black or green-black colors upon the production of spores (Genilloud, 2015). The *Micromonospora* strains produce a well-developed branch substrate mycelium (0.2-0.6 μm diameter), with nonmotile spores and usually absent aerial mycelium (**Figure 5 c**). The formation of single spores (0.7-1.5 μm) on the substrate mycelium is the main morphological characteristic of the genus *Micromonospora*. Its name refers to this characteristic (*Mikros* = pequeño, *mono* = una y *spora* = espora) (**Figure 5 d**). However, spores are also found in dense clusters on the surface or inside the substrate mycelium (Genilloud, 2015; Trujillo et al., 2014a).

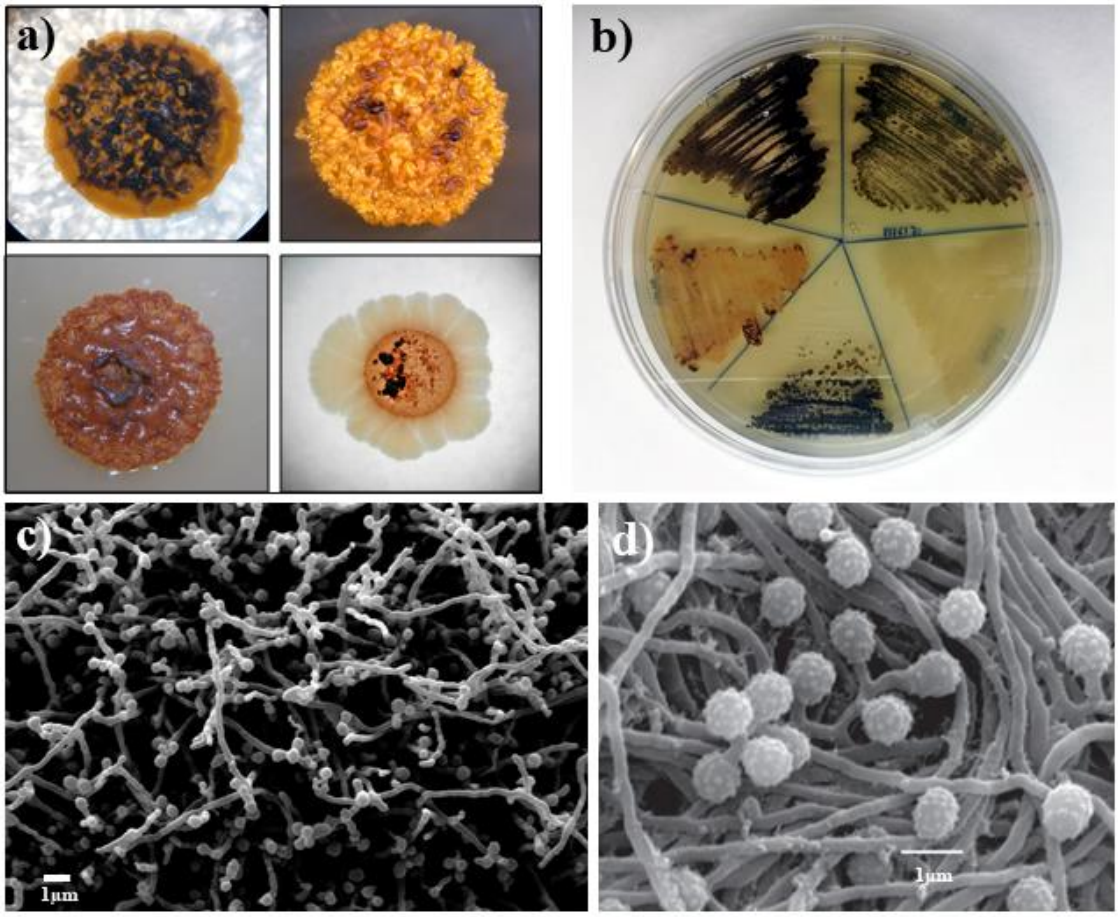


Figure 5. *Micromonospora* morphology. (**a**) *Micromonospora* colonies; (**b**) Pigmented *Micromonospora* strains; (**c,d**) Scanning electron micrographs of hyphas and spores.

They are widely distributed in many geographical sites worldwide such as soils (Li and Hong, 2016; Lee and Whang, 2017), aquatic habitats (freshwater and marine sediments), mangroves, sludge (Kroppenstedt et al., 2005; Thawai et al., 2005; Trujillo et al., 2005; Huang et al., 2008; Veyisoglu et al., 2016), and even in samples from Antarctic sandstone rock (Hirsch et al., 2004) and limestone quarry (Nimaichand et al., 2013). In the last decade, *Micromonospora* has been isolated from plant tissues, mainly from nitrogen fixing nodules both actinorhizal and legume plants (Valdés et al., 2005; Trujillo et al., 2006, 2007, 2010; Garcia et al., 2010; Carro et al., 2012a, 2013; Trujillo et al., 2015; Riesco et al., 2018) and rarely from roots and leaves (Kirby and Meyers, 2010; Kittiwongwattana et al., 2015; Thawai, 2015; Kaewkla et al., 2017). This genus has been reported as a normal and widespread occupant of actinorhizal plants, including the angiosperm species *Alnus viridis*, *Casuarina equisetifolia*, *Coriaria myrtifolia*, *Elaeagnus x ebbingei*, *Hippophae rhamnoides*, *Myrica gale*, and *Morella pensylvanica* (**Table 1**) (Valdés et al., 2005; Trujillo et al., 2006; Carro et al., 2013). In the study of Carro et al., (2013) obtained a high number of

Micromonospora isolates from *Alnus*, *Elaeagnus*, and *Hippophae* nodules and a much lower number in *Myrica*, *Morella*, and *Coriaria* nodules. In spite of it, *Micromonospora* strains were recovered from all plants sampled.

Table 1. Biogeographical and species distribution of *Micromonosporae* in nitrogen fixing nodules of legumes and actinorhizal plants sampled (Trujillo et al., 2015).

Host plant (Legumes)	**Common name**	**Geographical origin**	**Closest species identification (16S rRNA gene)**	**References**
Arachys sp.	Peanut	Nicaragua	*M. chaiyapumensis, M. endolithica*	Cerda, 2008
Cicer arietinum	Chickpea	Spain	ND	Trujillo et al., 2010
Glycine max	Soy	Nicaragua	ND	Trujillo et al., 2010
Lens culinarium	Lentil	Spain	ND	Trujillo et al., 2010
Lupinus angustifolius	Blue lupine	Spain	*M. aurantiaca, M. auratinigra, M. chaiyapumensis, M. coriariae, M. coxensis, M. echinospora, M. fulviviridis, M. lupini, M. matsumotoense, M. narathiwatensis, M. olivasterospora, M. sagamiensis, M. saelicesensis*	Trujillo et al., 2007; Rodríguez, 2008; Alonso de la Vega, 2010
Lupinus gredensis	Lupine	Spain	*M. chaiyapumensis, M. chersina, M. coxensis, M. echinofusca, M. echinospora, M. lupini, M. olivasterospora, M. saelicesensis, M. viridifaciens*	Alonso de la Vega, 2010
Lupinus sp.	Lupine	Germany	*M. saelicesensis*	Trujillo et al., 2010
Medicago sp.	Alfalfa	Australia, Spain	*M. aurantiaca, M. chokoriensis, M. lupini, M. saelicesensis, M. schwarzwaldensis, M. tulbaghiae, M. viridifaciens*	Martínez-Hidalgo et al., 2014
Mucuna sp.	Mucuna	Ecuador	ND	Trujillo et al., 2010
Ononis sp.	–	Spain	ND	Trujillo et al., 2010
Ornithopus sp.	–	Spain	ND	Trujillo et al., 2010
Phaseolus vulgaris	Bean	Nicaragua	*M. chaiyapumensis, M. chersina, M. endolithica*	Cerda, 2008
Pisum sativum	Sweet pea	Spain	*M. aurantica, M. auratinigra, M. chaiyapumensis, M. chersina, M. coerulea, M. coriariae, M. coxensis, M. fulviviridis, M. lupini, M. matsumotoense, M. pattaloongensis, M. saelicesensis, M. sagamiensis,, M. siamensis*	Carro, 2009; Carro et al., 2012a
Trifolium sp.	Clover	Spain	ND	Trujillo et al., 2010
Vicia sp.	Vetch	Spain	ND	Trujillo et al., 2010
Host plant (Actinorhizal	**Common name**	**Geographical origin**	**Closest species identification (16S rRNA gene)**	**References**
Alnus glutinosa	Alder	France	*M. cremea, M. coxensis, M. lupini, M. matsumotoense, M. olivasterospora, M. saelicesensis, M. siamensis*	Carro et al., 2013a
Alnus viridis	Alder	France	*M. chokoriensis, M. coriariae, M. lupini, M. matsumotoense, M. pisi, M. rifamycinica, M. saelicesensis*	Carro et al., 2013a
Casuarina equisetifolia	Coast sheoak	Mexico	*M. aurantiaca*	Valdés et al., 2005
Coriaria myrtifolia	Redoul	Spain, France	*M. coriarie, M. saelicesensis, M. peucetia*	Trujillo et al., 2006; Carro et al., 2013a
Elaeagnus x ebbingei	–	France	*M. aurantiaca, M. auratinigra, M. chaiyaphumensis, M. coriariae, M. coerulea, M. cremea, M. coxensis, M. equina, M. lupini, M. matsumotoense, M. mirobrigensis, M. peucetia, M. saelicesensis, M. siamensis*	Carro et al., 2013a
Hippophae rhamnoides	Sandthorne	France	*M. chaiyapumensis, M. chersina, M. coxensis, M. equina, M. lupini, M narathiwatensis, M. saelicesensis, M. siamensis, M. viridifaciens*	Carro et al., 2013a
Morella pensylvanica	–	France	*M. coriariae, M. cremea, M. olivasteraspora, M. peucetia, M. saelicesensis*	Carro et al., 2013a
Myrica gale		Canada	*M. lupini, M. tulbaghiae*	Carro et al., 2013a

In the case of legume plants, *Micromonospora* has been recovered from different wild species such as *Arachis hypogaea*, *Cicer arietinum*, *Glycine max*, *Lens culinaris*, *Lupinus angustifolius*, *Lupinus gredensis*, *Medicago sativa*, *Melilotus* sp., *Mucuna* sp., *Ononis* sp., *Ornithopus* sp., *Pisum sativum*, *Phaseolus* sp., *Trifolium* sp., and *Vicia* sp. (**Table 1**) (Cerda, 2008; Rodríguez, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Martínez-Hidalgo et al., 2014; Riesco et al., 2018). Several works have reported that the distribution of *Micromonospora* strains in the nitrogen-fixing nodules in both legumes and actinorhizal plants is not homogeneous and varies from nodule to nodule and plant to plant (Trujillo et al., 2010; Carro et al., 2012a). The first *Micromonospora* strain isolated from inside the nitrogen-fixing nodules was considered as a contaminant because it was assumed that the spores present in the external nodular tissues had resisted the sterilization process (Trujillo et al., 2010). However, subsequent isolates showed the absence of fast-growing sporulating microorganisms from externally sterilized nodules and the presence of *Micromonospora* inside the nodule. This strongly indicated that the *Micromonospora* strains had originated from the internal plant tissues. Until that time, *Micromonospora* was largely overlooked in this niche due to its slow growth (7-10 days) compared to rhizobial strains (3-5 days) (Trujillo et al., 2010). Despite the close relationship between *Micromonospora* and legumes, there is still few reports about how *Micromonospora* can colonize the internal root nodule tissues.

In recent years, the number of sequenced *Micromonospora* genomes has increased, enriching the genetic information of this genus. These sequenced genomes have shown several genomic traits potentially involved in the interaction between the plant and the bacteria (Alonso-Vega et al., 2012; Trujillo et al., 2014b; Carro et al., 2018). Different sequenced *Micromonospora* strains have shown genes involved in the production of IAA, ACC deaminase, siderophores and iron transport, trehalose, chitinases, acetoin, 2,3-butanediol and other secondary metabolites (Trujillo et al., 2014b; Carro et al., 2018). Some of these compounds that stimulate plant growth have been demonstrated in *in vitro* (**Figure 6**) (Trujillo et al., 2014b). Furthermore, different plant co-inoculation studies (*Micromonospora*-rhizobia) indicate that *Micromonospora* acts as a PGPB with a positive effect on the plant and increase in the number of nodules in comparison to single-strain treatments (**Figure 6**) (Cerda, 2008; Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). Although *Micromonospora* can promote plant growth and be recovered from nodules, it cannot fix nitrogen as hitherto, no genes involved in nitrogen fixation have been found (Carro et al., 2018).

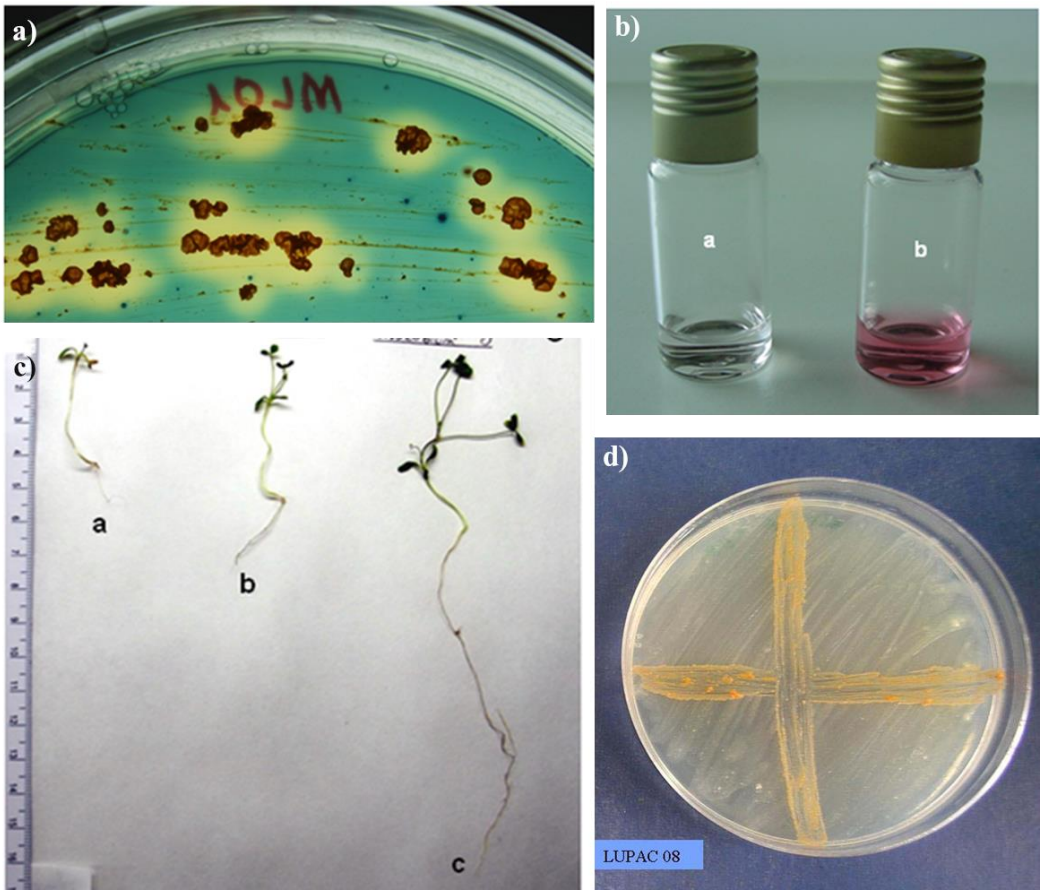


Figure 6. Plant growth promotion of *M. lupini* Lupac 08 and antagonism test. (**a**) Siderophores; (**b**) indole-3-acetic acid (a. negative control E. coli DH5α; b. Lupac 08); (**c**) Plant growth promoting effect of *M. lupini* Lupac 08 on clover plantlets (a. control; b. inoculated with *Rhizobium* sp. E11; c. co-inoculated with *Rhizobium* sp. E11 and *M. lupini* Lupac 08) (Trujillo et al., 2014b); (**d**) Simultaneous and joint growth of *M. lupini* Lupac 08 and rhizobia (Cerda, 2008).

The genus *Micromonospora* is well-known for its capacity to produce high numbers of hydrolytic enzymes, which can contribute to the organic matter turnover in different habitats (de Menezes et al., 2008, 2012). Sequenced genomes have shown to greater or lesser extent genes that code for hydrolytic enzyme production such as cellulases, xylanases, pectinases, amylases and chitinases. The production of these hydrolytic enzymes has been confirmed in the laboratory for some strains such as *M. lupini* Lupac 08 (**Figure 7**) (Trujillo et al., 2014b). However, this seems to be a paradox since *Micromonospora* shows a very high *in vitro* activity for cellulases and xylanases, however inoculation experiments indicate that the microorganism does not behave as a pathogen, on the contrary, *Micromonospora* appears to act as a plant growth-promoting bacterium (Cerda, 2008; Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). Nonetheless, the role of these enzymes in the legume-*Micromonospora* interaction is still unknown.

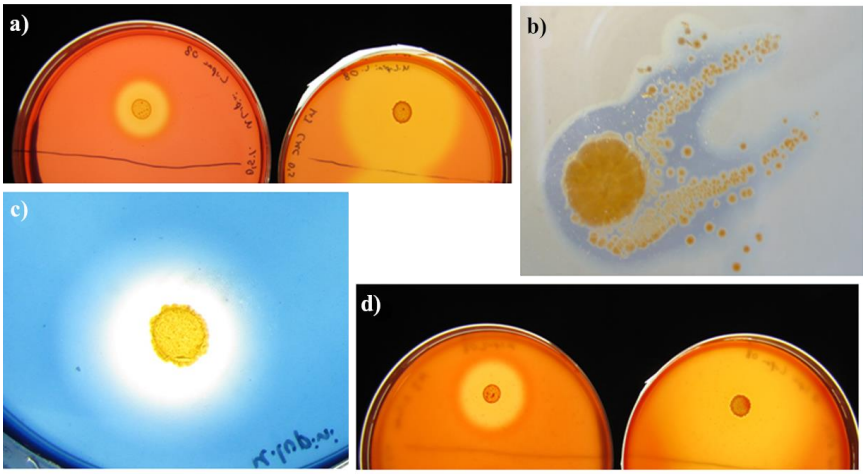


Figure 7. Hydrolitic enzymes producted by *M. lupini* Lupac 08. (**a**) Carboxymetheylcellulose hydrolysis at 4 (left) and 14 (right) days after inoculation; (**b**) Starch hydrolysis at 4 days after inoculation; (**c**) Chitin degradation at 7 days after inoculation; (**d**) Xylan degradation at 4 (left) and 14 (right) days after inoculation (Trujillo et al., 2014b).

1.6 Use of "Omics" tools to understand the plant-endophyte interaction

The information available on the molecular mechanisms that occur in the establishment of plant-endophyte relationships is still very limited. Two main reasons make this type of studies difficult. The first is the complex relationship between the host and its endophytes, while the second is the difficulty to imitate this type of association *in vitro* conditions, as well as studying the mechanisms in the plant. Complete comprehension of this ecological phenomenon can only be obtained by integrating different technologies called "omics", such as genomics, transcriptomics, proteomics and metabolomics (**Figure 8**) (Wani et al., 2015). Transcriptomics, proteomics and metabolomics can also be grouped under the name of "functional genomics", since they study the products of gene expression (Bunnik and Le Roch, 2013).

Genomics analyzes the set of genes that an organism contains in its chromosome or chromosomes. A genome can only provide information on what genetic characteristics of an organism can directly or indirectly influence its lifestyle, adaptation to endophytic life or related to PGP functions such as nitrogen fixation, phytohormones production, mineral acquisition, stress tolerance, root adhesion and other genes of interest (Taghavi et al., 2010; Kaul et al., 2016). The genome study of plant growth-promoting endophytic bacteria such as *Enterobacter* sp. 638 and *Azospirillum brasilense* CBG497 has allowed to identify functions essential for the successful colonization and endophytic association with their host (Taghavi

et al., 2010; Wisniewski-Dyé et al., 2012). In the case of *Micromonospora*, whole genome sequencing has not only provided a focus on its biotechnological and ecological potential, but also provided information at the taxonomic level (Carro et al., 2018; Riesco et al., 2018). However, the genome does not allow to determine which genes are active in different conditions, for this the functional genetics is approached (Fouts et al., 2008; Firrincieli et al., 2015; Martinez-Garcia et al., 2015).

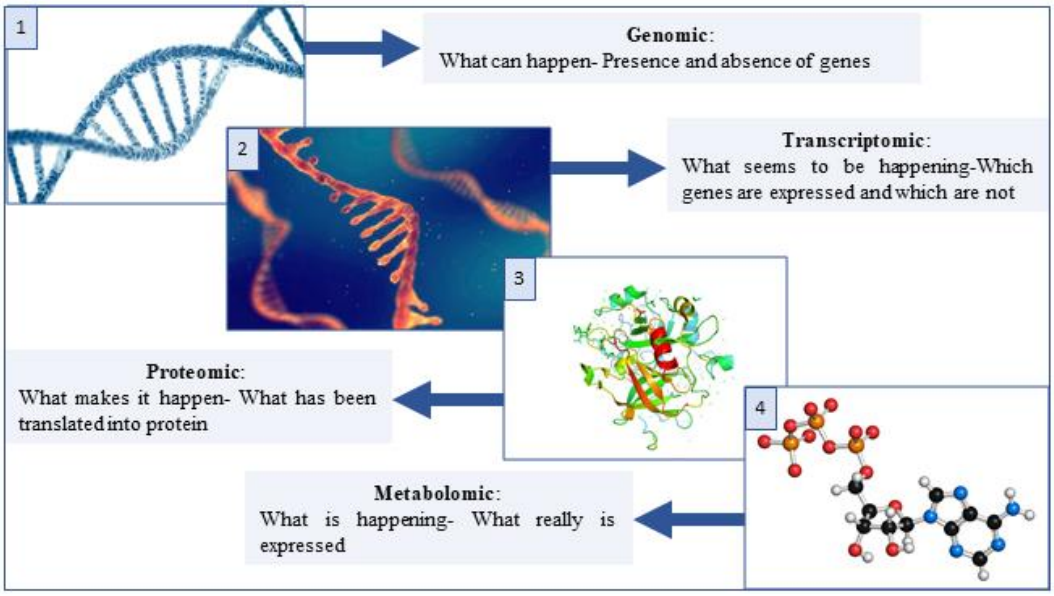


Figure 8. Scheme of the different disciplines that form the "omics".

Transcriptome refers to the complete set of messenger RNA (mRNA) and noncoding RNA (ncRNA) transcripts produced by a cell, that is, those genes that are expressed under certain conditions (Wang et al., 2009). An analysis of differentially expressed bacterial genes in the presence of specific plant species can provide data on the basic nature and the relationships established between the bacterium and the plant (Kaul et al., 2016). For example, the changes in the gene expression of endophytic bacteria such as *Azoarcus* sp. BH72, *Pseudomonas aeruginosa* PA01, *Bacillus amyloliquefaciens* FZB42 or *Frankia alni* ACN14a in presence of root exudates or within plant tissues (Mark et al., 2005; Alloisio et al., 2010; Fan et al., 2012; Shidore et al., 2012). The analysis of the transcriptomic profiles of these bacteria showed which genes were influenced by the compounds released by the plant and their ecological implication in the plant-bacteria relationship. For the transcriptome analysis of an organism, two technologies are usually used: RNA sequencing (RNA-Seq) and microarrays. RNA-Seq can detect differentially expressed genes by massive sequencing of RNA strands. For this, the RNA strands are fragmented, followed by the cDNA libraries construction and

their subsequent sequencing. In the case of microarrays, the procedure is different. First, a library of fluorochromes cDNAs is constructed that subsequently bind to specific probes. The expression is detected by the amount of emitted light that the detector manages to capture (Rensink and Buell, 2005; Manzoni et al., 2018).

Proteomics is the study of those genes that have been translated into proteins, but also large-scale proteins and their particular structure and function. Proteome analysis allows a dynamic image of the proteins expressed under certain conditions. This allows a vision of the processes that occur in an organism, including changes in expression levels, post-transcriptional modifications or the interaction between proteins (Wilkins et al., 1995; Maron et al., 2007). The study of influence of root exudates on the proteome of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42 is one of the few proteomic works focused on the variation of protein expression patterns when endophytic bacteria establish a relationship with their host (Kierul et al., 2015). However, the application of proteomics to investigate plant–microbe interactions is becoming more commonplace in recent years (Kav et al., 2007; Afroz et al., 2013). Two technologies are frequently used in proteomic studies: two-dimensional (2D) electrophoresis and liquid chromatography coupled to tandem mass spectroscopy (LC-MS/MS). 2D electrophoresis separates the proteins according to their isoelectric point and molecular mass, and the proteins are identified selectively, while LC-MS/MS characterizes proteins and small molecules in complex samples. Proteins digested in peptides are first separated according to peptide hydrophobicity in the chromatograph and subsequently based on their mass:charge (m/z) ratios in mass spectrophotometers. This increases the resolution of the resulting peaks, which are identified and thereby the proteins expressed by comparison with the databases (Mann and Pandey, 2001; Maron et al., 2007; Bhuyan et al., 2015).

Thus, omics technologies are excellent tools to study the *Micromonospora*-legume interaction to better understand the mechanisms during the establishment of the plant-microbe relationship, the communication mechanisms and the influence of the plant on bacterial behavior.

OBJECTIVES

2. Objectives

The main purpose of this work was to obtain information regarding the molecular interaction between *Micromonospora* and its host plant, as well as the capacity of *Micromonospora* in colonizing legumes other than its original host. In order to achieve this aim, the following specific objectives were:

1. To determine the presence of *Micromonospora* in different plant tissues (root, stem and leaves) besides the nitrogen-fixing nodules from *Lupinus angustifolius* and *Pisum sativum* plants by culture-dependent techniques.

2. To study the capacity of the strain *M. lupini* Lupac 08 to re-infect its original host and other legumes.

3. To locate *Micromonospora* cells inside nitrogen-fixing nodules through the use of different microscopy techniques.

4. To evaluate the effect of lupine root exudates on the intracellular proteome of different *Micromonospora* strains.

5. To use transcriptomic analyses to identify differentially expressed *Micromonospora* genes after exposure to the *Lupinus* to root exudates.

MATERIALS AND METHODS

3. Materials and methods

3.1 Isolation and characterization of *Micromonospora* strains from *Pisum* and *Lupinus* plants

3.1.1 Study site and sample collection

Fourteen legume plants (7 each) of wild pea (*Pisum sativum*) and blue lupine (*Lupinus angustifolius*) were collected in Salamanca (40º57′54″ N; 5º39′50″ W) and Cabrerizos (40º58'43" N; 5º36'46" W), Spain respectively (**Figure 9**). All plants were collected in April, just before reaching the flowering stage.

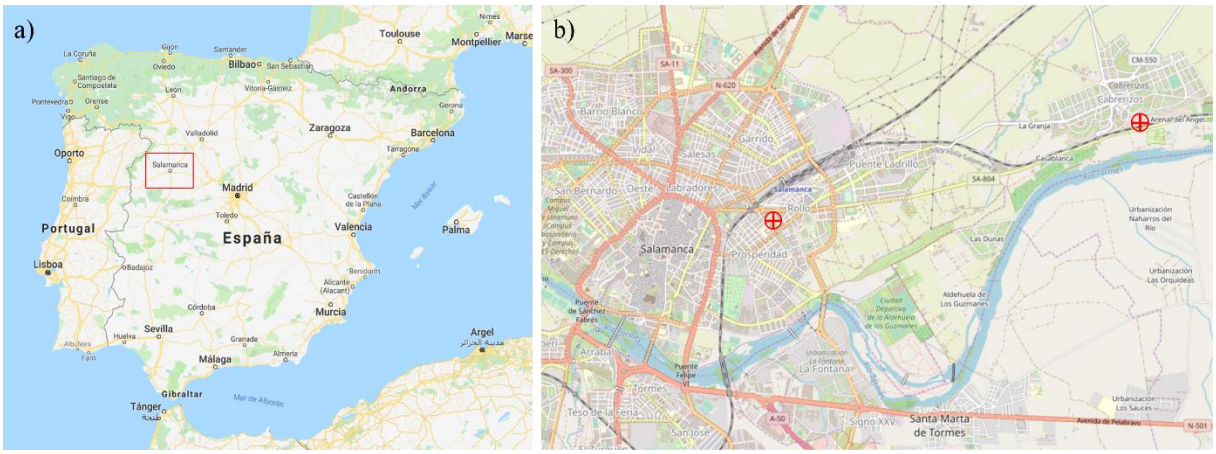


Figure 9. Sampling location map. (**a**) Spain map. (**b**) Aerial view of the sampling areas. The red rectangle in image (**a**) shows the area where the sampling areas are located. The symbol (⊕) in image (**b**) indicates the sampling places.

3.1.2 Isolation of microorganisms

The isolation of microorganisms from different plant tissues was carried out by selecting four nodules, four leaves, the stem and the roots per legume species. Before isolation, the nodules, leaves, roots and stems were washed under distilled water and surface sterilized.

The different plant tissues (roots, stems, leaves) were surface sterilized by immersing in 70% (v/v) ethanol for 1 min, transferred to 3.5% (v/v) sodium hypochlorite solution for 2 minutes (5 minutes for roots), and rinsed five times with sterile distilled water. Nodules were sterilized in 2.5% (w/v) $HgCl_2$ for 2 minutes and rinsed five times with sterile distilled water as well (Vincent, 1970). Samples were crushed with a sterile homogenizing pestle and the resulting slurry plated onto solid yeast extract mannitol (YMA) agar medium (Vincent, 1970) and yeast extract/humic acid (HA) agar (de la Vega, 2010) (**Appendix I**). Plates were incubated at 28ºC

for 3-4 weeks in the dark, with monitorization of growth every week. As negative control, sterilized surface plant tissues were placed on the same media plates to evaluate the absence of resistant microorganisms to the sterilization process.

After the incubation period, *Micromonospora*-like colonies on isolation plates were selected and sub-cultured on yeast extract/malt extract agar (ISP 2) medium (Shirling and Gottlieb, 1966) and SA1 agar (Trujillo et al., 2005) (**Appendix I)** to obtain pure cultures.

3.1.3. Maintenance and preservation of pure cultures of bacteria

The maintenance conditions of pure bacteria cultures for daily use was storage at 4 ºC for a short duration (2-3 weeks). Nevertheless, long-term preservation was performed at -80ºC in the 20% (v/v) glycerol cryoprotect agent.

3.1.4 Identification of isolates

3.1.4.1 DNA extraction

A small amount of bacterial biomass from fresh cultures on ISP 2 medium was resuspended in 300 µl sterile distilled water and precipitated by centrifugation at 12,000 rpm (Eppendorf centrifuge 5418) for 10 minutes. The supernatant was discarded, and the pellets were immediately stored at -20ºC. DNA extraction was performed using REDExtract-N-Amp Plant PCR kit (Sigma) according to the manufacturer's instructions with an additional purification step using phenol/chloroform (Trujillo et al., 2010). The procedure carried out was the following: First, the pellets were resuspended in 80 µl of *Extraction Solution* and incubated at 95 ºC for 10 minutes. An equal volume of *Dilution Solution* was added, continued by a treatment with 100 µl of phenol-chloroform-isoamyl alcohol (25:24:1) solution. The samples were then mixed with vortex until a milky suspension was formed. The suspension was centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the upper phase was collected and 100 µl of chloroform-isoamyl alcohol (24:1) solution was added, mixed and centrifuged at 13,000 rpm for 5 minutes. Finally, the supernatant was transferred to a new tube and stored at -20 ºC.

3.1.4.2 PCR amplification and sequencing of 16S rRNA genes

The identification of the isolates was performed by the amplification and sequencing of 16S ribosomal RNA (rRNA) gene, using the primers SF1 and 1522R (**Table 2**) and yielding a 1500 pb amplicon (Trujillo et al., 2010). The PCR reactions were done using the REDExtract-

N-Amp Plant PCR kit (Sigma) in a total volume of 25 µl. Each PCR reaction contained: 12 µl of Extract-N-Amp PCR Ready Mix (2X), 2.5 µl of *Extraction-Dilution solution* (1:1), 0.8 µl each primer (20 µM) and 1 µl of DNA template (3-5 µg). PCR amplification was carried out in a TProfessional Basic Thermal Cycler (Biometra), according to the following program: initial denaturation at 95 ºC for 9 minutes; 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 56 ºC for 1 minute and extension at 72 ºC for 1 minute. The final extension step was carried out at 72 ºC for 7 minutes.

Table 2. Primers used in the amplification and sequencing of 16S rRNA gene

Primers	Sequence	Location	Gene	Annealing
SF1	5'- AGAGTTTGATCMTGGCTCAG – 3'	27-47^{+}	16S	56ºC
SR2	5'- GWATTACCGCGGCKGCTG – 3'	501-519^{+}	16S	-
SR3	5'- CCGTCAATTCMTTTRAGTTT -3'	887-907^{+}	16S	-
SR4	5'- GGGTTGCGCTCGTTG – 3'	1505-1100^{+}	16S	-
1522R	5'-AAGGAGGTGATCCANCC-3'	1505-1522^{+}	16S	56ºC

The PCR reactions were loaded on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide, and electrophoresis was run in 1X Tris-acetate EDTA buffer (Millipore, Cork, Ireland) at 100 V for 1 hour (Bio-Rad powerPac 300 power supply). The amplicons were sequenced using the reverse primers SR2, SR3, SR4 and 1552R (**Table 2**) (Lane, 1991) for a complete and quality sequencing of 16S rRNA gene. The sequencing was performed by Sanger sequencing, the results of which were processed by Chromaspro 1.5 software (Technelysium, Australia) and assembled by Seqman software (DNAstar, Life Science). The results were compared to the EZ-biocloud database (https://www.ezbiocloud.net/identify) to determine the closest phylogenetic neighbors. Phylogenetic analysis of the strains was carried out using MEGA X software (Kumar et al., 2018) using neighbor joining, maximum-likelihood and maximum parsimony tree algorithms.

3.1.5 Screening for production of extracellular hydrolytic enzymes

3.1.5.1 Determination of the cellulolytic and hemicellulolytic activity

Cellulase activity was screened in the isolates using M3 agar medium (Rowbotham and Cross et al., 1997) (**Appendix I**) containing 0.5% (w/v) carboxymethylcellulose (CMC). The cultures were incubated for 7 and 14 days at 28ºC. This enzymatic activity was revealed by using 1% (w/v) Congo Red for 15 minutes, followed by washing with 1 M NaCl. Cellulolytic

activity was shown by an orange zone which appeared around the colony (Teather and Wood., 1982).

3.1.5.2 Determination of the of xylanolytic activity

The evaluation of xylanase activity was done by inoculating the isolates on M3 agar medium (**Appendix I**) with 0.5% (w/v) xylan. The plates were incubated for 4-7 days at 28ºC. The xylanolytic activity was revealed with 1% (w/v) Congo Red for 15 minutes, followed by destaining with 1 M NaCl. A positive reaction was observed as a yellow zone around the colony (Mateos et al., 1991).

3.1.5.3 Determination of pectinolytic activity

Pectinase activity was tested by growing the isolates on M3 agar medium (**Appendix I**) with 0.5% (w/v) pectin. The plates were incubated for 7-14 days at 28ºC. Pectinase production was revealed with Lugol (Fisher Scientific) for 10 minutes in dark, after which the plates were washed with distilled water. The positive strains showed a clear halo around the colony on a green background.

3.1.5.4 Determination of amylolytic activity

Amylolytic activity was determined by growing the isolates on M3 agar medium (**Appendix I**) with 0.5% (w/v) starch. The plates were incubated for 7-14 days at 28ºC. The amylolytic activity was revelated with Lugol (Fisher Scientific) for 10 minutes in dark, after which the plates were washed with distilled water. A positive reaction was displayed as a white halo around the colony on a dark blue background (Yamamoto, 1988).

3.1.5.5 Determination of chitinolytic activity

The production of chitinases was evaluated using M3 agar medium with 1% (w/v) colloidal chitin (**Appendix I**). The cultures were incubated for 60 days at 28ºC, monitorization of enzymatic activity performed every week. The positive strains showed a transparent halo around the colony.

Colloidal chitin was obtained by the following procedure (Hsu and Lockwood, 1974; Jagmann et al., 2010). Five grams of commercial chitin from shrimp shells (Sigma) were added to 100 ml of 37% (v/v) HCl and this mixture was stirred for 20 minutes, on an ice bath until a homogenous slurry was obtained. After stirring, the suspension was poured into 1250 ml of ice-cold deionized water for 10 minutes with stirring. The suspension was then filtered, and the chitin was washed repeatedly with sterile deionized water until a pH value of approximately 4 was reached. Subsequently, the pH was adjusted to 7 with 0.5 M NaOH and

the suspension was again filtered through a cellulose filter to collect the smallest chitin grain size. The final product was dried at 100ºC for 1h and then at 30ºC overnight and finely ground.

3.2 Monitoring the colonization and infection of legume nodules by *Micromonospora*

3.2.1 Bacterial strains and growth conditions

Mutant bacterial strains labeled with a green fluorescent protein (GFP) or red fluorescent protein (mCherry) were selected for their localization in plant tissues. The mutant strains used in this study were previously obtained in our laboratory (Benito et al., 2017) and are listed on **Table 3**. The *gfp*-tagged *Micromonospora lupini* (ML01-*gfp*) strain, a mutant of the wild strain Lupac 08, was selected as the working model strain. This strain was cultivated on SA1 agar supplemented with 25 µg/ml apramycin (**Appendix I**) at 28ºC for 7 days. The mCherry-tagged nitrogen-fixing rhizobia strains (*Sinorhizobium* (*Ensifer*) Rm1021-*mCh* and *Rhizobium* sp. E11-*mCh*) were grown on YMA supplemented with 60 µg/ml kanamycin, or on Tryptone yeast extract (TY) agar supplemented with 10 µg/ml tetracycline and 100 µg/ml streptomycin. However, the wild type strains *Bradyrhizobium* sp. CAR08, *Sinorhizobium* (*Ensifer*) Sm1021 and *Rhizobium* sp. E11 were cultivated on YMA agar (**Appendix I**) (Vincent, 1970). All rhizobial strains were incubated at 28ºC for 5 days.

Table 3. Mutant and wild type strains

Strain	Characteristics	Culture medium	Inoculation host
M. lupini ML01-*gfp*	*M. lupini* Lupac 08 egfp reporter gene	SA1+ Apr (25µg/ml)	*Lupinus, Medicago, Trifolium*
Sinorhizobium Rm1021-*mCh*	*S. meliloti* Rm1021(pM7604) carrying an mCherry reporter gene	TY+ Strep (100 µg/ml) + Tetra (10 µg/ml)	*Medicago*
Rhizobium sp. E11-*mCh*	*Rhizobium* sp. E11 (pM7607) carrying an mCherry reporter gene	YMA+Kn (60 µg/ml)	*Trifolium*
Bradyrhizobium sp. CAR08	Wild type	YMA	*Lupinus*
M. lupini Lupac 08	Wild type	SA1	-
Sinorhizobium Sm1021	Wild type	YMA	-
Rhizobium sp. E11	Wild type	YMA	-

3.2.2 *In vitro* antagonism assay between *Micromonospora* and different rhizobia

To determine the ability of strain *M. lupini* Lupac 08 to inhibit the growth of three rhizobia strains (*Bradyrhizobium* sp. CAR08, *Sinorhizobium* (*Ensifer*) sp. Sm1021 and *Rhizobium* sp. E11) and vice versa, the bacteria were grown following two different procedures based on the methodology of Gregor et al., 2003 and Haber and Ilan, 2013, with slight modifications.

Strains CAR08, Sm1021 and E11 were previously grown in YMA agar (**Appendix I**) for 5 days at 28 ºC, while strain Lupac 08 was grown in SA1 agar (**Appendix I**) for 7 days at 28 ºC. Bacterial suspensions of 1.8 $x10^9$ CFU/ml were prepared from these cultures. The rhizobial strains were spread evenly over surface of YMA agar (each on a separate plate) and left to dry at room temperature for 5 min. Subsequently, strain Lupac 08 was inoculated in form of a cross and the plates were incubated at 28 ºC for one week. On the other hand, Lupac 08 cells were inoculated as a 1.5-cm-wide strip in the center of the YMA plates. After 5 days of incubation at 28 ºC, the three rhizobial strains were streak inoculated in the margins where *Micromonospora* had grown. Bacterial growth and their interaction were observed 7 days after the inoculation of the rhizobial strains. YMA agar plates inoculated with individual target strains was used in all cases as a positive control.

3.2.3 Seed germination and infection assays

Lupinus albus (white lupin), *Medicago sativa* (alfalfa) and *Trifolium repens* (clover) plants were selected for the microscopy assays. Seeds were surface sterilized before germination through two different methods depending on the seed type. *Lupinus* seeds were sterilized using 2% (v/v) sodium hypochlorite for 12 min, followed by 5 washing steps with sterile distilled water. *Medicago* and *Trifolium* seeds were surface sterilized using 70% (v/v) ethanol for 30 seconds and immediately replaced by 2.5% (w/v) $HgCl_2$ for 2 minutes, followed by several rinses with sterile distilled water, and then placed on tap-water agar plates in the dark (**Appendix I**). After germination, *Medicago* and *Trifolium* seedlings were placed on square Petri dishes (120 × 120 mm) (**Figure 10**) containing nitrogen-free Rigaud and Puppo nutrient agar (**Appendix I**). In the case of *Lupinus*, the seedlings were planted in pots with vermiculite and watered with nitrogen-free Rigaud and Puppo solution (**Appendix I**) (Rigaud and Puppo, 1975). All seedlings were kept in phytotron with a photoperiod of 16h light and 8h dark at 21-22 ºC and 50-60% relative humidity.

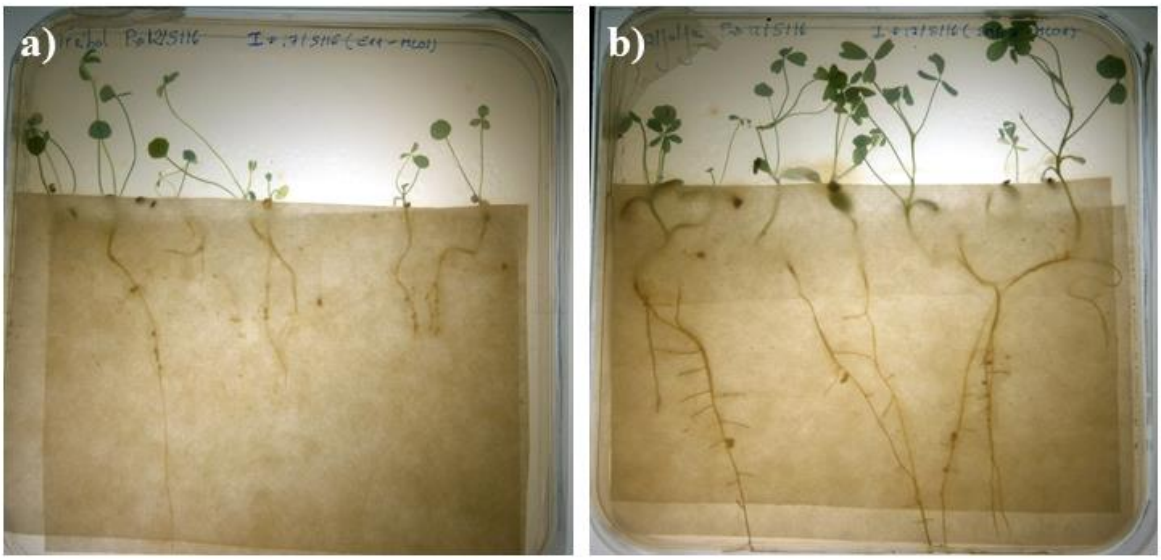


Figure 10. Growth of *Trifolium* and *Medicago* plants on square Petri dishes (120 × 120 mm). **(a)** *Trifolium* plants; **(b)** *Medicago* plants.

Upon the appearance of the first leaves, the plants were inoculated with the appropriate bacterial suspensions ($1.8x10^8$ cfu/ml). Overall, three different treatments were used:

1. Plants inoculated with GFP-tagged *Micromonospora lupini* (ML01-*gfp*)
2. Plants co-inoculated with *M. lupini* ML01-*gfp* and the appropriate rhizobia strains (*Bradyrhizobium* CAR08, *Sinorhizobium* Rm1021-*mCh* and *Rhizobium* sp. E11-*mCh*) to induce nodulation
3. Uninoculated plants, which served as negative controls.

3.2.4 Monitoring bacterial colonization by fluorescent and confocal microscopy

The monitorization of the root colonization by *Micromonospora* was performed in *Medicago* and *Trifolium* plants. Infected plant roots of *Medicago* and *Trifolium* were observed with fluorescence (Nikon Eclipse 80i) and confocal scanning laser (CLSM, Leica TCS model) microscopes 2 days after inoculation, and monitored every other day until the nodules were fully developed. The mature nodules were longitudinally sectioned for a better visualization of the internal infected tissues. Localization of *gfp* and *mCherry* fluorescence in the root and nodule tissues was performed by using standard filter settings (488 nm excitation and 515 to 560 nm emission for *gfp* expression, and 620 nm excitation and 620-660 nm emission for *mCherry*). Autofluorescence was evaluated by comparing the *gfp* image with the red

fluorescence channel (543 nm excitation and >570 nm emission) and by comparing the image with uninoculated plants. Co-inoculated *Lupinus* plants were grown for 4-5 weeks. For observation, nodules were longitudinally sectioned on a cryostat (Thermo HM560), mounted on glass slides, and viewed by CLSM as described above. Autofluorescence was reduced by staining the tissues with 5 μg/ml propidium iodide solution for 5 minutes.

3.2.5 Immunoelectron microscopy

Lupinus albus nodules were used to localize strain ML01-*gfp* by pre-embedding immunogold technique with antibodies raised against GFP following the procedures of Lujan et al., 1996 and Chen et al., 2003. A vibratome (Leica V1000) was employed to obtain 60 μm semithin sections from fixed agarose embedded nodules (4% (v/v) paraformaldehyde and 0,1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (PB)). Sections were washed in tris-buffered saline (TBS) (**Appendix I**) for 30 minutes, followed by blocking with 10% (v/v) normal goat serum (NGS) in 0.1 M TBS containing 0.9% (w/v) NaCl for 1 hour. The sections were labeled with the primary antibody raised against *gfp* in guinea pig (0.5-2 μg/ml diluted in 0.1 M TBS with 1% (v/v) NGS; Frontiers Institute, Japan) and incubated at 4ºC overnight. After three wash with TBS for 15 minutes each, 1.4 nm gold particles conjugated to goat anti-guinea pig antibodies (diluted 1:100 in TBS buffer containing 2% (v/v) NGS; Nanoprobes, NY). These were added in the samples and incubated for 2 hours in the dark. The nodule sections were washed again twice with TBS for 15 minutes each. Subsequently, they were washed two times with phosphate-buffered saline (PBS) (**Appendix I**) for 15 minutes each. After several PBS washes, sections were postfixed in 1% (v/v) glutaraldehyde (prepared in PBS) for 10 minutes. Sections were washed three times in double distilled water for 10 minutes, followed by a Silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes), incubated for 7 minutes in darkness. The reaction was stopped with distilled water, and the samples were washed first with distilled water 4 times for 10 minutes, followed by a wash with 0.1M PB (**Appendix I**) for 15 minutes. After these steps, the sections were prepared for observation under the microscope. At first, the sections were treated with 1% (v/v) osmium tetroxide in 0.1M PB and incubated for 30 minutes. This was followed by 5 washes with 0.1M PB for 8 minutes and one wash with MilliQ water for 5 minutes. Subsequently, the sections were incubated in 1% (w/v) uranyl acetate for 30 minutes, in the dark and shaking. The dehydration process was carried out with a series of ethanol solutions at different concentrations (50%, 70%, 90%, 96%, 100%, 100% ultrapure). Finally, the sections were incubated twice with

propylene oxide for 10 minutes. After this time, they were embedded in resin and incubated overnight and without agitation. The gold-silver-labeled sections were processed for transmission electron microscopy.

3.3 Effect of root exudates on the intracellular proteome of *Micromonospora*

3.3.1 Collection of root exudates

Lupinus albus seeds were surface sterilized and germinated as described previously in section 3.2.3. When seedlings had developed a main root of at least 7 cm in length, they were transferred into a 250 ml conical flask with 175 ml of autoclaved nitrogen-free Rigaud and Puppo nutrient solution (**Appendix I**) (Rigaud and Puppo, 1975). This was to facilitate axenic hydroponic growth conditions (**Figure 11)**. Seedings in the flask were incubated in a growth chamber with a photoperiod of 16h light and 8h dark at 21-22ºC and 50-60% relative humidity. After one week, the Rigaud and Puppo nutrient solution was replaced with fresh nutrient solution and incubated in the same growth conditions with gentle shaking at 25 rpm (MaxQ 2506 Reciprocating Shaker, Thermo Fisher). The exudates were collected every 7 days for 3 weeks, filtered through 0.45 μm membrane (Millipore), freeze-dried and suspended in sterile distilled water at a concentration of 40 mg/ml. All collected root exudates were stored at -20 ºC until their use. The sterility of the root exudates before and after filtration was tested by plating 100 μl on LB plates (**Appendix I**) and incubated at 30 ºC for 48 hours. In parallel, nitrogen-free Rigaud and Puppo nutrient solution (without exudates) was collected, freeze-dried and resuspended to the same concentration as the exudates collected.

Figure 11. The hydroponic system used for collection of *Lupinus* root exudates.

3.3.2 Growth of *Micromonospora* strains in the presence and absence of root exudates

The strains *M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^T and *M. cremea* CR30^T were chosen to evaluate the gene expression levels when they were grown in presence or absence of *Lupinus* root exudates. The three strains were selected for the following reasons: Lupac 09^T is type strain of *M. saelicesensis*, the most abundant species in isolates from *Pisum sativum* and *Lupinus angustifolius* tissues (section 3.1); Lupac 08 was the strain used in microscopy assays (section 3.2) and CR30^T strain without cellulolytic activity in *in vitro* tests.

The *Micromonospora* strains were grown under three different media conditions: ISP 2 broth (**Appendix I**), ISP 2 broth supplemented with 0.25 mg/ml of Rigaud and Puppo solution and ISP 2 broth supplemented with 0.25 mg/ml of lupine root exudates (collected in Rigaud and Puppo solution) (**Table 4**). The cultures were grown with shaking at 180 rpm at 28 ºC. Seven days later, the cultures were centrifuged at 12,000 rpm (Eppendorf centrifuge 5804 R) for 10 minutes at 4 ºC. Cell pellets were mixed with 5 ml "killing buffer" (**Appendix I**) to stop mRNA production (Fan et al., 2012; Zhang et al., 2015) and then centrifuged at 12,000 rpm for 3 minutes at 4 ºC. The pellets were treated once more with 1 ml "killing buffer" and then immediately washed a few times with saline solution. Finally, the pellets were stored at -80ºC until protein extraction.

Table 4. Protein samples and growth conditions of the strains

Sample	Strains	Growth conditions
Protein01	Lupac 08	ISP2
Protein02	Lupac 08	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
Protein03	Lupac 08	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)
Protein04	Lupac 09^T	ISP2
Protein05	Lupac 09^T	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
Protein06	Lupac 09^T	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)
Protein07	CR30^T	ISP2
Protein08	CR30^T	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
Protein09	CR30^T	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)

3.3.3 Determination of cellulolytic activity in presence and absence of plant

Strains Lupac 08, Lupac 09^T and CR30^T were screened for cellulase activity on M3 agar medium (Rowbotham and Cross et al., 1997) (**Appendix I**) containing 0.5% (w/v) carboxymethylcellulose (CMC). Plates were incubated at 28 ºC for 7 and 14 days, and later they were revealed using 1% (w/v) Congo Red for 15 minutes. Then faded plates were washed twice with 1 M NaCl. Colonies with cellulolytic activity displayed an orange zone around the colony (Teather and Wood., 1982).

The cellulolytic activity determination of the three *Micromonospora* strains in presence of live roots was evaluated using three different legumes: *Lupinus albus, Medicago sativa* and *Trifolium repens.* Seeds were surface sterilized and germinated as described previously in Section 3.2.3. The three *Micromonospora* strains were growth in ISP2 liquid medium at 28ºC in a rotatory shaker at 180 rpm for 7 days with the aim of obtaining enough biomass for the inoculation process. After incubation the cultures were centrifuged at 12,000 rpm (Eppendorf centrifuge 5804 R) for 10 minutes, and the cells were washed twice with 0.9% (w/v) saline solution. Immediately after, seedlings were inoculated by being dipped into the culture, softly swirled for 5 minutes and subsequently transferred to two culture mediums: M3 agar medium (**Appendix I**) and Rigaud and Puppo solid medium (**Appendix I**). Both mediums contained 0.5% (w/v) carboxymethylcellulose (CMC). As a negative control several plants were immersed in sterile 0.9% (w/v) saline solution for 5 minutes and subsequently transferred to the same culture medium as the inoculated plants. All plants were incubated for 7 and 14 days in a growth chamber with a photoperiod of 16h light and 8h dark at 21-22ºC and 50-60% relative humidity. In addition, the strains were grown in these two cellulose media but without plant and in the same growing conditions as the plant assays. The cellulolytic activity was revealed using 1% (w/v) Congo Red and 1 M NaCl.

3.3.4 Intracellular protein extraction

Total intracellular protein extraction was carried out by the procedure previously described by Diaz et al., 2013. The microbial biomass (~250 mg) was resuspended in 10.3% (w/v) saccharose and centrifuged at 13,000 rpm for 5 minutes (Eppendorf centrifuge 5418). The supernatant was discarded and the cell wall disruption was done with 0.4 mm silica pellets in a FastPrep instrument (MP-Biomedicals) at a speed setting of 6 M/second for 4 cycles of 15 seconds with 2 minutes of ice incubation between each cycle. The lysed cells were boiled

in a loading buffer (**Appendix I**) for 10 minutes and centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant contained the extracted proteins. All protein samples were stored at -20ºC until use.

Protein concentration was determined using the Qubit™ Protein Assay Kit and the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific), following the manufacturer's instructions.

3.3.5 One-dimensional gel electrophoresis (SDS-PAGE)

The proteins were separated according to their size by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Fisherbrand™ Vertical Gel Tank (FisherScientific). The separating gels were prepared with a 15% (v/v) polyacrylamide final concentration and stacking gels of 4% (v/v) (**Table 5**). The electrophoresis samples were prepared in a loading buffer (**Appendix I**) and heated in boiling water for 5 minutes just before electrophoresis. The gels were charged and run at 30mA/gel for 100 minutes in a 1x Tris-Glycine-SDS buffer (**Appendix I**). The low molecular weight protein marker used was the EZ-Rum^{TM} protein marker 116.0-14.4 KDa (Fisher bioreagents). Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 (Fisher BioReagents) (**Appendix I**) for 30 minutes and rinsed with a 5:1:4 methanol, acetic acid and distilled water solution (**Appendix I**) for 30-60 minutes in constant agitation.

Table 5. Composition of separating and stacking gels

1D gels composition				
	Separating gel (15%)		Stacking gel (4%)	
	Concentration	Volume	Concentration	Volume
ACR:BIS	30:0.8	5.0 ml	30:0.8	0.65 ml
Tris-HCl	1.5M pH 8.8	2.5 ml	0.5M pH 6.8	1.25 ml
Distilled water	-	2.5 ml	-	3.10 ml
SDS	10%	100 µl	10%	50 µl
APS	10%	50 µl	10%	25 µl
TEMED	-	5 µl	-	5 µl

3.3.6 Two-dimensional gel electrophoresis and protein visualization

Two-dimensional electrophoresis was performed in the proteomics facility of Centro de Investigación del Cáncer, CIC-University of Salamanca, Spain. The protein samples were dehydrated before delivery to the proteomics service. The protein dehydration was carried out by a treatment with methanol-chloroform, following the proteomic service instructions. Briefly, four volumes of methanol were added to the samples, and the mixture was vortexed. One volume of chloroform was then added, and the mixture was vortexed. Finally, three volumes of MilliQ water were added to the samples and mixed with vortex until the samples had a milky appearance. Samples were centrifuged at 12,000 rpm (Eppendorf centrifuge 5418) for 5 minutes and the upper phase was discarded. Following this, 3 volumes of methanol were added, mixed well with vortex and centrifuged at 12,000 rpm for 5 minutes, to remove the supernatant. Finally, the samples were allowed to dry for 10-15 minutes at room temperature.

Before performing two-dimensional (2D) electrophoresis, the protein samples precipitated with methanol-chloroform were resuspended rehydration buffer (**Appendix I**). Isoelectric Focusing (IEF) was the first step for 2D gel electrophoresis. The protein samples (4.66 μg/μl) were added to 18 cm IPG gel strips with a non-linear gradient of pH 3-11 (GE Healthcare). The IEF run conditions were the followings: 12 hours at 50 v, 1 hour at 500 v, 1 h at 1000 v, 30 minutes with a voltage gradient from 1000 to 8000 v and an accumulation of 50000 v at 8000 v/ h. The strips were treated with equilibration buffer (**Appendix I**) with 15 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) for 15 minutes, for denaturation and reduction of proteins. After this, the protein alkylation was carried out with equilibration buffer with 2.5% iodoacetamide. The second dimension of electrophoresis was realized in 18x20 cm at 10% (v/v) polyacrylamide 2D SDS-PAGE gels. The proteins were visualized with silver staining by a modified protocol described by Heukeshoven and Dernick, (1988). The gels were analyzed by SameSpots software (Totallabs).

3.3.7 Protein analysis by LC-MS/MS and data analysis

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed in the proteomics facility (CIC, Salamanca, Spain). The protein samples (2 μg) were concentrated in a 1D SDS-PAGE gel dyed with Coomassie Blue (Shevchenko et al., 1996). The concentrated proteins were digested with trypsin and the resulting peptides were desalinated

by C18 reversed phase microcolumns (Rappsilber et al., 2007). The peptides were resuspended in 0.5% (v/v) formic acid, 2% (v/v) acetonitrile in water, and analyzed by LC-MS/MS (nanouplc system (nanoacquity waters) connected to LTQ Orbitrap Velos Mass spectrometer (Thermo Fisher Scientific, Bremen, Germany)). The analysis was performed within the following parameters: The peptides were separated in a symmetry of C18 trap column (100 A 5 µm 180µm x 200 mm) with a 120 minutes gradient from 2% to 35% (v/v) acetonitrile, and in BEH C18 column (1.7µm 75ID 25cm).

Fragmented peptides were identified and quantified by Proteome Dicoverer program (version1.4.1.14) of Thermo Scientific using the MASCOT algorithm (Perkins et al., 1999). The local database was formed by Uniprot sequences of *Micromonospora lupini* Lupac 08 (UP000003448), *Micromonospora saelicesensis* Lupac 09^T (UP000198864), *Micromonospora cremea* $CR30^T$ (UP000185124), *Lupinus angustifolius* cv. Tanjil (UP000188354) and the common protein contaminants database (Mann and Wilm, 1994; Rappsilber et al., 2002). The *Lupinus angustifolius* cv. Tanjil (UP000188354) database was used with the objective of eliminating any lupin contaminant protein found in the *Micromonospora* samples. The filters applied to the results were fixed to a minimum of two peptides per protein and a high peptide confidence cut-off of 95% or 99%. The estimated false discovery rate (FDR) was set to 1% for all peptide and protein identifications (Choi and Nesvizhskii, 2008). The analysis was also performed with the MAXQUANT program (Cox and Mann, 2008) using the Andromeda search algorithm and the database formed by the Uniprot sequences and comparing the protein abundance through iBAQ intensity (Krey et al., 2014)

Proteins were considered differentially expressed if their levels of expression differed at least 1.5-fold from the control. In other words, if their Fold Change (FCH) was greater or equal to1.5 (Love et al., 2014). Subsequently, the obtained proteins were compared with the Lupac 08, Lupac 09^T and $CR30^T$ genomes by using the Uniprot and GenBank databases. The proteins identified were clustered into orthologous gene (COG) categories (Tatusov et al., 2001) and the reconstruction of the metabolic pathways was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa, 2002; Aoki-Kinoshita and Kanehisa, 2007).

3.4 Transcriptome profiling of *Micromonospora* under the effect of root exudates

3.4.1 RNA isolation and RNA-Seq

Total RNA was isolated from the cultures previously used for proteomic analysis (see section 3.3.2) (**Table 4**). After the three *Micromonospora* strains were grown in the presence and absence of exudates and treated with killing buffer, the bacterial pellets were immersed in RNAlater (Invitrogen™) with the aim of preventing RNA degradation and stored at -80ºC until RNA isolation. Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, USA) according to the manufacturer's instructions with some modifications of the first steps. The cell lysis with TE buffer supplemented with 3 mg/ml lysozyme was replaced with disruption using a mortar and pestle, freezing the samples immediately in liquid nitrogen and grinding it to a fine powder. The disrupted cells were then transferred into a new liquid-nitrogen-cooled tube and resuspended in 500 µl lysis buffer (Buffer RLT) supplemented with 10 µl β-mercaptoethanol per 1 ml Buffer RLT. The samples were mixed with vortex until a homogeneous suspension was obtained. If insoluble material was visible, the samples were centrifuged at 13,400 rpm (Eppendorf 5452 Minispin Centrifuge) for 2 minutes, using only the supernatant in subsequent steps. The RNA precipitation step was performed by adding 250 µl ethanol (96-100% (v/v)) to the lyse, mixing thoroughly by pipetting and incubating for 5 minutes at room temperature for optimal precipitation. The samples were then transferred to RNeasy mini columns placed in a 2 ml collection tube, including any precipitate that might have formed, and centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through. 300 µl Buffer RW1 was added into the RNeasy column and centrifuged at 10,000 rpm for 15 seconds to wash the column. Then, the samples were treated with TURBO DNA-free™ Kit TURBO™ DNase (Invitrogen™) to eliminate any DNA residues. 0.1 volume 10× TURBO DNase Buffer and 1 µl TURBO DNase were added to RNA samples, mixed gently and incubated for 20 minutes at 37ºC. To achieve the objective of deactivating DNase activity and eliminating it from the columns, 300 µl Buffer RW1 were added and the samples was centrifuged at 10,000 rpm for 15 seconds. The column was washed twice with 500 µl Buffer RPE (with absolute ethanol) and centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through. The columns were additionally centrifuged at full speed for 1 minute to eliminate all ethanol residues present in the column. The RNeasy columns were transferred into a new 1.5 ml collection tube and the RNA samples was eluted in 40 µl RNase-free water, deposited directly onto the RNeasy silica-gel membrane. After incubation for 1

minute at room temperature, the RNA samples were centrifuged at 10,000 rpm for 1 minute. The isolated RNA was stored at -80 ºC until use.

The RNA quality was checked on 1% (w/v) agarose gel and analyzed with the Agilent 2100 Bioanalyzer Platform (CIC, Salamanca, Spain). Before sequencing, the samples were precipitated in ethanol to avoid degradation. As a preliminary step, the RNA sample volume was measured. If the volume was less than 100 µl, it was adjusted with elution buffer to reach 100 µl. Immediately after, 1/10 volume of 3M sodium acetate (pH 5.2) was added to the samples and vortexed briefly. Immediately following this, 3 volumes of 100% (v/v) ethanol (calculated after the addition of salt) was added and the samples were vortexed briefly. Finally, the samples were place at -20ºC for over 30 minutes, with the aim that the RNA remain stable against any temperature change. The RNA-sequencing and alignment procedures were conducted by Chunlab (Seoul, South Korea). RNA sequencing was performed on the Illumina HiSeq 2500 platform using single-end 50 bp sequencing.

3.4.2 Transcriptome data analysis

RNA-Seq data obtained was aligned and annotated using the genomes of *M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^T and *M. cremea* $CR30^T$, with accession numbers NZ_CAIE00000000, NZ_FMCR00000000 and NZ_FSQT00000000 respectively.

Transcriptome data was visualized and analyzed using the CLRNASeq™ 1.00.06 software developed by Chunlab (South Korea). The relative transcript abundance was quantified as raw read counts and Relative Log Expression (RLE), since it had the lowest Coefficient of Variation (CV) (Anders and Huber, 2010; Anders et al., 2013). DESeq2, a Bioconductor component in the R package, was used for differential gene expression analysis of *Micromonospora* strains grown in the presence of root exudates and *Micromonospora* strains grown in the absence of root exudates (control sample) (**Table 6**). The genes with DESeq2 p-value (0.01-0.05), which were firstly selected, were significantly differentially expressed according to statistics. The second parameter used was fold change (FCH) (Love et al., 2014). The transcripts obtained were considered as significantly differentially expressed when they exhibited an FCH ≥1.5 in expression level. *Micromonospora* gene ontology analysis was performed by assigning Clusters of Orthologous Groups (COG) categories (Tatusov et al., 2000) and by using the Kyoto Encyclopedia of Genes a Genomes (KEGG) database (Kanehisa 2002; Aoki-Kinoshita and Kanehisa, 2007).

Table 6. RNA samples and growth conditions of the strains

Sample	Strains	Growth conditions
RNA01	Lupac 08	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
RNA02	Lupac 08	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)
RNA03	Lupac 09^T	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
RNA04	Lupac 09^T	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)
RNA05	CR30^T	ISP2+ Rigaud and Puppo solution (0.25 mg/ml)
RNA06	CR30^T	ISP2+ Rigaud and Puppo solution with exudates (0.25 mg/ml)

3.4.3 Real-time PCR

Semiquantitative real-time PCR was performed to validate the transcriptional profiling data obtained from RNA-Seq using Illumina HiSeq 2500 platform. The first step carried out was to obtain the strands of complementary DNA (cDNA) from single-stranded RNA. cDNA synthesis was obtained by reverse transcription with NZY first-stand cDNA synthesis kit (NZYTech, Lda, Portugal) according to the manufacturer's protocol. For each reaction, 10 µl of the NZYRT 2× Master Mix, 2 µl of NZYRT Enzyme Mix, 5 µl RNA (up to 4 µg) and 3 µl DEPC-treated H_2O was used. Adequate cDNA synthesis was carried out through the following steps: First, the reaction was incubated at 25 ºC for 10 minutes, followed by an incubation at 50 ºC for 30 minutes. The next step was to induce the inactivation reaction by applying heat of 85 ºC for 5 minutes and then chilling the sample on ice. In the last step, 1 µl of NZY RNase H (*E. coli*) was added and incubated at 37 ºC for 20 minutes. cDNA was stored at -20 ºC until further use. Oligonucleotide primers used (**TableX**) were designed by Primer3 0.4.0 software (Koressaar and Remm, 2007; Untergasser et al., 2012).

Real-Time PCR reactions were performed in a CFX96 Real-Time thermocycler (BioRad) using the NzySpeedy qPCR Green Master Mix (2X) (NZYTech, Lda, Portugal). For each reaction, 5 µl of the Master Mix, 0.5 µl of each primer (10 µM, forward and reverse), 4.2 µl of nuclease-free water and 0.5 µl of template cDNA (0.1-0.5 µg) was added. The PCR program included the following steps: An initial step of denaturation at 95 ºC for 3 minutes, followed by 40 cycles initiated by denaturation at 95 ºC for 15 seconds and an annealing at 60 ºC for 30 seconds. After this step the fluorescence was read. At the end of the program,

the temperature experienced an increase from 65 ºC to 95 ºC at a rate ramp of 0.1 ºC/seconds, allowing for the evaluation of melting curves.

Table 7. Primers used in RT-qPCR

Gene	Oligo Name	Sequence (5´-3´)	Length (nt)	GC-content (%)	Tm (ºC)	Tm óptima
rpoB	RTrpoB_F	GCACGGYAACAAGGGYGTCATCTC	24	62.50	62.79	60.00
rpoB	RTrpoB_R	ATGTTCATCCGGCTCGGCACACC	23	60.87	61.63	60.00
gyrB	RTgyrB_F	CGACTTCGACTTCCAGACCATCTAC	25	52.00	68.22	66.50
gyrB	RTgyrB_R	GTCTTGTGGATCGGGYTCTTSG	22	54.55	69.57	66.50
atpD	RTatpD_F	GACSGAGTCCGGCGTCATCGACAAG	25	64.00	63.21	60.00
atpD	RTatpD_R	CAGCACCTCCTGCTTCTTCACGTCSC	26	61.54	63.02	60.00
Endoglucanase	EgluL8_1-F	GTGGTTGTCACTGTCGATGTTCCT	24	50.00	67.97	65.50
Endoglucanase	EgluL8_1-R	AAGTTCCTCCGGGCTCTCAC	20	60.00	68.34	65.50
Endoglucanase	EgluL9_1-F	ACTGTCACTCTGCCCACGACGA	22	59.09	68.31	65.50
Endoglucanase	EgluL9_1-R	ACTCGGACTGGTTGCGGTAGGT	22	59.09	68.15	65.50
Chitinase	ChitiL8_1-F	CCAGATCGAGCCGTAGACCA	20	60.00	68.45	65.50
Chitinase	ChitiL8_1-R	CAGCGGCAACATCAACACTC	20	55.00	67.11	65.50
Chitinase	ChitiL9_1-F	GTACTGCATGTTCAGCCACCA	21	52.38	65.91	64.00
Chitinase	ChitiL9_1-R	CATCAACACCCTGTCCACCTC	21	57.14	66.40	64.00
Chitinase	ChitC30_1-F	ATGAAGGACAACGTCTACGAGAACC	25	48.00	65.07	64.00
Chitinase	ChitC30_1-R	GTCGAGCGAGCTGATGGTGATG	22	59.00	66.00	64.00
Glucokinase	GlukL8_1-F	ATGGTCATCTTCGGCGGCACCA	22	59.09	69.36	65.50
Glucokinase	GlukL8_1-R	CACCGAGGGCGTTGGAGTTGAG	22	63.64	68.05	65.50
Glucokinase	GlukL9_2-F	CGACGAGTTGGGCATCGACGTA	22	59.09	67.25	65.50
Glucokinase	GlukL9_2-R	GTACAGGTAGAGGACGTTGTCGCAG	25	56.00	67.25	65.50
Glucokinase	GlukC30_2-F	CTTTCGATGTCGCGAATGTCC	21	52.38	65.08	62.40
Glucokinase	GlukC30_2-R	CTGCTCGATCCGGAAGCTGT	20	60.00	65.14	62.40
Trehalose	TreaL8_1-F	CTTGACGTAGTTGTGCAGGA	20	50.00	61.67	61.30
Trehalose	TreaL8_1-F	CTGATGTCCAAGCACTGGAT	20	50.00	62.71	61.30
Trehalose	TreaL9_1-F	CAACTGGGAGCACGACCACAC	21	61.19	66.19	62.00
Trehalose	TreaL9_1-R	CCAGCCCGATGGCGATGAAC	20	65.00	66.31	62.00
ABC transporters	TrapL8_1-F	CGAGGGTCGCAACTACCTCCA	21	61.90	66.36	65.50
ABC transporters	TrapL8_1-R	CTGTCAACGCCACCACCGTC	20	65.00	66.32	65.50
ABC transporters	TrapL9_1-F	CCAGCAGCATGTCGATGAAG	20	55.00	67.20	65.50
ABC transporters	TrapL9_1-R	GTTCGGCTACGACCATCAGG	20	60.00	68.03	65.50
ABC transporters	TrapC30_1-F	GGTGTACGGATGGGCCGGGT	20	70.00	69.10	65.50
ABC transporters	TrapC30_1-R	TGCTCAACCTGCTCCAAGACCTCAA	25	52.00	68.78	65.50
Xylanase	XilL9_1-F	GGTTGTAGCCCTTGTCGATG	20	55.00	65.68	64.00
Xylanase	XilL9_1-R	CTCTTCACCCACCCGATCTT	20	55.00	66.46	64.00
Xylanase	XilC30_1-F	TGCTGGAGTTGTCGTTCACCC	21	57.14	65.33	64.00
Xylanase	XilC30_1-R	CAGGTCGGGTTCGTTCCACA	20	60.00	64.84	64.00
Pectinase	PecL9_1-F	TCACACAGATCGAGGTCTCC	20	55.00	62.15	60.00
Pectinase	PecL9_1-R	GACAAGGTCTTCCAGCACAA	20	50.00	63.07	60.00
Betaglucanase	BetagL8_1-F	TGCCGCCCATCGAGATCACCGAGAG	25	64.00	64.01	61.30
Betaglucanase	BetagL8_1-R	TCCCAGTTGTCCAGCAGCGACCAGA	25	60.00	63.53	61.30
Betaglucanase	BetagC30_1-F	ACTTCGTCGGTTACTCCGCCTC	22	59.00	66.73	64.00
Betaglucanase	BetagC30_1-R	ATCTCAAACGGCAGCAGCATCAC	23	52.17	66.60	64.00

Ct values were calculated by the threshold method with Bio-Rad CFX Manager software used to compare the expression between reference and target genes. The genes *gyr*B (B subunit of DNA gyrase), *rpo*B (β-subunit of RNA polymerase) and *atp*D (ATP synthase β-subunit) genes were chosen as references as their expression was not altered regardless of the growth conditions used. In the case of the target genes, six genes per strain were selected for their importance in the transcriptome analysis. Four technical replicates were carried out for

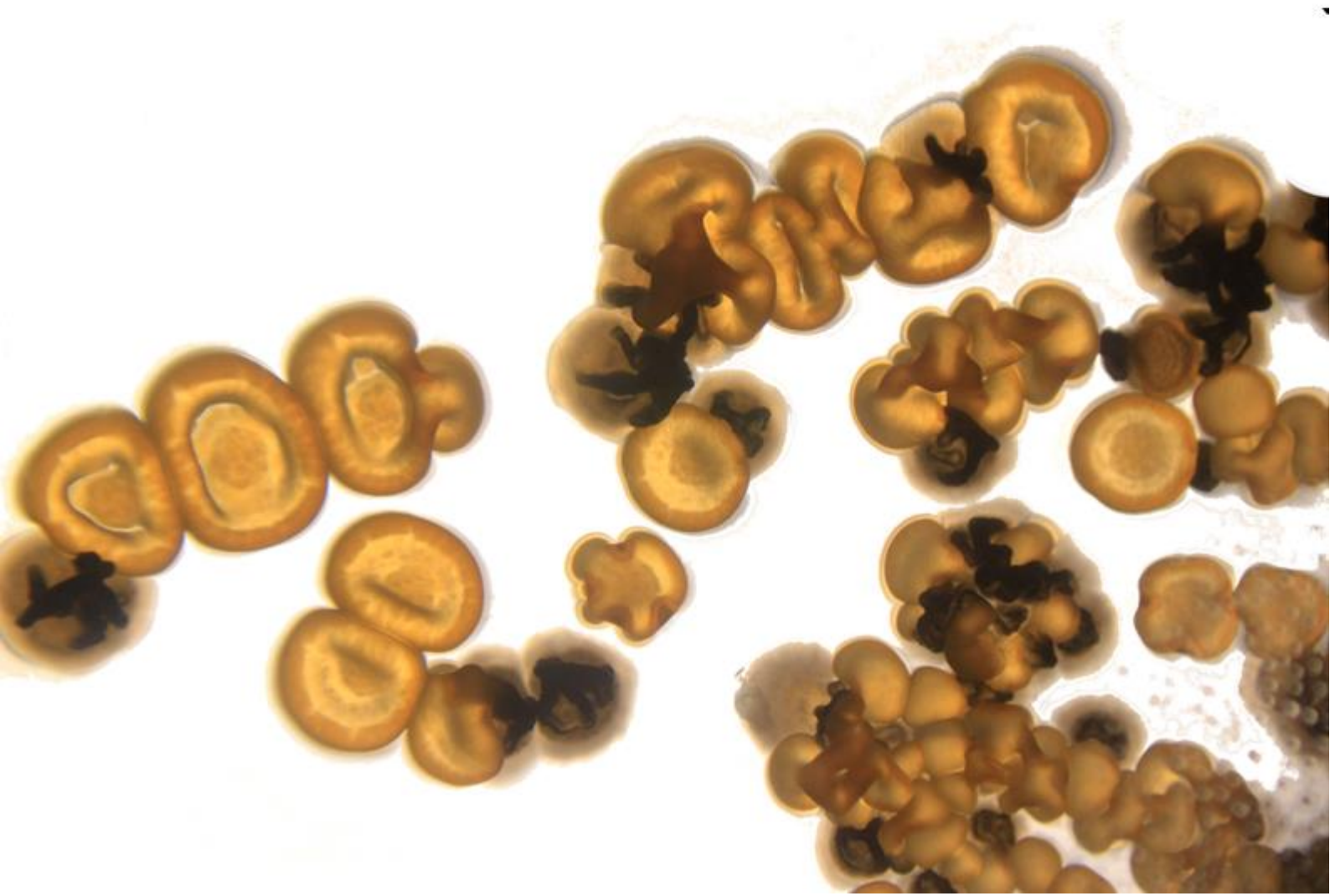
each reference and target gene. Quantification was analyzed based on the threshold cycle (Ct) values and the $2^{-\triangle\triangle Ct}$ Livak method (Livak and Schmittgen, 2001).

3.4.4 Evaluation of gene expression levels by real-time PCR of the *Micromonospora* strains after direct exposure to *Lupinus* root exudates

The same lupin seeds that were used in the root exudates collection were also used in this assay. *Lupinus* seeds were surface sterilized and germinated as described previously in the section 3.2.3. Between 7 to 10 days after germination, the seedlings were transferred into flasks with 175 ml of autoclaved nitrogen-free Rigaud and Puppo nutrient solution (**Appendix I**), where the root zone was in sterile conditions. In parallel, Lupac 08, Lupac 09^T and $CR30^T$ strains were grown in ISP 2 liquid medium (**Appendix I**) at 28ºC for a week, under shaking to obtain biomass to prepare inoculum. The plants were inoculated after an adaptation week to the hydroponic environment. First, the bacterial cells were recollected by centrifugation at 12,000 rpm (Eppendorf centrifuge 5804 R) for 3 minutes at 4 ºC and washed with the same nutrient solution utilized in hydroponic medium. The Rigaud and Puppo nutrient solution, where the lupine roots were submerged, were inoculated with a bacteria suspension of 2.1 $x10^8$ cfu/ml with the objective of exposing the strains directly to the exudates. The contact *Micromonospora*-exudates was incubated in a growth chamber with a photoperiod of 16h light and 8h dark at 21-22ºC and 50-60% relative humidity for 5 days with gentle shaking at 25 rpm (MaxQ 2506 Reciprocating Shaker, Thermo Fisher) for better contact. The bacterial cells were collected by centrifugation at 12,000 rpm for 3 minutes at 4 ºC. Finally, the samples were treated with "Killing buffer" (**Appendix I**) and immediately frozen with liquid nitrogen and stored at -80ºC. The RNA isolation, cDNA synthesis and RT-PCR were performed as described in sections 3.4.1 and 3.4.3. The genes evaluated in this assay were the same as those used in the validation of the transcriptome data.

CHAPTER 1

Diversity of *Micromonospora* strains isolated from different tissues of *Pisum* and *Lupinus* plants



4. Chapter 1. Diversity of *Micromonospora* strains isolated from different tissues of *Pisum* and *Lupinus* plants

4.1 Introduction

Microbes are widely distributed in the different ecosystems present on our planet including the most extreme habitats. The soil represents the richest reservoir of biological diversity, where microorganisms are in continuous interaction with other forms of life (Berendsen et al., 2012). The interactions between plant and bacteria occur in a specific soil region called rhizosphere, which is continually influenced by the plant root exudates (Kent and Triplett, 2002). Some bacteria not only colonize the rhizosphere but can also colonize the rhizoplane and the internal plant tissues. However, the bacterial density of internal plant tissues is significantly less than in soil. The number of bacterial cells in bulk soil and rhizospheric environments reaches10^7-10^9 CFU/g of soil, whereas the population densities in the rhizoplane and root endosphere range from 10^5 to 10^7 CFU/g of fresh weight. In other plant areas such as stems and leaves, the cultivable population densities are much lower, reaching 10^3-10^4 CFU/g of fresh weight (Benizri et al., 2001; Hallmann, 2001; Bais et al., 2006; Bulgarelli et al., 2013).

The endophytic bacteria are those capable of colonizing and residing at least part of their lives within plant tissues. They are considered non-pathogenic and even many of them improve plant growth or health (Sturz and Nowak, 2000; Hardoim et al., 2008). The endophytes can be translocated inside their plant hosts by passive and active mechanisms. Penetration can take place through cracks present on root emergence sites or created by pathogenic microorganisms, as well as by root hair tips and other bacterial specific mechanisms (Reinhold-Hurek and Hurek, 1998; Hardoim et al., 2008). However, endophytes present in the rhizosphere can encounter several obstacles before settling inside the plant. The plant immune system and the chemical signals present in the root exudates have a key role in the colonization of plant tissues (Turner et al., 2013). Furthermore, barriers such as the epidermis or endodermis can block further colonization since only a few bacteria are able to pass through the endodermis (Gregory, 2006). Several endophytes are able to cross the endodermis by the secretion of different hydrolytic enzymes such as cellulases and pectinases, without causing damage or visible symptoms to the plant (James et al., 2002).

Among the great bacterial diversity present in the soil, Actinobacteria represent approximately 20-30% of the rhizospheric microbial community (Bouizgarne and Ben

Aouamar, 2014). In recent years, several Actinobacteria have been isolated from both the rhizosphere and various plant tissues. *Frankia* was the first actinobacterial endophyte isolated from plant nodular tissues (Callaham et al., 1978). This genus is characterized as a nitrogen-fixing microorganism that induces root nodules on several angiosperm plants belonging to genera *Alnus*, *Myrica*, *Morella* and *Comptonia* (Huguet et al., 2005). In the last decade, endophytic actinobacteria other than *Frankia* such as *Micromonospora* or *Streptomyces* have been isolated from wild plants. The genus *Micromonospora* is a common inhabitant of the nodules and is widely distributed in different legumes and actinorhizal plants such as *Arachis hypogaea*, *Cicer arietinum*, *Glycine max, Lens culinaris, Lupinus angustifolius*, *Lupinus gredensis*, *Medicago sativa*, *Melilotus* sp., *Phaseolu*s sp., *Pisum sativum*, *Trifolium* sp., *Casuarina equisetifolia*, *Alnus glutinosa*, *Morella pensylvanica* or *Myrica gale* (Valdés et al., 2005; Cerda, 2008; Rodríguez, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2013, 2016). However, the isolation of this genus has been restricted to the rhizosphere, roots and nodules of legumes until now.

In this chapter, the aim was to determine the presence of *Micromonospora* in different plant tissues besides the nitrogen-fixing nodules of legumes. To this end, two wild plant species were sampled. Experiments were carried out for the selective isolation of *Micromonospora* strains. In addition, their ability to degrade different compounds present in the plant cell wall was evaluated.

4.2 Results

4.2.1 Endophytic bacteria isolated from *Pisum* and *Lupinus* plants

The isolation of endophytes belonging to the genus *Micromonospora* was performed by using plant tissues (nodules, roots, stems and leaves) from two wild legumes: *Pisum sativum* and *Lupinus angustifolius*. The culture media used for isolation were yeast extract-mannitol agar (YMA) and extract/humic acid (HA) agar (**Appendix I**). The first colonies were observed after an incubation period of seven days at 28°C. These colonies had a whitish and mucous appearance, typical characteristics of rhizobia or *Bacillus* (**Figure 12**). After 15 days of incubation, colonies with typical morphology of *Micromonosporaceae* (orange-pigmented colonies, filamentous and rough texture) began to appear (**Figure 12**), becoming perfectly visible after 4 weeks incubation.

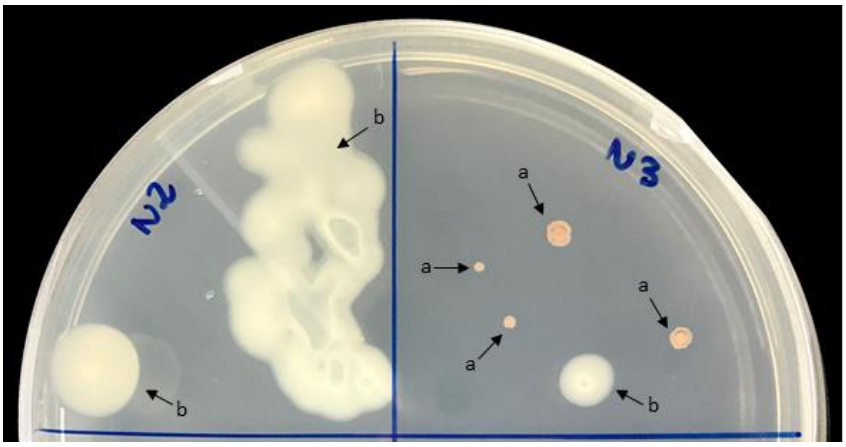


Figure 12. Isolation plate of *Lupinus* nodules in YMA medium. a. Colonies with typical morphology of *Micromonosporaceae.* b. Colonies with whitish and mucous appearance, typical characteristics of rhizobia or *Bacillus.*

A total of 248 and 273 colonies appeared on the isolation plates from *Lupinus* and *Pisum* tissues respectively, of which 44 and 107 strains were selected due to their morphological similarity to the genus *Micromonospora* (**Table 8**). In *Lupinus* plants, *Micromonospora*-like colonies were recovered from all tissues tested, but their abundance varied depending on the evaluated tissues. Leaves were the tissue with a greater number of isolates (21), in contrast stems were the tissues with the lowest number of isolates (1 isolate). In the case of *Pisum* plants, the strains with *Micromonospora* morphology were only isolated from leaves and nodules, with a similar number of isolates in these two tissues (~53 isolates) (**Table 8**).

Table 8. Number of *Micromonospora*-like strains isolated from different plant tissues and legumes

	Pisum		***Lupinus***	
Plant tissue	Total number of isolates per tissue type	Isolates with *Micromonospora* morphology	Total number of isolates per tissue type	Isolates with *Micromonospora* morphology
Nodules	91	54	56	12
Stems	8	0	16	1
Roots	82	0	120	10
Leaves	92	53	56	21
Total isolates	273	107	248	44

Most *Micromonospora*-like strains from *Pisum* and *Lupinus* nodules and *Lupinus* roots were isolated on the YMA medium. However, the strains from the leaves of both plants were isolated in the two media used, both YMA and HA (**Appendix II**). The absence of microbial colonies on control plates confirmed that isolates obtained were present inside the plant tissues.

4.2.2 Identification of *Micromonospora* strains by 16S rRNA

Sequencing of the 16S ribosomal RNA (rRNA) gene was performed for all 151 *Micromonospora*-like isolates. The sequences obtained were compared with the sequences deposited in the public database EZ-biocloud (www.ezbiocloud.net). The closest species of each isolate is listed on **Table 9**, together with the percentage identity and the plant tissue where strains were isolated. Most isolated strains belonged to the genus *Micromonospora*, except for four isolates from *Pisum* leaves. These non-*Micromonospora* strains showed a similarity percentage between 99.0-99.8% with *Pseudonocardia soli* (PSH10), *Micrococcus aloeverae* (PSH12), *Streptomyces alboniger* (PSH33) and *Nocardiopsis umidischolae* (PSH39). Subsequent studies did not include these four strains since they do not correspond to the genus *Micromonospora*.

Table 9. Similarity of the sequences of the 16S rRNA gene of the strains of this study compared with those deposited in the public database EZ-biocloud

Strain	Plant	Plant area	Identification	Percentage of identity
LAN01	6	N1	*Micromonospora yasonensis* DS3186^T	99.7
LAN02	6	N2	*Micromonospora echinospora* DSM 43816^T	99.9
LAN03	6	N3	*Micromonospora echinospora* DSM 43816^T	100.0
LAN04	6	N4	*Micromonospora echinospora* DSM 43816^T	99.9
LAN05	6	N3	*Micromonospora yasonensis* DS3186^T	99.7
LAN06	6	N4	*Micromonospora echinospora* DSM 43816^T	99.9
LAN07	6	N3	*Micromonospora chaiyaphumensis* DSM 45246^T	99.7
LAN08	6	N4	*Micromonospora echinospora* DSM 43816^T	99.9
LAN09	7	N2	*Micromonospora zamorensis* DSM 45600^T	99.6
LAN10	7	N1	*Micromonospora yasonensis* DS3186^T	99.7
LAN11	7	N1	*Micromonospora ureilytica* GUI23^T	100.0
LAN12	7	N3	*Micromonospora vinacea* GUI63^T	99.7
LAT01	1	T1	*Micromonospora saelicesensis* Lupac09^T	99.7
LAR01	7	R1	*Micromonospora saelicesensis* Lupac09^T	99.9
LAR02	7	R1	*Micromonospora yasonensis* DS3186^T	99.7
LAR03	7	R1	*Micromonospora saelicesensis* Lupac09^T	99.9
LAR04	7	R1	*Micromonospora kangleipakensis* MBRL 34^T	99.2
LAR05	5	R1	*Micromonospora saelicesensis* Lupac09^T	99.7
LAR06	7	R1	*Micromonospora vinacea* GUI63^T	99.6
LAR07	7	R1	*Micromonospora zamorensis* DSM 45600^T	100.0
LAR08	7	R1	*Micromonospora zamorensis* DSM 45600^T	100.0
LAR09	7	R1	*Micromonospora noduli* GUI43^T	99.9
LAR10	7	R1	*Micromonospora noduli* GUI43^T	99.9

LA: *Lupinus angustifolius*; **N**: nodule; **T**: stem; **R**: root.

Strain	Plant	Plant area	Identification	Percentage of identity
LAH01	6	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH02	6	H2	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH03	6	H1	*Micromonospora equina* Y22^T	99.4
LAH04	6	H2	*Micromonospora coerulea* DSM 43143^T	99.3
LAH05	1	H1	*Micromonospora citrea* DSM 43903^T	99.7
LAH06	1	H1	*Micromonospora saelicesensis* Lupac09^T	99.7
LAH07	1	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH08	5	H1	*Micromonospora noduli* GUI43^T	99.9
LAH09	4	H1	*Micromonospora zamorensis* DSM 45600^T	100.0
LAH10	4	H2	*Micromonospora inositola* DSM 43819^T	99.3
LAH11	4	H3	*Micromonospora saelicesensis* Lupac09^T	99.7
LAH12	4	H4	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH13	2	H1	*Micromonospora siamensis* DSM 45097^T	99.4
LAH14	2	H1	*Micromonospora noduli* GUI43^T	99.9
LAH15	2	H2	*Micromonospora pisi* DSM 45175^T	100.0
LAH16	5	H2	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH17	5	H3	*Micromonospora saelicesensis* Lupac09^T	99.9
LAH18	5	H4	*Micromonospora noduli* GUI43^T	100.0
LAH19	2	H1	*Micromonospora noduli* GUI43^T	100.0
LAH20	2	H1	*Micromonospora ureilytica* GUI23^T	99.9
LAH21	2	H1	*Micromonospora pisi* DSM 45175^T	100.0
PSN 01	2	N1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 02	2	N1	*Micromonospora noduli* GUI43^T	100.0
PSN 03	2	N3	*Micromonospora halotolerans* CR18^T	99.5
PSN 04	2	N3	*Micromonospora halotolerans* CR18^T	99.5
PSN 05	2	N3	*Micromonospora phytophila* SG15^T	99.7
PSN 06	2	N1	*Micromonospora luteiviridis* SGB14^T	99.3
PSN 07	3	N2	*Micromonospora noduli* GUI43^T	100.0
PSN 08	3	N2	*Micromonospora noduli* GUI43^T	100.0
PSN 09	3	N2	*Micromonospora echinofusca* DSM 43913^T	100.0
PSN 10	3	N2	*Micromonospora vinacea* GUI63^T	99.9
PSN 11	2	N3	*Micromonospora palomenae* NEAU-CX1^T	99.2
PSN 12	3	N1	*Micromonospora noduli* GUI43^T	100.0
PSN 13	3	N1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 14	3	N1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 15	5	N4	*Micromonospora noduli* GUI43^T	99.8
PSN 16	5	N4	*Micromonospora zamorensis* DSM 45600^T	99.3
PSN 17	5	N4	*Micromonospora noduli* GUI43^T	100.0
PSN 18	5	N4	*Micromonospora noduli* GUI43^T	100.0
PSN 19	6	N1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 20	6	N1	*Micromonospora noduli* GUI43^T	99.9

LA: *Lupinus angustifolius*; **PS**: *Pisum sativum*; **N**: nodule; **H**: leaf.

Strain	Plant	Plant area	Identification	Percentage of identity
PSN 21	6	N1	*Micromonospora noduli* GUI43^T	99.9
PSN 22	6	N1	*Micromonospora noduli* GUI43^T	99.9
PSN 23	6	N1	*Micromonospora siamensis* DSM 45097^T	100.0
PSN 24	6	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 25	6	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 26	6	N3	*Micromonospora noduli* GUI43^T	99.9
PSN 27	6	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 28	6	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 29	6	N2	*Micromonospora noduli* GUI43^T	99.9
PSN 30	6	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 31	1	N1	*Micromonospora saelicesensis* Lupac09^T	99.8
PSN 32	1	N1	*Micromonospora auratinigra* DSM 44815^T	99.4
PSN 33	3	N3	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 34	3	N3	*Micromonospora zamorensis* DSM 45600^T	100.0
PSN 35	3	N2	*Micromonospora saelicesensis* Lupac09^T	99.7
PSN 36	7	N3	*Micromonospora noduli* GUI43^T	100.0
PSN 37	7	N3	*Micromonospora noduli* GUI43^T	100.0
PSN 38	7	N3	*Micromonospora noduli* GUI43^T	99.8
PSN 39	7	N3	*Micromonospora vinacea* GUI63^T	100.0
PSN 40	7	N3	*Micromonospora noduli* GUI43^T	99.8
PSN 41	5	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 42	5	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 43	5	N2	*Micromonospora palomenae* NEAU-CX1^T	99.6
PSN 44	5	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 45	5	N2	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 46	2	N4	*Micromonospora palomenae* NEAU-CX1^T	99.8
PSN 47	2	N4	*Micromonospora vinacea* GUI63^T	100.0
PSN 48	2	N1	*Micromonospora echinospora* DSM 43816^T	99.9
PSN 49	6	N3	*Micromonospora noduli* GUI43^T	99.8
PSN 50	3	N3	*Micromonospora noduli* GUI43^T	99.7
PSN 51	3	N3	*Micromonospora vinacea* GUI63^T	100.0
PSN 52	1	N1	*Micromonospora siamensis* DSM 45097^T	100.0
PSN 53	1	N1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSN 54	1	N1	*Micromonospora noduli* GUI43^T	100.0
PSH 01	1	H1	*Micromonospora citrea* DSM 43903^T	99.7
PSH 02	1	H1	*Micromonospora palomenae* NEAU-CX1^T	99.7
PSH 03	1	H1	*Micromonospora vinacea* GUI63^T	99.7
PSH 04	1	H1	*Micromonospora phytophila* SG15^T	99.3
PSH 05	1	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSH 06	1	H1	*Micromonospora noduli* GUI43^T	100.0
PSH 07	1	H1	*Micromonospora noduli* GUI43^T	99.9
PSH 08	1	H2	*Micromonospora luteiviridis* SGB14^T	99.2

PS: *Pisum sativum*; **N**: nodule; **H**: leaf.

Strain	Plant	Plant area	Identification	Percentage of identity
PSH 09	1	H2	*Micromonospora noduli* GUI43^T	100.0
PSH 10	1	H2	*Pseudonocardia soli* NW8-21^T	99.1
PSH 11	2	H1	*Micromonospora noduli* GUI43^T	99.9
PSH 12	2	H1	*Micrococcus aloeverae* AE-6^T	99.8
PSH 13	2	H1	*Micromonospora halotolerans* CR18^T	99.4
PSH 14	2	H3	*Micromonospora echinospora* DSM 43816^T	100.0
PSH 15	2	H3	*Micromonospora coerulea* DSM 43143^T	99.3
PSH 16	2	H3	*Micromonospora inyonensis* DSM 46123^T	100.0
PSH 17	2	H3	*Micromonospora inyonensis* DSM 46123^T	100.0
PSH 18	2	H3	*Micromonospora noduli* GUI43^T	99.9
PSH 19	2	H3	*Micromonospora noduli* GUI43^T	100.0
PSH 20	5	H1	*Micromonospora inyonensis* DSM 46123^T	100.0
PSH 21	5	H1	*Micromonospora echinospora* DSM 43816^T	100.0
PSH 22	5	H1	*Micromonospora vinacea* GUI63^T	100.0
PSH 23	5	H2	*Micromonospora palomenae* NEAU-CX1^T	99.5
PSH 24	5	H2	*Micromonospora costi* CS1-12^T	99.2
PSH 25	5	H4	*Micromonospora vinacea* GUI63^T	99.8
PSH 26	5	H3	*Micromonospora zamorensis* DSM 45600^T	99.7
PSH 27	5	H3	*Micromonospora noduli* GUI43^T	100.0
PSH 28	3	H2	*Micromonospora coerulea* DSM 43143^T	99.4
PSH 29	3	H2	*Micromonospora palomenae* NEAU-CX1^T	100.0
PSH 30	3	H2	*Micromonospora vinacea* GUI63^T	99.8
PSH 31	3	H1	*Micromonospora luteiviridis* SGB14^T	99.6
PSH 32	3	H1	*Micromonospora noduli* GUI43^T	99.9
PSH 33	3	H2	*Streptomyces alboniger* NRRL B-1832^T	99.7
PSH 34	4	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSH 35	4	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSH 36	4	H1	*Micromonospora noduli* GUI43^T	100.0
PSH 37	4	H1	*Micromonospora coriariae* DSM 44875^T	99.5
PSH 38	4	H1	*Micromonospora saelicesensis* Lupac09^T	99.9
PSH 39	4	H2	*Nocardiopsis umidischolae* 66/93^T	99.5
PSH 40	4	H2	*Micromonospora noduli* GUI43^T	100.0
PSH 41	1	H2	*Micromonospora echinofusca* DSM 43913^T	99.5
PSH 42	6	H3	*Micromonospora noduli* GUI43^T	100.0
PSH 43	6	H3	*Micromonospora noduli* GUI43^T	100.0
PSH 44	6	H2	*Micromonospora noduli* GUI43^T	100.0
PSH 45	7	H3	*Micromonospora sediminis* CH3-3^T	100.0
PSH 46	7	H3	*Micromonospora echinofusca* DSM 43913^T	100.0
PSH 47	5	H4	*Micromonospora saelicesensis* Lupac09^T	99.9
PSH 48	5	H4	*Micromonospora noduli* GUI43^T	99.9
PSH 49	6	H1	*Micromonospora chokoriensis* DSM 45160^T	100.0
PSH 51	6	H1	*Micromonospora coerulea* DSM 43143^T	99.3
PSH 52	6	H3	*Micromonospora noduli* GUI43^T	99.9
PSH 53	6	H1	*Micromonospora phytophila* SG15^T	99.9
PSH 54	6	H1	*Micromonospora phytophila* SG15^T	99.9

PS: *Pisum sativum*; **H**: leaf.

Among the new strains belonging to genus *Micromonospora*, 23.1% and 27.2% of isolates were identified as *M. saelicesensis* and *M. noduli* respectively, being *M. noduli* the most isolated species. However, the abundance of these species was different depending on the type of plant tissue and legume species evaluated (**Figure 13**). In *Lupinus* nodules, the two most abundant species were *M. echinospora* and *M. yasonensis*, representing 41.7% and 25% of the isolates obtained respectively. In other tissues such as roots and leaves, *M. saelicesensis* was the most abundant species (~35%), followed by *M. noduli* (~20%). Nevertheless, among the strains isolated from *Pisum* plants, *M. noduli* and *M. saelicesensis* were the most prevalent species in both leaves and nodules. *M. noduli* was the most prevalent species in these two tissues, representing approximately 33% of all species (**Figure 13**).

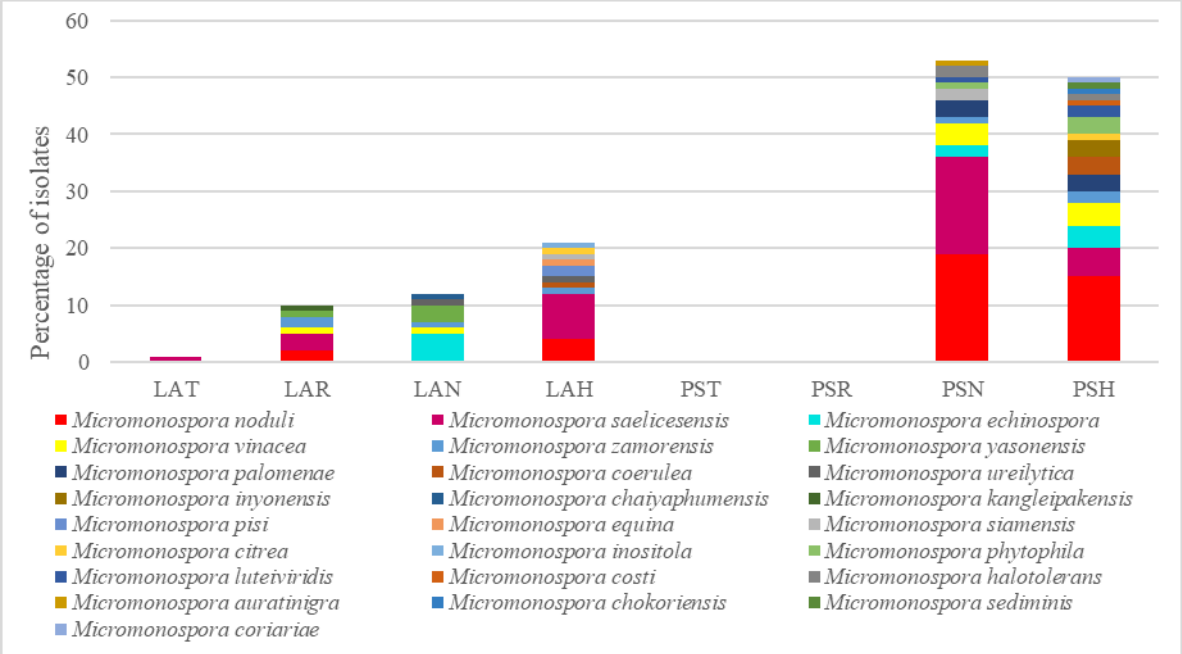


Figure 13. Percentage of *Micromonospora* species per legume and tissue. **LA**: *Lupinus angustifolius*; **PS**: *Pisum sativum*; **N**: nodule; **R**: root; **T**: stem; **H**: leaf.

The phylogenetic tree based on the maximum-likelihood method was constructed using the 16S rRNA sequences of the 147 isolates identified as *Micromonospora*. The phylogenetic tree represented in **Figures 14-16** showed that the isolated strains were distributed throughout it, displaying the high diversity found among the isolated strains. Approximately 85% of the strains were grouped in the same cluster. Within this cluster, a significant number of strains were branched with the type strains *M. noduli* GUI43^{T} and *M. saelicesensis* Lupac 09^{T}. Within the strains grouped with *M. noduli* GUI43^{T}, 50% were isolated from leaves of both *Pisum* and *Lupinus*, while the other 50% of the strains were isolated from *Pisum* nodules and

Lupinus roots. In the case of the isolates that formed the largest cluster with *M. saelicesensis* Lupac 09^T, most of them (~57%) were isolated from *Pisum* nodules, except 14 strains that were isolated from *Pisum* and *Lupinus* leaves and two strains from *Lupinus* roots. All strains grouped with *M. noduli* GUI43^T or with *M. saelicesensis* Lupac 09^T showed a percentage similarity between 99.9% and 100% with respect to these two type strains representing the two species. The remaining strains were distributed along the tree and grouped with different *Micromonospora* species with validly published names such as *M. zamorensis* CR38^T, *M. yasonensis* DS3186^T, *M. inositola* DSM4389^T or *M. pisi* GUI15^T, among other strains.

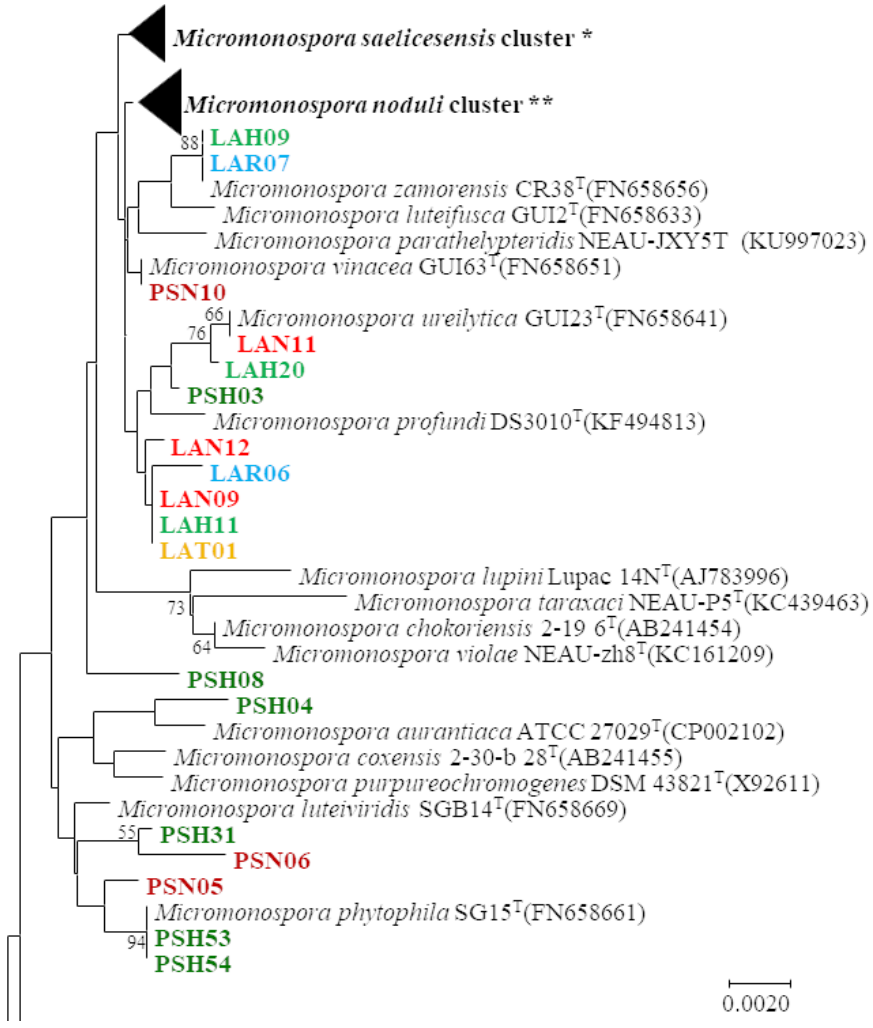


Figure 14. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains identified as *Micromonospora* and the currently recognized *Micromonospora* species. *Catellatospora citrea* type strain was used as outgroup. Bar, 0.002 substitutions per nucleotide position. Bootstrap percentages (1000 replicates) above 50% are shown at nodes.

***Micromonospora saelicesensis* cluster**: *Micromonopora saelicesensis* Lupac09^T (AJ783993)/ PSN13/ PSN14/ PSN19/ PSN20/ PSN21/ PSN22/ PSN24/ PSN25/ PSN26/ PSN27/ PSN28/ PSN29/ PSN30/ PSN33/ PSN35/ PSN41/ PSN42/ PSN44/ PSN45/ PSN53/ PSH05/ PSH25/ PSH26/ PSH34/ PSH35/ PSH38/ PSH47/ LAH01/ LAH02/ LAH06/ LAH07/ LAH12/ LAH16/ LAH17/ LAR03/ LAR05

****Micromonospora noduli* cluster:** *Micromonopora noduli* GUI43^T (FN658649)/ PSN02/ PSN07/ PSN08/ PSN12/ PSN15/ PSN17/ PSN18/ PSN21/ PSN22/ PSN26/ PSN36/ PSN37/ PSN48/ PSN49/ PSN50/ PSN54/ PSH06/ PSH07/ PSH11/ PSH18/ PSH19/ PSH27/ PSH30/ PSH32/ PSH36/ PSH40/ PSH42/ PSH43/ PSH44/ PSH48/ PSH52/ LAH08/ LAH14/ LAH18/ LAH19/ LAR01/ LAR09/ LAR10

Figure 14

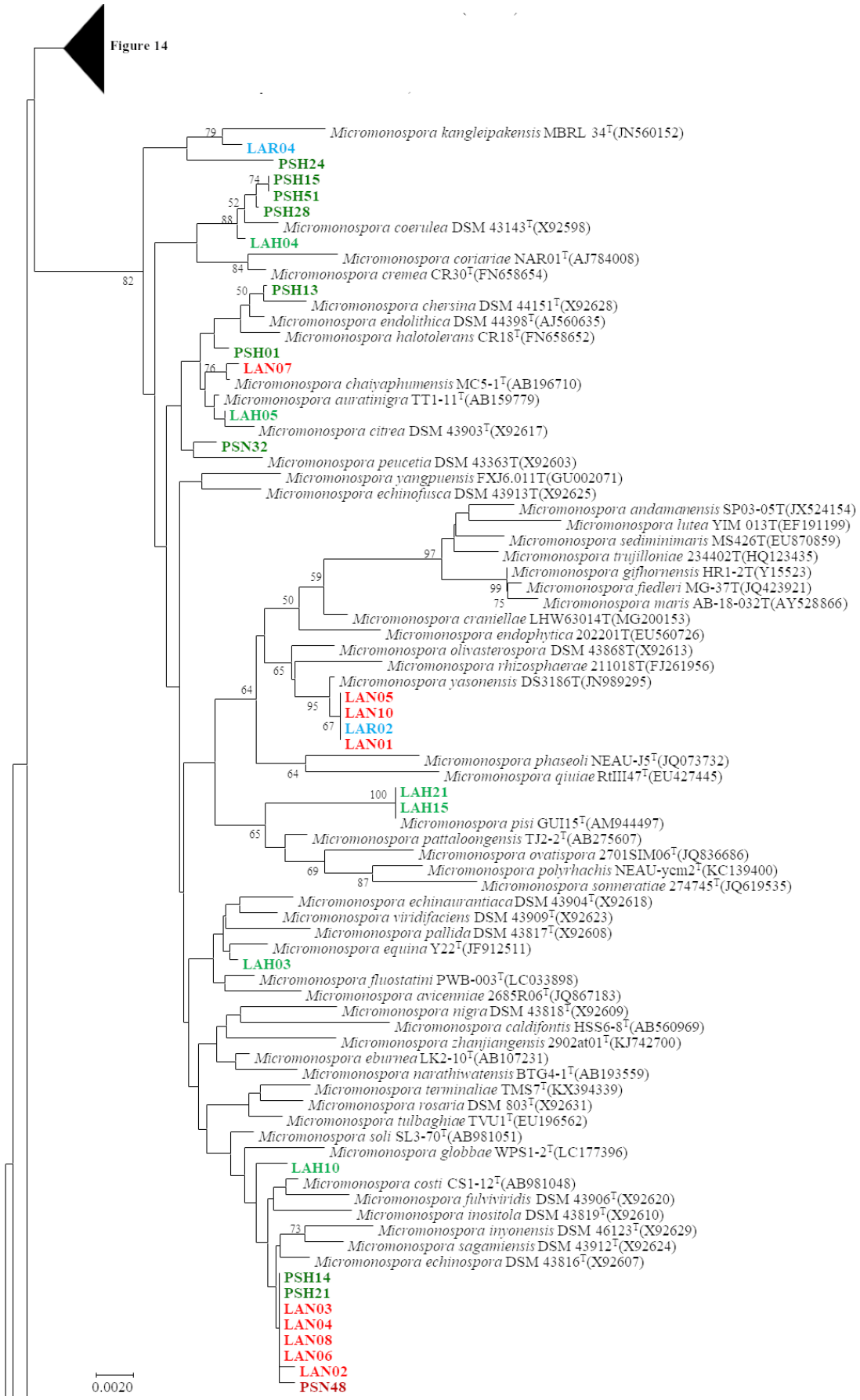

Micromonospora kangleipakensis MBRL 34T(JN560152)
LAR04
PSH24
PSH15
PSH51
PSH28
Micromonospora coerulea DSM 43143T(X92598)
LAH04
Micromonospora coriariae NAR01T(AJ784008)
Micromonospora cremea CR30T(FN658654)
PSH13
Micromonospora chersina DSM 44151T(X92628)
Micromonospora endolithica DSM 44398T(AJ560635)
Micromonospora halotolerans CR18T(FN658652)
PSH01
LAN07
Micromonospora chaiyaphumensis MC5-1T(AB196710)
Micromonospora auratinigra TT1-11T(AB159779)
LAH05
Micromonospora citrea DSM 43903T(X92617)
PSN32
Micromonospora peucetia DSM 43363T(X92603)
Micromonospora yangpuensis FXJ6.011T(GU002071)
Micromonospora echinofusca DSM 43913T(X92625)
Micromonospora andamanensis SP03-05T(JX524154)
Micromonospora lutea YIM 013T(EF191199)
Micromonospora sediminimaris MS426T(EU870859)
Micromonospora trujilloniae 234402T(HQ123435)
Micromonospora gifhornensis HR1-2T(Y15523)
Micromonospora fiedleri MG-37T(JQ423921)
Micromonospora maris AB-18-032T(AY528866)
Micromonospora craniellae LHW63014T(MG200153)
Micromonospora endophytica 202201T(EU560726)
Micromonospora olivasterospora DSM 43868T(X92613)
Micromonospora rhizosphaerae 211018T(FJ261956)
Micromonospora yasonensis DS3186T(JN989295)
LAN05
LAN10
LAR02
LAN01
Micromonospora phaseoli NEAU-J5T(JQ073732)
Micromonospora qiuiae RtIII47T(EU427445)
LAH21
LAH15
Micromonospora pisi GUI15T(AM944497)
Micromonospora pattaloongensis TJ2-2T(AB275607)
Micromonospora ovatispora 2701SIM06T(JQ836686)
Micromonospora polyrhachis NEAU-ycm2T(KC139400)
Micromonospora sonneratiae 274745T(JQ619535)
Micromonospora echinaurantiaca DSM 43904T(X92618)
Micromonospora viridifaciens DSM 43909T(X92623)
Micromonospora pallida DSM 43817T(X92608)
Micromonospora equina Y22T(JF912511)
LAH03
Micromonospora fluostatini PWB-003T(LC033898)
Micromonospora avicenniae 2685R06T(JQ867183)
Micromonospora nigra DSM 43818T(X92609)
Micromonospora caldifontis HSS6-8T(AB560969)
Micromonospora zhanjiangensis 2902at01T(KJ742700)
Micromonospora eburnea LK2-10T(AB107231)
Micromonospora narathiwatensis BTG4-1T(AB193559)
Micromonospora terminaliae TMS7T(KX394339)
Micromonospora rosaria DSM 803T(X92631)
Micromonospora tulbaghiae TVU1T(EU196562)
Micromonospora soli SL3-70T(AB981051)
Micromonospora globbae WPS1-2T(LC177396)
LAH10
Micromonospora costi CS1-12T(AB981048)
Micromonospora fulviviridis DSM 43906T(X92620)
Micromonospora inositola DSM 43819T(X92610)
Micromonospora inyonensis DSM 46123T(X92629)
Micromonospora sagamiensis DSM 43912T(X92624)
Micromonospora echinospora DSM 43816T(X92607)
PSH14
PSH21
LAN03
LAN04
LAN08
LAN06
LAN02
PSN48
0.0020

Figure 15. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains identified as *Micromonospora* and the currently recognized *Micromonospora* species. *Catellatospora citrea* type strain was used as outgroup. Bar, 0.002 substitutions per nucleotide position. Bootstrap percentages (1000 replicates) above 50% are shown at nodes.

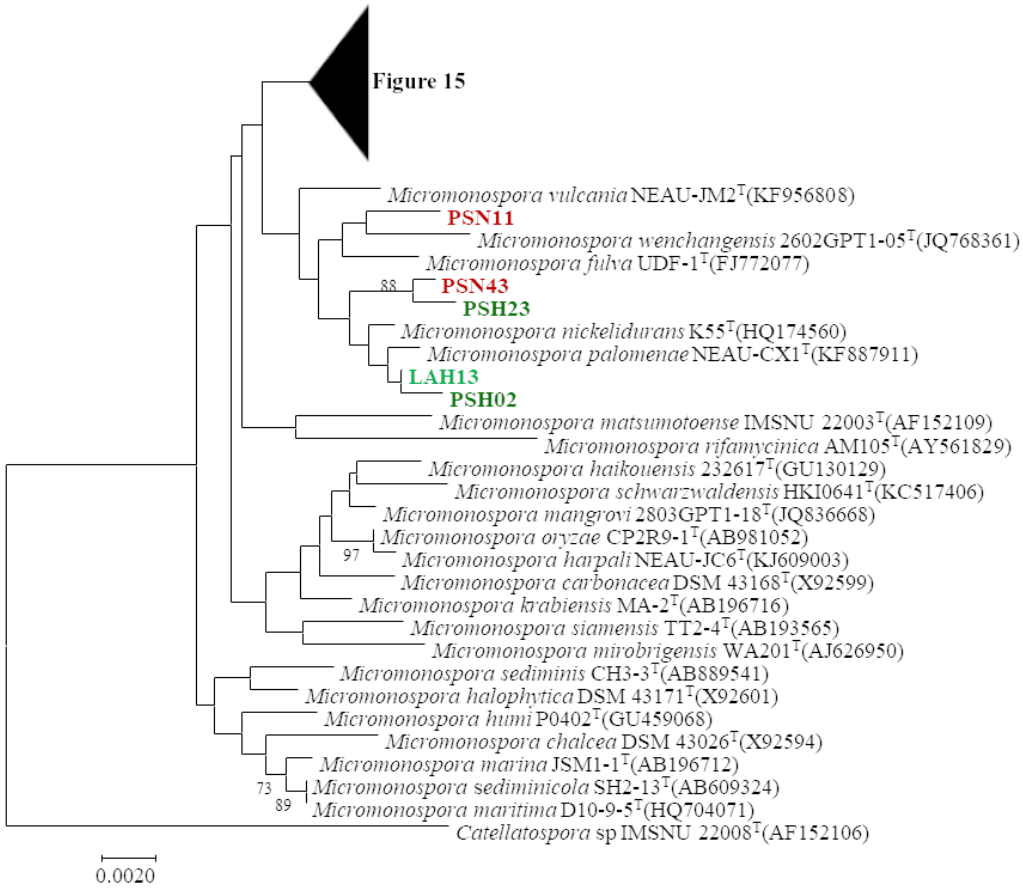


Figure 16. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains identified as *Micromonospora* and the currently recognized *Micromonospora* species. *Catellatospora citrea* type strain was used as outgroup. Bar, 0.002 substitutions per nucleotide position. Bootstrap percentages (1000 replicates) above 50% are shown at nodes.

4.2.3 Capacity of *Micromonospora* isolates to produce hydrolytic enzymes

The production of cellulases, xylanases, pectinases, amylases and chitinases was tested on 147 *Micromonospora* isolates (**Table 10**). All strains showed xylanase and pectinase production. The remaining enzymatic activities were positive in most strains, with some exceptions. The amylolytic activity was negative in the strain PSN16 isolated from nodules of *Pisum* and LAH21 from *Lupinus* leaves. Cellulase production was detected in most of the strains except in PSH04 and PSH24 isolated from *Pisum* leaves and PSN06 from nodules. However, chitinase activity was only observed in a small number of the tested strains. PSH04 was the most restricted strain for enzymes production showing negative results for cellulases and chitinases. Strains with positive activity for cellulases, xylanases, pectinases and amylases were visible seven days after inoculation. However, the positive results were visible later for chitinase production (15-30 days for some strains).

Table 10. Hydrolytic extracellular enzymes produced by each *Micromonospora* strain

Strain	Plant tissue	Cellulases	Xylanases	Pectinases	Amylases	Chitinases
LAN01	Nodules	+	+	+	+	-
LAN02		+	+	+	+	+
LAN03		+	+	+	+	+
LAN04		+	+	+	+	+
LAN05		+	+	+	+	+
LAN06		+	+	+	+	+
LAN07		+	+	+	+	-
LAN08		+	+	+	+	+
LAN09		+	+	+	+	+
LAN10		+	+	+	+	+
LAN11		+	+	+	+	+
LAN12		+	+	+	+	+
LAT01	Stems	+	+	+	+	+
LAR01	Roots	+	+	+	+	+
LAR02		+	+	+	+	+
LAR03		+	+	+	+	+
LAR04		+	+	+	+	-
LAR05		+	+	+	+	+
LAR06		+	+	+	+	+
LAR07		+	+	+	+	+
LAR08		+	+	+	+	+
LAR09		+	+	+	+	+
LAR10		+	+	+	+	+

LA: *Lupinus angustifolius*

Strain	Plant tissue	Cellulases	Xylanases	Pectinases	Amylases	Chitinases
LAH01	Leaves	+	+	+	+	+
LAH02		+	+	+	+	+
LAH03		+	+	+	+	+
LAH04		+	+	+	+	+
LAH05		+	+	+	+	+
LAH06		+	+	+	+	+
LAH07		+	+	+	+	+
LAH08		+	+	+	+	+
LAH09		+	+	+	+	+
LAH10		+	+	+	+	+
LAH11		+	+	+	+	+
LAH12		+	+	+	+	+
LAH13		+	+	+	+	+
LAH14		+	+	+	+	+
LAH15		+	+	+	+	-
LAH16		+	+	+	+	+
LAH17		+	+	+	+	+
LAH18		+	+	+	+	+
LAH19		+	+	+	+	+
LAH20		+	+	+	+	+
LAH21		+	+	+	-	+
PSN 01	Nodules	+	+	+	+	+
PSN 02		+	+	+	+	+
PSN 03		+	+	+	+	+
PSN 04		+	+	+	+	+
PSN 05		+	+	+	+	+
PSN 06		-	+	+	+	+
PSN 07		+	+	+	+	+
PSN 08		+	+	+	+	+
PSN 09		+	+	+	+	+
PSN 10		+	+	+	+	+
PSN 11		+	+	+	+	+
PSN 12		+	+	+	+	+
PSN 13		+	+	+	+	+
PSN 14		+	+	+	+	+
PSN 15		+	+	+	+	+
PSN 16		+	+	+	-	+
PSN 17		+	+	+	+	+
PSN 18		+	+	+	+	+
PSN 19		+	+	+	+	+
PSN 20		+	+	+	+	+
PSN 21		+	+	+	+	+
PSN 22		+	+	+	+	+
PSN 23		+	+	+	+	+
PSN 24		+	+	+	+	+
PSN 25		+	+	+	+	+
PSN 26		+	+	+	+	+
PSN 27		+	+	+	+	+
PSN 28		+	+	+	+	+
PSN 29		+	+	+	+	+
PSN 30		+	+	+	+	+

LA: *Lupinus angustifolius*; **PS**: *Pisum sativum*

Strain	Plant tissue	Cellulases	Xylanases	Pectinases	Amylases	Chitinases
PSN 31	Nodules	+	+	+	+	+
PSN 32		+	+	+	+	+
PSN 33		+	+	+	+	+
PSN 34		+	+	+	+	+
PSN 35		+	+	+	+	+
PSN 36		+	+	+	+	+
PSN 37		+	+	+	+	+
PSN 38		+	+	+	+	+
PSN 39		+	+	+	+	+
PSN 40		+	+	+	+	+
PSN 41		+	+	+	+	+
PSN 42		+	+	+	+	+
PSN 43		+	+	+	+	+
PSN 44		+	+	+	+	+
PSN 45		+	+	+	+	+
PSN 46		+	+	+	+	+
PSN 47		+	+	+	+	+
PSN 48		+	+	+	+	+
PSN 49		+	+	+	+	-
PSN 50		+	+	+	+	+
PSN 51		+	+	+	+	+
PSN 52		+	+	+	+	+
PSN 53		+	+	+	+	+
PSN 54		+	+	+	+	+
PSH 01	Leaves	+	+	+	+	+
PSH 02		+	+	+	+	+
PSH 03		+	+	+	+	+
PSH 04		-	+	+	+	-
PSH 05		+	+	+	+	+
PSH 06		+	+	+	+	+
PSH 07		+	+	+	+	+
PSH 08		+	+	+	+	+
PSH 09		+	+	+	+	+
PSH 11		+	+	+	+	+
PSH 13		+	+	+	+	+
PSH 14		+	+	+	+	+
PSH 15		+	+	+	+	+
PSH 16		+	+	+	+	+
PSH 17		+	+	+	+	+
PSH 18		+	+	+	+	+
PSH 19		+	+	+	+	+
PSH 20		+	+	+	+	+
PSH 21		+	+	+	+	+
PSH 22		+	+	+	+	+
PSH 23		+	+	+	+	+
PSH 24		-	+	+	+	+
PSH 25		+	+	+	+	+
PSH 26		+	+	+	+	+
PSH 27		+	+	+	+	+
PSH 28		+	+	+	+	-

PS: *Pisum sativum*

Strain	Plant tissue	Cellulases	Xylanases	Pectinases	Amylases	Chitinases
PSH 29	Leaves	+	+	+	+	+
PSH 30		+	+	+	+	+
PSH 31		+	+	+	+	+
PSH 32		+	+	+	+	+
PSH 34		+	+	+	+	+
PSH 36		+	+	+	+	+
PSH 37		+	+	+	+	+
PSH 38		+	+	+	+	+
PSH 40		+	+	+	+	+
PSH 41		+	+	+	+	+
PSH 42		+	+	+	+	+
PSH 43		+	+	+	+	+
PSH 44		+	+	+	+	+
PSH 45		+	+	+	+	+
PSH 46		+	+	+	+	+
PSH 47		+	+	+	+	+
PSH 48		+	+	+	+	+
PSH 49		+	+	+	+	+
PSH 51		+	+	+	+	-
PSH 52		+	+	+	+	+
PSH 53		+	+	+	+	+
PSH 54		+	+	+	+	+

PS: *Pisum sativum*

4.3 Discussion

4.3.1 Distribution of the genus *Micromonospora* in legume plant tissues

The presence of *Micromonospora* in legume plants has been documented in recent decades. This genus has been mainly isolated from nitrogen-fixing nodules of both legumes and actinorhizal plants (Valdés et al., 2005; Trujillo et al., 2006; Cerda, 2008; Rodríguez, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2013; Carro et al., 2016; Riesco et al., 2018). Our results demonstrated that *Micromonospora* can be isolated from surface-sterilized plant tissues other than nodules such as roots, stems and leaves. Until now, this genus had not been isolated from these plant tissues (except nodules) of *Lupinus angustifolius* and *Pisum sativum*. Saprophytic and endophytic actinobacteria, including *Micromonospora*, have been previously isolated from stems, roots and leaves of several non-legume plants belonging to the families *Acanthaceae*, *Amaryllidaceae*, *Cruciferae*,

Cyperaceae, Gramineae, *Meliaceae, Poaceace, Rubiaceae*, and *Zingiberaceae* (Okazaki al., 1995; Kizuka et al., 2002; Taechowisan et al., 2003; Kirby and Meyers, 2010; Kuncharoen et al., 2019). However, the distribution of *Micromonospora* in different plant tissues tested was not homogeneous and it varied from tissue to tissue and plant to plant. A greater number of isolates was obtained from nodules and leaves in comparison with those obtained from roots and stems. In the case of stems, the isolation of *Micromonospora* was testimonial, obtaining a single isolate. There is evidence that some endophytic bacteria are mainly recruited from the soil, which then ascends from the root to leaves via the xylem and phloem vessels. It is not surprising that endophytes present in the root can also be isolated in the leaves from the same plant (Chi et al., 2005; Rosenblueth and Martínez-Romero, 2006; Compant et al., 2010; Reinhold-Hurek and Hurek, 2011; Turner et al., 2013). The low presence of endophytes in stems may be because it is a transitional place in the bacterial dissemination from the roots to the aerial parts (James et al., 2002; Compant et al., 2005; Lacava et al., 2007; Reinhold-Hurek and Hurek, 2011). Furthermore, it has also been reported that endophytes capable of colonizing aerial vegetative plant parts, need to possess the physiological requirements to adapt and establish in different plant niches (Hallmann, 2001).

The 16S rRNA gene sequence analysis revealed that approximately 97% of the strains were identified with the genus *Micromonospora* as suggested by their morphological characteristics. The most abundant species were *M. noduli* and *M. saelicesensis*, representing 27.2% and 23.1% of the isolates respectively. The strains with high similarity (> 99%) with these two species were isolated from both *Pisum* and *Lupinus* plants, as well as from nodules, roots and leaves. The remaining strains belonged to 23 different *Micromonospora* species. The diversity of *Micromonospora* was independent of the plant tissue or legume where they were isolated. This was also observed in other previous works, where a great diversity of *Micromonospora* species were isolated from nodules of the same legume (Trujillo et al., 2010; Carro et al., 2012a; Carro et al., 2013). In addition, different members of the same species were isolated from different tissues and legumes, indicating that a species does not limit its presence to a single type of plant tissue. In terms of the bacterial species distribution in previous works, *M. saelicesensis* was the most abundant species in different legumes, but also in other legumes and even in actinorhizal plants (Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2012a; Carro et al., 2013).

The phylogenetic analysis carried out by the construction of the Maximum-likelihood tree based on 16S rRNA gene showed that the isolated strains were distributed throughout the

tree. Most of the isolates were grouped in the same cluster, which also included the type strains *M. noduli* GUI43^T and with *M. saelicesensis* Lupac 09^T. A large proportion of the type strains belonging to this cluster were defined by Carro., et al (2018) as members of group IV composed of strains isolated from ecto- and endo- rhizospheres and different soils. In addition, the results of the phylogenetic analysis suggest the existence of at least three candidates for new species. This is because several strains form an independent branch with respect to their closest type strain. Nevertheless, 16S rRNA gene sequences are not sufficiently divergent to distinguish between closely related strains. For this reason, it is necessary to perform additional studies. The amplification of other genetic markers such as the *gyr*B gene (B subunit of DNA gyrase), the *atp*D gene (ATP synthase β-subunit), the *rec*A gene (recombination protein RecA) and the *rpo*B gene (β-subunit of RNA polymerase); and especially whole genome sequencing can be used to define highly related species. For example, *M. noduli* GUI43^T and *M. saelicesensis* Lupac 09^T are two very close species, which according to the 16S rRNA gene cannot be differentiated. However genomic analysis demonstrated that they are two independent species (Carro et al., 2018; Riesco et al., 2018).

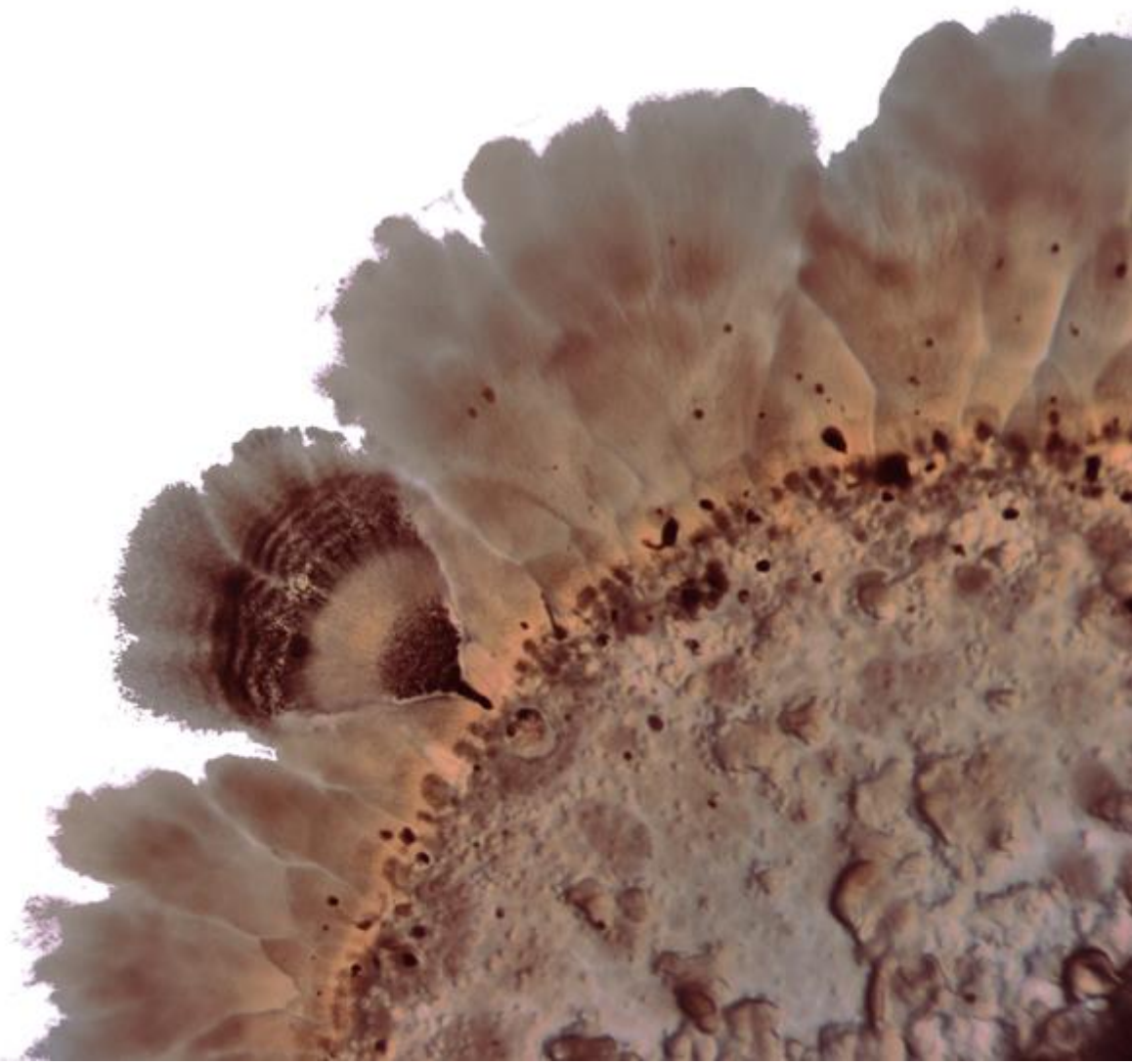
4.3.2. Hydrolytic enzymes produced by *Micromonospora* isolates

Micromonospora strains isolated in this work showed positive results in the production of different plant-polymer degrading enzymes such as cellulases, xylanases, pectinases, amylases and chitinases, independent of the plant tissue or legume where they were isolated. The genus *Micromonospora* is well-known for its capacity to degrade complex polysaccharides such as carboxymethylcellulase, pectin, xylan and starch (de Menezes et al., 2008, 2012; Trujillo et al., 2014b; Ichiwaki et al., 2017). The genome of the model strain *M. lupini* Lupac 08 revealed a significant percentage of putative genes related to degrading enzymes. Specifically, cellulolytic, xylanolytic, chitinolytic and pectinolytic activities were confirmed in the laboratory (Trujillo et al., 2014b). Other members of this genus, whose genome was also sequenced, revealed a diverse number of genes that code for this type of hydrolytic enzymes (Carro et al., 2018). In addition to the genus *Micromonospora*, other endophytic plant bacteria possess genes involved in cell wall degradation such as *Klebsiella pneumoniae* Kp342 and *Azoarcus* sp. BH72 (James et al., 2002; Krause et al., 2006; Reinhold-Hurek et al., 2006).

The production of hydrolytic enzymes has been widely observed in phytopathogenic bacteria and fungi. However, *Micromonospora* does not behave as a pathogen. On the contrary, different laboratory assays have confirmed that this genus promotes plant growth. In addition, *Micromonospora* not only assists plant development but also appears to interact in a tripartite relationship stimulating nodulation (Trujillo et al., 2014b). Plant-polymer degrading enzymes such as cellulases, xylanases and pectinases have been suspected to play a role in internal tissue colonization without causing damage to the plant health (Compant et al., 2005). The nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. *trifolii* produces a specific type of cellulase (CelC2). This cellulase is only expressed in the presence of the appropriate host, and it is essential for symbiotic infection of the legume host (Robledo et al., 2008). Another example is the case of *Azoarcus* sp. BH72, which is able to produce an endoglucanase involved in the infection of rice roots. Mutants of this strain with the inactivated endoglucanase gene revealed a significant reduction in the colonization of rice root internal tissues. This is evidence that *Azoarcus* sp. endoglucanase is an important determinant for successful endophytic colonization of rice roots (Reinhold-Hurek et al., 2006). This suggests that *Micromonospora* might use different enzymes for the internal colonization of plant tissues and later colonize the rest of plant tissues.

CHAPTER 2

Monitoring the colonization and infection of legume nodules by *Micromonospora* in co-inoculation experiments with rhizobia



5. Chapter 2. Monitoring the colonization and infection of legume nodules by *Micromonospora* in co-inoculation experiments with rhizobia

5.1 Introduction

Nitrogen is one of the most necessary elements for plant development and production. However, it is a critical limiting compound in soil. The nitrogen fixation by different rhizospheric bacteria reduce atmospheric nitrogen (N_2) to ammonia (NH_3), making it available to plants (de Bruijn, 2015). Nitrogen-fixing nodules are unique structures where atmospheric nitrogen fixation is possible. These structures are formed on the roots of legume and actinorhizal plants to establish a nitrogen-fixing symbiosis with either rhizobia or *Frankia* respectively. One remarkable feature of the legume-rhizobial symbiosis is its high level of specificity in that a rhizobial strain nodulates and fixes nitrogen with usually only a limited number of host plant species. This specificity is determined by several stages of chemical signaling between the symbiotic partners (Wang et al., 2012).

Nodular tissues are a favorable habitat not only for nitrogen-fixing bacteria, but also for the development of different bacterial species (Martinez-Hidalgo and Hirsch, 2017). Most studies of beneficial plant-microbe interactions focus on a single plant-microbe partnership at a time. However, several authors have reported that different endophytic non-rhizobial bacteria can co-exist with rhizobia inside the legume and actinorhizal nodule tissues (Sachs and Simms, 2008; Wu et al., 2011; Busby et al., 2016). The use of both dependent and independent culture techniques have revealed at least 12 different bacterial taxa represented by up to 32 different genera from surface-sterilized nodules of several wild legumes. The genera identified included *Bacillus*, *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Lactococcus* and *Xanthomonas* (Muresu et al., 2008; Lu et al., 2017). Many members of the phylum *Actinobacteria* have also been isolated from legume nodule tissues including strains of the genera *Agromyces* and *Microbacterium* (Zakhia et al., 2006; Muresu et al., 2008; Hoque et al., 2011), *Curtobacterium* (Sturz et al., 1997), and *Micromonospora* (Trujillo et al., 2006, 2007; Garcia et al., 2010; Trujillo et al., 2010; Carro et al., 2012a, 2013).

Micromonospora is a bacterial genus widely distributed in different environments, but its isolation from nitrogen-fixing nodules has been reported in recent years. The first isolation of *Micromonospora* strains from nitrogen-fixing nodules of the wild legume *Lupinus angustifolius* was reported in 2007 (Trujillo et al., 2007). Since then, the study of *Micromonospora* has focused on its ecology and interaction with plants, and the distribution

of this bacterium has been documented in a wide range of legumes and actinorhizal plants (Niner et al., 1996; Trujillo et al., 2010; Carro et al., 2013). Current data suggest that although *Micromonospora* species do not induce nodulation or fix nitrogen in association with a host plant, they provide many benefits to the plant by increasing the number of nodules, enhancing aerial growth and nutrient uptake (Solans et al., 2009; Martinez-Hidalgo et al., 2014; Trujillo et al., 2014a).

Plant growth is promoted by various mechanisms, including improved access to and uptake of minerals and nutrients, amelioration of soil toxicity, release of growth-stimulating phytohormones as well as modulation of plant hormone production, acquisition of nitrogen and phosphate via symbioses, and/or enhancement of the effects of symbioses (Francis et al., 2010). Studies based on *Micromonospora* strains isolated from alfalfa nodules suggest that the actinobacteria contribute to the nutritional efficiency of this legume (Martinez-Hidalgo et al., 2014), and several experimental data showed that *Micromonospora lupini* Lupac 08 is a plant growth-promoting bacterium (Trujillo et al., 2014b). The sequence of the genome of strain Lupac 08 has allowed the localization of genes that may help its survival in soils or in plant tissues. In addition, several genes that are involved in plant growth promotion, such as production of siderophores, phytohormones, degradation of chitin (for biocontrol), and the biosynthesis of trehalose, all appear to contribute to the welfare of the host plant (Trujillo et al., 2014b).

Although it is generally assumed that an endophytic bacterium is one that can be isolated from surface-sterilized plant tissues, several surface-inhabiting epiphytic bacteria can be resistant to sterilizing agents or be in structures that protect them against these chemicals. The use of different microscopy techniques, such as high-quality light microscopy and/or transmission electron microscopy (TEM) combined with immunological techniques and/or tagging with reporter genes, are a good strategy to confirm if a particular bacterium is truly endophytic (James, 2000; Rosenblueth and Martinez-Romero, 2006; Compant et al., 2010; Monteiro et al., 2012; Turner et al., 2013). Until now, the presence of *Micromonospora* in nodular tissues has only been reported through its isolation from legumes or actinorhizal plants and through the application of fluorescent *in situ* hybridization (FISH) and TEM techniques (Trujillo et al., 2010). Moreover, most inoculation experiments that analyze the effect of *Micromonospora* on a host plant and its interaction with rhizobia have been carried out using the same plant species from which the strains originated. However, no information is available as to whether any specificity exists in the *Micromonospora*-legume interaction.

Thus, the aim of this chapter was to determine the capacity of strain *M. lupini* Lupac 08, isolated from *Lupinus angustifolius* (Trujillo et al., 2007), to enter *Medicago* and *Trifolium* nodules and its original host, and to obtain information about the location of *Micromonospora* within nodule tissues.

5.2 Results

5.2.1 Antagonism assay

The capacity of *Micromonospora* to inhibit the growth of the rhizobial strains or vice versa was evaluated by co-cultivation of *M. lupini* Lupac 08 and three nitrogen-fixing bacteria (*Bradyrhizobium* sp. CAR08, *Sinorhizobium* (*Ensifer*) Sm1021 and *Rhizobium* sp. E11). The different assays showed that strain Lupac 08 is compatible with the three rhizobial strains tested (**Figure 17**). No growth inhibition or any other negative affect was observed when *Micromonospora* and the rhizobial bacteria were grown on the same agar plate and had physical contact (**Figure 17 a**). Similar results were obtained when the three nitrogen-fixing bacteria were inoculated on a plate where *Micromonopora* Lupac 08 was entering stationary phase and production of secondary metabolites was the possibility of producing secondary metabolites was higher (**Figure 17 b**).

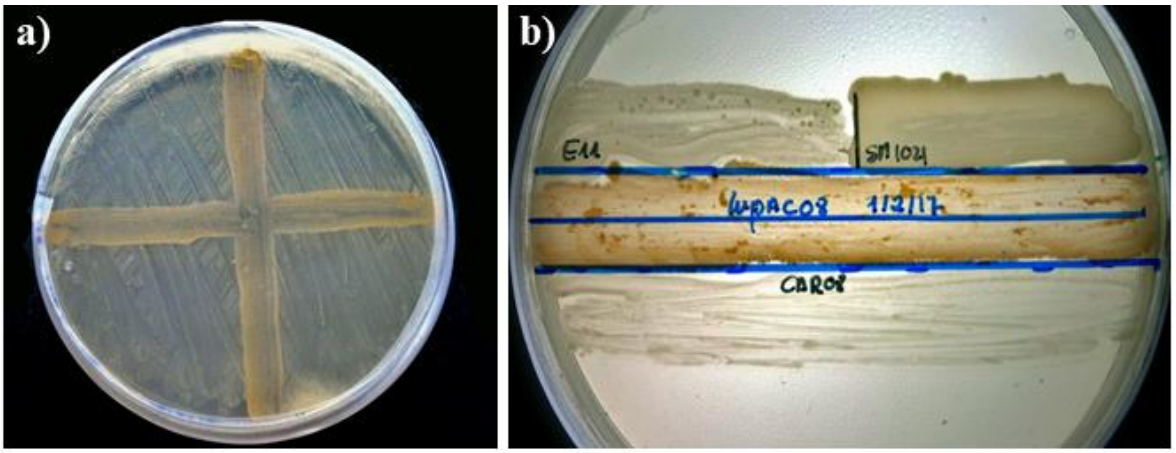


Figure 17. Antagonism assay between *Micromonospora* and rhizobial strains. (**a**) Simultaneous growth of *Micromonospora* with rhizobia (*Bradyrhizobium* sp. CAR08, *Sinorhizobium (Ensifer)* Sm1021 and *Rhizobium* sp. E11); (**b**) Growth of strains E11, Sm1021 and CAR08 in contact with *Micromonospora*.

5.2.2 Localization of *Micromonospora* in lupine nodules

The nodules produced by all co-inoculated *Lupinus* plants were pink in color, indicating effective nitrogen fixation. These co-inoculated plants also showed a high number of nodules than the plants only inoculated with *Bradyrhizobium* sp CAR08. Several root nodules (~35-40 days post inoculation (dpi)) were randomly selected and longitudinally sectioned to localize *M. lupini* ML01-*gfp* cells by CLSM. A large number of the selected nodules showed cells with green fluorescence, indicating the presence of *Micromonospora*. Bacterial cells located in the nodular tissues showed the same morphology as *Micromonospora* in pure culture preparations (**Figure 18**).

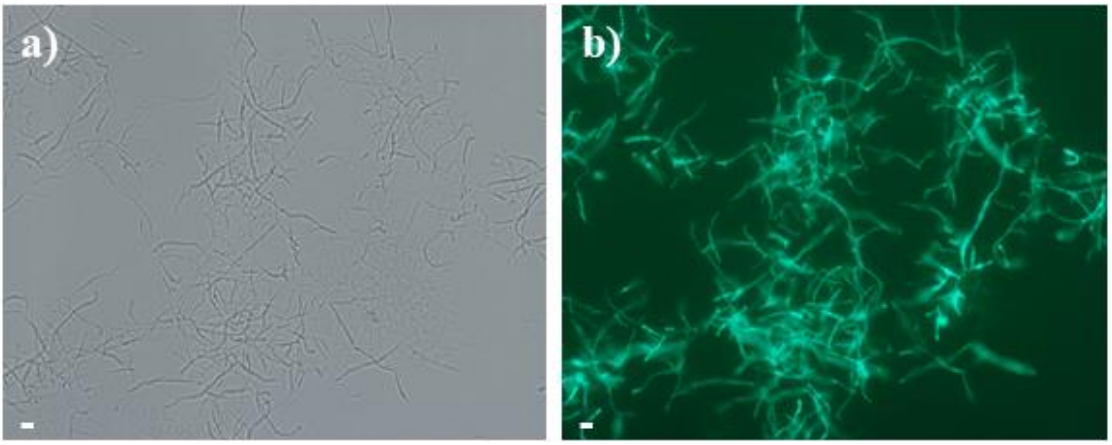


Figure 18. *Micromonospora* (ML01-*gfp*) cells pure culture observed by light (**a**) and fluorescent (**b**) microscopy. Bars: 2 μm.

The *Micromonospora* cells were successfully localized in several zones of the nodule, but they were especially prevalent in the infection and bacteroid zones (BZ; terminology described by González-Sama et al., 2004) (**Figure 19 a,b**). Although uninfected cells were not present in the central BZ of *Lupinus* nodules, *Micromonospora* cells were observed in those host cells that appeared devoid of *Bradyrhizobium*. This was more obvious in longitudinal nodule sections that were counterstained with 5 μg/ml propidium iodide to differentiate *Bradyrhizobium* sp. CAR08 from the *gfp*-tagged *Micromonospora* (**Figure 19 c-f**). As expected, the bradyrhizobia occupied the majority of the cells within the nodule tissue, and were clearly seen in the infection and bacteroid zones of the nodule, whereas *Micromonospora* cells were observed in fewer host cells, which were interspersed among the *Bradyrhizobium*-infected cells (**Figure 19 c**). The presence of both bacteria in the same cell was detected as yellow fluorescence (**Figure 19 f**) due to the coincidence of the green and red fluorescence (**Figure 19 d,e**).

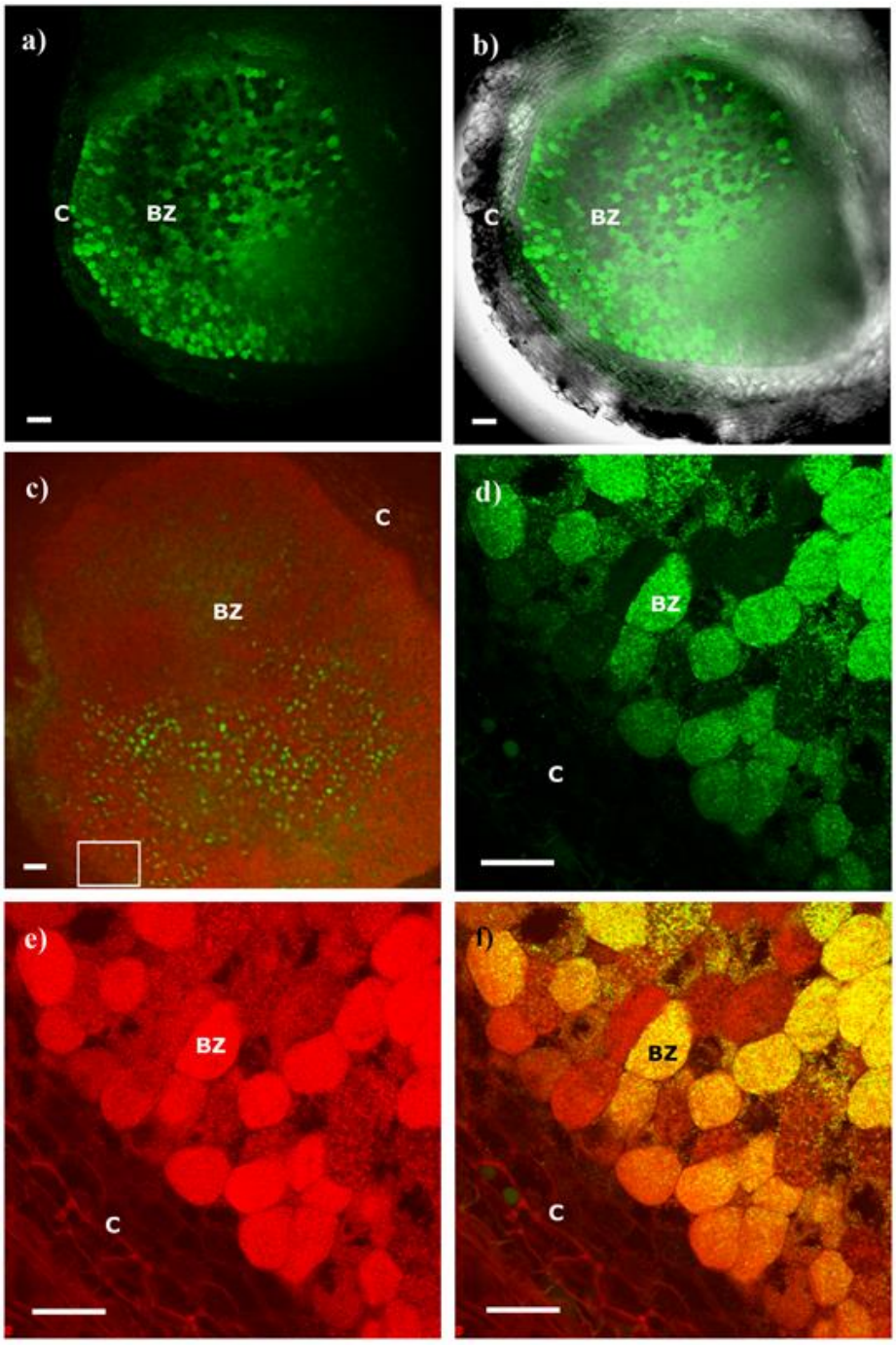


Figure 19. Longitudinal nodule sections of *Lupinus albus* coinoculated with *Bradyrhizobium* sp. CAR08 and *Micromonospora* ML01-*gfp* (21 dpi). (**a**) Green fluorescence signal captured by CLSM of infected cells containing *Micromonospora* ML01-*gfp*; **(b**) Overlay of light and fluorescence images of the nodule section; (**c**) Green fluorescence localization of ML01-*gfp* in a nodule section stained with propidium iodide and viewed by CLSM; (**d**) Higher magnification image captured with the green channel; (**e**) Higher magnification image captured with the red channel; (**f**) Composite image of both channels. The white rectangle in image c shows the area where images d-f were captured. C, cortex; BZ, bacteroid zone; dpi, days post inoculation. Bars: 100 μm (**a**, **b**, **c**); 40 μm (**d**, **e**, **f**).

Immunogold microscopy was used to confirm the presence of *Micromonospora* within the nodule cells due to its specificity. Pure cultures of the bacteria were observed by TEM (Jeol 1010, Japan) for comparison purposes. As expected, *Micromonospora* cells were seen as branched filaments or rod-shaped structures that corresponded to longitudinal and transverse sections, respectively (**Figure 20 a,b**). TEM preparations of nodules inoculated with *Bradyrhizobium* sp. CAR08 only were sectioned and served as controls. **Figures 20 c,d** illustrate infected cells containing bacteroids within their symbiosomes as well as uninfected plant cells.

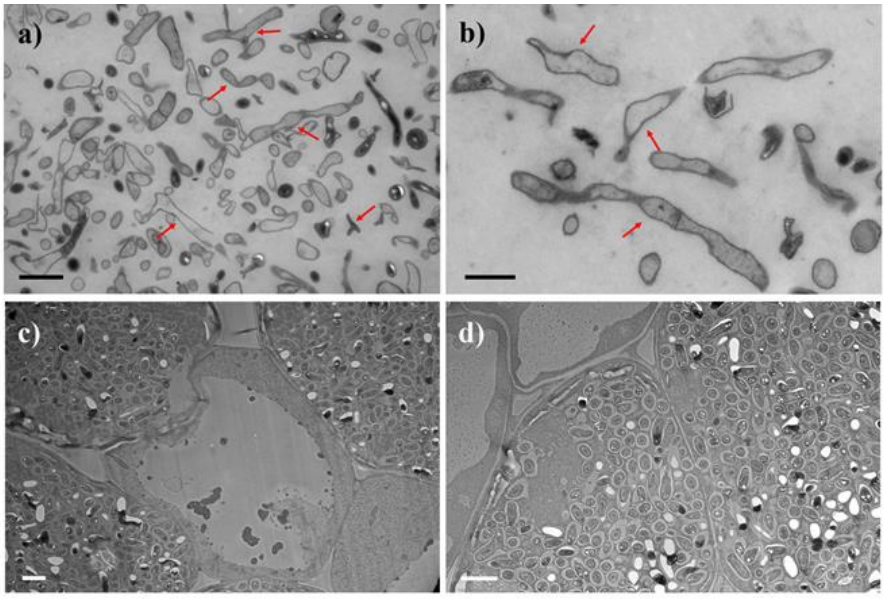


Figure 20. Transmission electron micrographs of *Micromonospora* pure cultures and nodular tissue infected with *Bradyrhizobium*. **(a**,**b)** *Micromonospora* ML01-*gfp* pure cultures (arrows, polymorphic *Micromonospora* cells); **(c**,**d)** Lupine nodule tissue infected with *Bradyrhizobium* sp. CAR08 only. Bar: 2 µm (**a**, **c**, **d**); 1 µm (**b**).

Nodule sections obtained from plants that were co-inoculated exhibited a structure similar to the control nodules with zones corresponding to the nodule cortex (C) and the infection zone and bacteroid zone (BZ) (**Figure 21 a**). Within these zones, bacteria-containing plant cells were seen, but also plant cells with an empty appearance, uninfected (**Figure 21 b**). *Micromonospora* hyphae were usually found in the latter cells, which at low magnification gave the impression of being "empty". **Figure 21 b** shows a *Micromonospora*-containing nodule cell, which is flanked by two host cells containing *Bradyrhizobium* bacteroids. The areas where the *gfp*-ML01 strain was found are marked with an asterisk enclosed by a circle. Within these cells, immunogold-labeled structures that resembled the cells of *Micromonospora* were observed (**Figure 21 c-f**). These structures were similar to those found

in the pure culture preparations (**Figure 21 a,b**), but were not detected in the nodules inoculated with *Bradyrhizobium* only. Unlike many rhizobia that undergo physical changes, the *Micromonospora* cells did not show any drastic morphological changes and many labeled cells resembled those that were observed in pure culture. In some cases, both types of bacteria were seen within the same plant cell (**Figure 19**), but *Micromonospora* was always found in lower numbers compared to the *Bradyrhizobium* bacteroids.

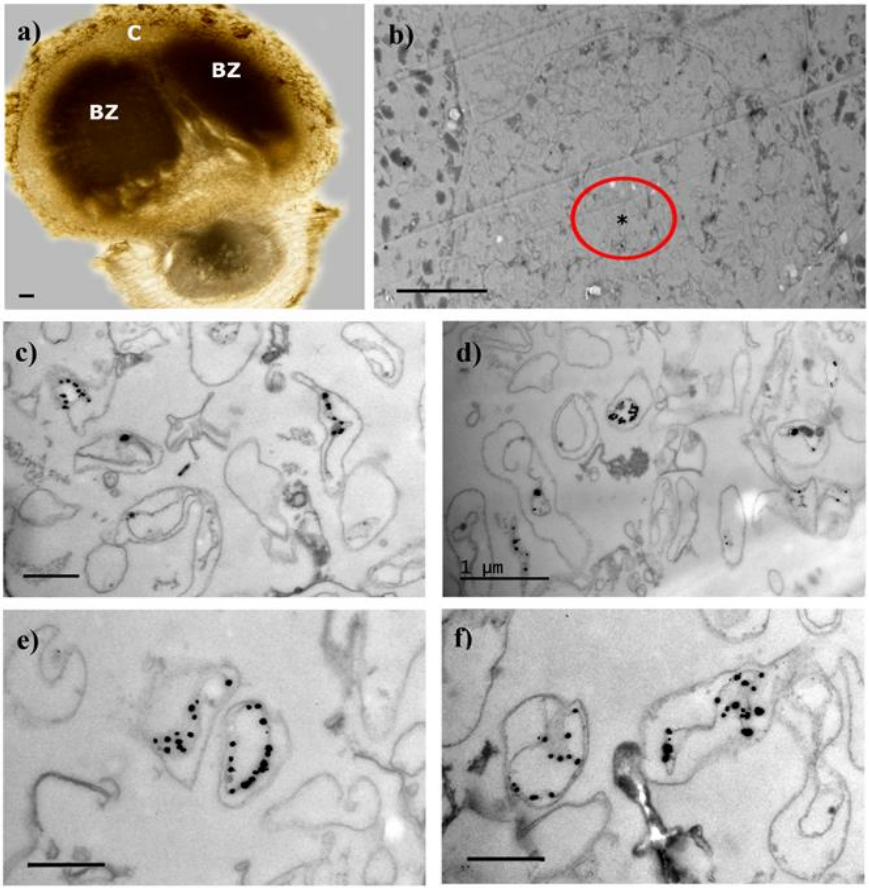


Figure 21. Immunoelectron microscopic images of lupine nodules infected with *Bradyrhizobium* CAR08 and *Micromonospora* ML01-*gfp* (21 dpi). (**a**) Light micrograph of a longitudinal nodule section; (**b)** Detail of an "empty" cell between two infected cells that contain bacteroids; (**c–f**) Labeled *Micromonospo*ra cells found in the area marked with an encircled asterisk in 21b. Bars: (**a**) 100 µm; (**b**) 10 µm; (**d**) 1 µm; 500 nm (**c, e, f**). C, cortex; BZ, bacteroid zone; dpi, days post inoculation.

5.2.3 Effect of *Micromonospora* on the root hairs of *Medicago* and *Trifolium*

The ability of the strain *M. lupini* Lupac 08 to infect legumes other than *Lupinus* was investigated. *Medicago* and *Trifolium* plants inoculated with *Micromonospora gfp*-labeled ML01 were observed under light and confocal microscopy. The presence of *Micromonospora* was detected throughout the entire root, especially on the root hairs. Root

hair deformations were observed as early as 2–3 days after inoculation. In both plants, *Micromonospora* was observed attached to the root surfaces where the deformations were located, principally on the apex of hairs (**Figure 22 a-h,j,k,m,n**). Root hairs deformed branching into L (**Figure 22 a,b,f,n**) and Y shapes (**Figure 22 d**) were observed at 2-3 dpi. However, as time progressed swollen root hair tips (**Figure 22 e,g**) and zig-zag forms (**Figure 22 k**) were observed, as well as, wiggling and curling forms (**Figure 22 h,m**) (6-10 dpi). After fifteen days post inoculation, most root hairs showed some of the deformations mentioned, with few exceptions. Root hairs of uninoculated *Trifolium* (**Figure 22 i**) and *Medicago* (**Figure 22 l**) control plants exhibited no deformation.

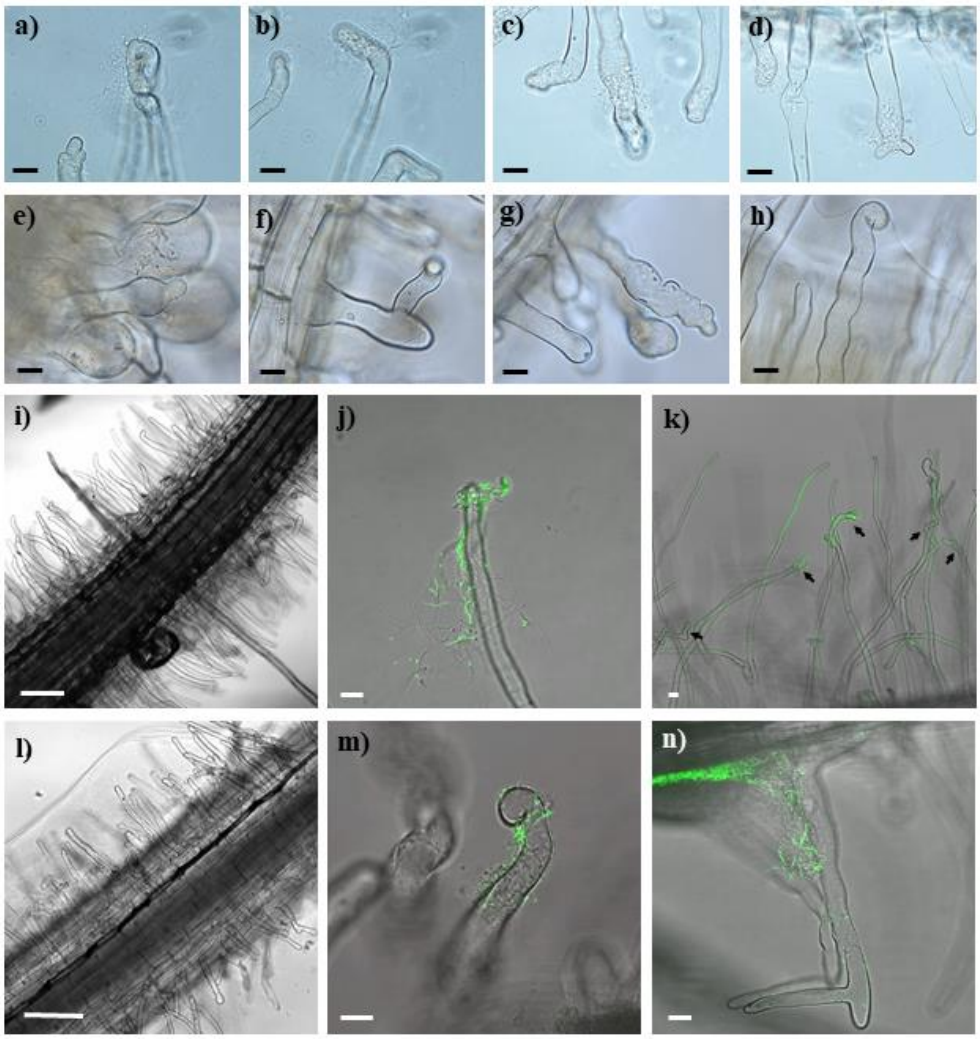


Figure 22. Effect of *Micromonospora* on the root hairs of *Trifolium* and *Medicago*. (**a-h**) Light microscopy of root hair deformations by *Micromonospora* effect at 3 dpi (**a-d**) and 6 dpi (**e-h**); (**i,l**) Control uninoculated plants of *Trifolium* and *Medicago* respectively; (**j,k**) CLMS micrographs of *Micromonospora* cells attached to a *Trifolium* hair root 3 dpi and 5 dpi; (**k**) *Trifolium* root hairs showing different deformations (arrows) 5 dpi with *Micromonospora*; (**m,n**) CLMS images of *Micromonospora* attached to *Medicago* root hairs**.** Arrowheads indicate deformed root hairs. Bars: (**a-d, j-h**) 8 µm; (**e-h, m-n**) 10 µm; (**i**) 100 µm; (**j**) 200 µm.

5.2.4 Infection of *Medicago* and *Trifolium* root nodules by *Micromonospora*

To determine the capacity of *Micromonospora* to penetrate and infect nodular internal tissues, *Medicago* and *Trifolium* plants were co-inoculated with the appropriate *mCherry*-tagged nitrogen-fixing rhizobia (*Sinorhizobium* (*Ensifer*) Rm1021-*mCh* and *Rhizobium* sp. E11-*mCh*, respectively), as well as *Micromonospora* ML01-*gfp*. The plants were monitored by light and CLSM microscopy every two days until approximately 25 dpi. Root tip deformations appeared 2 days after bacterial inoculation in *Trifolium* (**Figure 23 a-c**), whereas the changes in root hair deformation did not occur in *Medicago* until 6 dpi (**Figure 23 g-i**). Although green autofluorescence was detected in the plant tissues, *Micromonospora* cells were clearly observed attached to the root hairs (**Figure 23 g**). In both sets of plants, *Micromonospora* surrounded the youngest regions of the root, concentrating on the base and apices of the root hairs. Differences in root hair morphology were observed between the two plant species. Most *Trifolium* root hairs were branched or club-shaped 2–4 dpi, with the most visible deformations observed after 6 dpi (**Figure 23 a-c**). In the case of *Medicago*, in addition to branched or club-shaped forms (6-8 dpi) (**Figure 23 g**), root hairs became spiral in shape from 12 dpi (**Figure 23 i**). The spiral deformation was only detected in the co-inoculated plants with *Micromonospora* ML01-*gfp* and *Sinorhizobium* (*Ensifer*) Rm1021-*mCh*, when compared to plants inoculated with *Sinorhizobium* (*Ensifer*) Rm1021-*mCh*.

Nodule primordia were visible 3-5 dpi in *Trifolium* and after 7-9 days in *Medicago*. In both plants, the nodule primordia were covered with deformed root hairs and *Micromonospora* cells were attached to the hairs (**Figure 23 d**). *Micromonospora* ML01-*gfp* and *Rhizobium* E11-*mCh* were readily visible inside *Trifolium* young nodules 11 and 13 dpi, and both bacteria were co-localized as indicated by the yellow fluorescence, supporting the conclusion that both microorganisms were present (**Figure 23 e,f**). Comparably aged *Medicago* nodules were thicker, however, and the green and red fluorescence corresponding to *Micromonospora* ML01-*gfp* and *Sinorhizobium* (*Ensifer*) Rm1021-*mCh*, respectively, was detected in the intact nodules (**Figure 23 j-l**). Nodules were well developed in both plants after 15-20 days and fresh samples had a pink color indicating that nitrogen fixation was taking place. Mature nodules (~20 dpi) of both plant species exhibited the typical indeterminate structure: meristematic, infection, bacteroid, and senescent zones were identified. Although *Micromonospora* could be located in the developing nodules, when they increased their thickness, the location of the bacteria was more difficult to determine.

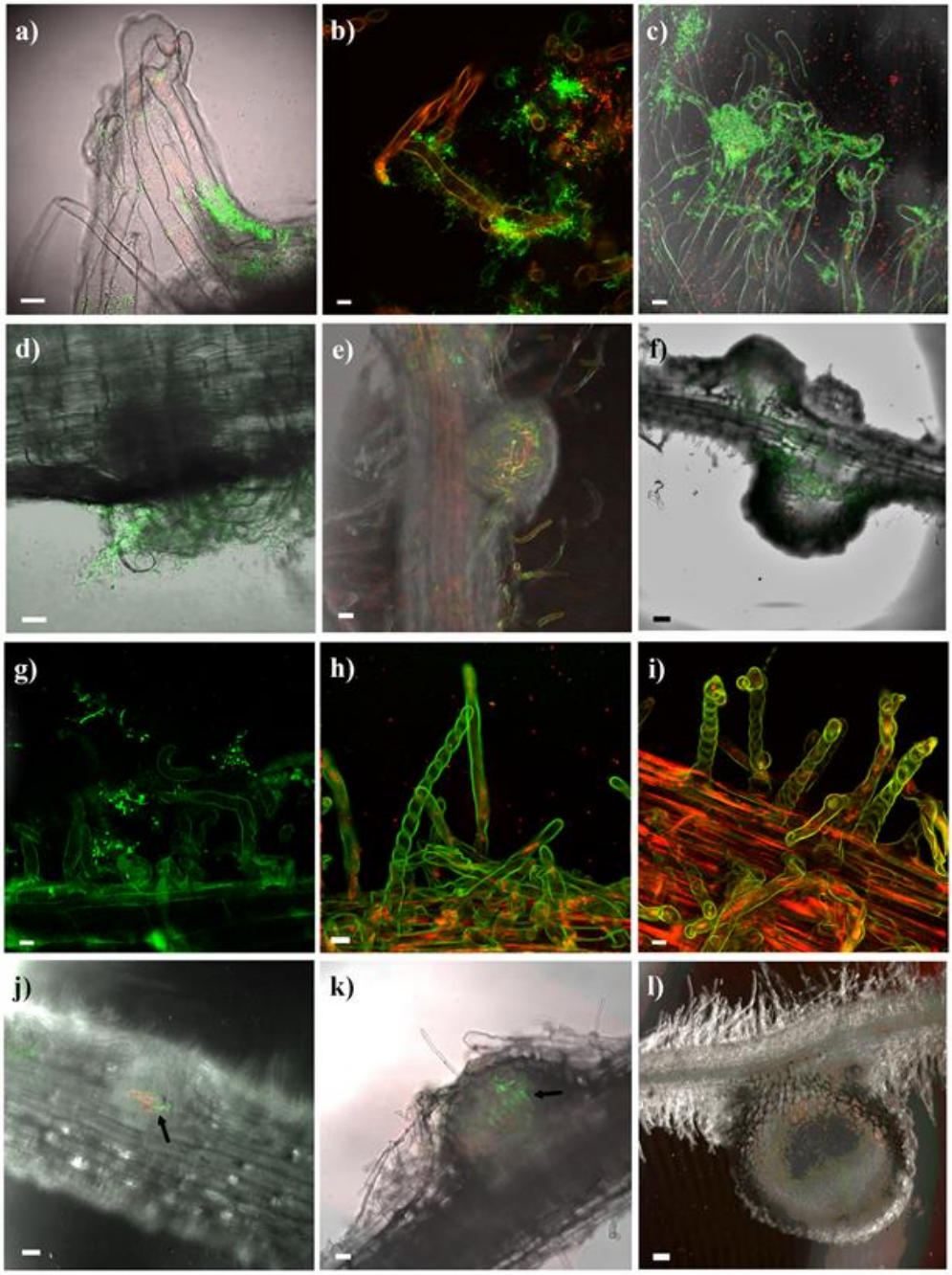


Figure 23. Infection and colonization of *Trifolium* and *Medicago* by *Micromonospora* ML01-*gfp* co-inoculated with strains *Rhizobium* sp. E11-*mCh* and *Sinorhizobium* sp. (*Ensifer*) Rm1021-*mCh* respectively, and observed by CLSM. (**a-c**) *Trifolium* root tip deformations observed 3, 5 and 7 dpi and surrounded by *Micromonospora* ML01-*gfp*. *Micromonospora* and *Rhizobium* sp. co-localized on the root hairs; (**d**) Nodule primordium and deformed root hairs observed in *Trifolium* 5 dpi; (**e-f**) Young *Trifolium* nodules observed 11 and 13 dpi. The fluorescent bacteria are visible within the internal tissues of the nodule; **(g)** Attachment of *Micromonospora* to *Medicago* root tips showing deformations 6 dpi. (**h, i**) *Medicago* root hair tips forming spiral shapes 10 and 12 dpi. (**j-l)** *Medicago* nodules at 11, 13 and 15 dpi with green and red fluorescence signals showing strings of bacteria. Because of the thickness of the tissue, the nodules themselves are slightly out of focus. For details see text. Bars: 8 µm (**a-c**); 10 µm (**d, h-i**); 60 µm (**e, f**); 75 µm (**j-l**). dpi, days post infection.

Longitudinal sections of 20-day old *Medicago* and *Trifolium* nodules were obtained for localizing *Micromonospora* by CLSM. In *Trifolium*, a green fluorescence signal (excitation 488-nm and 515- to 560-nm emission) was observed in the infection zone just below the meristematic area. Fluorescence expressed by *Micromonospora* ML01-*gfp* was clearly observed within the plant cells, whereas the uninfected cells showed no fluorescence apart from the autofluoresence emitted by the plant (**Figure 24 a**). *Rhizobium* sp. E11-*mCh* cells exhibited a bright red fluorescent signal (620 nm excitation and 620–660 nm emission) in the infected, bacteroid, and senescent zones of the nodule (**Figure 24 b**). In the most cases, both bacteria were found in the infection zone, cohabiting within the same host cell as indicated by yellow fluorescence (**Figure 24 c,d**), whereas in other instances, *Rhizobium* E11-*mCh* was the only occupant, especially in the senescent zone. In addition, *Micromonospora* was also located alone in some cells from the infection zone and to a lesser extent in cortex cells.

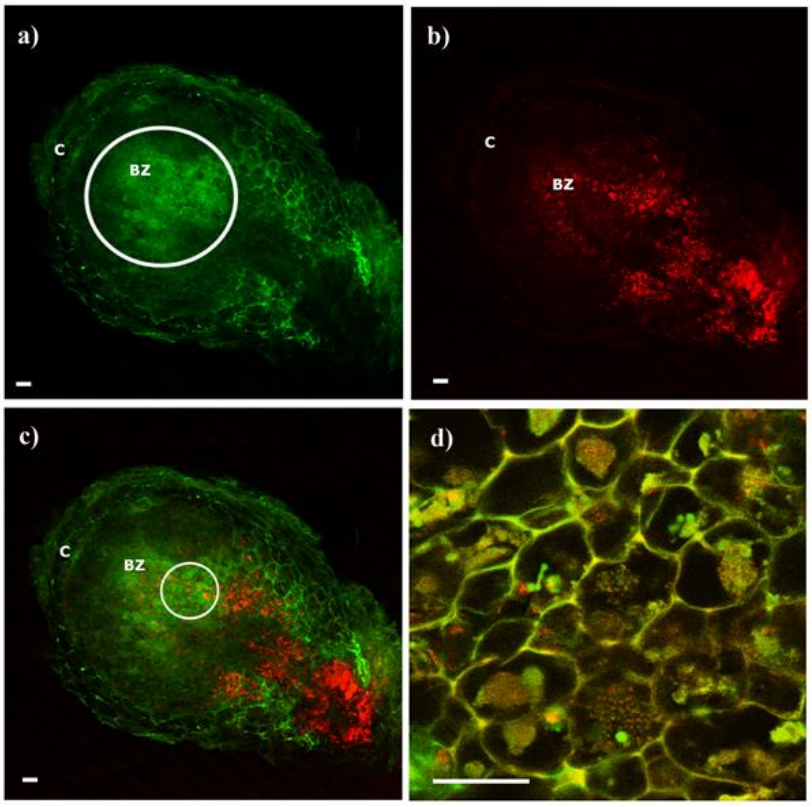


Figure 24. *Trifolium* longitudinal nodule section (20 dpi) showing the distribution of infected cells after co-inoculation with *Micromonospora* ML01-*gfp* and *Rhizobium* E11-*mCh* captured by CLSM. (**a**) Image obtained with the green channel for the localization of *Micromonospora*. The circled area indicates fluorescence emitted by *Micromonospora* concentrated in the bacteroid zone; (**b**) Image captured with the red channel for the localization of *Rhizobium*; (**c**) Combination of images a and b; (**d**) Detail of infected zone showing the co-localization of *Micromonospora* and *Rhizobium* in the host cells. The white circle in 24c shows the area where image (**d**) was captured. Bars: 60 μm (**a**–**c**); 20 μm (**d**). dpi, days post inoculation; C, cortex; BZ, bacteroid zone.

For *Medicago*, *Micromonospora* was localized across the nodule and infected cells were visible in all but the meristematic zone (**Figure 25 a**). As in *Trifolium* nodules, in some cases, both bacteria occupied the same host cells (**Figure. 25 c,d**). However, on other occasions both bacteria occupied independent cells. **Figure 25 a,b** show the distribution of the bacteria captured by the corresponding fluorescence channels (green for *Micromonospora* and red for *Sinorhizobium* (*Ensifer*)). A close up of the nodular tissue permitted a clear visualization of *Micromonospora* ML01-*gfp* cells (**Figure 25 e**).

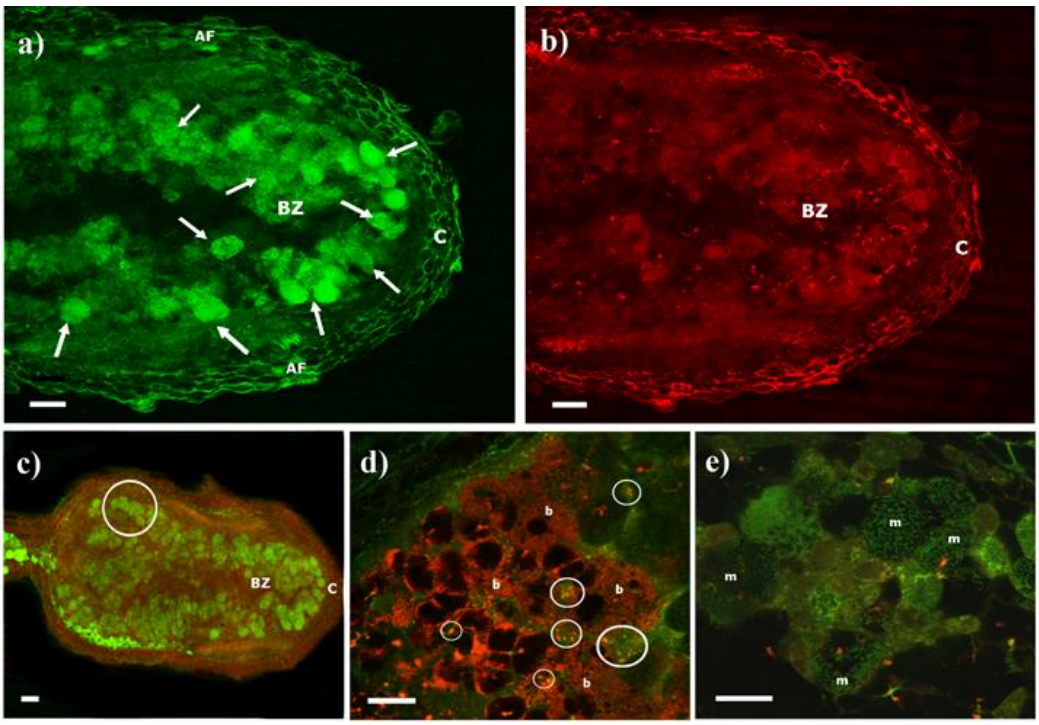


Figure 25. Longitudinal sections of a *Medicago* 20 dpi nodule showing the distribution of cells infected with *Micromonospora* ML01-*gfp* and *Sinorhizobium* (*Ensifer*) Rm1021-*mCh* captured by CLSM. (**a**) Detail of a nodule tip captured with the green channel for the localization of *Micromonospora*. Arrows indicate fluorescence emitted by *Micromonospora* to differentiate from autofluorescence emitted by the plant. (**b**) Detail of a nodule tip captured with the red channel for the localization of *Sinorhizobium* (*Ensifer*) Rm1021-*mCh*. (**c**) Composite image showing the distribution of green and red fluorescence. (**d**) Bacteroids occupying several plant cells, small circles show areas occupied by both bacteria. (**e**) *Micromonospora* hyphae inside host plant cells. The white circle in image 25c shows the area where images d and e were captured. Bars: 75 µm (**a**); 30 µm (**b**–**e**). dpi, days post inoculation; C, cortex; BZ, infection zone; AF, autofluorescence; b, bacteroids; m, *Micromonospora*.

5.3 Discussion

5.3.1 Root hair deformation of *Medicago* and *Trifolium* by *Micromonospora* effect

In general, plant tissue colonization by endophytic bacteria is comprised of the following steps: recognition, adherence, invasion, colonization and establishment of interactions (Bais et al., 2006). In this study, it was possible to observe *Micromonospora* attaching to the root hairs during the development of *Trifolium* and *Medicago* plants. These root hairs showed different deformations where the bacterium was present, being detected both in plants only inoculated with *Micromonospora* and plants co-inoculated with *Micromonospora* and the corresponding nitrogen fixing bacteria.

Several root hair deformations observed in this work have been found in plants inoculated with nitrogen-fixing bacteria such as *Frankia* or *Rhizobium*. In these cases, the bacterial contact with the flavonoids released by the plant to the rhizosphere activates signal transduction pathway that leads to symbiotic responses such as root hair deformations ("shephered crook" curling deformation), the infection threads formation and later the subsequent formation of nodule primordia and mature nodules (Cérémonie et al., 1999; Catoira et al., 2000; Cissoko et al., 2018). In the case of *Rhizobium leguminosarum* bv. trifolii, it was observed that the production of a low amount of plant cell wall- degrading enzymes may also be involved in the root hair deformation and in the plant tissue colonization without causing damage to the plant (Robledo et al., 2008). Solans et al., (2011) reported that different actinobacteria such as *Micromonospora*, *Streptomyces* and *Actinoplanes*, together with *Frankia*, produced deformations similar to those detected in this research work. In addition, they raise the possibility that phytohormones or other substances may take part in the root hair deformation and infection of root internal tissues. This may be due to the action of several phytohormones such as auxin, gibberellin or ethylene are involved in root hair development, but can also participate in their deformation (Miller et al., 1997; Libault et al., 2010; Spaepen and Vanderleyden, 2011; Jung and McCouch, 2013; Street et al., 2015; Swarup et al., 2007). Despite the studies carried out with *Micromonospora*, it is still unknown what triggers hair deformation when the bacterium is in contact with the root. A potential cause for the internal root tissue colonization by *Micromonospora* is that it uses the same colonization route as rhizobia. However, additional studies are needed to confirm this possibility.

5.3.2 The unspecific presence of *Micromonospora* within the legume nodular cells

Our results demonstrate that *Micromonospora* can colonize nodular tissues of three different legumes and strongly suggests that a non-specific relationship takes place between *Micromonospora* and the plant. This conclusion is based on data showing that strain *M. lupini* ML01-*gfp,* originally isolated from lupine, was able to colonize *Medicago* and *Trifolium* plants as well as *Lupinus*. This suggests that this actinobacterial strain has a broad host range. The ability of *Micromonospora* to infect different legume species contrasts with the symbiotic interactions between rhizobia and legumes and *Frankia* and actinorhizal plants, both of which are more restrictive (Pawlowski and Demchenko, 2012; Andrews and Andrews, 2016).

The evaluated plants did not show any negative effects related to the presence of *Micromonospora* and the nitrogen fixation process did not appear to be altered. The results also indicated that the different rhizobia were not inhibited by co-inoculation with the actinobacterium and that bacteroid development proceeded normally. Indeed, it has been reported that the growth of the co-inoculated plants with *Micromonospora* and rhizobia was better, such that in some cases a larger number of nodules per plant resulted (Trujillo et al., 2014b). Interestingly, when the nitrogen-fixing bacteria (*Bradyrhizobium* sp. CAR08, *Sinorhizobium* (*Ensifer*) Sm1021 and *Rhizobium* sp. E11) and *M. lupini* Lupac 08 were grown as a co-culture, no growth inhibition was observed by either bacterium even though this *Micromonospora* strain contains several genes that code for bacteriocins in their genome (Trujillo et al., 2014b). These genes may not be expressed when the two bacteria interact under the conditions tested, but more studies are needed.

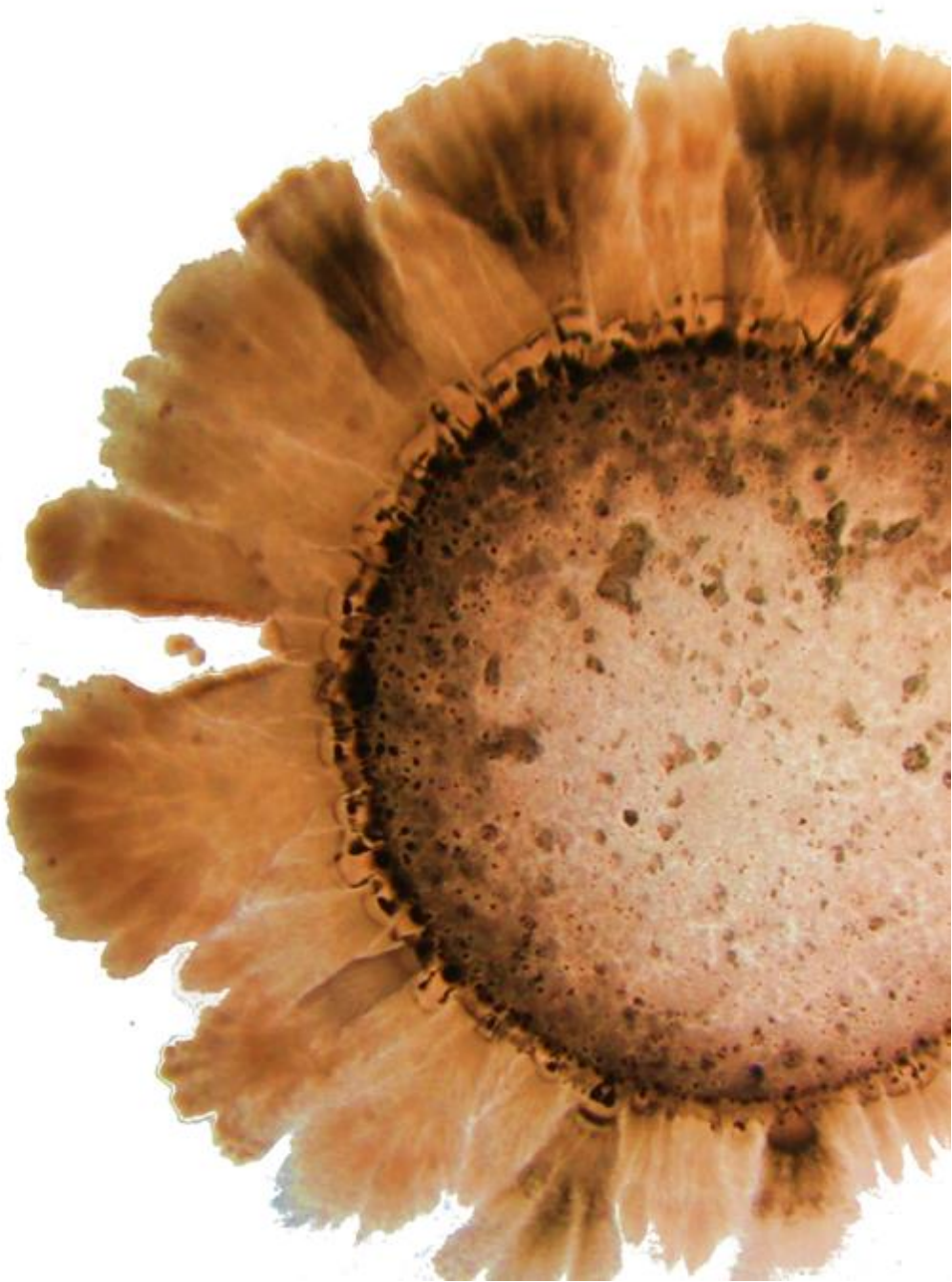
In previous reports, the systematic isolation of *Micromonospora* cells from nitrogen-fixing legume and actinorhizal nodules and the application of fluorescent *in situ* hybridization (FISH) and TEM techniques, have presented strong evidence that *M. lupini* Lupac 08 was a normal inhabitant of internal root nodule tissues and suggested a close interaction between the host plant and the bacterium (Valdés et al., 2005; Trujillo et al., 2010; Carro et al., 2012a; Carro et al., 2013; Trujillo et al., 2015). In this study, the unambiguous microscopic localization of *M. lupini* Lupac 08 was accomplished using a combination of tagged reporter genes and immunogold labeling. Furthermore, we have demonstrated that *Micromonospora* not only re-enters its original host, *Lupinus* sp., but also interacts with other legumes such as *Medicago* and *Trifolium*. In all plant samples studied, a plant growth-promoting effect as previously reported was confirmed (Trujillo et al., 2014b; Trujillo et al., 2015) and these

results are in line with those reported by other researchers (Tokala et al., 2002; Solans et al., 2009; Solans t al., 2011). Genomic analysis of strain Lupac 08 has revealed several features related to plant growth promotion, including the production of siderophores, phytohormones and other secondary metabolites, all of which may be involved in growth enhancement (Trujillo et al., 2014b).

By monitoring the colonization process, information about the distribution of *Micromonospora* in *Lupinus*, *Trifolium*, and *Medicago* nodules was obtained. In all cases, the infection zone was the main area where *Micromonospora* was found and the place where both bacteria were observed occupying the same plant cell. These results strongly suggest a tripartite interaction and the coexistence of non-rhizobial bacteria within nodule tissues (Tokala et al., 2002; Muresu et al., 2008) although at present a specific function cannot be attributed to *Micromonospora*. Furthermore, as compared to control plants inoculated with *Rhizobium* or *Sinorhizobium* (*Ensifer*) only, on average, the nodules appeared and developed 1-2 days earlier on the co-inoculated plants. It was previously reported that legumes co-inoculated with their compatible nitrogen fixer and associated "helper" *Micromonospora* developed a greater number of nodules in *Lupinus*, *Medicago*, and *Trifolium* (Trujillo et al., 2014b; Solans et al., 2009). Similar results were reported for actinorhizal plants and other "helper" bacteria (Knowton et al., 1980; Knowton et al., 1983; Solans et al., 2011). In the latter cases, however, these bacterial growth promoters were not considered endophytes because they were isolated from the external plant tissues or the rhizosphere. In our case, through the microscopy studies used and its capacity to re-infect disinfected seedlings, it can be confirmed that *Micromonospora* is a "true" endophytic bacterium, since it meets the criteria that defines an endophytic microorganism (Reinhold-Hurek and Hurek, 1998a).

CHAPTER 3

The effect of root exudates on the intracellular proteome of *Micromonospora*



6. Chapter 3. The effect of root exudates on the intracellular proteome of *Micromonospora*

6.1 Introduction

The rhizosphere is one of the most densely populated soil regions and most influenced by plant roots, where most plant-microbe interactions occur dynamically and on a constant basis (Schenk et al., 2012). One of the early steps in the establishment of the plant-microorganism interaction is the physical contact with root exudates. Some exudates can act as chemical signals, which enhance the capacity of the microorganisms to survive in the rhizosphere and establish a relationship with the plant. In addition, these signal molecules may induce the alteration of specific gene expression patterns in the rhizospheric microorganisms. Certain signals present in the exudates can influence their symbiotic, mutualistic or pathogenic behavior with the host and their ability to colonize plant tissues (Morrissey et al., 2004; Mark et al., 2005; Shidore et al., 2012).

Micromonospora is an actinobacterium present in the rhizo- and endosphere of plants. This genus has been isolated from different legumes and diverse plant tissues, among which the nitrogen-fixing nodules stand out (Cerda, 2008; Rodríguez, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010). Despite the common presence of *Micromonospora* in nodules, the members of this genus are not capable of nitrogen fixation or inducing nodules. However, plant co-inoculation studies have demonstrated that *Micromonospora* has a positive effect on plant growth, increasing the quantity of nodules and fresh shoot weight (Cerda, 2008; Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). The capacity of *Micromonospora* to colonize several leguminous plants has been monitored by microscopy recently (Benito et al., 2017). These studies demonstrated that *Micromonospora* is localized within the nodules and strongly indicate that a non-specific relationship exists between *Micromonospora* and the legume. In addition, its presence in the nodules does not affect either the nitrogen fixation process or the development of rhizobia within the nodule (Benito et al., 2017). Despite this, the role of *Micromonospora* in its interaction with plants and rhizobia, and its presence in nodular tissues is not yet completely understood.

Recently, whole-genome data analysis has provided a new framework for understanding several key functions in the plant-microbe interaction (Taghavi et al., 2010). However, the genome does not show which genes are induced or repressed in certain physiological and environmental conditions. Contrary to the static genome, the proteome behaves dynamically

depending on variations present in each environment (Fields, 2001). Proteins are the key agents which play the role of gene expression, strongly related to molecular processes and most biochemical reactions regulating the cellular physiology and behavior of an organism (Aebersold and Mann, 2003). However, few proteomic studies have focused on the change in protein expression patterns when endophytic bacteria are in direct contact with plant root exudates or other plant produced substances. The development of various proteomic technologies, such as two-dimensional (2D) gel electrophoresis and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), can assist in revealing the cellular events involved in plant- bacteria interactions (Afroz et al., 2013). 2D gel electrophoresis facilitates the separation of proteins with an identical molecular mass that differ in their isoelectric point, or proteins with similar isoelectric point values but with different molecular masses. Different protein spot patterns reveal the change in protein expression pattern between samples (Hixson et al., 2017). On the other hand, LC-MS/MS combines the separation function of liquid chromatography with two connected mass spectrometers to analyze peptides. That is, shorter sequences of amino acids that form proteins. Each protein has a mass spectrum with unique characteristics. This technique allows for more accurate identification of expressed proteins from a complex sample than other technologies (Karpievitch et al., 2010).

The exact role of *Micromonospora* in its interaction with plants, especially with legumes, is still unknown. To better understand the behavior of *Micromonospora* in relation to plants, the main objective of this chapter was to analyze the effect of *Lupinus albus* root exudates on the intracellular proteome of three *Micromonospora* strains: *M. saelicesensis* Lupac 09^{T}, *M. lupini* Lupac 08 and *M. cremea* CR30^{T}. These proteomic analyses were performed by 2D gel electrophoresis followed by LC-MS/MS.

6.2 Results

6.2.1 Two-dimensional protein maps

First, the expressed protein patterns were visualized by using 2D gel electrophoresis from bacterial samples grown under different conditions of presence and absence of root exudates. The majority of visible intracellular proteins present in the samples showed molecular masses from 31 to 71 kDa and were distributed across a pH gradient ranging from 3 to 7. These 2D gels revealed significant differences in the visible spots between the three growth conditions

(**Figure 26**). The control sample (ISP 2) gels (**Figure 26 a,d**) showed different spot patterns with respect to the other two growth conditions (**Figure 26 b-c, e-f**). This showed that the nutrient solution where exudates were collected also causes a change in protein expression. However, in order to observe the influence of exudates on expression patterns, the 2D gels corresponding to bacterial samples grown in ISP 2 medium supplemented with nutrient solution, were compared to those with nutrient solution supplemented with exudates. This comparison revealed different protein spot patterns with notable overlap of some spots present in the two gels (**Figure 26 b-c, e-f**). When the gels of the two growth conditions were compared, strain Lupac 09^{T} (**Figure 26 e-f**) displayed a greater influence of the exudates on its proteome compared to strain Lupac 08 (**Figure 26 b-c**). These results demonstrated that a 0.25 mg/ml final concentration of exudates affects the expression of *Micromonospora* proteins.

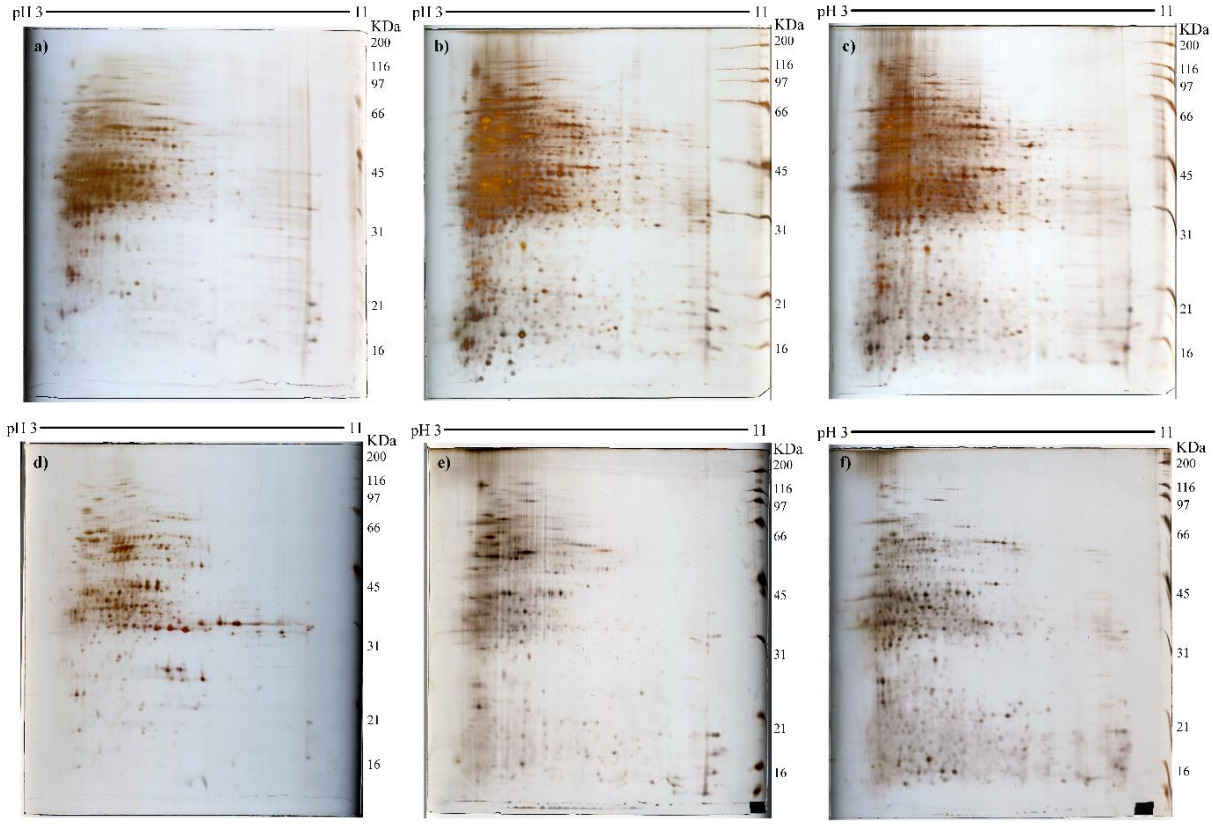


Figure 26. Two-dimensional electrophoresis gels of *Micromonospora* strains Lupac 08 and Lupac 09^{T} grown in the absence and presence of *Lupinus* root exudates. (**a**) Lupac 08 grown in ISP 2 medium; (**b**) Lupac 08 grown in ISP 2 medium+ nutrient solution; (**c**) Lupac 08 grown in ISP 2 medium+ root exudates collected in nutrient solution; (**d**) Lupac 09^{T} grown in ISP 2 medium; (**e**) Lupac 09^{T} grown in ISP 2 medium+ nutrient solution; (**f**) Lupac 09^{T} grown in ISP 2 medium+ root exudates collected in nutrient solution.

After observing the 2D polyacrylamide gels, the samples grown in ISP 2 medium supplemented with nutrient solution and with exudates were selected for the following tests. From this section onwards, the bacterial samples grown in ISP 2 broth supplemented with nutrient solution will be referred to as "without exudates" or "in the absence of exudates". Samples grown in ISP 2 supplemented with nutrient solution and root exudates will be referred to as "with exudates" or "in the presence of exudates". Samples grown without exudates were taken as control samples in order to eliminate the effect caused by the nutrient solution. Thus, only the proteins significantly altered by the *Lupinus* root exudates were analyzed.

6.2.2 Overall changes in the intracellular proteome expression in response to root exudates

As shown in the previous section, initial 2D gel analyses displayed differences in protein quantity patterns when the *Micromonospora* strains Lupac 08 and Lupac 09^T were grown in the presence or absence of root exudates. As the spots were not identified, the complex protein samples were analyzed by using a more sensitive technique, LC-MS/MS. In addition to the strains *M. lupini* Lupac 08 and *M. saelicesensis* Lupac 09^T, the proteome of the type strain *M. cremea* $CR30^T$ was also analyzed when grown in the same conditions as the two previous *Micromonospora* strains. In the LC-MS/MS data analysis, the statistically significant expressed proteins (SEPs) were selected (**Table 11**) by a false discovery rate (FDR) 1% and fold change (FCH) ≥1.5. Approximately 1555 proteins were globally detected in the *Micromonospora* strains. However, a total of 1106 (65.21%), 1147 (72.23%) and 862 (62.42%) proteins were significantly expressed in strains Lupac 08, Lupac 09^T and $CR30^T$ respectively. Among these significantly expressed proteins, other than the up- and down-regulated proteins, were only found in samples grown in the presence of exudates or in the absence of exudates, but not in both growth conditions (**Table 11**). These unique proteins represented between 12.38% (presence of exudates) and 25.2% (absence of exudates) of the identified proteins.

Table 11. Number of *Micromonospora* proteins with modulated expression upon exposure to *Lupinus* root exudates.

	Lupac 08	**Lupac 09^T**	**CR30^T**
Total proteins	1696	1588	1381
SEPs[a]	1106	1147	862
Up-regulated proteins	249	176	107
Down-regulated proteins	152	104	254
GPE unique proteins[b]	401	207	171
GAE unique proteins[c]	189	234	348

a. Statistically significant expressed proteins

b. Proteins only expressed in presence of root exudates

c. Proteins only expressed in absence of root exudates

The three *Micromonospora* strains responded in different ways to the root exudates, showing different percentages of affected proteins (**Figure 27**). Lupac 08 was the strain with the highest number of over-expressed proteins (249) compared to the other strains. With respect to under-expressed proteins, this strain showed 152 proteins in this expression category. Regarding unique proteins, 401 unique proteins were found when strain Lupac 08 was grown in the presence of exudates, compared to 189 in the control sample without exudates. When strain Lupac 09^T was exposed to root exudates, it displayed 176 up-regulated and 104 down-regulated proteins. In addition, this strain expressed 207 unique proteins in presence of exudates and 234 unique proteins in absence of exudates. In contrast to the previous strains, CR30^T presented the highest number of under-expressed proteins (254) and the lowest number of over-expressed proteins (107). Furthermore, this strain showed a greater number of unique proteins in the absence of exudates (348) compared to their growth in the presence of exudates (171).

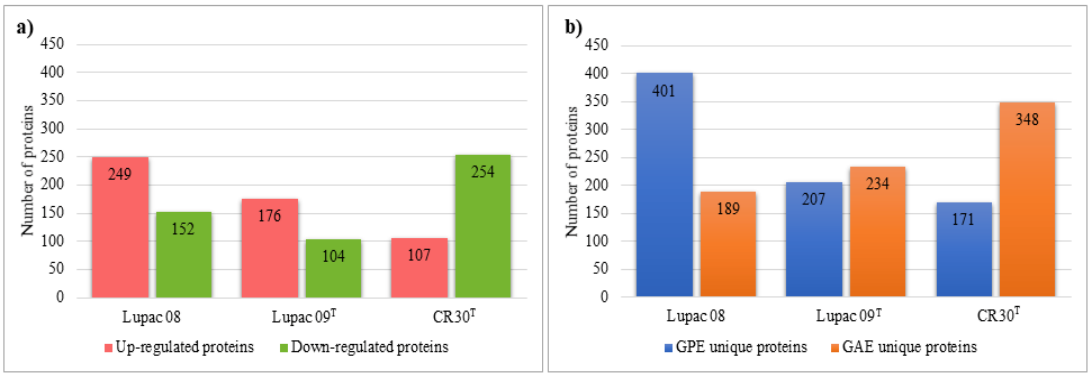


Figure 27. Effects of root exudates on protein expression. (**a**) Number of up- and down-regulated proteins in the presence of root exudates; (**b**) Number of unique proteins expressed in the presence (GPE) or absence (GAE) of root exudates.

The proteins identified in the analysis by LC-MS/MS showed different functional classification. Most of them were functionally well-annotated (~70%). However, approximately 30% of proteins displayed putative or hypothetical functions. Among the proteins with known functions, a significant proportion of them were involved in the transport and metabolism of amino acids, lipids, and carbohydrates; in addition to the production and conversion of energy (**Figure 28**).

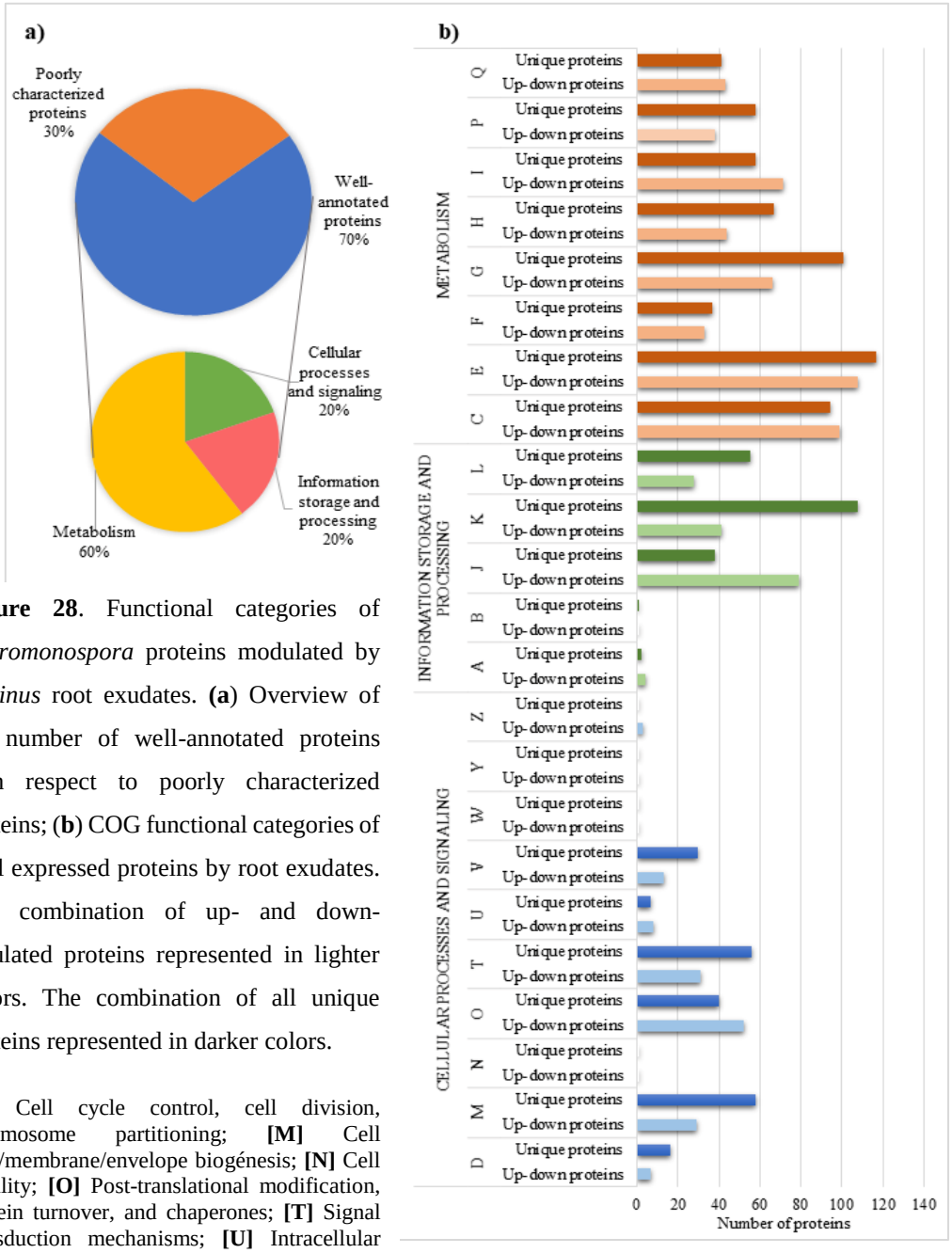


Figure 28. Functional categories of *Micromonospora* proteins modulated by *Lupinus* root exudates. **(a)** Overview of the number of well-annotated proteins with respect to poorly characterized proteins; (**b**) COG functional categories of total expressed proteins by root exudates. The combination of up- and down-regulated proteins represented in lighter colors. The combination of all unique proteins represented in darker colors.

[D] Cell cycle control, cell division, chromosome partitioning; **[M]** Cell wall/membrane/envelope biogénesis; **[N]** Cell motility; **[O]** Post-translational modification, protein turnover, and chaperones; **[T]** Signal transduction mechanisms; **[U]** Intracellular trafficking, secretion, and vesicular transport; **[V]** Defense mechanisms; **[W]** Extracellular structures; **[Y]** Nuclear structure; **[Z]** Cytoskeleton; **[A]** RNA processing and modification; **[B]** Chromatin structure and dynamics; **[J]** Translation, ribosomal structure and biogenesis; **[K]** Transcription; **[L]** Replication, recombination and repair; **[C]** Energy production and conversion; **[E]** Amino acid transport and metabolism; **[F]** Nucleotide transport and metabolism; **[G]** Carbohydrate transport and metabolism; **[H]** Coenzyme transport and metabolism; **[I]** Lipid transport and metabolism; **[P]** Inorganic ion transport and metabolism; **[Q]** Secondary metabolites biosynthesis, transport, and catabolism.

6.2.3 Functional characterization of proteins expressed in the presence and absence of exudates

A total of 1696, 1588 and 1381 proteins were identified in strains Lupac 08, Lupac 09^T and CR30^T respectively by LC-MS/MS. Among the identified proteins, approximately 23.64% of Lupac 08, 17.63% of Lupac 09^T, and 26.14% of CR30^T were significantly differentially expressed (**Table 11**). In other words, the same proteins were expressed in in the presence and absence of root exudates. This allowed us to obtain the fold change for each sample and its differential expression when the two growth conditions were compared. With respect to their functional annotations, approximately 76% of proteins were well-annotated. The majority of up- and down-regulated proteins were grouped on the metabolism functional category (**Figure 29**). All significantly differentially expressed proteins were mapped using the KEGG database to visualize which pathway are involved and their function.

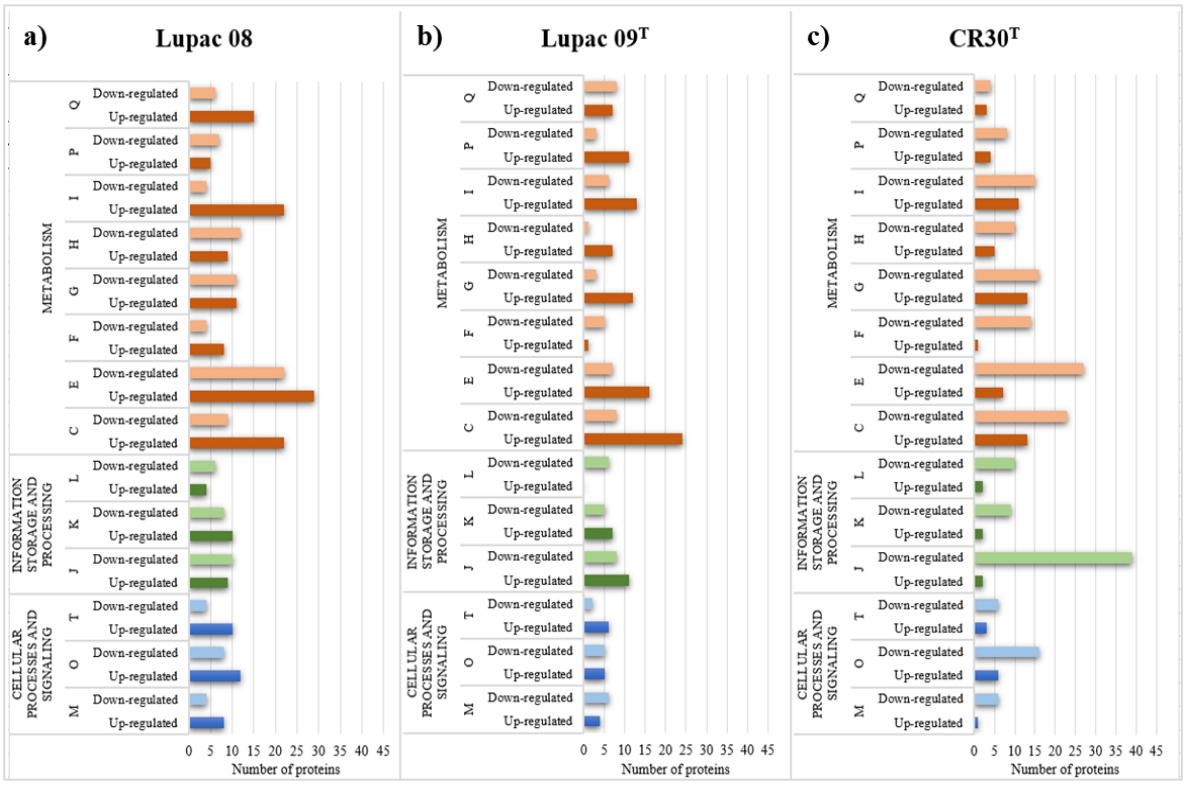


Figure 29. COG functional categories of up- and down-regulated proteins after exposed to root exudates. Up-and down-regulated proteins in the Lupac 08 (**a**), Lupac 09^T (**b**) and CR30^T (**c**) proteome.

[D] Cell cycle control, cell division, chromosome partitioning; **[M]** Cell wall/membrane/envelope biogenesis; **[N]** Cell motility; **[O]** Post-translational modification, protein turnover, and chaperones; **[T]** Signal transduction mechanisms; **[U]** Intracellular trafficking, secretion, and vesicular transport; **[V]** Defense mechanisms; **[W]** Extracellular structures; **[Y]** Nuclear structure; **[Z]** Cytoskeleton; **[A]** RNA processing and modification; **[B]** Chromatin structure and dynamics; **[J]** Translation, ribosomal structure and biogenesis; **[K]** Transcription; **[L]** Replication, recombination and repair; **[C]** Energy production and conversion; **[E]** Amino acid transport and metabolism; **[F]** Nucleotide transport and metabolism; **[G]** Carbohydrate transport and metabolism; **[H]** Coenzyme transport and metabolism; **[I]** Lipid transport and metabolism; **[P]** Inorganic ion transport and metabolism; **[Q]** Secondary metabolites biosynthesis, transport, and catabolism.

6.2.4 Plant-polymer degrading enzymes expressed in the *Micromonospora* proteome

The *Micromonospora* strains expressed a total of eight proteins related to plant-polymer degrading enzymes due to the influence of root exudates (**Table 12**). The hydrolytic enzymes belonged to the family of cellulases, amylases and chitosanases. However, the expression of these enzymes was different for each *Micromonospora* strain evaluated. Strain Lupac 08 showed several proteins involved in the production of β-glucosidases (BglB) (I0LCR6 and I0L805), α-glucosidases (MalZ) (I0L9D0 and I0LAV0), and β-N-acetylhexosaminidase (NagZ) (I0L9K9). Only I0LCR6, which was related to β-glucosidase production, was up-regulated with a fold change of 2.50. The rest of the proteins displayed a fold change of <0.50. Strain Lupac 09^T only showed one up-regulated protein involved in the production of α-glucosidase (MalZ) (A0A1C4ZGH1), which had a fold change of 1.50. In the case of CR30^T, unlike the other strains, no differentially expressed proteins related to hydrolytic enzymes were shown.

Table 12. Differently expressed proteins related to hydrolytic enzymes.

	Strain	Protein ID	Fold change	KEGG ID	Protein name	Function
Cellulase production						
	Lupac 08	I0LCR6	2.50	K05350	BglB	β-glucosidase [EC:3.2.1.21]
	Lupac 08	I0L805	0.50			
Amylase production						
	Lupac 08	I0L9D0	0.10	K01187	MalZ	α-glucosidase [EC:3.2.1.20]
	Lupac 08	I0LAV0	0.33			
	Lupac 09^T	A0A1C4ZGH1	1.50			
	CR30^T	A0A1N5V155	0.50	K00700	GBE1, GlgB	1,4-α-glucan branching enzyme [EC:2.4.1.18]
	Lupac 09^T	A0A1C4ZGH1	1.50	K01182	IMA, MalL	Oligo-1,6-glucosidase [EC:3.2.1.10]
Chitinase production						
	Lupac 08	I0L9K9	0.50	K01207	NagZ	β-N-acetylhexosaminidase [EC:3.2.1.52]

a. Fold change: Up-regulated (**red**) and down-regulated proteins (**green**).

6.2.5 Differentially expressed proteins involved in the stimulation of plant growth

Proteins related to plant growth promotion (PGP) were also altered by root exudates. The PGP activities expressed were involved in the production of indole acetic acid (IAA) and acetoin, the production and degradation of trehalose, the production and transport of siderophores and phosphate transport (**Table 13**). Strains Lupac 09^T and CR30^T showed proteins related to IAA and acetoin biosynthesis. Strain Lupac 09^T had two over-expressed proteins (A0A1C4UCL0 and A0A1C4X1Z8) and one down-expressed (A0A1C4XZJ7) protein related to the enzyme aldehyde dehydrogenase (AldH) involved in the IAA synthesis pathway. Strain CR30^T also displayed two over-expressed proteins (A0A1N5Z6C0 and A0A1N6AWJ1) associated with the production of aldehyde dehydrogenase (AldH) and

another over-expressed protein (A0A1N5VA91) in the production of amidase (AmiE), an enzyme also involved in the IAA synthesis. These proteins presented a fold change of approximately 2.27 for the induced proteins and 0.6 for the repressed protein. With respect to acetoin synthesis, Lupac 09^T displayed one up-regulated protein (A0A1C4TXZ4), while $CR30^T$ displayed one down-regulated protein (A0A1N5Z7L8). These had fold changes of 1.50 and 0.5 respectively. Moreover, two proteins (A0A1N5V155 and A0A1N5Z1H2) from $CR30^T$ which took part in trehalose synthesis were under-expressed. The protein A0A1N5V155 was involved in maltooligosyltrehalose trehalohydrolase (TreZ) and A0A1N5Z1H2 in trehalose 6-phosphate phosphatase (OtsB) production, which had a fold change of approximately 0.5.

Regarding the uptake of inorganic compounds such as iron or phosphate, only the Lupac 08 proteome revealed several over-expressed proteins (I0L490, I0L491, I0L493 and I0L4A5) entailed in the siderophore production such as enterochelin (EntE), bacillibactin (DhnE, DhnF) and pyochelin (PchG). The fold change of these proteins ranged from 1.78 to 6.0. In addition to proteins involved in the production of siderophores, specific iron uptake transport proteins were also differentially expressed. The two type strains, $CR30^T$ and Lupac 09^T, displayed one (A0A1N5VZ61) and three (A0A1C4ZW66, A0A1C4XTG0 and A0A1C4W565) over-expressed iron complex transport system substrate-binding proteins respectively (ABC.FEV.S). These over-expressed proteins had a fold change of 1.8. In addition to up-regulated proteins, the three *Micromonospora* strains displayed a down-regulated protein each (I0KXW7, A0A1C4TYE1 and A0A1N6ASP7) related to iron complex transport system ATP-binding (ABC.FEV.A). Meanwhile, strain $CR30^T$ also showed an under-expressed iron complex transport system permease protein (ABC.FEV.P) (A0A1N6B885). All these under-expressed proteins had a fold change of approximately 0.55. In addition to proteins related to iron transport, a total of five proteins involved in phosphate uptake were over-expressed. Strain $CR30^T$ had the highest number of proteins involved in phosphate transport (PhoP/PhoB, RegX3) showing four proteins induced by exudates (A0A1N5ZMK3, A0A1N5ZMK4, A0A1N6AL86, A0A1N5ZMK5); on the other hand, strain Lupac 09^T only displayed one up-regulated protein involved in the uptake of phosphate (PhoP) (A0A1C4YKL5).

Table 13. Differently expressed proteins associated with plant growth promotion activities

Strain	Protein ID	Fold change	KEGG ID	Protein name	Function
Indole-3-acetic acid (IAA) biosynthesis					
Lupac 09T	A0A1C4UCL0	1.67	K00128	ALDH	Aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
Lupac 09T	A0A1C4X1Z8	1.67			
Lupac 09T	A0A1C4XZJ7	0.60			
CR30T	A0A1N5Z6C0	3.50			
CR30T	A0A1N6AWJ1	3.00			
CR30T	A0A1N5VA91	1.50	K01426	AmiE	Amidase [EC:3.5.1.4]
Production and degradation of trehalose					
CR30T	A0A1N5V155	0.50	K01236	TreZ, GlgZ	Maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]
CR30T	A0A1N5Z1H2	0.58	K01087	OtsB	Trehalose 6-phosphate phosphatase [EC:3.1.3.12]
Acetoin/Butanodiol biosynthesis					
Lupac 09T	A0A1C4TXZ4	0.50	K01652	IlvB, IlvG, IlvI	Acetolactate synthase I/II/III large subunit [EC:2.2.1.6]
CR30T	A0A1N5Z7L8	1.50	K03366	ButA, BudC	Meso-butanediol dehydrogenase / (S,S)-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.-1.1.1.761.1.1.304]
Siderophore production					
Lupac 08	I0L490	6.00	K04780	DhbF	Nonribosomal peptide synthetase DhbF
Lupac 08	I0L491	1.78			
Lupac 08	I0L4A5	2.00	K02363	DhnE, DhnF	Bacillibactin biosynthesis
Lupac 08	I0L493	2.00	K12241	PchG	Pyochelin biosynthetic protein PchG
Iron uptake					
Lupac 09T	A0A1C4TYE1	0.60	K02013	ABC.FEV.A	iron complex transport system ATP-binding protein [EC:7.2.2.-]
CR30T	A0A1N6ASP7	0.50			
CR30T	A0A1N6B885	0.50	K02015	ABC.FEV.P	iron complex transport system permease protein
Lupac 09T	A0A1C4ZW66	1.75	K02016	ABC.FEV.S	iron complex transport system substrate-binding protein
Lupac 09T	A0A1C4XTG0	2.00			
Lupac 09T	A0A1C4W565	1.73			
CR30T	A0A1N5VZ61	1.77			
Phosphate uptake					
Lupac 09T	A0A1C4YKL5	2.67	K07658	PhoB1, PhoP	Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP
CR30T	A0A1N5ZMK5	1.67			
CR30T	A0A1N5ZMK5	1.67	K07657	PhoB	Two-component system, OmpR family, phosphate regulon response regulator PhoB
CR30T	A0A1N6AL86	1.50			
CR30T	A0A1N5ZMK5	1.67	K07776	RegX3	Two-component system, OmpR family, response regulator RegX3

a. Fold change: Up-regulated (**red**) and down-regulated proteins (**green**).

6.2.6 Differentially expressed proteins involved in the bacteria-plant communication

In addition to proteins related to plant growth promotion and enzymes involved in degradation of plant cell wall polysaccharides, different membrane transport proteins were also significantly differentially expressed in the presence of root exudates. These membrane transport proteins are listed in **Table 14**. Among ABC transport proteins affected by the root exudates, several proteins were related with the carbohydrate transport (N-acetylglucosamine, inositol, raffinose, ribose, etc.). Most of these transport proteins were over-expressed (26 proteins) except for eight proteins that were under-expressed. The

transport proteins that were over-expressed in the three *Micromonospora* strains were related with the transport of N-acetylglucosamine (ABC.NGC.S) (I0L812, A0A1C4Z0H0 and A0A1N5T7E2), N,N'-diacetylchitobiose (DasA) (I0LA93, A0A1C5AHC4 and A0A1N5YX65) and ribose (RbsA, RbsB, RbsC) (I0LA06, A0A1N6AGL4, A0A1N5YVC0, I0L002, A0A1C5AD71, A0A1C4ZV63, A0A1N6B599, A0A1N5YVW7, A0A1N5WNG1 and A0A1N5YWG6). However, not all the sugar transport proteins were up-regulated in the tested strains. Those that were related to the transport of α-glucoside (AglE) (A0A1C4ZRI9), inositol (IatP) (A0A1N5YWG6), D-xylose (XylG, XylH) (I0LBY3 and I0LBY4) and methyl-galactoside (MglC) (A0A1N5YWG6) were only over-expressed in one of the strains, but not in the other target strains. The putative multiple sugar transport system (GguA, GguB), unlike previous transporters, was up-regulated in the strains Lupac 08 and CR30^T. All these proteins had a fold change of approximately 2.0. The eight down-regulated proteins were involved in the transport of α-glucoside (AglG) (A0A1C4ZRR0), ribose (RbsA) (I0L034), D-xylose (XylG, XylH) (I0L034 and A0A1N6B641) and other saccharides (MsmE, ChvE) (I0L034, A0A1N6B641, I0L036 and A0A1C4Z0L1). In addition to carbohydrate transporters, several proteins related to the transport of molybdate, cobalt and nickel were also affected by the root exudates. The three *Micromonospora* strains showed down-regulated proteins (I0L5I1, I0KXW7, A0A1C4TYE1 and A0A1N6ASP7) for molybdate transport (ModA, ModF). Similarly, the nickel transport protein (NikD) from strain CR30^T was also down-regulated (A0A1N5ZF09). The fold change of these transport proteins ranged from 0.38 to 0.63. The cobalt/nickel transport protein (CbiN) was only over-expressed in strain Lupac 09^T (A0A1C4TZE6) with a fold change of 2.33.

Other proteins highlighted in the proteomic analysis were associated in the peptide/nickel, oligopeptide and branched-chain amino acid transport. Peptide/nickel transport proteins (ABC.PE) were differentially expressed in the three *Micromonospora* strains. Isolates Lupac 08 and Lupac 09^T showed two up-regulated proteins related to these transport proteins (I0LBU9, I0LBM2, A0A1C4WDN3 and A0A1C4YT12), with a fold change between 1.50 and 2.40. However, strain CR30^T displayed three up-regulated (A0A1N5ZEX0, A0A1N5ZV36 and A0A1N5W653) and four down-regulated proteins. These proteins from CR30^T proteome displayed a fold change between 1.50 and 4.50 for induced proteins, and <0.63 for repressed proteins. In the case of oligopeptide transport proteins (OppABD), each *Micromonospora* strain showed one over-expressed protein (A0A1C4YT12, A0A1N5ZEX0 and I0LBU9) with a fold change >1.50. CR30^T was the only strain that displayed three under-

expressed proteins (A0A1N5ZF37, A0A1N5ZVB4 and A0A1N5ZF09) related to oligopeptide transport. In addition to peptide and oligopeptide transport proteins, branched-chain amino acid transport proteins (LivFGHKM) were found among the differently expressed proteins in the three *Micromonospora* strains. Five, one and two proteins were over-expressed in Lupac 08 (I0LA06, I0L8I4, I0LCF4, I0LER6 and I0L8I5), Lupac 09^T (A0A1C5A637) and CR30^T (A0A1N5YVC0 and A0A1N5ZKB8) respectively, with a fold change of approximately 1.70. However, as in the previous cases, not all amino acid transporters were up-regulated. One protein was down-regulated in the strains CR30^T (A0A1N5YG43) and Lupac 08 (I0L034), with a fold change of <0.33.

Table 14. Differently expressed proteins involved in plant-bacteria interaction

ABC transporters

Strain	Protein ID	Fold change[a]	KEGG ID	Protein name	Function
Lupac 08	I0L5I1	0.38	K02020	ModA	Molybdate transport system substrate-binding protein
Lupac 08	I0KXW7	0.63	K05776	ModF	Molybdate transport system ATP-binding protein
Lupac 09^T	A0A1C4TYE1	0.60			
CR30^T	A0A1N6ASP7	0.50			
Lupac 09^T	A0A1C4TZE6	2.33	K02009	CbiN	Cobalt/nickel transport protein
CR30^T	A0A1N5ZF09	0.55	K15587	NikD, CntD	Nickel transport system ATP-binding protein [EC:7.2.2.11]
Lupac 08	I0LA06	2.00	K10548	ABC.GGU.A, GguA	Putative multiple sugar transport system ATP-binding protein [EC:3.6.3.17]
CR30^T	A0A1N5YVC0	1.50			
Lupac 08	I0L034	0.19			
Lupac 08	I0LBY4	2.00	K10547	ABC.GGU.P, GguB	Putative multiple sugar transport system permease protein
CR30^T	A0A1N6B641	0.50			
Lupac 08	I0L036	0.52	K10546	ABC.GGU.S, ChvE	Putative multiple sugar transport system substrate-binding protein
Lupac 08	I0L812	1.50	K10200	ABC.NGC.S	N-acetylglucosamine transport system substrate-binding protein
Lupac 09^T	A0A1C4Z0H0	1.50			
CR30^T	A0A1N5T7E2	1.50			
Lupac 09^T	A0A1C4ZRI9	1.50	K10232	AglE, GgtB	Alpha-glucoside transport system substrate-binding protein
Lupac 09^T	A0A1C4ZRR0	0.50	K10234	AglG, GgtD	Alpha-glucoside transport system permease protein
Lupac 08	I0LA93	3.33	K17329	DasA	N,N'-diacetylchitobiose transport system substrate-binding protein
Lupac 09^T	A0A1C5AHC4	1.50			
CR30^T	A0A1N5YX65	2.67			
CR30^T	A0A1N5YWG6	2.00	K17209	IatP	Inositol transport system permease protein
CR30^T	A0A1N5YWG6	2.00	K10541	MglC	Methyl-galactoside transport system permease protein
Lupac 09^T	A0A1C4Z0L1	0.60	K10117	MsmE	Raffinose/stachyose/melibiose transport system substrate-binding protein
Lupac 08	I0LA06	2.00	K10441	RbsA	Ribose transport system ATP-binding protein [EC:3.6.3.17]
Lupac 08	I0L034	0.19			
CR30^T	A0A1N6AGL4	1.75			
CR30^T	A0A1N5YVC0	1.50			
Lupac 08	I0L002	2.00	K10439	RbsB	Ribose transport system substrate-binding protein
Lupac 09^T	A0A1C5AD71	2.00			
Lupac 09^T	A0A1C4ZV63	1.50			
CR30^T	A0A1N6B599	2.00			
CR30^T	A0A1N5YVW7	1.67			
CR30^T	A0A1N5WNG1	1.75			
CR30^T	A0A1N5YWG6	2.00	K10440	RbsC	Ribose transport system permease protein

	CR30^{T}	A0A1N5WKL5	3.00	K10227	SmoE, MtlE	Sorbitol/mannitol transport system substrate-binding protein
	Lupac 08	I0LBY3	1.50	K10545	XylG	D-xylose transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 08	I0L034	0.19			
	Lupac 08	I0LBY4	2.00	K10544	XylH	D-xylose transport system permease protein
	CR30^{T}	A0A1N6B641	0.50			
Quorum sensing pathway						
	CR30^{T}	A0A1N5ZF09	0.55	K02031	ABC.PE.A	Peptide/nickel transport system ATP-binding protein
	Lupac 08	I0LBU9	1.50	K02032	ABC.PE.A1	Peptide/nickel transport system ATP-binding protein
	CR30^{T}	A0A1N5ZF09	0.55			
	CR30^{T}	A0A1N5ZEX0	1.50	K02033	ABC.PE.P	Peptide/nickel transport system permease protein
	CR30^{T}	A0A1N5ZV36	2.00			
	Lupac 08	I0LBM2	1.80	K02035	ABC.PE.S	Peptide/nickel transport system substrate-binding protein
	Lupac 09^{T}	A0A1C4WDN3	1.50			
	Lupac 09^{T}	A0A1C4YT12	2.40			
	CR30^{T}	A0A1N5W653	4.50			
	CR30^{T}	A0A1N5U2X5	0.63			
	CR30^{T}	A0A1N5ZVB4	0.63			
	Lupac 08	I0LA06	2.00	K01996	LivF	Branched-chain amino acid transport system ATP-binding protein
	Lupac 08	I0L034	0.19			
	Lupac 09^{T}	A0A1C5A637	1.57			
	CR30^{T}	A0A1N5YVC0	1.50			
	CR30^{T}	A0A1N5YG43	0.33			
	Lupac 08	I0LA06	2.00	K01995	LivG	Branched-chain amino acid transport system ATP-binding protein
	Lupac 08	I0L034	0.19			
	Lupac 09^{T}	A0A1C5A637	1.57			
	CR30^{T}	A0A1N5YVC0	1.50			
	Lupac 08	I0L8I4	1.67	K01997	LivH	Branched-chain amino acid transport system permease protein
	Lupac 08	I0LCF4	2.00	K01999	LivK	Branched-chain amino acid transport system substrate-binding protein
	Lupac 08	I0LER6	1.50			
	Lupac 08	I0L8I5	1.62			
	CR30^{T}	A0A1N5ZKB8	1.83			
	Lupac 09^{T}	A0A1C5A637	1.57	K01998	LivM	Branched-chain amino acid transport system permease protein
	Lupac 09^{T}	A0A1C4YT12	2.40	K15580	OppA, MppA	Oligopeptide transport system substrate-binding protein
	CR30^{T}	A0A1N5ZF37	0.62			
	CR30^{T}	A0A1N5ZVB4	0.63			
	CR30^{T}	A0A1N5ZEX0	1.50	K15581	OppB	Oligopeptide transport system permease protein
	CR30^{T}	A0A1N5ZF09	0.55	K15583	OppD	Oligopeptide transport system ATP-binding protein

a. Fold change: Up-regulated (**red**) and down-regulated proteins (**green**).

6.2.7 Functional characterization of proteins exclusively expressed in presence or absence of exudates

In addition to the proteins expressed both in the presence/ absence of lupin root exudates, other proteins were found in the LC-MS/MS analysis. These proteins were only expressed in the samples grown with exudates or in the samples grown without exudates, but not in both samples. For this reason, these proteins were named unique proteins. A total of 401 and 189 proteins in Lupac 08, 207 and 234 in Lupac 09^{T}, and 171 and 348 in CR30^{T} were only expressed in presence (GPE) or absence (GAE) of root exudates respectively (**Table 11**).

With respect to their functional annotations, approximately 66% of unique proteins had known function (**Figure 30**). A large proportion of these annotated proteins were involved in metabolic functions. As in the previous section (6.2.3), all unique proteins were mapped using the KEGG database.

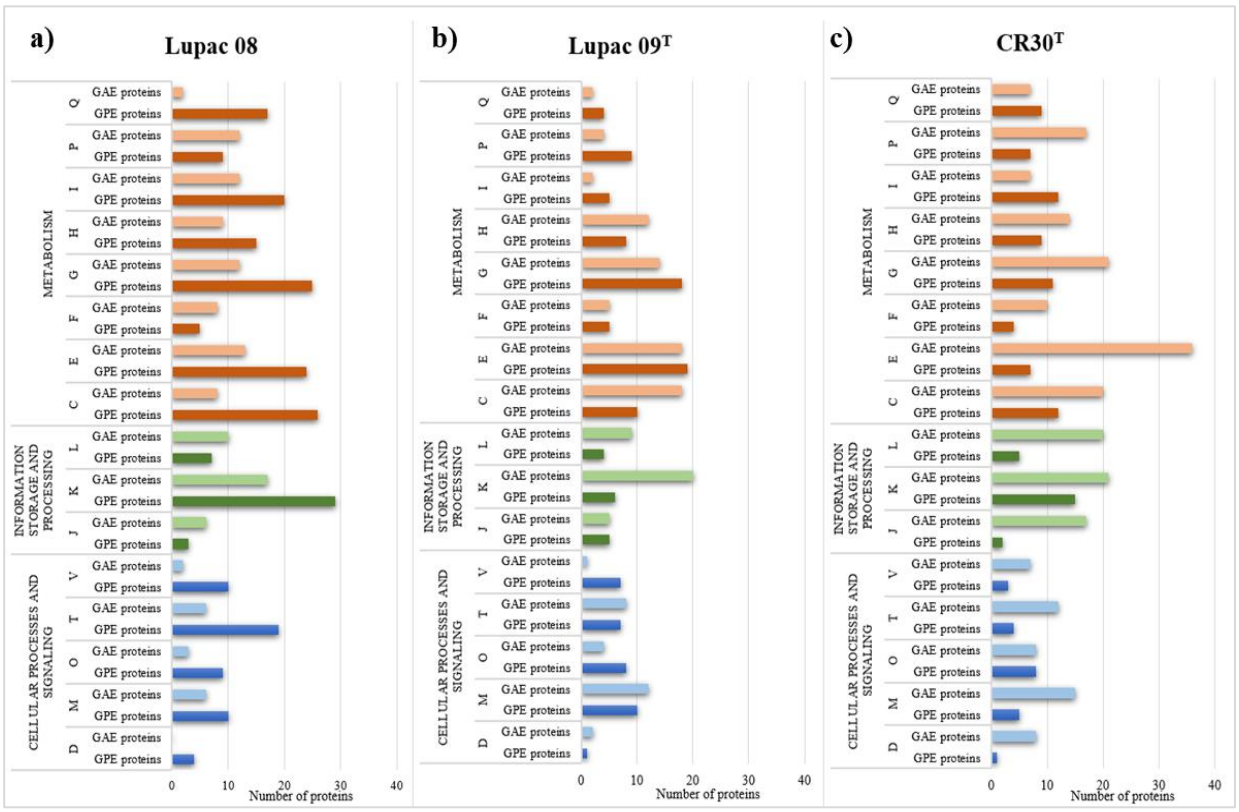


Figure 30. COG functional categories of unique proteins expressed in presence (GPE) and absence (GAE) of root exudates. Unique proteins expressed in the Lupac 08 (**a**), Lupac 09^T (**b**) and CR30^T (**c**) proteome.

[D] Cell cycle control, cell division, chromosome partitioning; **[M]** Cell wall/membrane/envelope biogenesis; **[N]** Cell motility; **[O]** Post-translational modification, protein turnover, and chaperones; **[T]** Signal transduction mechanisms; **[U]** Intracellular trafficking, secretion, and vesicular transport; **[V]** Defense mechanisms; **[W]** Extracellular structures; **[Y]** Nuclear structure; **[Z]** Cytoskeleton; **[A]** RNA processing and modification; **[B]** Chromatin structure and dynamics; **[J]** Translation, ribosomal structure and biogenesis; **[K]** Transcription; **[L]** Replication, recombination and repair; **[C]** Energy production and conversion; **[E]** Amino acid transport and metabolism; **[F]** Nucleotide transport and metabolism; **[G]** Carbohydrate transport and metabolism; **[H]** Coenzyme transport and metabolism; **[I]** Lipid transport and metabolism; **[P]** Inorganic ion transport and metabolism; **[Q]** Secondary metabolites biosynthesis, transport, and catabolism.

6.2.8 Unique proteins involved in plant-polymer degrading enzymes

As in the case of proteins expressed both in the presence and in the absence of root exudates, the *Micromonospora* strains showed several annotated unique proteins, such as hydrolytic enzymes (**Table 15**). Among the unique proteins expressed in GPE conditions, Lupac 08 showed three proteins involved in the production of xylan 1,4- β-xylosidases (XynB) (I0L6N5), α-L-arabinofuranosidases (AbfA) (I0L793) and β-N-acetylhexosaminidases

(NagZ) (I0KXW3). Strain Lupac 09^T expressed two proteins annotated as β-glucosidase (BglX) (A0A1C4TYQ6) and β-N-acetylhexosaminidases (NagZ) (A0A1C4TYB0), while CR30^T only displayed one GPE protein associated with the production of α-amylases (AmyA) (A0A1N6B7W3). On the other hand, the strains Lupac 09^T and CR30^T showed unique proteins expressed in GAE conditions related to several hydrolytic enzymes. This was in contrast with Lupac 08, which did not show any protein expressed in this growth condition. The enzyme β-glucosidase (BglB) was present among GAE proteins from Lupac 09^T and CR30^T (A0A1N5U3Y9 and A0A1N5ZN22). However, Lupac 09^T also expressed two proteins annotated as α-amylase (AmyA) (A0A1C4XSR6) and hexosaminidase (HEXA_B) (A0A1C5ACP7).

Table 15. Unique proteins related to hydrolytic enzymes

	Strain	Protein ID	Type of expression[a]	KEGG ID	Protein name	Function
Cellulase production						
	Lupac 09^T	A0A1C4TYQ6	With exudates	K05349	BglX	Beta-glucosidase [EC:3.2.1.21]
	Lupac 09^T	A0A1N5U3Y9	Without exudates	K05350	BglB	Beta-glucosidase [EC:3.2.1.21]
	CR30^T	A0A1N5ZN22	Without exudates			
Amylase production						
	Lupac08	I0L3C3	With exudates	K00700	GBE1, GlgB	1,4-alpha-glucan branching enzyme [EC:2.4.1.18]
	Lupac 09^T	A0A1C4YYI8	With exudates			
	CR30^T	A0A1N6B7H5	Without exudates			
	Lupac 09^T	A0A1C4XSR6	Without exudates	K01176	AMY, amyA, malS	alpha-amylase [EC:3.2.1.1]
	CR30^T	A0A1N6B7W3	With exudates			
Xylanase production						
	Lupac08	I0L6N5	With exudates	K01198	XynB	Xylan 1,4-beta-xylosidase [EC:3.2.1.37]
	Lupac08	I0L793	With exudates	K01209	AbfA	Alpha-L-arabinofuranosidase [EC:3.2.1.55]
Chitinase production						
	Lupac08	I0KXW3	With exudates	K01207	NagZ	Beta-N-acetylhexosaminidase [EC:3.2.1.52]
	Lupac 09^T	A0A1C4TYB0	With exudates			
	Lupac 09^T	A0A1C5ACP7	Without exudates	K12373	HEXA_B	Hexosaminidase [EC:3.2.1.52]

a. Type of expression: Proteins expressed only in the presence of exudates (**with exudates**) and proteins expressed only in the absence of exudates (**without exudates**).

6.2.9 Unique proteins involved in plant growth promotion

In addition to proteins related to plant polymer degradation, some unique proteins were annotated with functions related to the plant-bacteria interaction. Several unique proteins expressed in GPE conditions were involved in different PGP activities (**Table 16**). Strain Lupac 08 showed two proteins (I0L3C3 and I0L910) entailed in synthesis and degradation of trehalose, one protein (I0LAE7) in IAA synthesis and other two (I0L2I7 and I0L7D7) in acetoin synthesis. Specifically, these proteins were associated with the production of maltooligosyltrehalose trehalohydrolase (TreZ) (I0L3C3), trehalose 6-phosphate synthase (TPS) (I0L910), nitrilase (NIT) (I0LAE7) and (R,R)-butanediol dehydrogenase (ButA, ButB, ButC) (I0L2I7 and I0L7D7). Strain Lupac 09^T also displayed proteins (A0A1C4YYI8,

A0A1C4U878 and A0A1C4XID9) involved in the same PGP activities as Lupac 08, and one protein (A0A1C4WL14) involved in the ACC deaminase synthesis. However, CR30^T showed fewer proteins in the GPE condition than previous strains related to growth promotion. It displayed one protein (A0A1N6ABK3) entailed in the trehalose degradation (α-trehalose phosphorylase (TreP)) and two proteins (A0A1N6BB15 and A0A1N6AJL1) in the IAA synthesis (aldehyde dehydrogenase (AldH) and amidase (AmiE)). On the other hand, among unique proteins expressed in GAE conditions, strains Lupac 09^T and CR30^T expressed one (A0A1C4U685) and two proteins (A0A1N5VJ32 and A0A1N6AH43) respectively involved in aldehyde dehydrogenase (AldH) production. This enzyme takes part in the IAA synthesis. In the case of strains Lupac 08 and CR30^T showed proteins related to the synthesis and degradation of trehalose, expressed as maltooligosyltrehalose trehalohydrolase (TreZ) (A0A1N6B7H5), trehalose 6-phosphate phosphatase (OtsB) (I0KWU1) and α-trehalose phosphorylase (TreP) (I0KVM7). In addition, CR30^T, unlike the other two *Micromonospora* strains, had one protein (A0A1N6ATT5) involved in acetoin synthesis, and one (A0A1N5UQ14) related to siderophore production.

In addition to the proteins involved in the production of compounds that can improve plant growth, other unique proteins were expressed in the presence and absence of exudates (GPE and GAE conditions) related to the iron and phosphate transport. In the case of iron transporters, globally, most unique proteins were expressed in the absence of exudates. CR30^T was the strain with the highest number of proteins expressed in GAE conditions annotated as iron complex transport system (ABC.FEV.A, ABC.FEV.P, ABC.FEV.S) (A0A1N6B7R9, A0A1N6B7K5, A0A1N5UJS8 and A0A1N6A766). Lupac 08 also showed three proteins (I0L5Q7, I0L0L5 and I0L0L4) related to the iron complex transport system (ABC.FEV.A, ABC.FEV.P). However, not all proteins were expressed GAE conditions, Lupac 08 expressed two proteins (I0L7H1and I0L7X3) involved in the iron (III) transport (AfuC, FbpC) and one (I0L7H9) involved in the transport system iron complex transport system (ABC.FEV.A) in GPE conditions. Lupac 09^T, like Lupac 08, displayed one protein (A0A1C4W5E3) annotated as iron complex transport system (ABC.FEV.A). In the case of phosphate uptake all proteins related to this function were expressed in the presence of exudates. Lupac 08 showed two proteins (I0LCV1 and I0KX70) implicated in phosphatase synthesis response regulator (PhoB, PhoP), while the strain CR30^T presented one protein (A0A1N5ZPC3) related to phosphate transport (PstS).

Table 16. Unique proteins related to plant-growth promotion activities

	Strain	Protein ID	Type of expression[a]	KEGG ID	Protein name	Function
Indole-3-acetic acid (IAA) biosynthesis						
	Lupac08	I0LAE7	With exudates	K01501	E3.5.5.1	Nitrilase [EC:3.5.5.1]
	Lupac 09^T	A0A1C4U685	Without exudates	K00128	ALDH	Aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
	CR30^T	A0A1N6BB15	With exudates			
	CR30^T	A0A1N5VJ32	Without exudates			
	CR30^T	A0A1N6AH43	Without exudates			
	Lupac 09^T	A0A1C4U878	With exudates	K01426	AmiE	Amidase [EC:3.5.1.4]
	CR30^T	A0A1N6AJL1	With exudates			
1-Aminocyclopropane-1-Carboxylate (ACC) deaminase						
	Lupac09^T	A0A1C4WL14	With exudates	K01505	AcdS	1-aminocyclopropane-1-carboxylate deaminase [EC:3.5.99.7]
Production and degradation of trehalose						
	Lupac08	I0L3C3	With exudates	K01236	TreZ, GlgZ	Maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]
	Lupac 09^T	A0A1C4YYI8	With exudates			
	CR30^T	A0A1N6B7H5	Without exudates			
	Lupac08	I0L910	With exudates	K16055	TPS	Trehalose 6-phosphate synthase/phosphatase [EC:2.4.1.153.1.3.12]
	Lupac08	I0KWU1	Without exudates	K01087	OtsB	Trehalose 6-phosphate phosphatase [EC:3.1.3.12]
	Lupac08	I0KVM7	Without exudates	K05342	E2.4.1.64	Alpha,alpha-trehalose phosphorylase [EC:2.4.1.64]
	CR30^T	A0A1N6ABK3	With exudates			
Acetoin/Butanodiol biosynthesis						
	Lupac08	I0L2I7	With exudates	K00004	BDH, ButB	(R,R)-butanediol dehydrogenase / meso-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.41.1.1.- 1.1.1.303]
	Lupac 09^T	A0A1C4XID9	With exudates			
	Lupac08	I0L7D7	With exudates	K03366	ButA, BudC	Meso-butanediol dehydrogenase / (S,S)-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.-1.1.1.761.1.1.304]
	CR30^T	A0A1N6ATT5	Without exudates	K01652	IlvB, IlvG, IlvI	Acetolactate synthase I/II/III large subunit [EC:2.2.1.6]
Siderophore production						
	CR30^T	A0A1N5UQ14	Without exudates	K04780	DhbF	Nonribosomal peptide synthetase DhbF
Iron uptake						
	Lupac08	I0L7H1	With exudates	K02010	AfuC, FbpC	Iron(III) transport system ATP-binding protein [EC:7.2.2.7]
	Lupac08	I0L7X3	With exudates			
	Lupac08	I0L7H9	With exudates	K02013	ABC.FEV.A	Iron complex transport system ATP-binding protein [EC:7.2.2.-]
	Lupac 09^T	A0A1C4W5E3	With exudates			
	Lupac08	I0L5Q7	Without exudates			
	Lupac08	I0L0L5	Without exudates			
	CR30^T	A0A1N6B7R9	Without exudates			
	Lupac08	I0L0L4	Without exudates	K02015	ABC.FEV.P	Iron complex transport system permease protein
	CR30^T	A0A1N6B7K5	Without exudates			
	CR30^T	A0A1N5UJS8	Without exudates			
	CR30^T	A0A1N6A766	Without exudates			
Phosphate uptake						
	Lupac08	I0LCV1	With exudates	K07658	PhoB1, PhoP	Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP
	Lupac08	I0KX70	With exudates	K07657	PhoB	Two-component system, OmpR family, phosphate regulon response regulator PhoB
	CR30^T	A0A1N5ZPC3	With exudates	K02040	PstS	Phosphate transport system substrate-binding protein

a. Type of expression: Proteins expressed only in the presence of exudates (**with exudates**) and proteins expressed only in the absence of exudates (**without exudates**).

6.2.10 Unique proteins related to the plant-*Micromonospora* interaction

In addition to the PGP properties, different membrane transporters were expressed in GPE and GAE conditions. Some of these transport proteins were involved in the transport of molybdenum, zinc, cobalt and nickel, in addition to peptides, oligopeptides and branched-chain amino acid. The majority of these proteins were expressed in GAE conditions, although several proteins were also expressed in the GPE conditions. All proteins annotated as transport proteins are given in **Table 17**. Among unique proteins that were only expressed in GPE conditions, strain Lupac 08 displayed one protein (I0LDX4) involved in nickel transport (NikD) and molybdate transport (ModF) (I0L7H9). In this growth condition, Lupac 08 also expressed six peptide/nickel transporters (ABC.PE) (I0L547, I0LDX4, I0LDX3, I0LBV2, I0LDX6 and I0LDX5), four oligopeptide transporters (OppBCDF) (I0LBV2, I0LDX5, I0LDX4 and I0LDX3) and one branched-chain amino acid transporter (LivM) (I0L8I3). Lupac 08 was the strain that showed a greater number of expressed proteins per action of root exudates for nickel and molybdate transport. However, strain Lupac 09^T, unlike strain Lupac 08, did not present proteins related to the metal transport proteins. This type strain only showed two proteins (A0A1C4WJX4 and A0A1C4XA06) related to peptide/nickel transport (ABC.PE). Strain CR30^T, like Lupac 08, showed two proteins (A0A1N5WNH8 and A0A1N5ZK34) involved in branched-chain amino acid transport (LivFG). In the case of proteins expressed in GAE conditions, Lupac 08 displayed two proteins (I0L5I2 and I0L5I3) involved in molybdate transport (ModC, ModD) and one protein related to cobalt/nickel (CbiQ) (I0KXS1) and branched-chain amino acid (LivK) (I0LC22) transport. Strain Lupac 09^T expressed one protein related to zinc (ZnuA) (A0A1C5AGN5), molybdate (ModA) (A0A1C4W3D7) and peptide/nickel transport (ABC.PE) (A0A1C4WJY8). However, this strain also showed three proteins (A0A1C4XMI2, A0A1C5A5P1 and A0A1C5A685) related to branched-chain amino acid transport (LivFGH). Strain CR30^T also displayed three proteins (A0A1N5VXY2, A0A1N5VXJ0 and A0A1N5W0G0) related to molybdate transport (ModA, ModB, ModC). Moreover, this type strain showed one expressed protein in each of the following transporters: branched-chain amino acid (LivFG) (A0A1N5U4P8), peptide/nickel (ABP.PE) (A0A1N5TCS6) and cobalt/nickel (CbiM) (A0A1N6ARE0).

Table 17. Unique proteins related to plant-bacteria interaction.

	Strain	Protein ID	Type of expression[a]	KEGG ID	Protein name	Function
ABC transporters	CR30[T]	A0A1N5WNH8	With exudates			
	Lupac 09[T]	A0A1C4XMI2	Without exudates	K10548	ABC.GGU.A, GguA	Putative multiple sugar transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 08	I0L035	Without exudates			
	Lupac 09[T]	A0A1C4YB89	Without exudates	K10547	ABC.GGU.P, GguB	Putative multiple sugar transport system permease protein
	CR30[T]	A0A1N5YAH6	With exudates			
	Lupac 09[T]	A0A1C4Z057	Without exudates	K10200	ABC.NGC.S	N-acetylglucosamine transport system substrate-binding protein
	CR30[T]	A0A1N5Z3Y9	With exudates	K10233	AglF, GgtC	Alpha-glucoside transport system permease protein
	Lupac 08	I0L6N4	With exudates	K17326	BxlE	Xylobiose transport system substrate-binding protein
	Lupac 08	I0KWJ8	With exudates			
	Lupac 09[T]	A0A1C4UDQ8	Without exudates			
	CR30[T]	A0A1N6AGG3	Without exudates	K15770	CycB, GanO	Arabinogalactan oligomer / maltooligosaccharide transport system substrate-binding protein
	Lupac 08	I0KWJ8	With exudates			
	Lupac 09[T]	A0A1C4UDQ8	Without exudates			
	CR30[T]	A0A1N6AGG3	Without exudates	K10108	MalE	Maltose/maltodextrin transport system substrate-binding protein
	CR30[T]	A0A1N5WNH8	With exudates	K10542	MglA	Methyl-galactoside transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 08	I0L6N4	With exudates			
	Lupac 09[T]	A0A1C4W2S5	Without exudates exudates	K10117	MsmE	Raffinose/stachyose/melibiose transport system substrate-binding protein
	Lupac 08	I0L7X3	With exudates			
	Lupac 08	I0L7H1	With exudates		MsmX, MsmK, MalK, SugC, GgtA, MsiK	
	Lupac 08	I0L7H0	With exudates	K10112		Multiple sugar transport system ATP-binding protein
	CR30[T]	A0A1N5WNH8	With exudates			
	CR30[T]	A0A1N5U4P8	Without exudates	K10441	RbsA	Ribose transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 09[T]	A0A1C4XIH1	With exudates			
	CR30[T]	A0A1N5U4S7	With exudates	K10439	RbsB	Ribose transport system substrate-binding protein
	CR30[T]	A0A1N5U4V7	With exudates			
	Lupac 08	I0L035	Without exudates	K10440	RbsC	Ribose transport system permease protein
	CR30[T]	A0A1N5U4V7	With exudates	K10560	RhaP	Rhamnose transport system permease protein
	CR30[T]	A0A1N5WAG0	With exudates	K10561	RhaQ	Rhamnose transport system permease protein
	CR30[T]	A0A1N5WAG0	With exudates	K10559	RhaS	Rhamnose transport system substrate-binding protein
	CR30[T]	A0A1N5WNH8	With exudates	K10562	RhaT	Rhamnose transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 09[T]	A0A1C4WJX6	Without exudates	K10227	SmoE, MtlE	Sorbitol/mannitol transport system substrate-binding protein
	Lupac 09[T]	A0A1C4Z1B1	Without exudates	K10238	ThuG, SugB	Trehalose/maltose transport system permease protein
	CR30[T]	A0A1N5WNH8	With exudates			
	Lupac 09[T]	A0A1C4XMI2	Without exudates	K10545	XylG	D-xylose transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 08	I0L035	Without exudates			
	Lupac 09[T]	A0A1C4YB89	Without exudates	K10544	XylH	D-xylose transport system permease protein
Quorum sensing pathway	Lupac08	I0L547	With exudates			
	Lupac08	I0LDX4	With exudates			
	Lupac 09[T]	A0A1C4WJX4	With exudates			
	Lupac 09[T]	A0A1C4XA06	With exudates			Peptide/nickel transport system ATP-binding protein
	Lupac 09[T]	A0A1C4WJY8	Without exudates	K02031	ABC.PE.A	
	Lupac08	I0LDX3	With exudates			
	Lupac08	I0LDX4	With exudates			
	Lupac08	I0L547	With exudates			Peptide/nickel transport system ATP-binding protein
	Lupac 09[T]	A0A1C4XA06	With exudates	K02032	ABC.PE.A1	
	Lupac08	I0LBV2	With exudates			
	Lupac08	I0LDX6	With exudates	K02033	ABC.PE.P	Peptide/nickel transport system permease protein
	Lupac08	I0LDX4	With exudates			
	Lupac08	I0LDX5	With exudates			
	Lupac 09[T]	A0A1C4WJX4	With exudates	K02034	ABC.PE.P1	Peptide/nickel transport system permease protein

	CR30^{T}	A0A1N5TCS6	Without exudates	K02035	ABC.PE.S	Peptide/nickel transport system substrate-binding protein
	Lupac 09^{T}	A0A1C4XMI2	Without exudates			
	CR30^{T}	A0A1N5WNH8	With exudates			
	CR30^{T}	A0A1N5ZK34	With exudates			Branched-chain amino acid transport system ATP-binding protein
	CR30^{T}	A0A1N5U4P8	Without exudates	K01996	livF	
	Lupac 09^{T}	A0A1C4XMI2	Without exudates			
	CR30^{T}	A0A1N5WNH8	With exudates			Branched-chain amino acid transport system ATP-binding protein
	CR30^{T}	A0A1N5U4P8	Without exudates	K01995	livG	
	Lupac09^{T}	A0A1C5A5P1	Without exudates	K01997	livH	Branched-chain amino acid transport system permease protein
	Lupac08	I0LC22	Without exudates			Branched-chain amino acid transport system substrate-binding protein
	Lupac 09^{T}	A0A1C4ZVY7	With exudates	K01999	livK	
	Lupac08	I0L8I3	With exudates			Branched-chain amino acid transport system permease protein
	Lupac 09^{T}	A0A1C5A685	Without exudates	K01998	livM	
	Lupac08	I0LBV2	With exudates	K15581	oppB	Oligopeptide transport system permease protein
	Lupac08	I0LDX5	With exudates	K15582	oppC	Oligopeptidetransport system permease protein
	Lupac08	I0LDX4	With exudates	K15583	oppD	Oligopeptide transport system ATP-binding protein
	Lupac08	I0LDX3	With exudates	K10823	oppF	Oligopeptide transport system ATP-binding protein

a. Type of expression: Proteins expressed only in the presence of exudates (**with exudates**) and proteins expressed only in the absence of exudates (**without exudates**).

Like the up- and down- regulated proteins, some unique proteins were also involved in the carbohydrate transport (N-acetylglucosamine, D-xylose, raffinose, ribose, etc.). The total number of proteins expressed between GPE and GAE conditions was very close (approximately 19 proteins). The unique proteins which have a function of carbohydrate transport are grouped in **Table 17**. In GPE conditions, CR30^{T} was the strain with the highest number of transporters expressed under these growth conditions. This strain expressed different proteins related to the transport of N-acetylglucosamine (ABC.NGC.S) (A0A1N5YAH6), xylobiose (BxlE) (A0A1N5WNH8), α-glucoside (AglF) (A0A1N5Z3Y9), arabinogalactan oligomer/maltooligosaccharide (CycB, GanO) (A0A1N5WNH8), maltose/maltodextrin (MalE) (A0A1N5U4S7), methyl-galactoside (MglA) (A0A1N5U4V7), raffinose/stachyose/melibiose (MsmE) (A0A1N5U4V7) and ribose (RbsA) (A0A1N5WNH8). CR30^{T} also showed the proteins A0A1N5WAG0 and A0A1N5WNH8, which were related to multiple sugar transport protein. In the case of Lupac 08, several proteins involved in the transport of ribose (RbsA, RbsB, RbsC) (I0L6N4 and I0KWJ8), and rhamnose were expressed in GPE conditions (RhaP, RhaQ, RhaS, RhaT) (I0L6N4, I0L7X3, I0L7H1, I0L7H0 and I0L0D3). The type strain Lupac 09^{T} only had one transport protein expressed in GPE conditions, whose function was to transport D-xylose (XylG) (A0A1C4XIH1). In GAE conditions, Lupac 09^{T} was the strain with the highest number of transporters expressed, contrary to the previous conditions. This strain expressed

proteins were entailed in the transport of maltose/maltodextrin transport system substrate-binding protein (MalE) (A0A1C4XMI2 and A0A1C4YB89), raffinose/stachyose/melibiose (MsmE) (A0A1C4Z057), ribose (RbsA, RbsC) (A0A1C4UDQ8), sorbitol/mannitol (SmoE, MtlE) (A0A1C4W2S5), trehalose/maltose (ThuG, SugB) (A0A1C4WJX6) and D-xylose (XylG, XylH) (A0A1C4Z1B1, A0A1C4XMI2 and A0A1C4YB89). In these same conditions, Lupac 08 expressed the protein I0L035, which is implicated in the transport of different substrates such as N-acetylglucosamine (ABC.NGC.S) and arabinogalactan oligomer/maltooligosaccharide (CycB and GanO). CR30^T also showed a low number of transport proteins expressed in this condition. It only expressed two proteins (A0A1N6AGG3 and A0A1N5U4P8) related to putative multiple sugar transport (GguA).

6.2.11 Cellulolytic activity in the presence of legume roots

Cellulase production by *Micromonospora* strains Lupac 08, Lupac 09^T and CR30^T was evaluated using M3 agar medium supplemented with 0.5% (w/v) carboxymethylcellulose (CMC). Strains Lupac 08 and Lupac 09^T produced cellulases, represented by a yellow halo around the colony at 7 days after inoculation. However, strain CR30^T did not present any halo at 7 or 14 days after incubation, producing a negative result for cellulose degradation in this condition (**Figure 31 a**).

The cellulolytic activity was also evaluated in the presence of live roots of three legumes: *Lupinus albus*, *Medicago sativa* and *Trifolium repens*. The roots of these plants were dipped into the bacteria suspension and subsequently were placed on M3 agar medium and Rigaud and Puppo solid medium, both supplemented with 0.5% (w/v) carboxymethylcellulose (CMC). The three strains, including CR30^T, showed production of cellulases in the presence of roots (**Figure 31 d-i**). The cellulolytic activity in the presence of the *Lupinus* roots was detected in the three *Micromonospora* strains at 7 days of incubation (**Figure 31 j-l**). However, this did not happen in the presence of *Medicago* or *Trifolium* plants. Strain CR30^T showed very low activity after a week of contact. However, after 14 days, the cellulase production was perfectly visible (**Figure 31 f, i**). It should be noted that CR30^T in the absence of roots did not produce cellulases (**Figure 31 a**). However, in the case of strains Lupac 08 and Lupac 09^T, the cellulolytic activity was detected in all legume species after 7 days incubation (**Figure 31 d-e, g-h**).

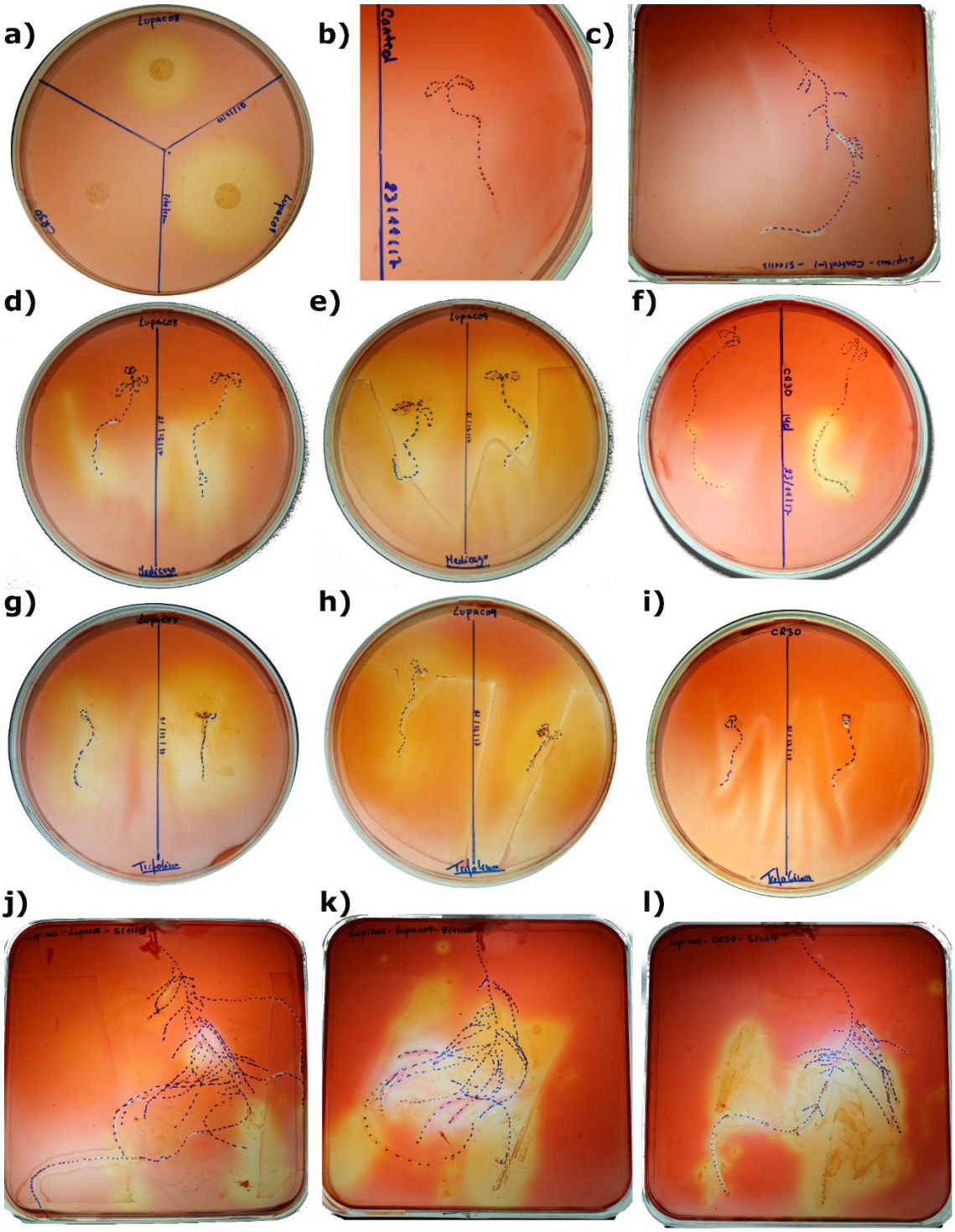


Figure 31. Cellulolytic activity in the presence and absence of live legume roots. (**a**) Cellulolytic activity in the absence of live roots; (**b-c**) Negative control, uninoculated plants; (**d-f**) Production of cellulases in the presence of *Medicago* roots by: (**d**) Lupac 08, (**e**) Lupac 09^{T} and (**f**) CR30^{T}; (**g-i**) Production of cellulases in the presence of *Trifolium* roots by: (**g**) Lupac 08, (**h**) Lupac 09^{T} and (**i**) CR30^{T}; (**j-l**) Production of cellulases in the presence of *Lupinus* roots by: (**j**) Lupac 08, (**k**) Lupac 09^{T} and (**l**) CR30^{T}.

6.3 Discussion

6.3.1. Global changes in the proteome of *Micromonospora* by root exudates

In this study, the proteins were analyzed by 2D protein gel electrophoresis and LC-MS/MS. 2D electrophoresis permitted a global view of proteins affected in response to root exudates when *Micromonospora* strains were grown in the presence or absence of *Lupinus* root exudates. The spots were not identified, nevertheless, the protein extracts were analyzed by LC-MS/MS. LC-MS/MS is complementary to 2D electrophoresis, but is a more sensitive and efficient method to identify proteins in a complex sample. Furthermore, it generates a greater amount of information, with a higher speed of the analysis and greater identification (Hanna et al., 2000; Mastronunzio et al., 2009). For these reasons, this technique was selected for our samples. Even though the information provided by these two techniques could not be compared since the spots from 2D gels were not identified, the combined information showed that the root exudates from *Lupinus* influenced the proteome of the three *Micromonospora* strains analyzed.

In the proteomic data obtained, Lupac 08 was the strain with the highest number of up-regulated proteins and unique proteins expressed in the presence of exudates (GPE), while $CR30^T$ displayed the opposite. Strain $CR30^T$ showed the greatest number of down-regulated proteins and unique proteins expressed in the absence of exudates (GAE). However, this strain also presented the lowest number of over-expressed and unique proteins expressed in GPE conditions. The unique proteins are of significance as they reflect that set of proteins have been more strongly influenced by the presence (or absence) of exudates to such a degree that they have only been expressed in this specific growth condition. In addition, the presence of unique proteins together with the over- and under-expressed protein demonstrate that *Lupinus* root exudates have influenced the *Micromonospora* proteome.

6.3.2 Hydrolytic enzymes and their role in plant tissue colonization

Among proteins which expression was altered by root exudates, proteins involved in the production of plant-polymer degrading hydrolytic enzymes were highlighted. The three *Micromonospora* strains displayed a greater number of proteins only expressed in presence or absence of exudates for these hydrolytic enzymes, in contrast with the proteins expressed in both growth conditions (where fold change was ≥ 1.5). Among the analyzed strains, $CR30^T$ displayed a lower number of up-regulated proteins and unique proteins only expressed in the

presence of root exudates. Globally, the altered proteins were involved in the degradation of polymeric plant cell wall components, such as cellulose, hemicellulose, chitin or starch. Nevertheless, these hydrolytic enzymes may also take part in the bacterium-plant relationship. For example, β-glucosidase plays important roles in several biological processes related to plant. It is not only involved in the cellulose degradation, but also in cell wall remodeling, lignification and chemical defense in plants, the establishment of pathogenic or symbiotic relationships, and in the activation of phytohormones and metabolic intermediates (Collins et al., 2007; Gilbert et al., 2008; Singh et al., 2016). This enzyme was expressed in Lupac 08 and Lupac09^T in the presence of exudates, unlike in strain CR30^T. This contrasts with the results obtained on the plate tests. In these assays, strain CR30^T showed cellulase production when it was in direct contact with the live root of three legumes (*Trifolium*, *Medicago* and *Lupinus*). β-N-acetylhexosaminidase, another enzyme expressed in Lupac 08 and Lupac 09^T, is involved in important biological processes catalyzing the hydrolysis of terminal N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides (Val-Cid et al., 2015). This hydrolysis process is related to the chitin degradation pathway together with other enzymes and plays a key role in plant defense systems against chitin-containing pathogens, such as fungi, oomycetes or insects (Swiontek Brzezinska et al., 2014; Veliz et al., 2017). Furthermore, β-N-acetylhexosaminidase may be involved in bacterial cell wall regeneration and growth promotion, assisting bacterial establishment in the plant (Litzinger et al., 2010; Ankati et al., 2018). In the cases of amylases, in addition to participating in starch degradation, these may also degrade different cell wall components of fungi together with other hydrolytic enzymes, such as chitinases. The joint action of chitinases and amylases help protect the plant against pathogens (Bull et al., 2002; Compant et al., 2005a; Saraf et al., 2014; Mhlongo et al., 2018).

Until a few years ago, there was a view that the role of hydrolytic enzymes was to allow the plant tissue colonization by phytopathogens. However, it has been demonstrated that some plant endophytes are also able to produce hydrolytic enzymes without causing damage to the host. An example is the plant-beneficial rhizobacterium *Bacillus amyloliquefaciens* FZB42 and *B. amyloliquefaciens* SQR9, which showed over-expression of plant-polymer degrading enzymes when they were exposed to maize root exudates (Kierul et al., 2015; Zhang et al., 2015). Another example is *Rhizobium leguminosarum* bv. Trifolii, an endophytic bacterium that produces the cellulase CelC2 in the presence of its host. This cellulase (CelC2) can erode the non-crystalline tip of the white clover host root hair wall, but not in other legumes such

as alfalfa (Robledo et al., 2008). In the case of *Micromonospora*, this is the first study about the effect of root exudates in its intracellular proteome. In previous works, the genome of different *Micromonospora* strains were sequenced. In the genome of strain *M. lupini* Lupac 08, a high number of putative plant-degrading enzyme genes were identified. Moreover, this strain has shown very high *in vitro* activity for cellulases, xylanases, pectinases, amylases and chitinases (Trujillo et al., 2014b). The genome of other *Micromonospora* strains also displayed a high number of these hydrolytic enzymes, with some exceptions, such as CR30^{T}. This strain shows a low number of genes that code for cellulases in its genome (Carro et al., 2018). The role of these hydrolytic enzymes in *Micromonospora* may play an important role in infection and colonization of plants, protection against possible pathogens and in the establishment of the bacterium-plant relationship. However, additional tests are needed to confirm the function of the hydrolytic enzymes expressed by effect of the exudates in this work.

6.3.3 The effect of root exudates on the bacterium-plant relationship

Most plant-microorganism interactions take place in the rhizosphere. In this soil region, root exudates play a fundamental role in the type of relationships established between the two organisms (Schenk et al., 2012). Some rhizospheric bacteria can produce compounds that bolster the health and development of plants, stimulated by the presence of these exudates (Berendsen et al., 2012). The *Micromonospora* strains analyzed in this study expressed several proteins related to plant growth promoting compounds by the lupin root exudates. These proteins were related to the production of siderophores, IAA, ACC deaminase, acetoin, and the synthesis and degradation of trehalose, but also in the transportation of phosphate and nickel. Most of them were over-expressed or only expressed in the presence of exudates. On the other hand, the molybdate and iron transport proteins were down-regulated or only expressed in the absence of exudates, with Lupac 08 being the strain with the highest number of genes under-expressed and only expressed in GAE conditions. As can be observed, root exudates potentiate the expression of proteins involved in plant growth promotion, although with some exceptions. Siderophores, one of the PGP characteristics present among the up-regulated proteins, are produced by many bacteria beneficial to plants. The siderophores act as ferric ion transport vehicles into microbial cells (Butler and Theisen, 2010), and may enhance the development of the plant increasing root and shoot biomass when host plants are inoculated with this bacterium (Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). In

addition, the production of these iron-chelating compounds by endophytic bacteria can contribute to protect the host plant against pathogenic infections by binding the available iron and limiting access to iron for plant pathogen microorganisms (Höfte and Bakker et al., 2007; Glick, 2015). Strains Lupac 08 and Lupac 09^T displayed induced proteins by lupin root exudates involved in the chitin degradation pathway and the production and/or transport of iron. The combined expression of proteins related to the production of siderophores and chitinases by these *Micromonospora* strains may provide more efficient protection against phytopathogens that affect the health of the plant, even in the early stages of root colonization. In the case of phosphorous, together with iron and other compounds, is an essential element for the growth of all living organisms, including plants (Bergkemper et al., 2016). This macronutrient is one of the major limiting elements in the soil due to its insolubility. Several microorganisms, such as PGPB, play a fundamental role in the phosphate availability for plants. They are able to transform insoluble phosphate in the form of PO_4^{3-}, the form that can be absorbed by plants (Bergkemper et al., 2016). Over-expression of phosphate transporters in the three *Micromonospora* strains tested, especially by strain $CR30^T$, shows the possible role of *Micromonospora* in providing this compound to the plants, stimulated by the presence of root exudates.

In the proteomes analyzed, in addition to iron and phosphate transporters, proteins related to other PGPB characteristics were expressed. As mentioned above, several proteins associated with the synthesis and degradation of trehalose were also expressed. This disaccharide can act as an osmoprotectant and improve plant abiotic stress tolerance (Garg et al., 2002). In addition, it may be involved in nodule growth regulation, which could explain the coexistence of *Micromonospora* and rhizobia in the internal tissues of legumes (Aeschbacher et al., 1999; Barraza et al., 2013). Other PGP characteristics expressed in the analyzed proteins were the microbial production of plant hormones, such as IAA and acetoin. Root exudates are composed of different types of low carbon molecules that may serve as precursors for the biosynthesis of phytohormones, among them, tryptophan (Haichar et al., 2014). Tryptophan is a precursor for IAA synthesis, so the expression of this phytohormone may be due to the presence of tryptophan in the *Lupinus* exudates. IAA and acetoin can enhance plant health and growth, and act as plant signaling molecules, allowing communication between the bacteria and the plant (Fahad et al., 2015; Mhlongo et al., 2018). In addition, IAA together with ACC deaminase contribute to the modulation of ethylene as a stress response in the host plant (Arshad and Frankenberger, 1991; Glick et al., 1998; Glick, 2004). The ethylene

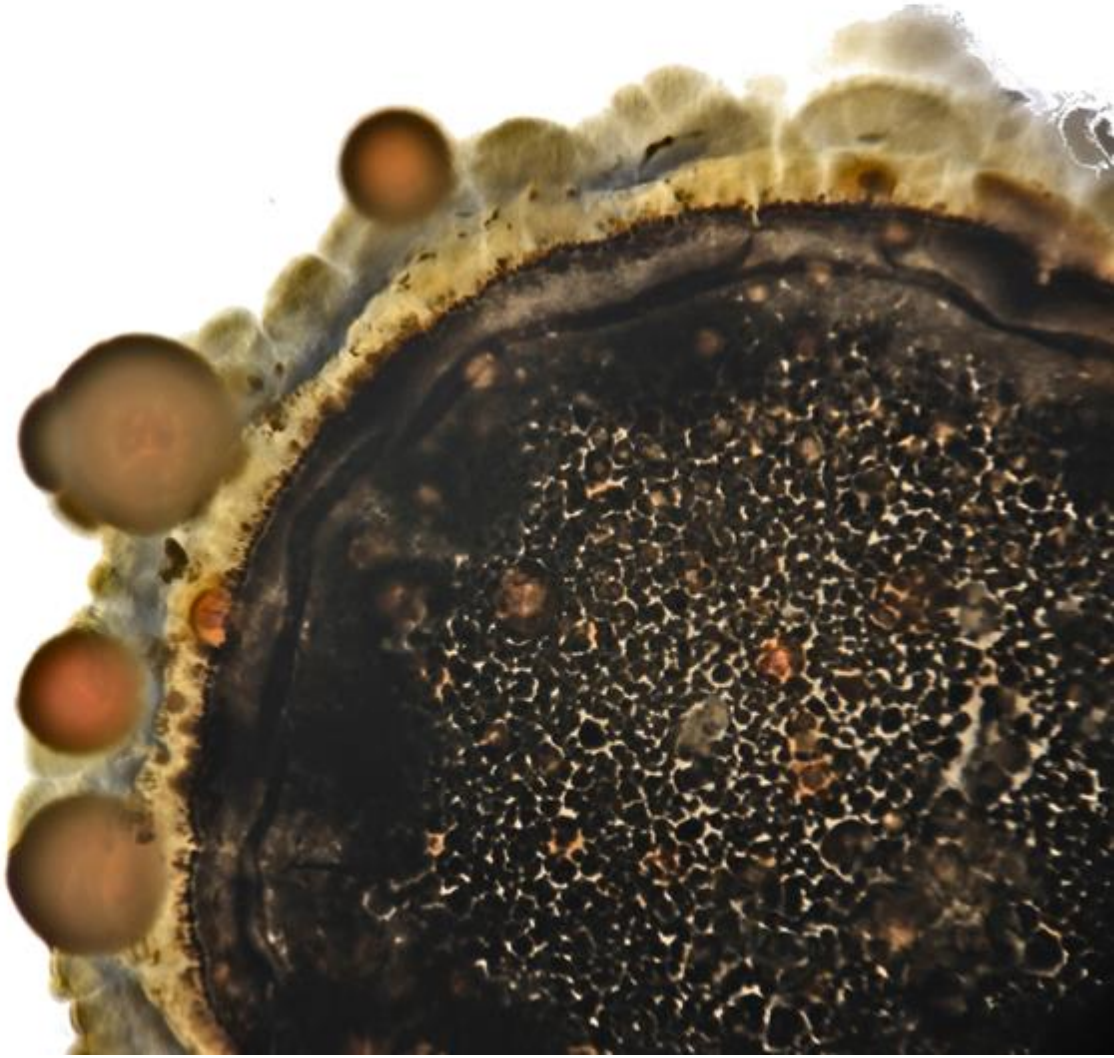
precursor, ACC, also exudes from roots, which the rhizospheric bacteria can use as carbon and energy recourse and reduce stress due to excess ethylene (Haichar et al.,2012). Some PGP activities have been detected in *Micromonospora* in *in vitro* tests previously, such as the IAA and siderophore production in Lupac 08 (Trujillo et al., 2014b). Moreover, different genes related to siderophore, trehalose, IAA and ACC deaminase have been found in the genome of *Micromonospora* strains other than Lupac 08 (Trujillo et al., 2014b; Carro et al., 2018). High expression of PGP-related proteins in the presence of root exudates may be a specific response to signaling molecules derived from plants. This may indicate that many of these compounds are produced in the early stages of plant colonization, when the bacteria come into contact with the legume root exudates.

Molecules present in root exudates may serve as carbon and energy sources for surrounding microorganisms and therefore, these may be attracted to the plant roots (Shidore et al., 2012). Microorganisms living in endophytic association with the plants need to exchange nutrients and molecular signals (Chibucos and Tyler, 2009). Bacteria have a large diversity of transporters that allow the exchange of metabolites and nutrients produced by the plant (Andrés-Barrao et al., 2017). In the proteomes analyzed, several transport proteins were expressed in the presence and absence of root exudates involved in the transport of nutrients, trace elements and signal molecules. Proteins related to the transport of carbohydrates, such as N-acetylglucosamine (ABC.NGC.S), ribose (RbsA, RbsB, RbsC), α-glucoside (AglE, AglG), D-xylose (XylG, XylH) or N,N'-diacetylchitobiose (DasA) were induced in the *Micromonospora* strains in response to root exudates. It has been reported that *Lupinus* plants release different sugars, organic acids and amino acids into the soil (Egle et al., 2003; Vranova et al., 2013). The sugars may be stimulating the protein expression of polysaccharide and monosaccharide transporters in *Micromonospora*. It has been described that rhizospheric bacteria can utilize these components as carbon sources for their proliferation and their subsequent colonization of roots before the establishment of an endophytic relationship (Mark et al., 2005; Bais et al., 2006). In addition to the compounds mentioned, the exudates may include several molecular signals, which function is to attract the bacteria to the plant roots. An exchange of signals between the plant and the bacteria is necessary to form an endophytic, symbiotic or pathogenic relationship (Bais et al., 2006). The ABC transporter RbsB associated with the ribose transport, over-expressed by exudates in the three strains under study, can also act as a primary recipient of chemoattractants released by the plant and subsequently initiate the events that alter the behavior of the recipient bacteria (Macnab,

1987; Boos and Lucht 1996; Stock and Surette 1996). These events are related to the chemotaxis towards the origin of the molecular signals captured, that is, the plant roots. This may indicate that the three *Micromonospora* strains (Lupac 08, Lupac 09^T and $CR30^T$) have received signals from the plant that can attract them to the root and establish a relationship. In addition to this ribose transporter, among the analyzed proteins in the *Micromonospora* strains, peptide/nickel, branched-chain amino acid, and oligopeptide transport proteins were activated in the presence of the *Lupinus* root exudates. The root exudates of several legumes can be rich in amino acids and oligopeptides (Carvalhais et al., 2011). It was not surprising that membrane transporters involved in the transport of oligopeptides across the cell wall were activated in the presence of these compounds. However, the transport of these molecules may be involved in the uptake of signaling peptides present in the exudates, which regulate the communication process between plant and bacteria (Lazazzera, 2001; Kierul et al., 2015). Branched-chain amino acid transport has also been detected in proteomic studies of endophytic microorganisms, such as *Frankia*, *Bacillus*, *Azorhizobium*, *Sinorhizobium* and *Bradyrhizobium*. Amino acid and oligopeptide transport in symbiosis may be an important component of bacterium-plant interactions, due to its importance in the signal exchange between the two organisms (Djordjevic, 2004; Sarma and Emerich, 2006; Mastronunzio et al., 2009; Kierul et al., 2015). An efficient exchange of molecular signals may facilitate plant tissue chemotaxis and colonization by endophytic bacteria and establish a beneficial relationship between the two organisms.

CHAPTER 4

Transcriptome profiling of *Micromonospora* responses to root exudates



7. Chapter 4. Transcriptome profiling of *Micromonospora* responses to root exudates

7.1. Introduction

The different interactions between plants and microorganisms occur continuously in the rhizosphere, where the plants exert selective pressure on the microbial community. Microbes can live in the rhizosphere, rhizoplane and endosphere of the plant depending on their ability to colonize and live inside or outside the plant tissues (Dudeja et al., 2012; Hacquard et al., 2017). Many microorganisms associated with plants such as endophytes can have a positive effect on plant health and development through phytohormone production, nutrient acquisition or protection against phytopathogens. Additionally, some bacterial endophytes can perform biological nitrogen fixation, increasing its availability for the plant (Lebeis, 2014; Rosenblueth and Martínez-Romero, 2006; Gaiero et al., 2013 Santi et al., 2013; Kandel et al., 2017).

Root exudates released by plants are one of the key factors influencing bacterial colonization and plant-bacteria interactions. Some of the exudate components can initiate cross-talk interactions with surrounding microbes, inhibiting or increasing the potential of bacterial growth and affecting their ability to colonize the plant tissues (Bais et al., 2006; Kandel et al., 2017). Chemical signals present in exudates may influence the bacterial specific gene expression patterns, which in turn may affect their behavior with respect to plants. The changes in the gene expression of endophytic bacteria by root exudates have been studied mainly in Gram-negative bacteria such as *Azoarcus* sp. BH72, *Pseudomonas aeruginosa* PA01, *Pseudomonas putida* KT2440, *Burkholderia phymatum* STM815, *Cupriavidus taiwanensis* LMG19424 and *Rhizobium mesoamericanum* STM3625 (Mark et al., 2005; Shidore et al., 2012; Neal et al., 2012; Klonowska et al., 2018). However, few studies have focused on the expression changes produced by root exudates in Gram-positive bacteria, where the most studied genus is *Bacillus* (Fan et al., 2012; Zhang et al., 2015; Xie et al., 2015; Yi et al., 2017). Within the phylum *Actinobacteria*, only the *Frankia* symbiotic transcriptome has been analyzed (Alloisio et al., 2010).

The genus *Micromonospora* is an actinobacterium widely distributed in different environments, even in plant tissues such as nitrogen fixing nodules from different legumes and actinorrhizal plants (Valdés et al., 2005; Trujillo et al., 2006, 2007, 2010; Garcia et al., 2010; Carro et al., 2012a, 2013a). It has been described that this genus maintains a beneficial

relationship with its host plant when it resides in the internal plant tissues **(**Trujillo et al., 2015; Benito et al., 2017). However, its ecological role in relation to the plant is still unknown. Transcriptome studies of members of the genus *Micromonospora* can assist in discovering what occurs when the bacterium interacts with the plant. Unlike the genome, the transcriptome can show which genes are induced and repressed at different stages of the organism's development (Manzoni et al., 2016). Over the last two decades, gene expression analysis has been commonly performed using microarrays. This technology is based on measuring the level of hybridization between a specific probe and its target molecule. Hybridization between these two components is indicated with a measurable fluorescent signal, showing the gene expression levels of different genes analyzed (Koltai and Weingarten-Baror, 2008). On the other hand, RNA sequencing (RNA-Seq) is the direct sequencing of transcripts by high-throughput next-generation sequencing technologies (Zhao et al., 2014). Within the last ten years, the RNA-Seq has gradually replaced microarrays (Wolff et al., 2018). This is because RNA-Seq shows a greater detection of low abundance transcripts and a broader dynamic range than microarrays. In addition, RNA-Seq does not suffer from associated hybridization-based limitations, such as cross-hybridization, limited detection range of individual probes and non-specific hybridization. RNA-Seq does not depend on genome annotation for prior probe selection since allows sequencing and subsequent analysis of most genes expressed in the transcriptome (Zhao et al., 2014; Wolff et al., 2018). In addition, a significant decrease in costs has allowed RNA-Seq to be more commonly used by researchers today.

The main aim of this chapter was to determine the influence of *Lupinus albus* root exudates in the regulation of gene expression through transcriptomic analysis by RNA-Seq in three *Micromonospora* strains: *M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^{T} and *M. cremea* CR30^{T}.

7.2. Results

7.2.1. Global changes in the transcriptome of Lupac08, Lupac09^{T} and CR30^{T} strains in response to root exudates

The transcriptional change analysis of *Micromonospora* strains in response to *Lupinus* root exudates was assessed by RNA-Seq. The RNA-Seq generated between 30-45 million reads for each sample, of which 70–90% were confirmed to be valid after filtering reads with Phred

quality scores of <20 using FASTX-Toolkit version 0.0.13.2. The differentially expressed genes (DEGs) of *M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^T and *M. cremea* CR30^T in the presence of lupin root exudates were identified by the DESseq2 analysis, with criteria of p-value ≤0.05 and fold change (FCH) ≥1.5. Only those genes that fulfilled both filter conditions were considered significantly differentially expressed and were chosen for the following analysis. The target strains displayed different responses to root exudates. The number of DEGs was 656 (9.7 %), 687 (10.4 %) and 1050 (14.5 %) for Lupac 08, Lupac 09^T and CR30^T respectively (**Table 18, Figure 32**). In the case of strain Lupac 08, out of the 656 DEGs 398 were up-regulated and 258 were down-regulated. Among the altered genes from this strain, 329 (~50%) were related to a known function and the other 50% of genes coded proteins with unknown or hypothetical functions. On the other hand, the strain Lupac 09^T responded to the root exudates with 319 up-regulated and 368 down-regulated genes. Unlike Lupac 08, Lupac 09^T had more genes that could be related to functions were well-annotated (422); genes with putative or unknown function (265), represented <39% of the differentially regulated genes. In the case of strain CR30^T, 1050 genes were significantly expressed, being 458 up-regulated and 592 down-regulated genes. Among these up- and down-regulated genes, 580 (~55%) were annotated with known functions; genes with putative or unknown function constituted 44%.

Table 18. Number of *Micromonospora* genes with modulated expression upon exposure to *Lupinus* root exudates

	Lupac08	**Lupac09^T**	**CR30^T**
Genes total	6757	6578	7251
DEG[a]	656 (9.71%)	687 (10.44%)	1050 (14.48%)
Up-regulated genes	398	319	458
Down-regulated genes	258	368	592

a. Significant differentially expressed genes

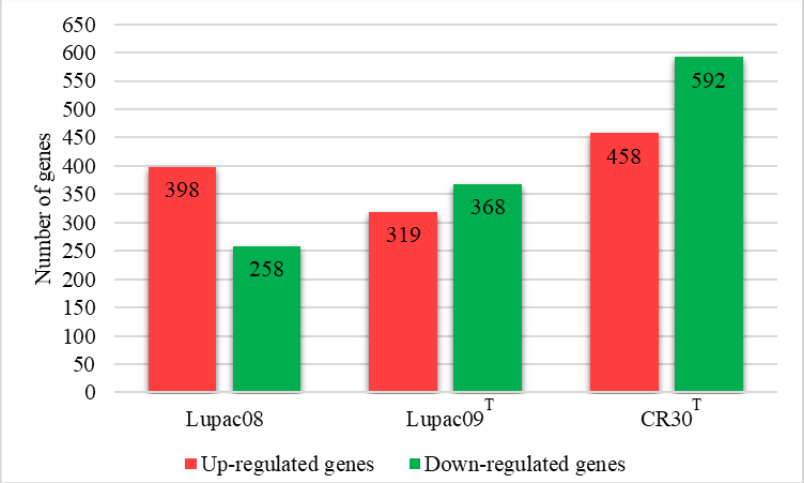


Figure 32. Number of up- and down-regulated genes by the presence of lupin root exudates.

7.2.2. Validation of RNA-Seq data by real-time PCR

Six differentially altered genes per strain were selected for validation of the transcriptome data by semiquantitative real-time PCR (RT-qPCR). The values were normalized according to three reference genes: *gyr*B (B subunit of DNA gyrase), *rpo*B (β-subunit of RNA polymerase) and *atp*D (ATP synthase β-subunit). The target genes displayed a similar fold change to that obtained by RNA-Seq, with a small variation of the values depending on the reference gene used in the normalization (**Appendix III**). This means that all RT-qPCR data demonstrated strong correlation of gene expression in comparison with RNA-Seq results, validating the transcriptome data (**Figure 33**).

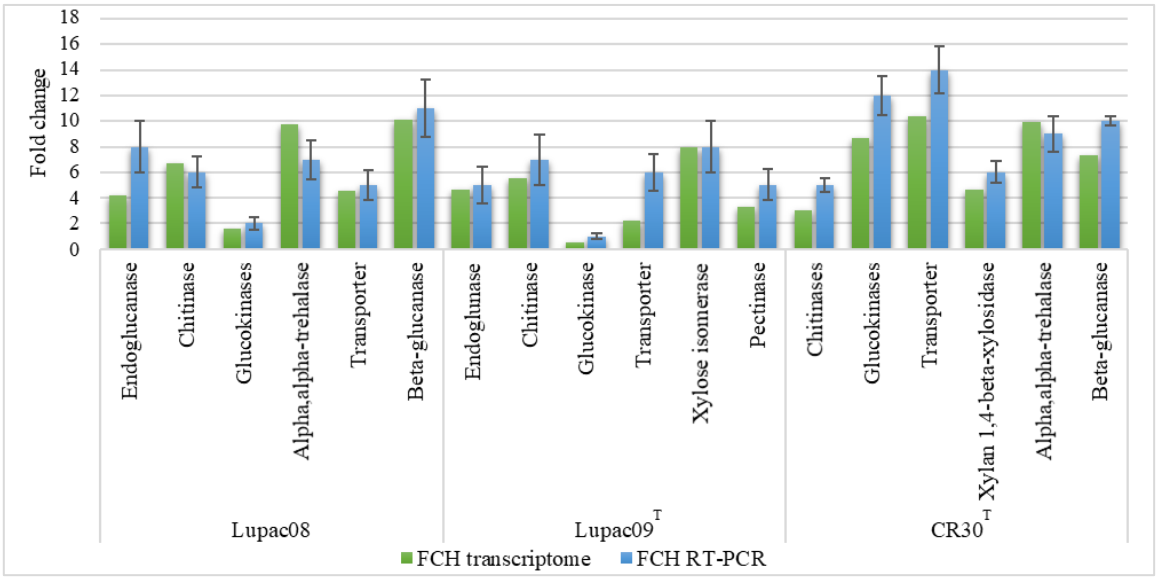


Figure 33. Validation of transcriptome data. Comparison of the expression levels obtained in the transcriptome with those obtained by RT-PCR.

7.2.3. Differentially expressed genes with functional classification in contact with root exudates

Among the significantly differentially expressed genes by lupin root exudates highlighted several functional COG categories whose function was related to metabolism (60%), information storage and processing (23%), and cellular processes and signaling (17%) (**Figure 34**). Within these COG categories, a significant proportion of genes were involved in the transport and metabolism of amino acids (E), lipids (I) and carbohydrates (G) and in the production and conversion of energy (C). Especially in the down-regulated genes, the functional category related to translation, ribosomal structure and biogenesis (J) stood out itself among the other categories (**Figure 35 a-c**). These functional COG categories corresponded to approximately 58% of altered genes with known function. In order to have a deeper understanding of the relationships among expressed genes, they were mapped in the KEGG database. The KEGG pathways with the highest number of up- and down-regulated genes were: starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, glyoxylate and dicarboxylate metabolism, ABC transporters, two-component system and *quorum* sensing. In addition to these categories, ribosome and oxidative phosphorylation categories stood out among the down-regulated genes in the three *Micromonospora* strains.

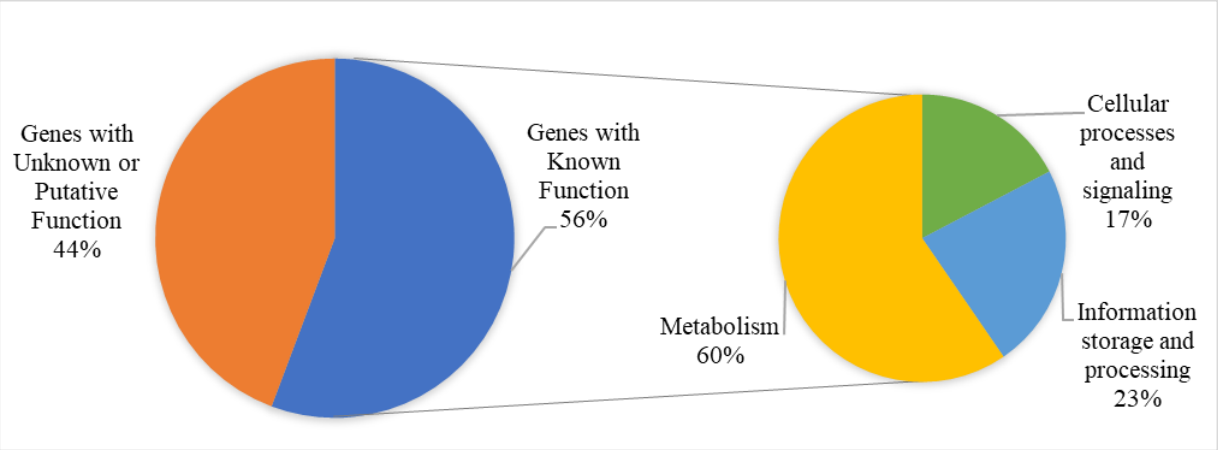


Figure 34. Overview of the number of well-annotated genes with respect to poorly characterized proteins

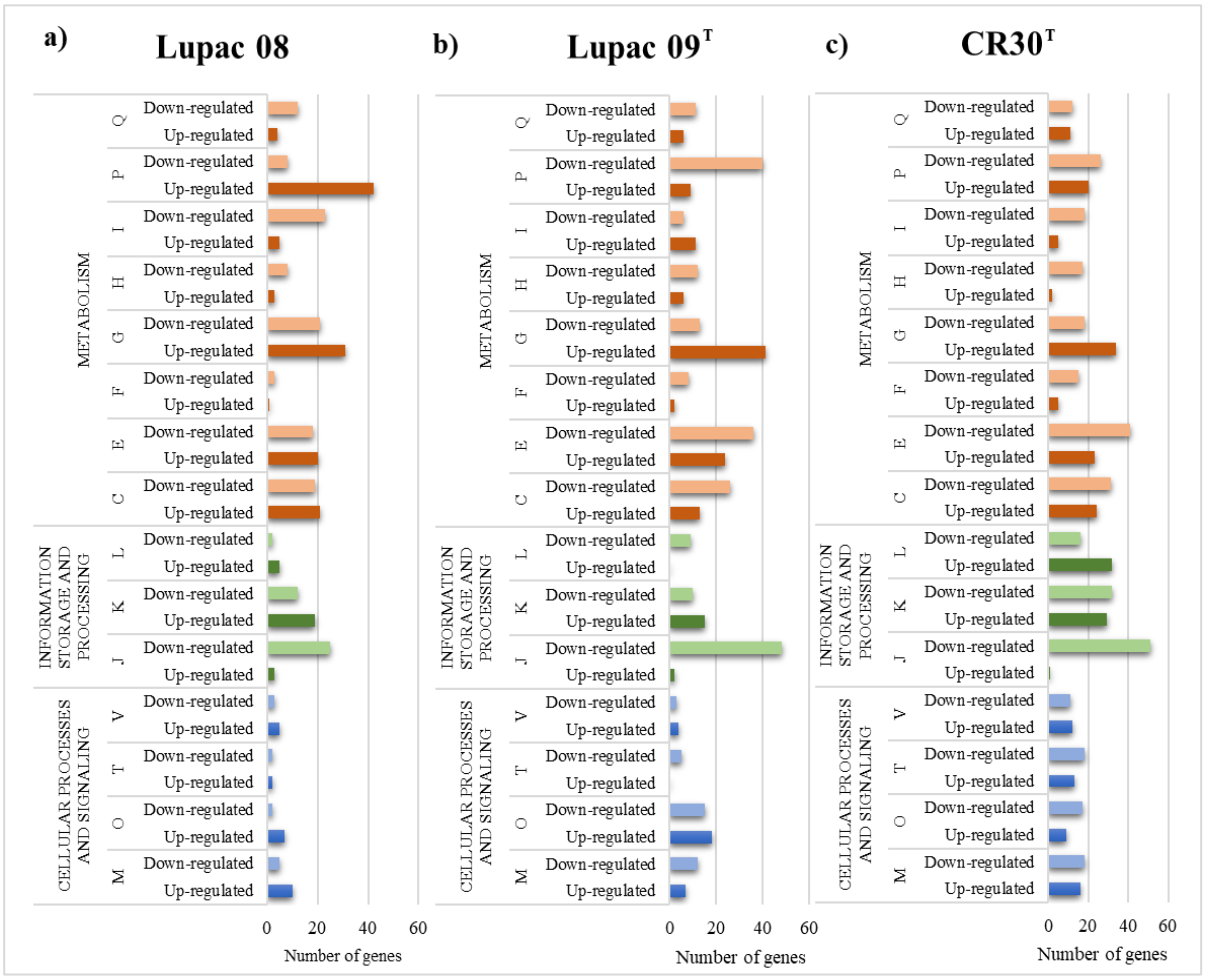


Figure 35. Functional categories of *Micromonospora* genes altered by *Lupinus* root exudates. (**a-c**) COG functional categories of expressed genes by root exudates from the transcriptome of Lupac 08 (**a**), Lupac 09^T(**b**) and CR30^T (**c**).

[D] Cell cycle control, cell division, chromosome partitioning; **[M]** Cell wall/membrane/envelope biogenesis; **[N]** Cell motility; **[O]** Post-translational modification, protein turnover, and chaperones; **[T]** Signal transduction mechanisms; **[U]** Intracellular trafficking, secretion, and vesicular transport; **[V]** Defense mechanisms; **[W]** Extracellular structures; **[Y]** Nuclear structure; **[Z]** Cytoskeleton; **[A]** RNA processing and modification; **[B]** Chromatin structure and dynamics; **[J]** Translation, ribosomal structure and biogenesis; **[K]** Transcription; **[L]** Replication, recombination and repair; **[C]** Energy production and conversion; **[E]** Amino acid transport and metabolism; **[F]** Nucleotide transport and metabolism; **[G]** Carbohydrate transport and metabolism; **[H]** Coenzyme transport and metabolism; **[I]** Lipid transport and metabolism; **[P]** Inorganic ion transport and metabolism; **[Q]** Secondary metabolites biosynthesis, transport, and catabolism.

7.2.4. Effect of root exudates in the expression of hydrolytic enzyme genes

The three *Micromonospora* strains responded to the root exudates differently, expressing genes that coded for several plant polysaccharide-degrading enzymes. All these genes are listed in **Table 19**. A total of 14 significant expressed genes were involved in the cellulose and hemicellulose degradation pathway. In detail one, two and six genes, that corresponded to the strains CR30^T, Lupac 09^T and Lupac 08 respectively, encoded for enzymes related to

the production of several endoglucanases and β-glucosidases. In relation to the endoglucanases (EC 3.2.1.4), one and two genes were up-regulated in Lupac 08 and Lupac 09^T respectively (MILUP08_RS10420, GA0070561_RS15115, GA0070561_RS01460) with a fold change >3.5. MILUP08_RS10420 from Lupac 08 was the gene with the highest expression (6.80-fold). The strains CR30^T and Lupac 08, showed significant expression for β-glucosidase (*bglB*, *bglX*), with one and three genes up-regulated respectively (BUS84_RS05405, MILUP08_RS26450, MILUP08_RS23660, MILUP08_RS19950), whose fold change was between 4.2 and 10.2. Strain Lupac 08 had again, the highest expression for this enzyme (MILUP08_RS26450). However, not all genes involved in cellulose degradation were over-expressed. Lupac 08 was the only strain which displayed under-expressed genes for cellulases. This strain showed one down-regulated gene that coded for an endoglucanase and another one for cellulose 1,4-β-cellobiosidase (CBH1) (MILUP08_RS33810 and MILUP08_RS33880 respectively), with a fold change <0.55. Besides the genes involved in the cellulose degradation, several altered genes participated in xylan degradation (**Table 19**). Strain CR30^T had two genes (BUS84_RS10310 and BUS84_RS10315) that encoded for xylan 1,4-β-xylosidase (*xynB*), with a fold change >3. In the case of strains Lupac 08 and Lupac 09^T, they did not show differentially expressed gene related to xylan 1,4-β-xylosidase. However, these strains displayed one over-expressed gene each for the enzyme α-N-arabinofuranosidase (*abf*A) (MILUP08_RS31550 and GA0070561_RS19245), with expression levels of 8.6 and 3.6 respectively. On the other hand, the arabinoxylan arabinofuranohydrolase (*xyn*D) enzyme was significantly over-expressed only in Lupac 09^T (GA0070561_RS27505), with a fold change of 4.9.

In addition to cellulases and xylanases, other enzymes involved in plant-polymer degradation were differentially influenced by the lupin root exudates. This occurred in the case of the enzymes: pectate lyase, glucoamylase and maltose α-D-glucosyltransferase/α-amylase, involved in the pectin or starch degradation pathways. Strains Lupac 08 and Lupac 09^T displayed one over-expressed gene each (MILUP08_RS22255 and GA0070561_RS01190) related to the production of pectate lyase (*pel*), with a fold change >3. Within the amylases family, glucoamylase (SGA1) was only found in strain Lupac 08, with an expression level of 12.83 (MILUP08_RS07430). However, maltose α-D-glucosyltransferase/α-amylase (*treS*) was down-regulated in strain Lupac 09^T, with a fold change of 0.34 (GA0070561_RS25165) (**Table X2**).

Table 19. Differently expressed genes related to plant-polymer degrading enzymes

	Strain	Gene ID	Fold change[a]	KEGG ID	Gene name	Function
Cellulase production						
	Lupac08	MILUP08_RS10420	6.80	K01179	E3.2.1.4	Endoglucanase [EC:3.2.1.4]
	Lupac08	MILUP08_RS33810	0.56			
	Lupac 09^T	GA0070561_RS15115	4.65			
	Lupac 09^T	GA0070561_RS01460	3.69			
	Lupac08	MILUP08_RS26450	10.15	K05350	*bgl*B	β-glucosidase [EC:3.2.1.21]
	Lupac08	MILUP08_RS23660	7.38			
	Lupac08	MILUP08_RS19950	4.22			
	CR30^T	BUS84_RS05405	7.31	K05349	*bgl*X	β-glucosidase [EC:3.2.1.21]
	Lupac08	MILUP08_RS33880	0.59	K01225	CBH1	Cellulose 1,4-β-cellobiosidase [EC:3.2.1.91]
Amylase production						
	Lupac08	MILUP08_RS07430	12.83	K01178	SGA1	Glucoamylase [EC:3.2.1.3]
	Lupac 09^T	GA0070561_RS25165	0.34	K05343	*tre*S	Maltose α-D-glucosyltransferase / α-amylase [EC:5.4.99.163.2.1.1]
Chitinase production						
	Lupac08	MILUP08_RS33605	8.43	K01183	EC:3.2.1.14	Chitinase [EC:3.2.1.14]
	Lupac08	MILUP08_RS10410	6.69			
	Lupac 09^T	GA0070561_RS27280	6.26			
	Lupac 09^T	GA0070561_RS23495	5.50			
	Lupac 09^T	GA0070561_RS28930	5.35			
	CR30^T	BUS84_RS30800	6.19			
	CR30^T	BUS84_RS24385	3.04			
	CR30^T	BUS84_RS21465	0.15			
	Lupac 09^T	GA0070561_RS02005	6.06	K12373	HEXA_B	Hexosaminidase [EC:3.2.1.52]
	Lupac 09^T	GA0070561_RS08665	3.24			
	CR30^T	BUS84_RS35165	0.42			
	CR30^T	BUS84_RS07835	4.08			
	Lupac 09^T	GA0070561_RS07620	10.29	K01233	*csn*	Chitosanase [EC:3.2.1.132]
Pectinase production						
	Lupac08	MILUP08_RS22255	5.29	K01728	*pel*	Pectate lyase [EC:4.2.2.2]
	Lupac 09^T	GA0070561_RS01190	3.28			

a. Fold change: Up-regulated (red) and down-regulated genes (green)

Other groups of hydrolytic enzymes that were differentially expressed in response to the root exudates were chitinases (EC:3.2.1.14), hexosaminidase (HEXA_B) and chitosanase (*csn*) involved in different steps of the chitin degradation pathway (**Table 19**). Eight genes related to the production of chitinase (EC:3.2.1.14) were expressed in the transcriptome of the three *Micromonospora* strains. A total of seven genes were over-expressed (MILUP08_RS33605, MILUP08_RS10410, GA0070561_RS27280, GA0070561_RS23495, GA0070561_RS28930, BUS84_RS30800, and BUS84_RS24385), constituting of two, three and two genes from Lupac 08, Lupac 09^T and CR30^T respectively. These over-expressed genes related to chitinase production (EC:3.2.1.14) had a fold change between 3.04 and 8.43, where the gene with the highest over-expression was MILUP08_RS33605 from Lupac 08. Only the strain CR30^T showed under-expression for this enzyme (BUS84_RS21465), with a fold change of 0.15. The expression of hexosaminidase (HEXA_B) was significantly detected in the strains Lupac 09^T and CR30^T; strain Lupac 09^T showed two over-expressed

loci (GA0070561_RS02005 and GA0070561_RS08665), whereas CR30^T over-expressed one gene (BUS84_RS07835) and under-expressed another (BUS84_RS35165). The over-expressed genes had a fold change >3.20, but the fold change of the under-expressed gene was 0.42. Finally, chitosanase (*csn*) was only up-regulated in strain Lupac 09^T (GA0070561_RS07620), with an expression level >10 (**Table 19**).

7.2.5 Differentially expressed genes associated with plant growth promotion

In addition to genes related to the hydrolytic enzyme production, root exudates also had an effect on the regulation of genes that coded for various characteristics involved in the growth and development of the plant. All these genes are listed in **Table 20**. Among genes related to plant growth promotion (PGP), the pathway of production and degradation of trehalose, was highlighted. In this pathway three genes were over-expressed for two enzymes: α-trehalase (*treA*) in the strains Lupac 08 (MILUP08_RS10885), and CR30^T (BUS84_RS05160), and maltooligosyltrehalose trehalohydrolase (*treZ*) in strain Lupac 08 (MILUP08_RS12600). These genes displayed a very high expression, with a fold change of approximately 9.8 for α-trehalase and 14.4 in the case of maltooligosyltrehalose trehalohydrolase. However, not all genes related to the synthesis and degradation of trehalose were up-regulated. Several genes were under-expressed, and these included GA0070561_RS25165 (Lupac 09^T) that coded for maltose α-D-glucosyltransferase (*treS),* and BUS84_RS17285 (CR30^T) that coded for trehalose 6-phosphate synthase (*otsA*). The expression levels of these genes were <0.3. In addition to the trehalose-related pathway, genes involved in the acetoin and 2,3-butanediol synthesis pathway were also differentially expressed in the transcriptome of strains Lupac 09^T and CR30^T. They showed an under-expression of 0.3-fold in genes encoded for several enzymes involved in different biosynthesis steps, that included (R,R)-butanediol dehydrogenase, meso-butanediol dehydrogenase, diacetyl reductase and acetolactate synthase I/II/III large subunit.

In the case of ABC transporters, out of the 49 significantly altered genes, 26 were involved in iron uptake. Despite the high number of genes for these types of transport proteins, Lupac 08 was the only strain which showed over-expressed genes involved in the transport of this metal. The remaining strains (Lupac 09^T and CR30^T) showed under-expressed genes for these transporters. All genes are listed in the **Table 20**. In detail, strain Lupac 08 showed six up-regulated genes coding for iron complex transport system (ABC.FEV.A, ABC.FEV.P and

ABC.FEV.S), and one for iron (III) transport system (*afuC*, *fbpC*). Overall expression levels were approximately 2.4 and 13.8 respectively. However, this strain also showed one down-regulated gene for each cited iron transporter. In the case of Lupac 09^T, it displayed a total of ten down-regulated genes for the iron complex transport system (ABC.FEV.A, ABC.FEV.P and ABC.FEV.S), and one for iron (III) transport system (*afuC, fbpC*). Similar to Lupac 09^T, strain $CR30^T$ also displayed several under-expressed genes related to the iron complex transport system (ABC.FEV.A, ABC.FEV.P and ABC.FEV.S), and iron (III) transport system (*afuC, fbpC*). The expression levels were low for these iron ABC transporters expressed in all strains, with a fold change <0.6. In addition to the iron transporters, molybdate ABC transporters (*modABC*) were also differentially expressed (**Table 21)**. The up-regulation of genes that coded for the ModA molybdate transport system substrate-binding protein was only observed in strain Lupac 08 (MILUP08_RS20015), with an expression level of 5.3. *mod*C genes were over-expressed in isolates Lupac 08 and $CR30^T$ (MILUP08_RS20025 and BUS84_RS11620) with a fold change >2.8. In contrast, three genes that coded for ModA and ModB transport proteins were down-regulated in strains Lupac 09^T and $CR30^T$, with a low expression of 0.3-fold. Other membrane transport proteins worth mentioning among plant growth promoting bacteria features were those involved in the phosphate transport system, which comprised four transport proteins: PstA, PstB, PstC and PstS (**Table 20**). Strain Lupac 08 showed one over-expressed gene for each PstA, PstC and PstS transport proteins (MILUP08_RS32060, MILUP08_RS32055 and MILUP08_RS32050), with a fold change between 1.8 and 3.8. On the contrary, strain $CR30^T$ displayed a very low expression (~0.4-fold) in all phosphate transport proteins except in PstA, which was not found among the differentially expressed genes (BUS84_RS20880, BUS84_RS20870 and BUS84_RS20865). Strain Lupac 09^T did not show any genes related to these phosphate transport proteins. Furthermore, another five genes encoding for other phosphate transporters (*pho*B, *reg*X3, *sen*X3) were differentially expressed; one gene was over-expressed in strains $CR30^T$ and Lupac 09^T (BUS84_RS10890 and GA0070561_RS27350) and three genes were only under-expressed in strain $CR30^T$ (BUS84_RS15705, BUS84_RS20595 and BUS84_RS20600).

Table 20. Differently expressed genes involved in plant-growth promotion activities

	Strain	Gene ID	Fold change[a]	KEGG ID	Gene name	Function
Production and degradation of trehalose						
	Lupac08	MILUP08_RS10885	9.72	K01194	TREH, *treA*, *treF*	Alpha,α-trehalase [EC:3.2.1.28]
	CR30^T	BUS84_RS05160	9.93			
	Lupac08	MILUP08_RS12600	14.43	K01236	*treZ*, *glgZ*	Maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]
	Lupac 09^T	GA0070561_RS25165	0.34	K05343	*treS*	Maltose α-D-glucosyltransferase / α-amylase [EC:5.4.99.163.2.1.1]
	CR30^T	BUS84_RS17285	0.13	K00697	*otsA*	Trehalose 6-phosphate synthase [EC:2.4.1.15 2.4.1.347]
Acetoin/Butanodiol biosynthesis						
	Lupac 09^T	GA0070561_RS30445	0.44	K00004	BDH, *butB*	(R,R)-butanediol dehydrogenase / meso-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.4 1.1.1.- 1.1.1.303]
	Lupac 09^T	GA0070561_RS30935	0.24	K00100	*bdhAB*	Butanol dehydrogenase [EC:1.1.1.-]
	Lupac 09^T	GA0070561_RS00415	0.37	K01652	*ilvB*, *ilvG*, *ilvI*	Acetolactate synthase I/II/III large subunit [EC:2.2.1.6]
	CR30^T	BUS84_RS30085	0.29			
	Lupac 09^T	GA0070561_RS00410	0.31	K01653	*ilvH*, *ilvN*	Acetolactate synthase I/III small subunit [EC:2.2.1.6]
	CR30^T	BUS84_RS30090	0.24			
Siderophore production						
	Lupac08	MILUP08_RS20345	2.89	K13745	*ddc*	L-2,4-diaminobutyrate decarboxylase [EC:4.1.1.86]
Iron uptake						
	Lupac08	fecE	2.56	K02013	ABC.FEV.A	Iron complex transport system ATP-binding protein [EC:3.6.3.34]
	Lupac08	MILUP08_RS20350	1.56			
	Lupac08	MILUP08_RS12910	0.58			
	Lupac 09^T	fecE	0.06			
	Lupac 09^T	GA0070561_RS10100	0.07			
	Lupac 09^T	GA0070561_RS27425	0.09			
	Lupac 09^T	GA0070561_RS31255	0.17			
	CR30^T	fecE	0.29			
	Lupac08	MILUP08_RS20360	2.89	K02015	ABC.FEV.P	Iron complex transport system permease protein
	Lupac08	MILUP08_RS20365	2.22			
	Lupac 09^T	GA0070561_RS10110	0.15			
	Lupac 09^T	GA0070561_RS10115	0.15			
	Lupac 09^T	GA0070561_RS16645	0.47			
	Lupac 09^T	GA0070561_RS16650	0.30			
	CR30^T	BUS84_RS04725	0.46			
	CR30^T	BUS84_RS09750	0.48			
	Lupac08	MILUP08_RS12115	2.25	K02016	ABC.FEV.S	Iron complex transport system substrate-binding protein
	Lupac08	MILUP08_RS20355	3.12			
	Lupac 09^T	GA0070561_RS16660	0.15			
	Lupac 09^T	GA0070561_RS10105	0.10			
	Lupac 09^T	GA0070561_RS27415	0.15			
	Lupac08	MILUP08_RS29775	13.76	K02010	*afuC*, *fbpC*	Iron(III) transport system ATP-binding protein [EC:3.6.3.30]
	Lupac08	MILUP08_RS11490	0.29			
	Lupac 09^T	GA0070561_RS16115	0.03			
	CR30^T	BUS84_RS18745	0.01			
	CR30^T	BUS84_RS33395	0.42			
Phosphate uptake						
	Lupac08	MILUP08_RS32060	3.80	K02038	*pstA*	Phosphate transport system permease protein
	CR30^T	BUS84_RS20880	0.10	K02036	*pstB*	Phosphate transport system ATP-binding protein [EC:7.3.2.1]
	Lupac08	MILUP08_RS32055	1.78	K02037	*pstC*	Phosphate transport system permease protein
	CR30^T	BUS84_RS20870	0.23			
	Lupac08	MILUP08_RS32050	1.96	K02040	*pstS*	Phosphate transport system substrate-binding protein
	CR30^T	BUS84_RS20865	0.04			
	CR30^T	BUS84_RS10890	1.99	K01113	*phoD*	Alkaline phosphatase D [EC:3.1.3.1]
	CR30^T	BUS84_RS15705	0.64			
	CR30^T	BUS84_RS20595	0.23	K07776	*regX3*	Two-component system, OmpR family, response regulator RegX3
	Lupac 09^T	GA0070561_RS27350	1.62	K07768	*senX3*	Two-component system, OmpR family, sensor histidine kinase SenX3 [EC:2.7.13.3]
	CR30^T	BUS84_RS20600	0.19			

a. Fold change: Up-regulated (red) and down-regulated genes (green)

7.2.6 Transcriptional responses to root exudates involved in the communication between bacteria and plants

Different genes related to carbohydrate transport were significantly expressed by effect of the compounds exuded by the *Lupinus* plants (**Table 21**). These genes were involved in the transport of cellobiose (*ceb*EF), N,N'-diacetylchitobiose (*das*C), arabinogalactan oligomer/maltooligosaccharide (*gan*Q), maltose/maltodextrin (*mal*E), ribose (*rbs*ABC), raffinose/stachyose/melibiose (*msm*EG) and D-xylose (*xyl*F). All genes that coded for transport of cellobiose (MILUP08_RS31865 and MILUP08_RS31870), N,N'-diacetylchitobiose (MILUP08_RS10890), arabinogalactan oligomer/maltooligosaccharide (BUS84_RS11015), raffinose/stachyose/melibiose (GA0070561_RS27830, MILUP08_RS31875 and MILUP08_RS23865) and maltose/maltodextrin (GA0070561_RS12420) were up-regulated, showing a fold change >7.0. Strain Lupac 08 had the largest number of genes involved in sugar transport (**Table 21**). However, not all genes related to these carbohydrate transports were up-regulated in the Lupac 09^T and CR30^T transcriptome. These strains showed one down-regulated gene each related to D-xylose transport system (*xyl*F) (GA0070561_RS18115 and BUS84_RS19380). In the case of ribose (*rbs*ABC) transport proteins, five genes were up-regulated (GA0070561_RS15420, GA0070561_RS12045, GA0070561_RS15425, BUS84_RS11280 and BUS84_RS08720) and four were down-regulated (GA0070561_RS18120, MILUP08_RS27195, BUS84_RS16255 and BUS84_RS02840). The type strains Lupac 09^T and CR30^T showed several genes both over-expressed and under-expressed, while Lupac 08 only showed one under-expressed gene.

With respect to the *quorum* sensing systems, several genes involved in different steps of these systems were found. Different transport systems were significantly expressed: transport system proteins of peptides/nickel, oligopeptides and branched-chain amino acids (**Table 21**). A total of twelve genes that coded for peptide/nickel transport system proteins (ABC.PE.APS) were significantly expressed in the three *Micromonospora* strains. All these genes were over-expressed with a fold change between 3.7 and 12.4. The strain with the highest number of genes involved in peptide/nickel transport was CR30^T with five up-regulated genes. In the case of branched-chain amino acid transport system proteins (*liv*FGKM), the strains Lupac 09^T and CR30^T displayed two over-expressed genes for each one, while Lupac 08 only one over-expressed gene. Besides over-expressed genes, one and four genes were under-expressed in the Lupac 08 and CR30^T transcriptome respectively. The

fold change was >2.4 for the up-regulated genes, and <0.6 for the down-regulated loci. Of the three transport systems highlighted on the *quorum*-sensing systems, oligopeptide transport system (*opp*A, *opp*B, *opp*F) was present in only three expressed genes, one from CR30^T and two from Lupac 08. These genes were up-regulated with a fold change between 2.0 and 16.4.

Table 21. Differently expressed genes related to plant-bacteria communication

	Strain	Gene ID	Fold change[a]	KEGG ID	Gene name	Function
ABC transporters						
	Lupac08	MILUP08_RS20015	5.29	K02020	*mod*A	Molybdate transport system substrate-binding protein
	Lupac 09^T	modA	0.14			
	CR30^T	BUS84_RS09560	0.09			
	Lupac 09^T	GA0070561_RS09870	0.25	K02018	*mod*B	Molybdate transport system permease protein
	Lupac08	MILUP08_RS20025	2.85	K02017	*mod*C	Molybdate transport system ATP-binding protein [EC:3.6.3.29]
	CR30^T	BUS84_RS11620	3.25			
	CR30^T	BUS84_RS27935	6.01	K05816	*ugp*C	Sn-glycerol 3-phosphate transport system ATP-binding protein [EC:7.6.2.10]
	Lupac08	MILUP08_RS23020	0.03			
	Lupac08	MILUP08_RS31865	7.15	K10240	*ceb*E	Cellobiose transport system substrate-binding protein
	Lupac08	MILUP08_RS31870	8.42	K10241	*ceb*F	Cellobiose transport system permease protein
	Lupac08	MILUP08_RS10890	8.49	K17331	*das*C	N,N'-diacetylchitobiose transport system permease protein
	CR30^T	BUS84_RS11015	7.99	K15772	*gan*Q	Arabinogalactan oligomer / maltooligosaccharide transport system permease protein
	Lupac 09^T	GA0070561_RS12420	9.62	K10108	*mal*E	Maltose/maltodextrin transport system substrate-binding protein
	Lupac 09^T	GA0070561_RS27830	10.04	K10117	*msm*E	Raffinose/stachyose/melibiose transport system substrate-binding protein
	Lupac08	MILUP08_RS31875	8.18	K10119	*msm*G	Raffinose/stachyose/melibiose transport system permease protein
	Lupac08	MILUP08_RS23865	4.97			
	Lupac08	MILUP08_RS10650	0.34	K10112	*msm*X, *msm*K, *mal*K, *sug*C, *ggt*A, *msi*K	Multiple sugar transport system ATP-binding protein
	Lupac 09^T	GA0070561_RS15420	2.69	K10441	*rbs*A	Ribose transport system ATP-binding protein [EC:3.6.3.17]
	Lupac 09^T	GA0070561_RS18120	0.47			
	Lupac 09^T	GA0070561_RS12045	4.32	K10439	*rbs*B	Ribose transport system substrate-binding protein
	Lupac08	MILUP08_RS27195	0.17			
	CR30^T	BUS84_RS16255	0.26			
	CR30^T	BUS84_RS02840	0.24			
	Lupac 09^T	GA0070561_RS15425	2.07	K10440	*rbs*C	Ribose transport system permease protein
	CR30^T	BUS84_RS11280	11.38			
	CR30^T	BUS84_RS08720	2.36			
	Lupac 09^T	GA0070561_RS18115	0.48	K10543	*xyl*F	D-xylose transport system substrate-binding protein
	CR30^T	BUS84_RS19380	0.32			
Quorum sensing pathway						
	Lupac08	MILUP08_RS15065	12.37	K02031	ABC.PE.A	Peptide/nickel transport system ATP-binding protein
	CR30^T	BUS84_RS02770	6.72			
	CR30^T	BUS84_RS10325	5.94			
	CR30^T	BUS84_RS02785	3.70	K02032	ABC.PE.A1	Peptide/nickel transport system ATP-binding protein
	Lupac08	MILUP08_RS15055	9.08	K02033	ABC.PE.P	peptide/nickel transport system permease protein
	CR30^T	BUS84_RS02080	8.02			
	CR30^T	BUS84_RS02780	5.89			
	Lupac08	MILUP08_RS30245	7.84	K02034	ABC.PE.P1	Peptide/nickel transport system permease protein
	Lupac08	MILUP08_RS15060	4.56			
	Lupac 09^T	GA0070561_RS20985	2.23			
	Lupac 09^T	GA0070561_RS11270	6.15	K02035	ABC.PE.S	Peptide/nickel transport system substrate-binding protein
	Lupac 09^T	GA0070561_RS20995	4.21			
	Lupac 09^T	GA0070561_RS00495	1.98	K14051	*gmr*	C-di-GMP phosphodiesterase Gmr [EC:3.1.4.52] cyclic di-3',5'-guanylate + H2O = 5'-phosphoguanylyl(3'->5')guanosine
	Lupac 09^T	GA0070561_RS10760	7.38	K01218	*gmu*G	Mannan endo-1,4-β-mannosidase [EC:3.2.1.78]
	CR30^T	BUS84_RS10450	14.48	K01996	*liv*F	Branched-chain amino acid transport system ATP-binding protein
	Lupac08	MILUP08_RS31345	0.59			
	CR30^T	BUS84_RS10455	4.47	K01995	*liv*G	Branched-chain amino acid transport system ATP-binding protein
	Lupac08	MILUP08_RS31350	0.21			
	Lupac 09^T	GA0070561_RS10875	3.93	K01999	*liv*K	Branched-chain amino acid transport system substrate-binding protein
	Lupac08	MILUP08_RS31355	0.64			
	Lupac08	MILUP08_RS24735	0.14			
	CR30^T	BUS84_RS14240	0.10			
	Lupac08	MILUP08_RS32395	11.98			
	Lupac 09^T	GA0070561_RS19075	2.44	K01998	*liv*M	Branched-chain amino acid transport system permease protein
	Lupac08	MILUP08_RS30235	16.37	K15580	*opp*A, *mpp*A	Oligopeptide transport system substrate-binding protein
	Lupac08	MILUP08_RS30240	8.63	K15581	*opp*B	Oligopeptide transport system permease protein
	CR30^T	BUS84_RS10720	2.02	K10823	*opp*F	Oligopeptide transport system ATP-binding protein
	Lupac08	MILUP08_RS22255	5.29	K01728	*pel*	Pectate lyase [EC:4.2.2.2]
	Lupac 09^T	GA0070561_RS01190	3.28			

a. Fold change: Up-regulated (red) and down-regulated genes (green)

7.2.7 The influence of exudates on translation, ribosomal structure and biogenesis

The three *Micromonospora* strains showed a large number of down-regulated genes in response to the root exudates involved in translation, ribosomal structure and biogenesis (**Figure 35**). When these genes were mapped in the KEGG database, all were related with the ribosomal translation path. There were 22, 35 and 36 repressed genes in the strains Lupac 08, Lupac 09^T and CR30^T respectively involved in this pathway. These genes were annotated as large (L2-36) and small (S2-19) ribosomal subunit proteins, with fold changes that were ≤0.2 (**Table 22**).

Table 22. Differently expressed genes associated with ribosomal processes

Ribosome

Strain	Gene ID	Fold change[a]	KEGG ID	Gene name	Function
Lupac08	MILUP08_RS30930	0.21	K02886	RP-L2, MRPL2, *rpl*B	Large subunit ribosomal protein L2
Lupac 09^T	GA0070561_RS18585	0.24			
Lupac08	MILUP08_RS30945	0.21	K02906	RP-L3, MRPL3, *rpl*C	Large subunit ribosomal protein L3
Lupac 09^T	GA0070561_RS18600	0.27			
CR30^T	BUS84_RS19820	0.08			
Lupac08	MILUP08_RS30940	0.24	K02926	RP-L4, MRPL4, *rpl*D	Large subunit ribosomal protein L4
Lupac 09^T	GA0070561_RS18595	0.27			
CR30^T	BUS84_RS19815	0.12			
Lupac08	MILUP08_RS30885	0.22	K02931	RP-L5, MRPL5, *rpl*E	Large subunit ribosomal protein L5
Lupac 09^T	GA0070561_RS18540	0.16			
Lupac08	MILUP08_RS30870	0.24	K02933	RP-L6, MRPL6, *rpl*F	Large subunit ribosomal protein L6
Lupac 09^T	GA0070561_RS18525	0.15			
CR30^T	BUS84_RS19745	0.10			
Lupac08	MILUP08_RS31000	0.30	K02935	RP-L7, MRPL12, *rpl*L	Large subunit ribosomal protein L7/L12
Lupac 09^T	GA0070561_RS18650	0.11			
CR30^T	BUS84_RS19870	0.13			
CR30^T	BUS84_RS23115	0.12	K02939	RP-L9, MRPL9, *rpl*I	Large subunit ribosomal protein L9
Lupac08	MILUP08_RS31005	0.32	K02864	RP-L10, MRPL10, *rplJ*	Large subunit ribosomal protein L10
Lupac 09^T	GA0070561_RS18655	0.13			
CR30^T	BUS84_RS19875	0.08			
CR30^T	BUS84_RS19895	0.13	K02867	RP-L11, MRPL11, *rpl*K	Large subunit ribosomal protein L11
Lupac08	MILUP08_RS30620	0.19	K02871	RP-L13, MRPL13, *rpl*M	Large subunit ribosomal protein L13
CR30^T	BUS84_RS19510	0.05			
Lupac 09^T	GA0070561_RS18550	0.15	K02874	RP-L14, MRPL14, *rpl*N	Large subunit ribosomal protein L14
Lupac 09^T	GA0070561_RS18505	0.17	K02876	RP-L15, MRPL15, *rpl*O	Large subunit ribosomal protein L15
Lupac 09^T	GA0070561_RS18565	0.21	K02878	RP-L16, MRPL16, *rpl*P	Large subunit ribosomal protein L16
Lupac 09^T	GA0070561_RS18450	0.23	K02879	RP-L17, MRPL17, *rpl*Q	Large subunit ribosomal protein L17
CR30^T	BUS84_RS19670	0.09			
Lupac08	MILUP08_RS30865	0.28	K02881	RP-L18, MRPL18, *rpl*R	Large subunit ribosomal protein L18
Lupac 09^T	GA0070561_RS18520	0.12			
CR30^T	BUS84_RS19740	0.13			
CR30^T	BUS84_RS30585	0.12	K02884	RP-L19, MRPL19, *rpl*S	Large subunit ribosomal protein L19
CR30^T	BUS84_RS16920	0.08	K02888	RP-L21, MRPL21, *rpl*U	Large subunit ribosomal protein L21
Lupac09	GA0070561_RS18575	0.21	K02890	RP-L22, MRPL22, *rpl*V	Large subunit ribosomal protein L22
Lupac08	MILUP08_RS30935	0.23	K02892	RP-L23, MRPL23, *rpl*W	Large subunit ribosomal protein L23
Lupac 09^T	GA0070561_RS18590	0.23			
Lupac 09^T	GA0070561_RS18545	0.15	K02895	RP-L24, MRPL24, *rpl*X	Large subunit ribosomal protein L24
CR30^T	BUS84_RS26885	0.10	K02897	RP-L25, *rpl*Y	Large subunit ribosomal protein L25
Lupac08	MILUP08_RS27790	0.11	K02899	RP-L27, MRPL27, *rpm*A	Large subunit ribosomal protein L27
CR30^T	BUS84_RS16915	0.06			

	Lupac 09^{T}	rpmB	0.41	K02902	RP-L28, MRPL28, *rpm*B	Large subunit ribosomal protein L28
	CR30^{T}	BUS84_RS30365	0.05			
	Lupac 09^{T}	GA0070561_RS18560	0.19	K02904	RP-L29, *rpm*C	Large subunit ribosomal protein L29
	Lupac08	MILUP08_RS30855	0.19	K02907	RP-L30, MRPL30, *rpm*D	Large subunit ribosomal protein L30
	Lupac 09^{T}	GA0070561_RS18510	0.14			
	CR30^{T}	BUS84_RS19730	0.14			
	CR30^{T}	BUS84_RS18050	0.05	K02909	RP-L31, *rpm*E	Large subunit ribosomal protein L31
	CR30^{T}	BUS84_RS19925	0.11	K02913	RP-L33, MRPL33, *rpm*G	Large subunit ribosomal protein L33
	CR30^{T}	BUS84_RS23285	0.23	K02914	RP-L34, MRPL34, rpmH	Large subunit ribosomal protein L34
	CR30^{T}	BUS84_RS28245	0.07	K02919	RP-L36, MRPL36, *rpm*J	Large subunit ribosomal protein L36
	CR30^{T}	BUS84_RS19695	0.13			
	Lupac08	MILUP08_RS08350	0.31	K02967	RP-S2, MRPS2, *rps*B	Small subunit ribosomal protein S2
	Lupac 09^{T}	rpsB	0.15			
	CR30^{T}	BUS84_RS30730	0.08			
	Lupac08	MILUP08_RS30915	0.32	K02982	RP-S3, *rps*C	Small subunit ribosomal protein S3
	Lupac 09^{T}	GA0070561_RS18570	0.19			
	Lupac 09^{T}	GA0070561_RS18460	0.20	K02986	RP-S4, *rps*D	Small subunit ribosomal protein S4
	CR30^{T}	BUS84_RS19680	0.09			
	Lupac08	MILUP08_RS30860	0.31	K02988	RP-S5, MRPS5, *rps*E	Small subunit ribosomal protein S5
	Lupac 09^{T}	GA0070561_RS18515	0.14			
	CR30^{T}	BUS84_RS19735	0.12			
	Lupac08	MILUP08_RS00930	0.18	K02990	RP-S6, MRPS6, *rps*F	Small subunit ribosomal protein S6
	Lupac 09^{T}	GA0070561_RS07100	0.24			
	CR30^{T}	BUS84_RS23130	0.09			
	Lupac 09^{T}	GA0070561_RS18620	0.33	K02992	RP-S7, MRPS7, *rps*G	Small subunit ribosomal protein S7
	CR30^{T}	BUS84_RS19840	0.09			
	Lupac08	MILUP08_RS30615	0.24	K02996	RP-S8, *rps*H	Small subunit ribosomal protein S8
	Lupac08	MILUP08_RS30875	0.19	K02994	RP-S8, *rps*H	Small subunit ribosomal protein S8
	Lupac 09^{T}	rpsH	0.14			
	CR30^{T}	BUS84_RS19750	0.08			
	Lupac 09^{T}	GA0070561_RS18245	0.48	K02996	RP-S9, MRPS9, *rps*I	Small subunit ribosomal protein S9
	CR30^{T}	BUS84_RS19505	0.06			
	Lupac 09^{T}	GA0070561_RS18605	0.27	K02946	RP-S10, MRPS10, *rps*J	Small subunit ribosomal protein S10
	CR30^{T}	BUS84_RS19825	0.08			
	Lupac08	MILUP08_RS30815	0.29	K02948	RP-S11, MRPS11, *rps*K	Small subunit ribosomal protein S11
	Lupac 09^{T}	GA0070561_RS18465	0.17			
	CR30^{T}	BUS84_RS19685	0.07			
	Lupac 09^{T}	GA0070561_RS18625	0.31	K02950	RP-S12, MRPS12, *rps*L	Small subunit ribosomal protein S12
	CR30^{T}	BUS84_RS19845	0.09			
	Lupac08	MILUP08_RS30820	0.29	K02952	RP-S13, *rps*M	Small subunit ribosomal protein S13
	Lupac 09^{T}	GA0070561_RS18470	0.29			
	CR30^{T}	BUS84_RS19690	0.11			
	Lupac08	rpsN	0.24	K02954	RP-S14, MRPS14, *rps*N	Small subunit ribosomal protein S14
	Lupac 09^{T}	GA0070561_RS18535	0.18			
	Lupac 09^{T}	GA0070561_RS13500	0.40	K02956	RP-S15, MRPS15, *rps*O	Small subunit ribosomal protein S15
	CR30^{T}	BUS84_RS31050	0.15			
	Lupac08	MILUP08_RS08250	0.17	K02959	RP-S16, MRPS16, *rps*P	Small subunit ribosomal protein S16
	Lupac 09^{T}	GA0070561_RS13070	0.18			
	CR30^{T}	BUS84_RS30565	0.11			
	Lupac 09^{T}	GA0070561_RS18555	0.20	K02961	RP-S17, MRPS17, *rps*Q	Small subunit ribosomal protein S17
	CR30^{T}	BUS84_RS23120	0.08	K02963	RP-S18, MRPS18, *rps*R	Small subunit ribosomal protein S18
	Lupac 09^{T}	GA0070561_RS18580	0.23	K02965	RP-S19, *rps*S	Small subunit ribosomal protein S19
	CR30^{T}	BUS84_RS16630	0.05	K02968	RP-S20, *rps*T	Small subunit ribosomal protein S20

a. Fold change: Up-regulated (**red**) and down-regulated genes (**green**)

7.2.8 Evaluation of *Micromonospora* gene expression after direct exposure to Lupin root exudates by RT-qPCR

The six genes selected per strain (related to endoglucanase, chitinase, glucokinases, alpha, α-trehalase, transporter, β-glucanase, xylose isomerase, pectinase and xylan 1,4-β-xylosidase) used in the validation of the transcriptome data were also used to measure the change in gene expression after exposing *Micromonospora* directly to *Lupinus* root exudates (different conditions from those used in the transcriptome analysis). The RT-qPCR data showed that the direct *Micromonospora*-exudates contact exerted a higher up-regulation in comparison with the transcriptome data (**Figure X3, Appendix IV**). Almost all evaluated genes displayed a significant increase in their expression; even the gene GA0070561_RS18130 (glucokinase) from Lupac 09^T, which was down-regulated in the transcriptome, its expression increased 24.29-fold. The increased expression ranged from 1.5-fold to 68.8-fold, where BUS84_RS24385 (chitinase), GA0070561_RS01190 (pectate lyase) and MILUP08_RS10420 (endoglucanase) were the most up-regulated genes in CR30^T, Lupac 09^T and Lupac08 respectively. The least up-regulated genes were BUS84_RS10315 (xylan 1,4-β-xylosidase) and BUS84_RS04400 (glucokinase) from CR30^T, and GA0070561_RS24610 (xylan 1,4-β-xylosidase) from Lupac 09^T. The only gene where expression decreased was BUS84_RS05160 (α-trehalase) from CR30^T. This decrease in expression was 2.6-fold less than in the transcriptome data. In the case of cellulases (endoglucanase and β-glucosidase) and chitinases, the expression increased between 3.7-fold and 35.1-fold in cellulases, and 15.7-fold and 68.8-fold in chitinases with respect to transcriptome data.

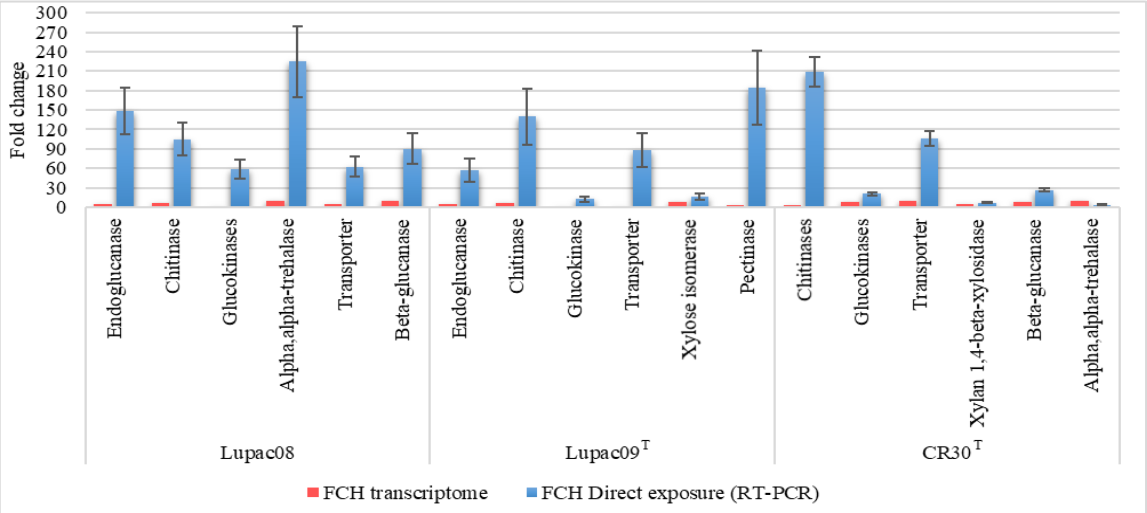


Figure 36. Differential expression by exposing *Micromonospora* to root exudates by two different methods. **Red**: Growth of *Micromonospora* in culture medium supplemented with 0.25 mg/ml of exudates. **Blue**: Direct exposure of *Micromonspora* to exudates released by the plant to the hydroponic media.

7.3. Discussion

7.3.1 Transcriptional responses of *Micromonospora* to *Lupinus* root exudates

The transcriptomic profiling showed a significant influence of root exudates on the gene expression of the three *Micromonospora* study strains (*M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^T and *M. cremea* CR30^T). More than 9.7% of the total transcriptome was significantly altered in response to lupin root exudates. A proportion of the genes with modified expression (56%) coded for proteins with known function. These well-annotated genes were mostly involved in metabolic processes, especially carbohydrate transport and metabolism, amino acid transport and metabolism, and energy production and conversion. This can be explained because the plant root exudates are commonly composed of a complex mixture of different compounds that include monosaccharides, amino acids, and organic acids and these compounds can be used as carbon and energy sources by the rhizospheric bacteria (Egle et al., 2003; Chaparro el al., 2013; Vranova et al., 2013; Valentinuzzi et al., 2015). Similar results have been obtained in other plant-bacteria interactions where the bacterial transcriptomes were also analyzed in response to the influence by the host root exudates (Mark et al., 2005; Shidore et al., 2012; Fan et al., 2012; Zhang et al., 2015; Xie et al., 2015; Yi et al., 2017; Klonowska et al., 2018). The responsiveness of the gene expression due to the exudates released by the plant is an adaptation strategy to recognize and/or respond to plant-signals, often improving the nutrient acquisition, the colonization of the plant tissues and the optimal establishment in the niche (Yi et al., 2017).

7.3.2. The role of hydrolytic enzymes in the plant-*Micromonospora* interaction

Plant cell walls are mainly composed of cellulose and hemicellulose, in addition to other less abundant polymers such as pectin and starch. The complex composition of plant tissues makes them very difficult to hydrolyze enzymatically by the microorganisms. It is for this reason that a complex combination of enzymes is necessary for their degradation (Yarbrough et al., 2009; Medie et al., 2012). The transcriptome profiles showed that the strains Lupac 08, Lupac 09^T and CR30^T had several significantly expressed genes especially related to the production of cellulases and chitinases, but also xylanases, amylases and pectinases. All *Micromonospora* strains displayed several up-regulated genes that coded for different cellulases, including *M. cremea* CR30^T. This strain did not show cellulolytic activity in *in vitro* tests without live plant roots, while the two remaining strains produced this enzyme

with and without plant roots (section 6.2.11, chapter 3). Strain Lupac 08 showed the highest number of differentially expressed genes related to cellulases, and it displayed greater expression levels for endoglucanases and β-glucosidase. The over-expression of genes involved in cellulose degradation was also observed in the following cases: endophytic strain *Bacillus amyloliquefaciens* SQR9 grown in the presence of maize root exudates (Zhang et al., 2015), nitrogen-fixing bacterium *Bulkholderia phymatum* STM815 in response to *Mimosa pudica* root exudates (Klonowska et al., 2018) and non-pathogenic endophytic strain *Azoarcus* sp. BH72 in contact with rice seedlings (Reinhold-Hurek et al., 2006). This may suggest that cellulases are involved in colonization and penetration into plants by being activated in response to root exudates (Compant et al., 2005; Reinhold-Hurek et al., 2006; Robledo et al., 2008). Several endophytes are able to cross the endodermis by the secretion of different hydrolytic enzymes such as cellulases, without causing damage or visible symptoms to the host plant (James et al., 2002). The *Micromonospora* strains may be expressing genes that code for cellulases, since the interaction with the root exudates precedes the plant tissue colonization and the establishment of the plant-bacteria relationship. On the other hand, several genomic analyses have also shown that many bacterial genes encode for a cellulase, which plays a part in cellulose biosynthesis. This reveals that cellulases are not restricted to cellulose degradation (Medie et al., 2012), as originally described for the bacterium *Gluconacetobacter xylinus* (Wong et al., 1990). The cellulose biosynthesis can be involved in the adhesion step and the root surface colonization (Robertson et al., 1988; Laus, 2005).

In addition to genes coding for cellulases, several genes related to the production of xylan 1,4-β-xylosidases, α-N-arabinofuranosidases and pectate lyases were also over-expressed in presence of the *Lupinus* root exudates. These enzymes have been detected along with cellulases in the initial steps of the symbiotic plant-bacteria interaction, allowing endophytic bacteria to colonize internal plant tissues (Compant et al., 2005). In the particular case of pectate lyases, it has been previously described that it is involved in the adhesion, invasion and colonization of the interspatial region between the plant root cells (Taghavi et al., 2010). Some symbiotic bacteria produce pectate lyases in the initial steps of symbiosis with the plant, since pectin degradation products may act as signals affecting the plant-bacteria interactions (Hugouvieux-Cotte-Pattat et al., 2014). This enzyme has been detected during the establishment of symbiosis between rhizobia with its host, acting as a signal molecule in the rhizobia-legume communication (Xie et al., 2012; Hugouvieux-Cotte-Pattat et al., 2014).

Over-expression of genes that code for pectate lyase by *Micromonospora* in contact with lupin root exudates may also be related to bacterial-plant communication, since this over-expression may be due to molecular signals present in root exudates. In addition, the production of pectate lyase by *Micromonospora* may be involved in the colonization of plant tissues, acting together with other enzymes (cellulases or xylanases) to create a path of entry to internal root tissues.

Chitin is one of the most abundant polymers in the biosphere, the second after cellulose. This biopolymer is found in many organisms, especially in insects, fungi and crustaceans (Daulagala and Allan-Atkins 2015; Cohen-Kupiec and Chet 1998). Several over-expressed genes related to chitin degradation were found in the transcriptomic profiles of the three *Micromonospora* strains, especially in strain Lupac 09^T, followed by $CR30^T$. Chitinase production has been observed particularly in microorganisms that have the potential to act as biocontrol against phytopathogenic fungi (Swiontek Brzezinska et al., 2014; Veliz et al., 2017). The synergistic action of chitinases together with other hydrolytic enzymes such as β-glucanases and amylases has shown greater efficiency in protection against fungal cells (Mauch et al., 1988; Mhlongo et al., 2018). However, chitinases may also regulate several processes related to growth and development of the healthy plants, in addition to participating in the establishment of the symbiosis between endophytic microorganisms and plants (Litzinger et al., 2010; Ankati et al., 2018). Several studies have described that different types of chitinases can hydrolyze the lipochitooligosaccharides (Nod factors) produced by rhizobia when they come into contact with the flavonoids released by the legume roots, regulating the nodulation process (Goormachtiget al., 1998; Schultze et al., 1998; Cullimore et al., 2001; Kasprzewska, 2003). It has also been observed that these enzymes may be involved in the activation of a signaling pathway related to the first steps of root colonization by arbuscular mycorrhizal fungi (Genre et al., 2013). The ability to degrade complex polysaccharides such as cellulose and chitin is a common feature in *Micromonospora* strains (Kawamoto, 1984; Jendrossek et al., 1997; Gacto et al., 2000; Gasmi et al., 2019; Trujillo et al., 2014b). *Micromonospora* may produce chitinases to inhibit fungal pathogens, or to induce the plant defense mechanism, providing a benefit to its host. Besides, over-expression of chitinase-related genes may be involved not only in plant protection but also in establishing the endophytic relationship with the plant.

7.3.3. Genes involved in plant growth promotion influenced by root exudates

Many of the bacteria found in the rhizosphere are able to provide compounds that facilitate plant development through direct and indirect mechanisms (Olanrewaju et al., 2017). *Micromonospora* strains displayed several genes related to plant growth promotion (PGP) in their genomes (Trujillo et al., 2014b; Carro et al., 2018). However, not all PGP genes were significantly differentially expressed in the evaluated strains. Several genes involved in the synthesis and degradation of trehalose were significantly regulated in the transcriptomic profiles. Trehalose is a low molecular weight sugar found in root exudates from different plant species, such as lettuce (Neumann, 2014). The up-regulation of genes related to the synthesis and degradation of trehalose pathway may be due to the presence of this compound in the exudates produced by *Lupinus*, which is an essential signal disaccharide in plants. This disaccharide can act as an osmoprotectant for many organisms that are subjected to environmental stress, such as high salinity, low temperature or drought (Duan et al., 2013). Different mutants of *Pseudomonas* sp. UW4, where the trehalose synthesis pathway was modified, showed that the trehalose production played a synergistic role in protecting tomato plants from the growth inhibitory effects of high salinity (Orozco-Mosqueda et al., 2019). Similar results were observed in a genetically engineered *Azoarcus* strain, where improved trehalose biosynthesis increased both drought tolerance and plant biomass in maize plants (Rodríguez-Salazar et al., 2009). In the case of the interaction between *Phaseolus vulgaris* and *Rhizobium*, trehalose not only played a role in tolerance against abiotic stress, but also enhanced germination, quality, and grain yield. In addition, trehalose is a common reserve disaccharide in the nitrogen-fixing nodules, present at high concentrations for the onset of nitrogen fixation and bacteroid survival (Streeter,1985; Farías-Rodriguez et al., 1998; Aeschbacher et al., 1999; Altamirano-Hernández et al., 2007; Barraza et al., 2013). In the last decade, the importance of trehalose metabolism in establishing plant-microbe relationships has been highlighted, not only in legume-rhizobia interactions, but also among plant-endophytic bacteria, plant-mycorrhizae and plant-pathogens (Müller et al., 2001; Brodmann et al., 2002; Foster et al., 2003; Ocón et al., 2007; Nehls, 2008; Wilson et al., 2010).

Other PGP activities were also expressed in the transcriptomic profiles. Among the strains analyzed, Lupac 08 was the only strain that showed up-regulated genes related to ABC transporters of iron, phosphate and molybdenum. This could be possible since Lupac 08 may be more efficient in obtaining and transporting these inorganic compounds in the steps preceding colonization, when the bacterium is in contact with the root exudates. In the case

of siderophores, the production of these iron-chelating compounds has been detected *in vitro* assays in Lupac 08 (Trujillo et al., 2014b). The genome of this strain has revealed several genes related to specific iron uptake transporters, the secretion of different siderophores and the synthesis of siderophore receptors (Trujillo et al., 2014b). The over-expression of siderophores in response to the root exudates was also detected in the transcriptome of the endophytic strains *Bacillus amyloliquefaciens* SQR9 and *Burkholderia phymatum* STM815 (Zhang et al., 2015; Klonowska et al., 2018). Siderophores are iron-chelating compounds that sequestering iron from soil and providing the nutrient to the plant, stimulating its growth (Rungin et al., 2012). Besides, siderophore-producing endophytic bacteria can limit the iron availability for other organisms, protecting the host plant against phytopathogens (Höfte and Bakker et al., 2007; Glick, 2015). In the transcriptome of strain Lupac 08, several genes involved in iron uptake transport and chitin degradation pathway were up-regulated by lupin root exudates. The combined expression of these genes by *Micromonospora* may provide more efficient protection against phytopathogens that affect the health of the plant, as well as improve plant development by increasing root and shoot biomass when host plants are inoculated with this bacterium (Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). In addition to iron transporters, several phosphate and molybdenum transporters were also expressed. Phosphate and molybdenum are essential elements that contribute to the health, establishment and development of plants. Specifically, phosphorus is one of the essential macronutrients required for plant growth and productivity (Goldstein, 1986), while molybdenum is used by specific plant enzymes that participate in reduction and oxidation reactions (Williams and Frausto da Silva, 2002). However, their availability for plants is very limited, so endophytic bacteria such as *Micromonospora* could potentially contribute to the bioabsorption of these compounds by the plant, stimulated by radical exudates present in the rhizosphere.

7.3.4. Influence of root exudates in plant-microorganism communication

In order to establish an effective symbiotic relationship between plants and endophytic bacteria, chemical communication between the two organisms must be established. Through the exchange of molecular signals, the activation of the immune response by the plant is avoided, allowing colonization of the plant by the endophyte (Mhlongo et al., 2018). Plant root colonization by beneficial endophytic bacteria involves *quorum* sensing (QS), which is a microbial cell-to-cell communication mechanism through which the plant cells and/or the

bacterial cells produce, secrete and detect chemical signals (autoinducers) (Atkinson and Williams, 2009; Ng and Bassler, 2009; Jimenez and Federle et al., 2014). QS signaling in Gram-positive bacteria is mediated by peptides known as autoinducing polypeptides (AIPs), involved in the signaling and regulation of gene expression, and possibly in cross-talk directly or indirectly with their host (Polkade et al., 2016; Verbeke et al., 2017). The transcriptome data showed up-regulated genes related to different peptide/nickel transport system proteins (ABC.PE.APS) and oligopeptide transport system proteins (*opp*A, *opp*B and *opp*F). It has been reported that when the secreted AIPs reach a certain threshold concentration in the extracellular medium, theses peptide signals can be transported into the bacterial cytoplasm through different transport systems, such as oligopeptide permease systems (Opp or Ami) (Lazazzera et al., 1997; Slamti and Lereclus, 2002; Fontaine et al., 2010; Chang et al., 2011; Jimenez and Federle et al., 2014). Other transport systems were also significantly regulated in the Lupac 08, Lupac 09^T and $CR30^T$ transcriptomes, which function was the transport of branched-chain amino acids (*livF*GKM). The branched-chain amino acid transport systems (Bra/LIV) are related to the transport of different amino acids, such as proline, serine, leucine, histidine or gamma-aminobutyric acid (GABA) (Hildebrandt et al., 2015). Some of these amino acids may act as signaling molecules, precursors for the synthesis of phytohormones or other secondary metabolites with signaling function (Szabados and Savoure, 2010; Timm et al., 2012, Häusler et al., 2014; Hildebrandt et al., 2015). The branched-chain amino acid transport system has been observed between several strains of *Rhizobium* and their host plant (Hosie et al., 2002; Haudecoeur et al., 2009). Amino acid, peptide and oligopeptide transporters may have an important role in the bacterium-plant interactions. An efficient exchange of signals between the two organisms can allow the endophytic bacteria to establish themselves earlier in the plant tissues (Djordjevic, 2004; Sarma and Emerich, 2006; Mastronunzio et al., 2009; Kierul et al., 2015). It is not surprising the over-expression of genes related to these membrane transporters in the presence of root exudates because legume root exudates can be rich in amino acids and oligopeptides (Carvalhais et al., 2011). However, the role of some genes involved in the QS pathway is not well understood and further studies are required.

The exudates released by the plant roots not only contain molecular signals that are recognized by the rhizospheric bacteria, but also contain low molecular weight compounds such as sugars, amino acids and organic acids (Egle et al., 2003; Vranova et al., 2013). The sugars present in exudates may be stimulating the gene expression of polysaccharide and

monosaccharide transporters in *Micromonospora*. In the three *Micromonospora* strains were found several up-regulated genes related to the transport of different carbohydrates such as cellobiose (*ceb*EF), N,N'-diacetylchitobiose (*das*C), arabinogalactan oligomer/maltooligosaccharide (*gan*Q), maltose/maltodextrin, ribose (*rbs*ABC), and raffinose/stachyose/melibiose (*msm*EG). These sugar groups can act as carbon and energy sources during bacterial colonization of plant tissues (Mark et al., 2005; Bais et al., 2006). In the case of the ABC transporter *rbs*B involved in the ribose transport, it has been described that it can also act as a primary recipient of chemoattractants released by the plant and subsequently initiate the bacterial chemotaxis towards the host plant which signals have been captured (Macnab, 1987; Boos and Lucht 1996; Stock and Surette 1996). An efficient exchange of molecular signals such as peptides, oligopeptides, amino acids or carbohydrates may facilitate plant tissue chemotaxis and colonization by *Micromonospora* to establish in the rhizosphere, rhizoplane or endosphere of legumes.

7.3.5. Reduction of ribosomal translation by the action of root exudates

The three *Micromonospora* strains exposed to root exudates showed down-regulation of genes related to large and small subunit ribosomal proteins (**Table 22**). In other works, it has also been observed a large number of down-regulated genes related to protein synthesis as in the case of *Burkholderia phymatum* STM815 grown in presence to *Mimosa pudica* root exudates (Klonowska et al., 2018) or *Bacillus amyloliquefaciens* FZB42 exposed to root exudates collected from maize plants grown under nitrogen-deficient conditions (Carvalhais, et al., 2013). It has been described that under-expression of these genes may be due to different environmental stresses, such as high salinity and low Mg^{2+} concentration (Starosta et al., 2014; Pontes et al., 2016), or to changes that occur during the transition to the stationary growth phase (Piir et al., 2011). In the particular case of the transition between the exponential and the stationary phase, it has been reported that nutrient deprivation such as amino acids can cause the suppression of ribosomal protein synthesis (Hand and Hardewig, 1996; Yus et al., 2009). This event corresponds to a change in bacterial metabolism towards survival, preventing bacteria from excessively investing biosynthetic resources in ribosome synthesis which process involves high energy cost (Krásný and Gourse, 2004). In our samples, the under-expression of the genes coding for ribosomal proteins may not be due to saline stress because the same concentration of Rigaud and Puppo solution (0.25 mg/ml) was added both in the control and the target samples. The only difference between the control

samples and the target samples was that nutrient solution added in the target samples contained the root exudates collected from *Lupinus*. However, samples grown in the presence of root exudates could have reached the stationary phase faster than the control samples. This is because the presence of the lupin root exudates to the target sample may have provided nutrients to the bacteria, accelerating their metabolism and growth. However, there is currently little information related to the under-expression of genes involved in ribosomal protein synthesis. Its study would be interesting since some compounds present in the root exudates could interfere in the bacterial protein synthesis and in the behavior of these cells with respect to the plant.

FINAL DISCUSSION

8. Final discussion

The results obtained throughout this investigation showed information about the molecular interaction between *Micromonospora* and the host plant and its ability to colonize different legumes and plant tissues. Therefore, we can answer the questions posed at the beginning of this research: Can *Micromonospora* be isolated from plant tissues other than nitrogen-fixing nodules? Has *Micromonospora* the capacity to enter and colonize legumes other than its original host? Can root exudates from *Lupinus* plants alter of specific gene expression patterns in *Micromonospora* and influence its interaction with the host?

This work demonstrates that *Micromonospora* can be isolated not only from nitrogen-fixing nodules but also from different legume tissues such as leaves, stems and roots. In previous works, *Micromonospora* has been occasionally isolated from stems, roots and leaves of several non-leguminous plants, but not from legumes (Okazaki al., 1995; Kizuka et al., 2002; Taechowisan et al., 2003; Kirby and Meyers, 2010; Kuncharoen et al., 2019). In addition, different members of the same species were isolated from different tissues, indicating that a species does not limit its presence to a single type of plant tissue. In terms of the bacterial species distribution, *M. saelicesensis* was again the most abundant species in the different tissues sampled and the only bacterial species present in all plant tissues analyzed. In previous works, *M. saelicesensis* was also the most abundant species in different legumes and even in actinorhizal plants (Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2012a; 2013). The presence of *Micromonospora* in different tissues such as stems and leaves may be due to its passage from the root to the upper tissues through the plant vascular vessels (xylem and phloem), which has been observed in different endophytic bacteria (Compant et al., 2010; Reinhold-Hurek and Hurek, 2011; Turner et al., 2013). In addition, the high number of *Micromonospora* isolates from leaves and nodules may suggest that *Micromonospora* is probably well adapted to live in these tissues. It has been reported that endophytes capable of colonizing aerial vegetative plant parts, need to possess the physiological requirements to adapt and establish in different plant niches (Hallmann, 2001).

Up until now, the presence of *Micromonospora* in nitrogen-fixing nodules has only been reported through its isolation or visualization by different techniques such as fluorescent *in situ* hybridization (FISH) and transmission electron microscopy (TEM), and using the same or different plant species from which the strains originated (Alonso de la Vega, 2010; Trujillo et al., 2010). In this work, the localization of *Micromonospora* in specific parts of the nodule was achieved using a tagged strain, *M. lupini* Lupac 08. Furthermore, it was also

demonstrated that this strain is not only able to re-infect the internal root tissues of its original host (*Lupinus*), but also that of other legumes such as *Medicago* and *Trifolium*, suggesting that a broad host range (Benito et al., 2017). The ability of *Micromonospora* to infect different legume species contrasts with the symbiotic interactions between rhizobia and legumes and *Frankia* and actinorhizal plants, both of which are more restrictive (Pawlowski and Demchenko, 2012; Andrews and Andrews, 2016). In addition, this work describes the colonization process of *Micromonospora* on the root surface and root hairs to its location in the nodular primordia and mature nodules, together with rhizobia. In the case of root nodules, they appeared and developed 1-2 days earlier on the co-inoculated plants with *Micromonospora* and rhizobia, compared to those inoculated only with rhizobia. This may indicate that *Micromonospora* acts as a "helper" in the nodulation process by producing a positive effect in the early stages of nodule development and by increasing the number of nodules (Trujillo et al., 2014b; Solans et al., 2009). In mature nodules, a co-existence of *Micromonospora* and rhizobia was observed in the infection zone, where bacteroid development proceeded normally. In addition, the study plants did not show any negative effects related to the presence of *Micromonospora* and the nitrogen fixation process did not appear to be altered. These results strongly suggest a tripartite interaction and the coexistence of non-rhizobial bacteria such as *Micromonospora* within nodule tissues (Tokala et al., 2002; Muresu et al., 2008) reinforcing its role as a helper bacterium although its specific function still waits to be elucidated.

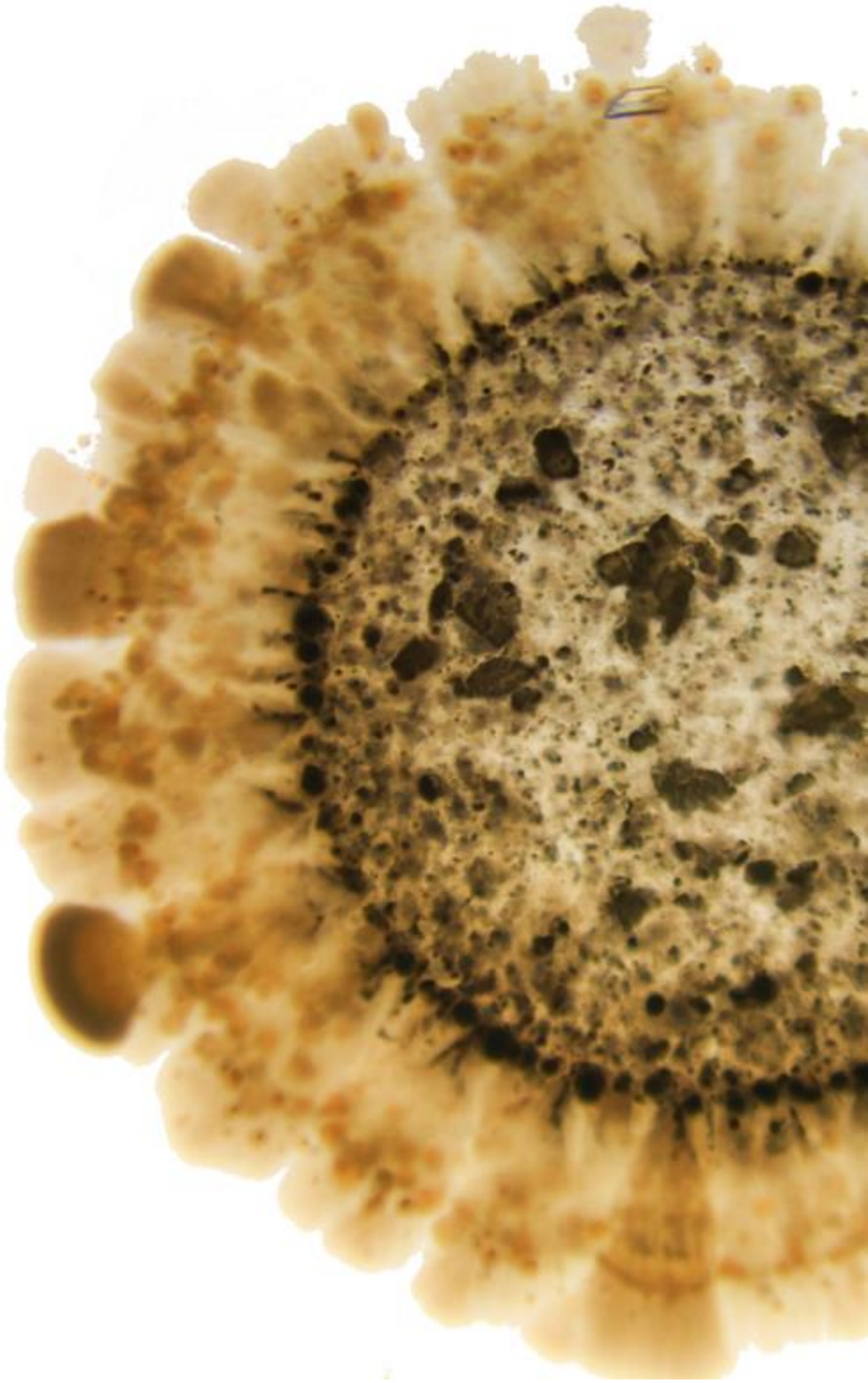
The capacity of *Micromonospora* to colonize different legumes may be due to an exchange of signals between the plant and the bacterium. This process is carried out by the release of signal molecules by the plants that can modify bacterial behavior (Mark et al., 2005; Shidore et al., 2012). The study of the plant-*Micromonospora* interaction has focused mainly on the effect that *Micromonospora* has on the plant and whether the bacterium acts as a plant growth promoter (Trujillo et al., 2015). However, the influence that the plant has on *Micromonospora* had not been studied until now. This work explored how root exudates from a legume such as *Lupinus albus* could alter the gene and protein expression in three *Micromonospora* strains (*M. lupini* Lupac 08, *M. saelicensensis* Lupac 09^{T} and *M. cremea* CR30^{T}). The transcriptomic and proteomic profiles obtained in this work, provided a valuable insight about how *Micromonospora* reacts to compounds released by the plant and what events may be occurring during this interaction when *Micromonospora* comes into contact with plant root exudates. Among proteins and genes differentially regulated in the presence

of root exudates, a high number may be related to the plant tissues colonization (cellulases, xylanases, pectinases), phytohormone production (acetoin, IAA), nutrient mobilization for the plant (Fe and P), the absorption of signal molecules (carbohydrates, peptides, oligopeptides and branched-chain amino acids), and the plant protection against phytopathogens (chitinases, siderophores) and other adverse conditions (trehalose). Similar results have been observed in other endophytic bacteria exposed to the root exudates from their host (Carvalhais et al., 2013; Kierul et al., 2015; Zhang et al., 2015; Liu et al., 2017). Overall, the proteome and transcriptome have produced new information about the genetic and functional responses of *Micromonospora* under the effect of the host root exudates, and have provided a molecular basis for further investigations into the mechanisms underlying nonspecific symbiosis between *Micromonospora* and legumes.

An outstanding feature of *Micromonospora* that was observed in this work was its capacity to produce different plant-polymer hydrolytic enzymes, such as cellulases. In the isolated strains from different tissues of *L. angustifolius* and *Pisum sativum* plants, cellulolytic activity was observed in 98% of strains when this activity was tested in *in vitro* assays. In the case of proteomic and transcriptomic studies, strains Lupac 08 and Lupac 09^T displayed over-expressed proteins and genes related to enzymes belonging to the cellulase family in response to root exudates. In the particular case of strain CR30^T, it did not show any significantly up-regulated protein related to cellulase production, unlike in the transcriptome that showed one over-expressed gene related to the β-glucanase production. In addition, strain CR30^T displayed cellulase production in the presence of live lupin roots while it did not produce any cellulases in *vitro tests* on agar plates which contained carboxymethylcellulose, unlike the other two strains tested which showed cellulase activity. The genes that code for cellulases may be considered appropriate candidates for future mutagenesis works in order to study the effect of individual candidate genes on the growth and development of several plants. This is because the function of cellulases is still unknown in the establishment of the legume-*Micromonospora* relationship. Different authors have described that cellulases, together with other enzymes, produced by endophytes may be involved in adhesion and colonization of the host's internal tissues (Compant et al., 2005; Reinhold-Hurek et al., 2006), but also in cell wall remodeling, lignification and chemical defense in plants, the activation of phytohormones, protection against possible pathogens and in the establishment of the bacterium-plant relationship (Compant et al., 2005; Collins et al., 2007; Gilbert et al., 2008; Taguchi et al., 2010; Singh et al., 2016). Many questions still remain to be answered in the

interaction of *Micromonospora* and legumes, but this work confirms that this bacterium plays an important role. Understanding the ecology of this endophytic bacterium and its molecular interactions will have an impact in plant growth and crop yields and therefore in economics and the environment.

CONCLUSIONS



9. Conclusions

The results obtained throughout this investigation allow us to come to the following conclusions:

1. *Micromonospora* can be isolated from surface-sterilized plant tissues other than nodules such as roots, stems and leaves of *Lupinus angustifolius* and *Pisum sativum* plants. The presence of this actinobacterium in different plant tissues is therefore not limited to internal nodular tissues.

2. The bacterial diversity of the genus *Micromonospora* in the plant tissues of *Pisum sativum* and *Lupinus angustifolius* is very high, being *M. noduli* and *M. saelicensensis* the most abundant species.

3. *Micromonospora* strains isolated from plant tissues show positive results in the production of different plant-polymer degrading enzymes such as cellulases, xylanases, pectinases, amylases and chitinases, independently of the plant tissue or legume where they were isolated.

4. Strain *M. lupini* Lupac 08 not only re-infects its original host (*Lupinus* sp.) but also interacts with other legumes such as *Medicago* and *Trifolium.* In addition, *M. lupini* Lupac 08 is localized within nodules of the three legumes and strongly suggests that a non-specific relationship takes place between *Micromonospora* and the plant.

5. The different rhizobia are not inhibited by co-inoculation with *Micromonospora* and the bacteroid development proceeded normally within the nodule.

6. Transcriptomic and proteomic profiles show that *Lupinus* root exudates can alter gene and protein expression in the three target strains: *M. lupini* Lupac 08, *M. saelicesensis* Lupac 09^{T} and *M. cremea* CR30^{T}.

7. Transcriptomic and proteomic responses of *Micromonospora* to the presence of *Lupinus* root exudates depend on the strain, showing a different proportion of over-expressed and under-expressed genes and proteins between strains.

8. The significantly regulated genes and proteins by the effect of root exudates on transcriptomic and proteomic profiles can be related to the plant-bacterial interaction, especially those involved in the plant-polymer degrading enzyme production, plant-growth promotion activities and the plant-bacterium communication.

9. *Lupinus* root exudates promote over-expression of genes and proteins in *Micromonospora* involved in plant-polymer degradation such as cellulases. Strain CR30^{T}, which did not show cellulolytic activity in *in vitro* conditions, displayed one over-expressed gene related to the β-glucanase production in presence of root exudates.

10. *Micromonospora* strains exposed directly to the root exudates of *Lupinus* causes an over-regulation of genes involved in the plant-polymer degrading enzyme production, compared to the *Micromonospora* growth in ISP 2 broth supplemented with 0.25 mg/ml of lupine root exudates.

11. *Micromonospora* is able to produce cellulases in the presence of live roots of different legumes (*Trifolium, Medicago* and *Lupinus*) without causing damage to the plant. Even strain CR30^{T}, which showed no cellulolytic activity in the absence of live roots, showed cellulase production in the presence of roots of the three legumes.

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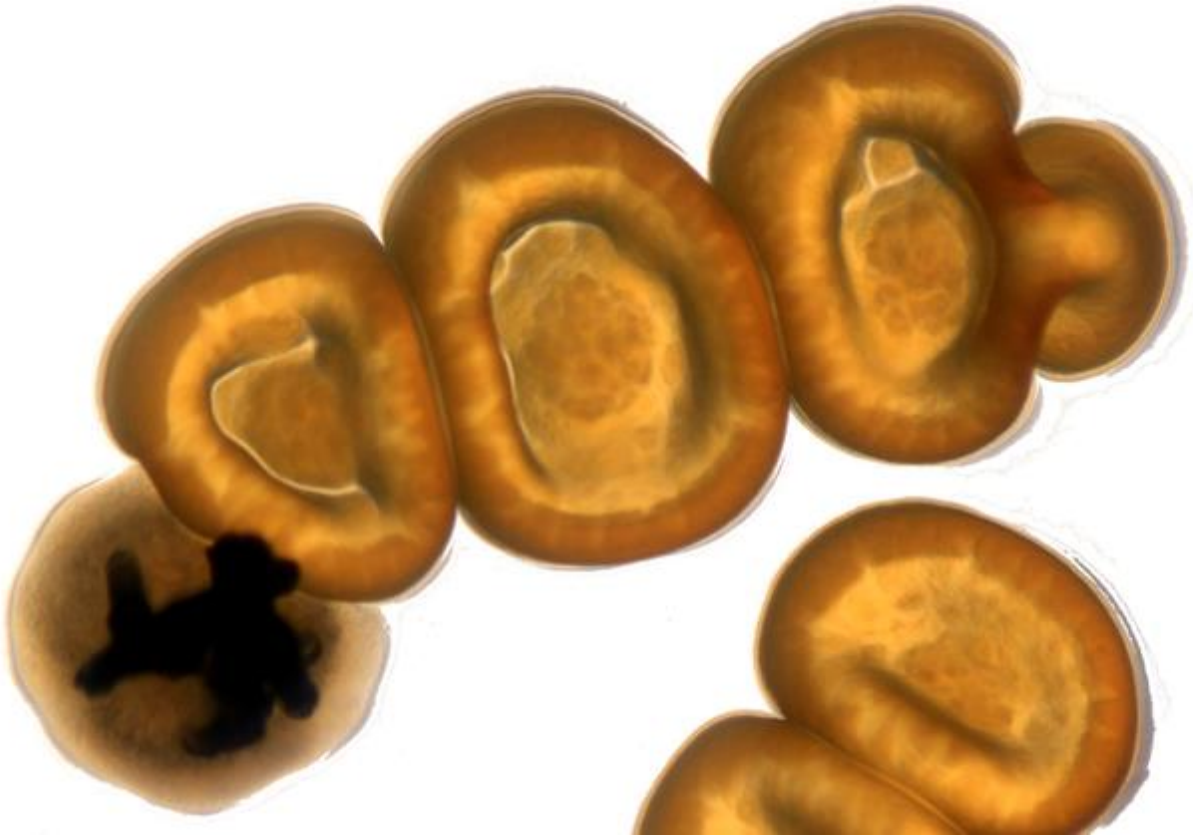

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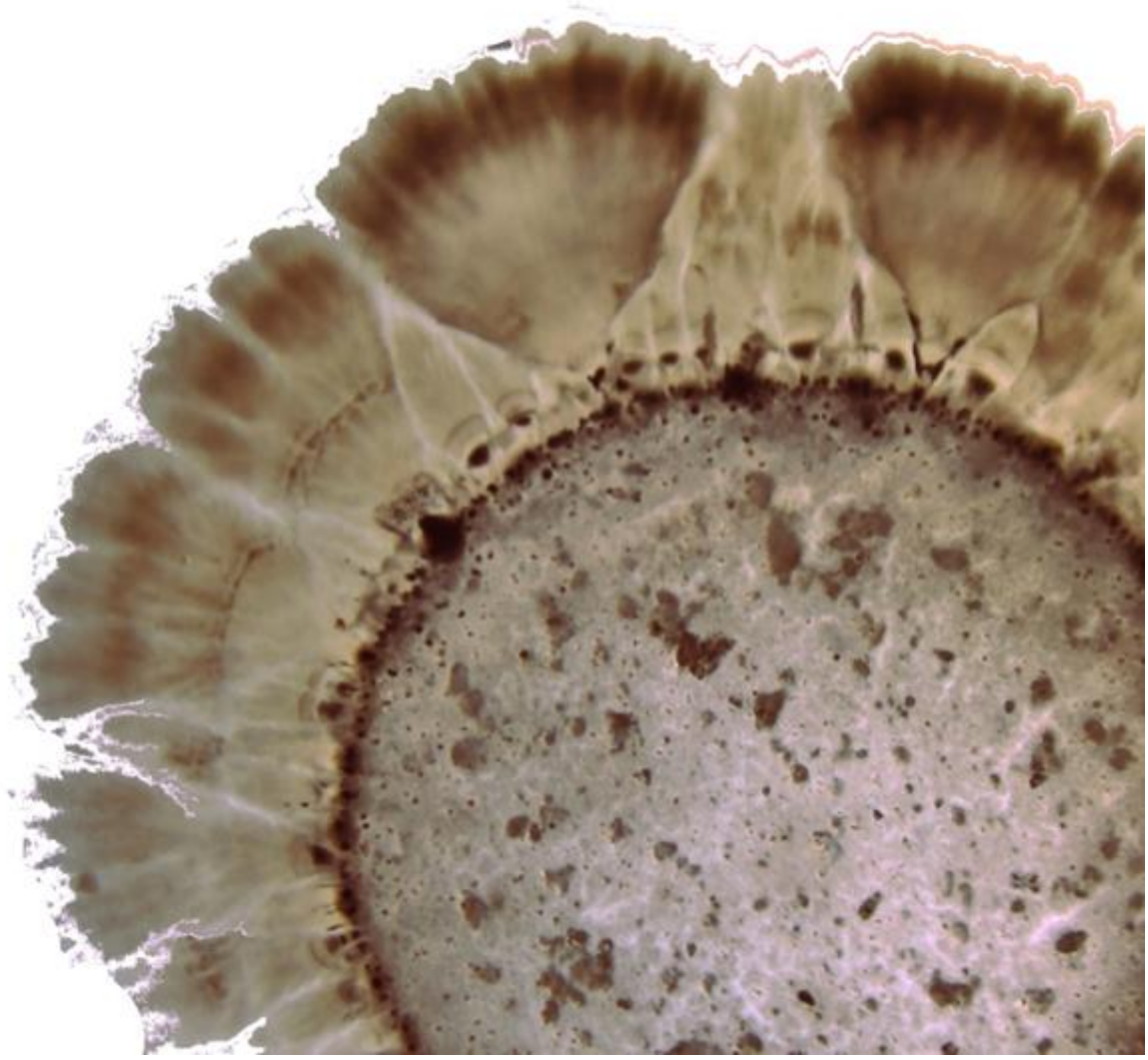
APPENDIXES



Appendix I: Media, buffers and solutions

Media

All media used in this work were sterilized in an autoclave for 20 minutes at 15 psi pressure (121ºC).

Yeast Mannitol Agar (YMA) medium (Vincent, 1970)	
Mannitol (Applichem)	10 g
Yeast extract (Applichem)	3 g
K_2HPO_4	0.2 g
$MgSO_4$	0.2 g
NaCl	0.1 g
Agar	18 g
Distilled water-1000 ml	
pH 6.8-7.0	

Extract/ humic acid (HA) agar (de la Vega, 2010)	
Yeast extract (Applichem)	1 g
Humic acid	0.2 g
Agar	18 g
Distilled water-1000ml	

ISP 2 medium (Shirling and Gottlieb, 1966)	
Glucose (Applichem)	4 g
Yeast extract (Applichem)	4 g
Malt extract (Difco)	10 g
$CaCO_3$	2 g
Agar	18 g
Distilled water-1000 ml	
pH 7.2	

Luria Bertani (LB) medium (Bertani, G)	
Tryptone (Difco)	10 g
Yeast extract (Applichem)	5 g
NaCl	10 g
Agar	18 g
Distilled water-1000 ml	
pH 7.0	

TY medium	
Tryptone-peptone (Difco)	1 g
Yeast extract (Applichem)	0.6 g
$CaCl_2$	0.1 g
Agar	18 g
Distilled water-1000ml	

Trap-water agar	
Trap-water	100 ml
Agar	1 g

Minimal salt medium (M3) (Rowbotham and Cross et al., 1997)	
Component	Mass
KH_2PO_4	0.46 g
Na_2HPO_4	0.73 g
KNO_3	0.1 g
NaCl	0.3 g
$MgSO_4 \cdot 7H_2O$	0.02 g
$CaCO_3$	0.02 g
Sodium propionate	0.2 g
$FeSO_4 \cdot 7H_2O$	200 µg
$ZnSO_4 \cdot 7H_2O$	180 µg
$MnSO_4 \cdot 4H_2O$	4 µg
Agar	18 g
Distilled water-1000 ml	
pH 7.2	

Add 0.5% (w/v) CMC, 0.5% (w/v) xylan, 0.5% (w/v) pectin, 0.5% (w/v) starch or 1% (w/v) colloidal chitin as required

Buffer and solutions

All buffer used in this work were sterilized in an autoclave for 20 minutes at 15 psi pressure (121ºC), unless otherwise indicated.

Nitrogen-free Rigaud and Puppo solution (Rigaud and Puppo, 1975)	
Component	Mass
Na_2MoO_4	0.004 g
H_3BO_3	0.018 g
$MnSO_4 \cdot 4H_2O$	0.02 g
$ZnSO_4$	0.003 g
$CuSO_4 \cdot 5H_2O$	0.002 g
$CaCl_2$	0.003 g
$Na_2FeEDTA$	0.025 g
Distilled water-1000 ml	

Nitrogen-free Rigaud and Puppo nutrient agar: Add 18g/l of agar to the solution before autoclaving

Killing buffer (Fan et al., 2012; Zhang et al., 2015)	
Component	Concentration
Tris-HCL	20 mM
$MgCl_2$	5 mM
NaN_3	20 mM
Agar	18 g
Distilled water-1000ml	
pH 7.5	

Phosphate-buffered saline (PBS) pH 7.4		
Component	Mass (g)	Molarity (M)
NaCl	4 g	0.137
KCl	100 mg	0.0027
Na_2HPO_4	720 mg	0.01
KH_2PO_4	120 mg	0.0018
pH 7.4		
Distilled water	Until 500 ml	

0.1 M phosphate buffer (PB) pH 7.2			
1) Stock preparation			
Component	Mass (g)	Volume H2O elix (ml)	Molarity (M)
Sodium phosphate dibasic	35.5	500	0.5
Sodium phosphate monobasic (anhydrous)	30.0	500	0.5
2) 0.1 M sodium phosphate solution			
Component	Volume sodium phosphate solution (ml)	Volume H2O elix (ml)	Molarity (M)
Sodium phosphate dibasic (0.5M)	80	400	0.1
Sodium phosphate monobasic (anhydrous) (0.5M)	30	150	0.1
3) 0.1 M phosphate buffer (PB) pH 7.2 preparation			
Bring the 0.1 M sodium phosphate dibasic solution to pH 7.2 by adding as much as needed of the 0.1 M sodium phosphate monobasic solution			

Tris-buffered saline (TBS)		
Component	Mass (g)	Molarity
Tris-HCl	6.05	50 mM, pH 7.5
NaCl	8.76	150 mM
Distilled water	Until 1000 ml	

Loading buffer (4X)	
Component	Concentration
Tris-HCl	250 mM pH 6.8
DTT	50 mM
SDS	8% (w/v)
Glycerol	40% (v/v)
Bromophenol blue	0.05 % (w/v)

1x Tris-Glycine-SDS buffer	
Component	Mass
Tris-base	3 g
Glycine	14.4 g
SDS	1 g
Distilled water-1000ml	

Methanol:Acetic: Water (5:1:4) solution	
Component	Volume(ml)
Methanol	500
Acetic acid	100
Distilled water	400
Total Volume	1000

Protein rehydration buffer	
Component	Concentration
Urea	9 M
Thiourea	2 M
CHAPS	4% (w/v)
DTT	50 mM
DeStreak	1.20 % (w/v)
IPG buffer	0.5% (v/v)

Equilibration buffer	
Component	Concentration
Tris-HCl	375 mM, pH 8.8
Urea	6 M
SDS	2% (w/v)
Glicerol	20% (v/v)

Appendix II. Isolated strains from different plant tissues and culture media

Strain	Plant	Plant area	Isolation medium
LAN01	6	Nodule 1	YMA
LAN02	6	Nodule 2	YMA
LAN03	6	Nodule 3	YMA
LAN04	6	Nodule 4	YMA
LAN05	6	Nodule 3	YMA
LAN06	6	Nodule 4	YMA
LAN07	6	Nodule 3	YMA
LAN08	6	Nodule 4	YMA
LAN09	7	Nodule 2	YMA
LAN10	7	Nodule 1	YMA
LAN11	7	Nodule 1	YMA
LAN12	7	Nodule 3	YMA
LAT01	1	Stem 1	HA
LAR01	7	Root 1	YMA
LAR02	7	Root 1	YMA
LAR03	7	Root 1	YMA
LAR04	7	Root 1	YMA
LAR05	5	Root 1	YMA
LAR06	7	Root 1	YMA
LAR07	7	Root 1	YMA
LAR08	7	Root 1	YMA
LAR09	7	Root 1	YMA
LAR10	7	Root 1	YMA
LAH01	6	Leave 1	HA
LAH02	6	Leave 2	HA
LAH03	6	Leave 1	HA
LAH04	6	Leave 2	HA
LAH05	1	Leave 1	HA
LAH06	1	Leave 1	HA
LAH07	1	Leave 1	HA
LAH08	5	Leave 1	YMA
LAH09	4	Leave 1	YMA
LAH10	4	Leave 2	YMA
LAH11	4	Leave 3	YMA
LAH12	4	Leave 4	HA
LAH13	2	Leave 1	HA
LAH14	2	Leave 1	HA
LAH15	2	Leave 2	YMA
LAH16	5	Leave 2	HA
LAH17	5	Leave 3	HA
LAH18	5	Leave 4	HA
LAH19	2	Leave 1	HA
LAH20	2	Leave 1	YMA
LAH21	2	Leave 1	YMA

Strain	Plant	Plant area	Isolation medium
PSH 01	1	Leave 1	HA
PSH 02	1	Leave 1	HA
PSH 03	1	Leave 1	HA
PSH 04	1	Leave 1	HA
PSH 05	1	Leave 1	HA
PSH 06	1	Leave 1	YMA
PSH 07	1	Leave 1	YMA
PSH 08	1	Leave 2	HA
PSH 09	1	Leave 2	HA
PSH 10	1	Leave 2	YMA
PSH 11	2	Leave 1	HA
PSH 12	2	Leave 1	HA
PSH 13	2	Leave 1	HA
PSH 14	2	Leave 3	HA
PSH 15	2	Leave 3	HA
PSH 16	2	Leave 3	YMA
PSH 17	2	Leave 3	YMA
PSH 18	2	Leave 3	YMA
PSH 19	2	Leave 3	YMA
PSH 20	5	Leave 1	HA
PSH 21	5	Leave 1	HA
PSH 22	5	Leave 1	HA
PSH 23	5	Leave 2	YMA
PSH 24	5	Leave 2	YMA
PSH 25	5	Leave 4	YMA
PSH 26	5	Leave 3	HA
PSH 27	5	Leave 3	HA
PSH 28	3	Leave 2	YMA
PSH 29	3	Leave 2	YMA
PSH 30	3	Leave 2	YMA
PSH 31	3	Leave 1	YMA
PSH 32	3	Leave 1	HA
PSH 33	3	Leave 2	YMA
PSH 34	4	Leave 1	YMA
PSH 35	4	Leave 1	YMA
PSH 36	4	Leave 1	YMA
PSH 37	4	Leave 1	YMA
PSH 38	4	Leave 1	YMA
PSH 39	4	Leave 2	HA
PSH 40	4	Leave 2	YMA
PSH 41	1	Leave 2	HA
PSH 42	6	Leave 3	YMA
PSH 43	6	Leave 3	YMA
PSH 44	6	Leave 2	HA
PSH 45	7	Leave 3	YMA
PSH 46	7	Leave 3	YMA
PSH 47	5	Leave 4	YMA
PSH 48	5	Leave 4	YMA
PSH 49	6	Leave 1	YMA
PSH 51	6	Leave 1	YMA
PSH 52	6	Leave 3	YMA
PSH 53	6	Leave 1	YMA
PSH 54	6	Leave 1	YMA

Strain	Plant	Plant area	Isolation medium
PSN 01	2	Nodule 1	YMA
PSN 02	2	Nodule 1	YMA
PSN 03	2	Nodule 3	YMA
PSN 04	2	Nodule 3	YMA
PSN 05	2	Nodule 3	YMA
PSN 06	2	Nodule 1	YMA
PSN 07	3	Nodule 2	YMA
PSN 08	3	Nodule 2	YMA
PSN 09	3	Nodule 2	YMA
PSN 10	3	Nodule 2	YMA
PSN 11	2	Nodule 3	YMA
PSN 12	3	Nodule 1	YMA
PSN 13	3	Nodule 1	YMA
PSN 14	3	Nodule 1	YMA
PSN 15	5	Nodule 4	YMA
PSN 16	5	Nodule 4	YMA
PSN 17	5	Nodule 4	YMA
PSN 18	5	Nodule 4	YMA
PSN 19	6	Nodule 1	YMA
PSN 20	6	Nodule 1	YMA
PSN 21	6	Nodule 1	YMA
PSN 22	6	Nodule 1	YMA
PSN 23	6	Nodule 1	YMA
PSN 24	6	Nodule 2	YMA
PSN 25	6	Nodule 2	YMA
PSN 26	6	Nodule 3	HA
PSN 27	6	Nodule 2	YMA
PSN 28	6	Nodule 2	YMA
PSN 29	6	Nodule 2	YMA
PSN 30	6	Nodule 2	YMA
PSN 31	1	Nodule 1	YMA
PSN 32	1	Nodule 1	YMA
PSN 33	3	Nodule 3	YMA
PSN 34	3	Nodule 3	YMA
PSN 35	3	Nodule 2	YMA
PSN 36	7	Nodule 3	YMA
PSN 37	7	Nodule 3	YMA
PSN 38	7	Nodule 3	YMA
PSN 39	7	Nodule 3	YMA
PSN 40	7	Nodule 3	YMA
PSN 41	5	Nodule 2	YMA
PSN 42	5	Nodule 2	YMA
PSN 43	5	Nodule 2	YMA
PSN 44	5	Nodule 2	YMA
PSN 45	5	Nodule 2	YMA
PSN 46	2	Nodule 4	YMA
PSN 47	2	Nodule 4	YMA
PSN 48	2	Nodule 1	YMA
PSN 49	6	Nodule 3	HA
PSN 50	3	Nodule 3	YMA
PSN 51	3	Nodule 3	YMA
PSN 52	1	Nodule 1	YMA
PSN 53	1	Nodule 1	YMA
PSN 54	1	Nodule 1	YMA

Appendix III: Validation of transcriptome data by RT-PCR

Strains	ID gene	Gene	FCH transcriptome	2-ΔΔCq= Gene-*gyr*B	2-ΔΔCq= Gene-*atp*D	2-ΔΔCq= Gene-*rpo*B	Average 2-ΔΔCq	Desviation
Lupac08	MILUP08_RS10420	Endoglucanase	4.22	10.79	6.68	8.95	8.81	2.06
	MILUP08_RS10410	Chitinase	6.69	4.74	6.61	7.02	6.12	1.22
	MILUP08_RS30500	Glucokinases	1.62	1.87	2.62	2.83	2.44	0.50
	MILUP08_RS10885	Alpha, α-trehalase	9.72	5.80	8.13	8.69	7.54	1.53
	MILUP08_RS15060	Transporter	4.56	4.19	5.82	6.40	5.47	1.15
	MILUP08_RS26450	β-glucanase	10.15	8.36	11.75	12.50	10.87	2.21
Lupac09^{T}	GA0070561_RS15115	Endoglunase	4.65	4.14	5.08	6.90	5.37	1.40
	GA0070561_RS23495	Chitinase	5.51	5.84	7.15	9.73	7.57	1.98
	GA0070561_RS18130	Glucokinase	0.51	0.61	0.75	1.02	0.79	0.21
	GA0070561_RS20985	Transporter	2.23	4.32	5.34	7.20	5.62	1.46
	GA0070561_RS24610	Xylose isomerase	7.95	5.90	7.24	9.81	7.65	1.99
	GA0070561_RS01190	Pectinase	3.28	3.60	4.42	5.98	4.67	1.21
CR30^{T}	BUS84_RS24385	Chitinases	3.04	3.48	4.29	4.59	4.12	0.57
	BUS84_RS04400	Glucokinases	8.65	9.63	11.72	12.59	11.31	1.52
	BUS84_RS02785	Transporter	10.39	11.03	13.61	14.53	13.06	1.81
	BUS84_RS10315	Xylan 1,4-β-xylosidase	4.66	5.70	6.48	7.39	6.52	0.85
	BUS84_RS05160	Alpha,α-trehalase	9.93	7.49	9.44	10.11	9.01	1.36
	BUS84_RS05405	β-glucanase	7.31	9.46	10.09	10.09	9.88	0.36

Appendix IV: Data obtained by RT-PCR after exposing *Micromonospora* directly to the root exudates released by *Lupinus*

Strains	ID gene	Gene	FCH transcriptome	2-ΔΔCq= Gene-gyrB	2-ΔΔCq= Gene-atpD	2-ΔΔCq= Gene-rpoB	Average 2-ΔΔCq	Desviation
Lupac08	MILUP08_RS10420	Endoglucanase	4.22	118.42	137.32	187.63	147.79	35.77
	MILUP08_RS10410	Chitinase	6.69	84.59	96.95	133.61	105.05	25.49
	MILUP08_RS30500	Glucokinases	1.62	47.14	54.71	74.78	58.88	14.28
	MILUP08_RS10885	Alpha,α-trehalase	9.72	179.81	208.17	284.98	224.32	54.41
	MILUP08_RS15060	Transporter	4.56	50.38	57.32	79.63	62.44	15.28
	MILUP08_RS26450	β-glucanase	10.15	72.38	82.39	116.40	90.39	23.07
Lupac09^{T}	GA0070561_RS15115	Endoglunase	4.65	37.58	62.83	72.13	57.52	17.88
	GA0070561_RS23495	Chitinase	5.51	91.04	152.68	175.14	139.62	43.54
	GA0070561_RS18130	Glucokinase	0.51	8.12	13.56	15.55	12.41	3.85
	GA0070561_RS20985	Transporter	2.23	58.13	95.23	110.30	87.89	26.85
	GA0070561_RS24610	Xylose isomerase	7.95	10.73	17.85	20.52	16.37	5.06
	GA0070561_RS01190	Pectinase	3.28	120.71	201.01	232.00	184.57	57.43
CR30^{T}	BUS84_RS24385	Chitinases	3.04	183.90	215.01	228.45	209.12	22.85
	BUS84_RS04400	Glucokinases	8.65	17.78	20.86	22.12	20.25	2.24
	BUS84_RS02785	Transporter	10.39	92.76	107.49	115.32	105.19	11.45
	BUS84_RS10315	Xylan 1,4-β-xylosidase	4.66	6.04	7.14	7.51	6.90	0.77
	BUS84_RS05405	β-glucanase	7.31	23.52	27.21	29.29	26.67	2.92
	BUS84_RS05160	Alpha,α-trehalase	9.93	3.38	3.93	4.20	3.84	0.42