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Bacterial Endophytes: Unexplored Sources Of Biotechnologically Relevant Molecules

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1. Introduction

1.1 Endophytic bacteria

1.1.1 Definition, origin and functions

Plants and microorganisms have been living in association for such a long time, in fact arbuscular mycorrhizal mutualism is believed to have had a key importance in the terrestrialization process and in the evolution and diversification of plant phototrophs (Tacon 1998), (Heckman, Geiser, and Eidell 2001). Microorganisms can be associated with plants externally, colonizing the rhizosphere or the phyllosphere; in this case they are called *epiphytes* (from Greek, *epi-phyton*, *on plants*). Microorganisms can also colonize leaves, stems and roots internally, which is the case of endophytes (from Greek, *endon-phyton*, *within plants*). Most of the microorganisms that inhabit plants are beneficial and sometimes neutral (Mendes, Garbeva, and Raaijmakers 2013). De Bary was the first to describe the presence of non-pathogenic microorganisms in plant tissues while using a microscope to observe plant tissues. A first definition of endophytic microorganisms was provided by De Bary in 1866 as *any organism that grows within plant tissues* (De Bary 1866). Nowadays, the most shared definition for endophytes underlines the non-pathogenic nature of such organisms: *endophytes are any organism that at some part of its life cycle, colonizes the internal plant tissues without causing any type of harm to the host plant* (Patriquin and Dobereiner 1978). Endophytes can be furtherly defined, distinguishing between *obligate* and *facultative* ones (Rosenblueth and Martínez-Romero 2006). Obligate endophytes are dependent on the plant metabolism for survival (Hardoim, van Overbeek, and van Elsas 2008), while facultative endophytes live outside the host plant for a certain stage of their lives (Navarrete 2010). It has been evidenced that endophytic bacterial communities are mainly constituted by facultative endophytes (Hardoim et al. 2008). Different microorganisms can establish associations with plants, including bacteria and fungi. Regarding bacterial endophytes, Actinobacteria, Proteobacteria, and Firmicutes are the most commonly reported bacterial phyla (Golinska et al. 2015). More than 200 different bacterial genera have been observed in plant-associated microbiome, as *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Brevibacterium*, *Microbacterium*, *Pseudomonas*, and *Xantomonas*.

Endophytic bacteria generally derive from the surrounding soil as originally proposed by Galippe in the early 1877, when he postulated that soil microorganisms were able to penetrate plant tissues (Galippe 1887). From the surrounding soil, especially rhizosphere,

bacteria can enter plant tissues switching to an endophytic lifestyle. Plant root exudates represent signals for bacterial chemotactic movement from rhizosphere to roots. In order to penetrate plant roots, bacteria need to attach to the root surface and successively move along the root surface searching for a suitable point for penetration, such as tissue wounds, germinating radicles, emergence points of lateral roots or root elongation and differentiation zones (Reinhold-Hurek et al. 2018), (Sturz et al. 2010). Figure 1 represents soil-inhabiting bacteria colonization of plant's rhizosphere and roots, pointing out bacterial concentration at an emergence point of lateral roots.

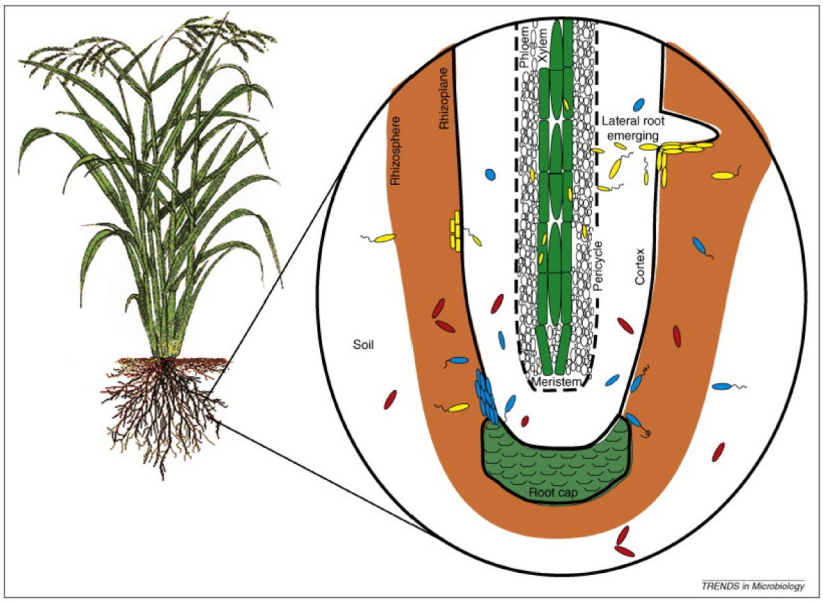

Figure 1. Root colonization by endophytic bacteria. Soil-inhabiting bacteria may become endophytic by chance (via colonization of natural wounds or following root invasion by nematodes). Such bacteria are considered passenger endophytes (red cells) and are often restricted to the root cortex tissue. Opportunistic endophytes (blue cells) show particular root colonization characteristics (chemotactic responses). However, as occurs with passenger endophytes, opportunistic endophytes are confined to particular plant tissues (the root cortex). Competent endophytes (yellow cells) are proposed to have all properties of opportunistic endophytes and, in addition, be well adapted to the plant environment (Hardoim et al. 2008).

The bacterial ability to produce degrading enzymes, such as endoglucanase, helps penetrating the host cell wall and entering plants internal tissues (Reinhold-Hurek et al. 2006). Once inside plants tissues, bacteria need to adapt to different environmental conditions (pH, osmotic pressure, carbon source, availability of oxygen) and to overcome plant defense responses (Gerber et al. 2006). Endophytic bacteria can either establish at the entrance point of plant tissues or spread within the plant (Hurek et al. 1994) (Hallmann, Mahaffee, and Kloepper 2011), by means of twitching motility (Böhm, Hurek, and Reinhold-Hurek 2007), flagella (Buschart et al. 2012), and the production of cell wall-degrading enzymes (Compant, Clément, and Sessitsch 2010), colonizing intercellular spaces (Patriquin and Dobereiner 1978) and the vascular system (Hurek et al. 1994), (Bell, Dickie, Harvey & Chan, 1995). A hint on the origin of endophytic bacteria can be provided by the analysis of their genome organization (Andreote, Gumiere, and Durrer 2014): bacteria presenting large genomes are believed to live within soils, where the environmental conditions are more variable; bacteria living inside plant tissues instead are considered to have smaller genomes, since plant organs may offer more stable conditions, compared to the soil (Reinhold-Hurek, Nowak, and Sessitsch 2013).

The plant host and the bacterial endophyte create an interaction, which is generally considered to be mutualistic, where the microorganism acquires nutrients and a niche to colonize and helps the host plant with increased stress tolerance, control of pathogens, induction of systemic resistance and improved nutrient uptake (Sturz et al. 2010).

Some studies have addressed the question of which genetic characteristics are required for a bacterial cell to determine, at a certain point of its lifecycle, the establishment of endophytic lifestyle. Mitter and colleagues (Mitter et al. 2013) analyzed genome sequences of different bacterial endophytes and evidenced common traits shared by the different analyzed genomes, as the detoxification of reactive oxygen species (ROS), plant polymer-degrading enzymes, and prominent quorum-sensing. On the other hand, the analyzed endophytes showed to adopt different strategies for the expression of some other important phenotypes for the endophytic lifestyle, as the acquisition of iron and communication strategies. The comparative genomic studies of Mitter and colleagues have also evidenced that seven out of the eight analyzed genomes show relatively stable genomes, suggesting that horizontal gene transfer is not the main mechanism adopted for adapting to variable environmental conditions.

The exact role of endophytes within plant tissues has not been fully understood yet, although it is well-established that in many cases endophytes are beneficial to plants (Schlaeppi and Bulgarelli 2015), (Wani et al. 2015). The interaction between plants and

microorganisms probably began when higher plants first appeared on the earth and it is possible that, at some point of this ancient association, endophytic microorganisms developed genetic systems allowing them to transfer information to the plant host, resulting in the evolution of biochemical pathways, for example, for phytohormones production. This scenario could explain the occurrence of many plant-beneficial endophytic phenotypes. The most common functions observed for bacterial endophytes are listed below:

- Uptake of nutrients, such as nitrogen, phosphorus, sulphur, magnesium, and calcium (Aydın and Fikrettin 2006)
- Plant growth promoting activity by phytohormone biosynthesis (Spaepen, Vanderleyden, and Remans 2007), 1-aminocyclopropane-1-carboxylate deaminase activity (ACC) (Glick et al. 2007), nitrogen fixation (Doty et al. 2009), siderophore production (Duijff et al. 1999)
- Prevention of pathogenic effects of parasitic microorganisms (Pérez-García, Romero, and De Vicente 2011), (Weller 2007)
- Plant tolerance to pollution or stresses (Ryan et al. 2008), (Lugtenberg and Kamilova 2014)
- Increased plant ability to adapt to variable environmental conditions (Bell et al. 1995)
- Accelerated seedling emergence (Hardoim et al. 2008)

Plant growth promotion activity was highlighted for many bacterial endophytes within cereals (Celador-Lera, Jiménez-Gómez, Menéndez, and Rivas 2018), and generally occurs due to a combination of different mechanisms, such as improvement of the host's nutrient status, promoting of the root surface area and increasing of the availability of nutrients to the plants. Furthermore, endophyte's potential to improve plant growth was also observed to persist when the isolates were reinoculated to the same plant host (Pérez-Montaño et al. 2014). Figure 2 (Celador-Lera et al. 2018) graphically represents the main mechanisms adopted by bacterial endophytes which determine plant growth promotion.

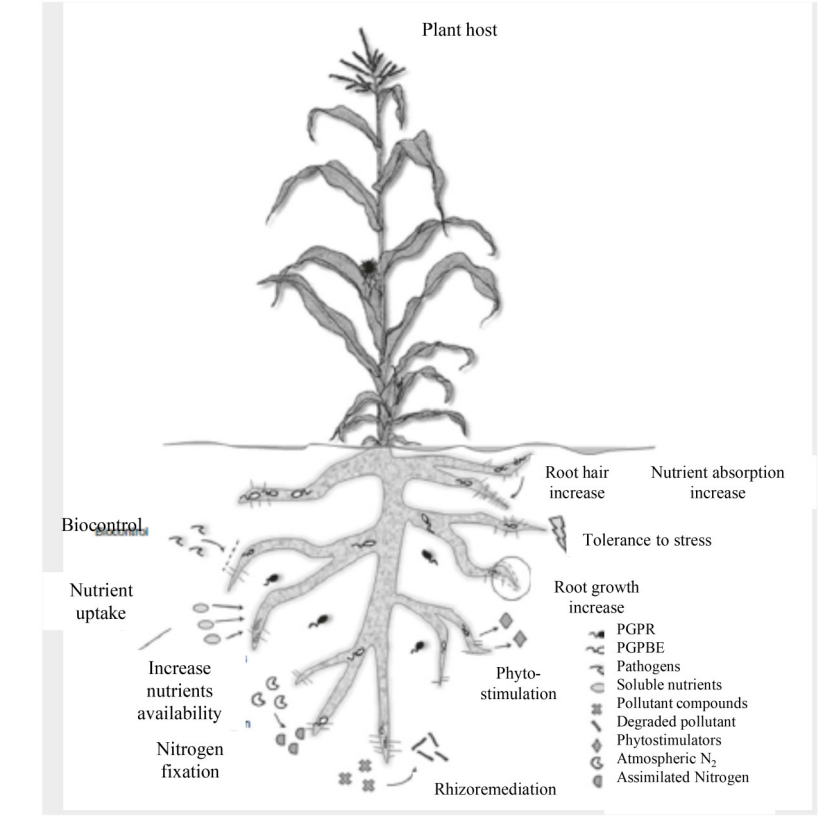

Figure 2. Schematic representation of plant growth-promoting mechanisms of bacterial endophytes (Celador-Lera et al. 2018).

1.1.2 The plant holobiont

The main endophytic functions show that these microorganisms may strongly influence the host plant physiology. Consequently, it was suggested that plants should no longer be seen as standalone organisms, but should be considered together with their microbiome, as a unique entity, the so-called holobiont (Pérez-Montaño et al. 2014). An evidence of the relevance of endophytes for plants physiology is also demonstrated by the difficult of culturing transplants of different species in the absence of bacteria and fungi (Hardoim et al. 2008). Furthermore, the holobiont vision (Pérez-Montaño et al. 2014) implies that evolution acts on it as a single dynamic entity, determining variation in the host or in the microbiota genomes (Zilber-Rosenberg and Rosenberg 2008). Relatively rapid responses to environmental changes could be determined by variations in microbial communities resulting in the adaptation of the whole holobiont. Adaptation and evolution would then be subjected to the hologenome plasticity and to the capacity of cooperation between its components (Zilber-Rosenberg and Rosenberg 2008).

1.1.3 Isolation and identification of endophytic bacterial strains from different sources

The first step for the isolation of endophytes is to properly select the host plant. Generally, endophytes are isolated from a certain host plant, considering its biology, age, endemism, ethnobotanical history and environmental setting (Strobel 2003). In this way, we establish a rationale for the exploration of endophytes. For example, we could wonder if the therapeutic properties of a medicinal plant could be related to the presence of endophytic bacteria or, as well, if the detoxification potential of hyperaccumulator plants are due to the endophytic bacterial population. Thus, the isolation of endophytes may help answering to specific biological questions and may lead to the discovery of interesting endophytes from the taxonomical and/or phenotypic point of view. For example, endophytic bacteria from medicinal plants may participate directly or indirectly in the production of molecules with therapeutic properties. As well, endophytes from hyperaccumulator plants may present the ability of degrading toxic compounds. Once the plant is selected, small portions are harvested and stored at 4°C until isolations procedures begin. The isolation of bacterial endophytes proceeds from the sterilization of the surface of plants' parts, in order to eliminate epiphytic bacteria. Disinfection of plant surfaces is usually achieved by treatment with sodium hypochloride, ethanol or similar agents (Balandreau, Lyon, and Lyon 2001). It

is important to properly choose the surface sterilization treatment to eliminate epiphytes without compromising inner tissues and consequently endophytes. Regarding taxonomic characterization of endophytes, it can be performed on cultivable bacterial communities or on total bacterial communities. Analyses on cultivable populations are influenced by growth media, growth conditions and cultivability of strains. The composition of cultivable bacterial communities is assayed by the amplification and sequencing of 16S rRNA gene from a single isolate. On the contrary, molecular approaches overcome the limitations of classic isolation methods. In this case, amplification and sequencing of 16S rRNA genes are performed on total DNA isolated from the plant or specific plant parts, determining the composition of total bacterial communities. Other molecular techniques, such as DGGE, TGGE and T-RFLP, can be applied to study population structure and dynamics.

Furthermore, bacterial endophytic communities can be screened for the presence of specific phenotypes by amplifying certain genes, such as the *nif* genes required for nitrogen fixation or genes involved in the degradation of toxic molecules. Endophytic bacteria have been isolated from a wide variety of plants, both monocotyledonous and dicotyledonous plants, ranging from woody tree species to herbaceous crops (Lodewyckx et al. 2010). Different plants showed to be simultaneously colonized by a variety of bacterial endophytes, comprising more than 90 different bacterial genera. However, specific plant groups as medicinal and hyperaccumulator plants still lack deep exploration of their bacterial microbiome. Considering the importance of such microorganisms on the plant physiology, it is extremely necessary to further characterize these bacterial communities from both taxonomic and phenotypic point of views.

1.1.4 How can research on endophytes be beneficiary?

Today there is the urgent need for new bioactive molecules with antibiotic, antimycotic and anti-parasitic properties, in order to address the alarming issues of antibiotic-resistance, increasing fungal infections by weakened-immune system patients and low availability of drugs for malaria, leshmaniasis, trypanosomiasis and filariasis. Another important issue worldwide is the need to reduce environmental pollution, for example by enhancing remediation processes and degrading toxic compounds.

The metabolic potential of endophytes and plant-associated microbiome has been well-evidenced with many pharmaceuticals being isolated from the plant microbiome, as the well-known Taxol. In fact, natural products play a key role in drugs discovery. From 1981 to 2010, 26% of the new chemical entities introduced to the market, were natural products and/or their derivatives. In 2010 the percentage of new chemicals with natural origin

reached the percentage of 50% (Köberl et al. 2013). Endophytes have also shown great potential in the environmental field, demonstrating to be able enhance phytodepuration, by degrading toxic compounds as phenolic pollutants, herbicides, pharmaceutical products, and others (Ho et al. 2012), (Germaine et al. 2010), (Ijaz et al. 2015), (Sauvêtre et al. 2018). These evidences do not imply that microorganisms' potential has been deeply explored and cannot offer anything more. On the contrary, their metabolic potential has only been poorly investigated, as demonstrated by the recent genomic revolution (Brader et al. 2014), (Miao and Davies 2010). In particular, bacterial biodiversity has also been poorly explored: only 5% of the estimated number of bacterial species has been documented (Adewale et al. 2015). Thus, both bacterial metabolic repertoire and biodiversity can still offer many exciting and useful possibilities. Since there is still much to discover, why should we venture into the untapped world of the plant-associated microbiome? Research on microbial endophytes and plant-associated microorganisms can be beneficial for many reasons; the most notable ones are:

- Microbial endophytes can produce compounds similar to those produced by the plant host itself, so that they constitute a cost-effective and renewable source of novel natural molecules (Shaanker 2009)
- Endophytic infection has shown to be able to profoundly influence the bioactive constituent profile of medicinal plants. For example, the endophytic *Pseudonocardia* YIM 63111 strain determines the up-regulation of genes involved in artemisin synthesis (Li et al. 2012), as well as alkamide production by the medicinal plant *Echinacea purpurea* was demonstrated to be modulated by bacterial endophytic strains isolated from the same plant species (Maggini, De Leo, et al. 2017)
- Secondary metabolites produced by endophytes may have reduced cell toxicity since they are produced within an eukaryotic system, the plant host, which naturally selects low-toxic compounds, in order to preserve its tissues integrity (Strobel 2003)
- The recent genomics revolution has led to the development of new technologies addressing natural products biosynthesis from endophytes. For example, biosynthetic gene clusters can be analyzed without having complete genome sequences (Zazopoulos et al. 2003), (Wilkie et al. 2001), thanks to the whole-genome sequence mining (Lautru et al. 2005) and genome scanning

- The use of new natural drugs of endophytic origin not only address the antibiotic-resistance problem, but also prevents the occurrence of pathogens' resistance. Natural therapeutic compounds usually consist in a blend of different active substances, which would require many mutations in the pathogen's genome to determine resistance
- Isolation of bioactive compounds from endophytes do not affect environmental biodiversity as the harvesting of plants, necessary to obtain therapeutic material directly from plants
- Endophytes have also gained attention as biocatalysts in chemical transformations of natural products and drugs due to their ability to modify chemical structures with a high degree of stereospecificity and to produce enzymes that facilitate the production of compounds of interest (Pimentel et al. 2011)
- Endophytic microorganisms have been characterized with high resistance to heavy metals and antimicrobials and ability to degrade toxic compounds (Germaine et al. 2004), (Germaine et al. 2010), suggesting their possible application to improve phytodepuration (Siciliano et al. 2001), (Barac et al. 2004).

1.2 Diversity and composition of bacterial endophytic communities

The study of plant-associated microbiome from a taxonomical point of view is of primary importance towards a better comprehension of the interactions existing between the plant host and the associated microorganisms. These analyses further contribute to the use of endophytes in many fields, such as agriculture, industry and pharmaceutics/clinics.

1.2.1 Bacterial communities associated to medicinal plants

In the last decades, the growing evidence of metabolic potential of medicinal plants-associated microbiome has led to its characterization. However, despite the growing attention medicinal plants microbiome has gained recently, there is still much to be explored since few plants have been studied so far. Table 1 illustrates different medicinal plants and their associated bacterial communities.

Table 1. Bacterial communities associated to different species of medicinal plants.

Host plant	Compartment	Dominant bacterial endophytes	References
Fritillaria thunbergii	Rhizospheric and non-rhizospheric soil	Proteobacteria Actinobacteria Acidobacteria Bacteroidetes	(J.-Y. Shi et al. 2011)
Rumex patientia	Non-rhizospheric soil Rhizosphere	Proteobacteria Acidobacteria Bacteroidetes Proteobacteria Bacteroidetes Acidobacteria	(Qi, Wang, and Xing 2012)
Origanum vulgare	Roots and rhizosphere	*Pseudomonas, Stenotrophomonas, Bacillus*	(Bafana 2013)
Lavandula angustifolia	Rhizosphere Roots Stem Leaves	*Bacillus, Pseudomonas, Microbacterium* *Rhizobium, Pseudomonas, Stenotrophomonas* *Pseudomonas* *Pseudomonas, Pantoea, Microbacterium*	(Emiliani et al. 2014)
Echinacea purpurea *Echinacea angustifolia*	Rhizosphere Roots Stem/leaves Rhizosphere Roots Stem/leaves	*Pseudomonas* *Pseudomonas* *Staphylococcus* *Pseudomonas* *Pseudomonas* *Staphylococcus*	(Chiellini et al. 2014)
Aloe vera	Root Stem Leaves	Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes	(Adewale et al. 2015)
Aloe vera	Root Stem Leaves	*Pseudomonas, Bacillus, Enterobacter* Enterobacter, Bacillus, Pseudomonas *Bacillus, Pseudomonas*	(Akinsanya et al. 2015)
Sapindus saponaria L.	Rhizosphere	Actinobacteria, Proteobacteria, Proteobacteria	(Polonio et al. 2016)
Curcuma longa L.	Rhizome	*Bacillus, Pseudomonas, Clavibacter*	(Kumar et al. 2016)
Panax notoginseng	Rhizosphere healthy plants	Proteobacteria, Cyanobacteria, Actinobacteria,	(Tan 2017)

		Acidobacteria *Pseudomonas, Rhodoplanes, Candidatus Solibacter, Streptomyces*	
	Rhizosphere diseased plants	Proteobacteria, Cyanobacteria, Actinobacteria, Acidobacteria *Pseudomonas, Rhodoplanes, Candidatus Solibacter, Streptomyces*	
	Roots healthy plants	Cyanobacteria, Proteobacteria, Actinobacteria, Acidobacteria *Pseudomonas, Candidatus Solibacter, Rhodoplanes, Streptomyces*	
	Roots diseased plants	Cyanobacteria, Proteobacteria, Actinobacteria, Acidobacteria *Pseudomonas, Candidatus Solibacter, Rhodoplanes, Streptomyces*	
Thymus vulgaris	Rhizosphere, root and leaves	Proteobacteria, Firmicutes *Bacillus, Pseudomonas*	(Checcucci et al. 2017)
Thymus citriodorus	Rhizosphere, root and leaves	Proteobacteria, Firmicutes *Bacillus, Pseudomonas*	
Panax ginseng Meyer	Root, stem and leaves	Proteobacteria, Firmicutes *Pseudomonas, Bacillus*	(Chowdhury et al. 2017)

In the analyzed medicinal plants shown in Table 1, Proteobacteria phylum and *Pseudomonas* genus emerge as the most dominant taxa. The genus *Bacillus* is also well-represented. *Pseudomonas* and *Bacillus* have been documented as the most common plant growth promoting-rhizobacteria which can determine enhance of biomass, nitrogen and phosphorus uptake, and crop yield (Haldar and Roy 2011). On the other hand, Tan and colleagues (Tan 2017) evidenced that *Pseudomonas* genus was more abundant among rhizospheric and root-associated microbiome of *Panax notoginseng* diseased plants than in healthy plants, suggesting that such genus could be related to the pathogenic effects that continuous cropping determine in *P. notoginseng*. Furthermore, research on medicinal plants microbiome allows to discover bacterial genera not yet associated to an endophytic-lifestyle, such as *Chryseobacterium*, *Sphingobacterium*, *Macrococcus*, *Shigella* and *Klebsiella* from *Aloe vera* plants, described by Akinsanya and colleagues (Akinsanya et al. 2015).

An important aspect that emerges from the analyses of different medicinal plants microbiome is the specificity of the bacterial communities associated to different plant compartments. Such specificity has been highlighted among the endophytic communities of different plant tissues (roots, stem and leaves) from *Echinacea* spp. (Chiellini et al. 2014) and *Lavandula angustifolia* plants (Emiliani et al. 2014), and also between rhizospheric and non-rhizospheric soil (Qi et al. 2012). *Echinacea* spp. and *L. angustifolia* showed different bacterial communities composition within the analyzed plant tissues. As well, non-rhizospheric and rhizospheric soil from *Rumex patientia* shared only 2.76% of the associated bacterial taxa.

The taxonomic composition of the plant microbiota can be also related to the essential oil produced by the host medicinal plant. Such evidence emerged from the analysis of endophytic bacterial communities associated to two phylogenetically close medicinal plants, *Thymus vulgaris* e *T. citriodorus* (Checcucci et al. 2017). The plants microbiome showed different tolerance levels to the plant's essential oil, which may determine the selection of certain bacterial taxa.

Also, continuous cropping and plant disease can influence the structure of plants microbiome. Tan and colleagues (Tan 2017) reported that both these factors determine a reduction of bacterial diversity in rhizospheric and root communities of *P. notoginseng*.

Very interesting evidences came from the analysis of the microbiome associated to medicinal plants cultivated in a desert farm under organic management in Egypt (Köberl et al. 2013). The microbiome associated to the three medicinal plants, *Matricaria chamomilla* L., *Calendula officinalis* L., and *Solanum distichum* Schumach. and Thonn., turned out to

be different from the microbiome present in plants from other soils (i.e. desert soil uninfluenced by anthropogenic activity and humid soils). The microbiome of the analyzed medicinal plants, grown in the desert farm, were enriched with high abundance of Gram-positive bacteria from native desert soil, which were mainly represented by *Firmicutes*. *Bacillus* and *Paenibacillus* were the most predominant genera and are characterized by drought-resistance and pathogen-suppression. On the other hand, the plants microbiome presented reduced extremophilic bacterial groups, such as *Acidimicrobium* and *Rubellimicrobium* (Köberl et al. 2011). Regarding bacteria exhibiting nitrogen-fixing phenotypes, each medicinal plant presented a specific root associated diazotrophic microbiome. The rhizosphere microbiomes of both *M. chamomilla* and *C. officinalis* showed to be dominated by potentially root-nodulating rhizobia, acquired from the soil. *S. distichum* instead showed a rhizospheric microbiome dominated by free-living nitrogen fixers most likely transmitted among plants, since these were not detectable in soils. Furthermore, total bacterial communities were very similar between *M. chamomilla* and *C. officinalis*. The similarities occurring between *M. chamomilla* and *C. officinalis* microbiomes, in comparison to *S. distichum,* could be due to the sharing of the same family *Asteraceae* by the first two, from which derives a similar bioactive metabolites profile. The distinctive and intriguing evidence that emerged from this study on medicinal plants growing in a desert soil is that the plants tend to select from soil those microbial populations which can provide a better fitness to the plant, as Firmicutes phylum, characterized by high resistance in arid environments.

Research on actinobacterial communities associated to medicinal plants highlighted that the most represented genus is *Streptomyces* (Nalini and Prakash 2017). Strains belonging to *Streptomyces* genus are deeply analyzed because of their notable metabolic potential, in fact most of antibiotics of natural origin (as Neomycin, Cypemycin, Grisemycin, Bottromycins and Chloramphenicol) are produced by *Streptomyces* strains (Köberl et al. 2011). Actinobacterial endophytic communities' distribution and biological diversity are influenced by ecological environment and characterized by high functional diversity (Nalini and Prakash 2017).

1.2.2 Bacterial endophytic communities associated to plants' seeds

Seeds consist in a very important phase of the life cycle of spermatophytes, since they can persist for a long time in a dormant state and germinate, developing a new plant when favorable conditions are met. Interestingly, not only seeds can remain alive for many years, but also the microorganisms residing within plant seeds can persist for such a long time.

López and colleagues pointed out that bacterial endophytes in *Medicago sativa* seeds remained alive for more than 5 years, since harvesting and analyses carried by them required this period of time (Luis et al. 2018). Seed-associated microorganisms play a role in seed preservation and preparation for germination, facilitating this process (Chee-sanford et al.). Once germination starts, seed exudates can attract bacteria from the surrounding environment, so that seed-associated microbiome can be constituted both by endophytes already present within seeds, transmitted from parental line, and by recruitment of microorganisms in the surrounding environment (Nelson 2004). Seed-microbiome was described as contributing to many plant functions, such as:

- Direct plant growth promotion by nitrogen fixation; mobilization of nutrients such as phosphorus and iron by production of organic acids and siderophores; production of auxins and cytokinins; suppression or reduction of ethylene stress by ACC deaminase (Weyens, Lelie, and Taghavi 2009);
- Indirect plant growth promotion by preventing the growth or activity of phytopathogens through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of toxins and induction of plant defense mechanisms (Weyens et al. 2009).

Seed-borne endophytes are particularly interesting since they can be transmitted from generation to generation. Thus, by means of seeds, the parental line can assure the transmission of beneficial symbionts to progeny. In fact, seed-microbiome should be considered as a reservoir of important genes, that integrate the plant second metabolism.

Despite the biological relevant and intriguing nature of seed-associated endophytes, these still remain the less explored portion of the plant microbiome. In fact, information on their communities' assembly is still very scarce (Klaedtke et al. 2016).

Seed endophytes have been described with specific phenotypes that favor the colonization and persistence within seeds. In fact, such specific phenotypes are rarely found among endophytes isolated from root, shoots or other plant tissues (Shahzad et al. 2018). The main phenotypes associated with seed bacterial endophytes are:

- Tolerance to a high osmotic pressure (Elbeltagy et al. 2012), (Mano et al. 2007)
- favorable during seed maturation, characterized by accumulation of starch and loss of water
- Endospore formation, which offers protection from environment changes within seeds (Mano et al. 2007), (Compant et al. 2011)

- Amylase activity, that allows utilization of starch and resumption of growth after long-term survival within seeds (Mano et al. 2007)
- Utilization of phytate, the main storage form of phosphorus in seeds (López-López et al. 2010)
- Motility, which enables migration into seeds, described for almost all seed endophytes from rice seeds (Elbeltagy et al. 2012), (Okunishi, S., Sako, K., Mano, H., Imamura, A., Morisaki 2005)
- Phosphorus solubilization, acetoin secretion, nitrogen fixation, highlighted for endophytes from seeds of different maize genotypes (Johnston-Monje and Raizada 2011)
- ACC deaminase activity and antibiosis, also described among maize seed endophytes (Johnston-Monje and Raizada 2011)

Colonization of plant seeds by bacteria is not only favored by specific phenotypes, as described above, but diversity of bacterial genera also seems to be important. The early stages of seed development were mainly related to the presence of Gram-negative bacterial endophytes, such as *Methylobacterium* spp. and *Sphingomonas* spp., while matured seeds were mainly colonized by Gram-positive isolates, for example *Bacillus* spp. and *Curtobacterium* spp. (Mano et al. 2007).

The composition of seed-associated bacterial microbiome has been analyzed for different plant species. The most dominant phylum observed in seed bacterial endophytic communities is Proteobacteria, mainly γ-Proteobacteria. Actinobacteria and Firmicutes phyla are also very represented, while Bacteroidetes is the least represented. Such composition is similar to the composition of bacterial endophytic communities from plant tissues, where Proteobacteria is generally the dominant phylum and Bacteroidetes is less represented (Rosenblueth and Martínez-Romero 2006). It could suggest that the plant microbiome derives from the seed microbiome. Concerning bacterial genera, the most described in the seed-associated microbiome of many plants are *Bacillus* and *Pseudomonas*. Other well-represented genera are *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter*. Table 2 shows a summary of bacterial endophytes isolated from seeds of different plant species.

Table 2. Bacterial endophytes associated to seeds of different plant species.

Seed host	Dominant bacterial endophytes	References
Vitis vinifera	Firmicutes	(Compant et al. 2011)
Eucalyptus	Actinobacteria, Firmicutes, α-Proteobacteria	(Ferreira et al. 2008)
Oryza sativa	Actinobacteria, Bacteroidetes, Firmicutes, α- Proteobacteria, β-Proteobacteria, γ-Proteobacteria	(Mukhopadhyay et al. 1996) (Elbeltagy et al. 2012) (Aguilar-flores, Valle, and Pe 2001) (Cottyn et al. 2000) (Okunishi, S., Sako, K., Mano, H., Imamura, A., Morisaki 2005) (Mano et al. 2007) (Tripathi et al. 2018) (Kaga et al. 2009) (Ruiza et al. 2011) (Hardoim et al. 2012)
Pachycereurs pringlei	Firmicutes, γ-Proteobacteria	(Puente, Li, and Bashan 2009)
Coffea arabica	Actinobacteria, Firmicutes, β- Proteobacteria, γ-Proteobacteria	(Vega et al. 2005)
Brassica napus	Actinobacteria, Bacteroidetes, Firmicutes, β- Proteobacteria, γ-Proteobacteria	(Persson, Meijer, and Alstro 2003)
Zea mays	Actinobacteria, Bacteroidetes, Firmicutes, α- Proteobacteria, β-Proteobacteria, γ-Proteobacteria	(Dunleavy 2018) (Rijavec et al. 2007) (Johnston-Monje and Raizada 2011) (Liu, Zuo, and Xu 2012) (Liu et al. 2012)
Fraxinus	Actinobacteria, Firmicutes, γ-Proteobacteria	(Donnarumma et al. 2011)
Glycine max	Actinobacteria, Firmicvutes, γ-Proteobacteria	(Kremer 1987)

		(Dunleavy 2018) (Oehrle et al. 2000)
Triticum aestivum *Elymus trachycaulus* *Agropyron fragile*	Actinobacteria, Firmicutes, γ-Proteobacteria	(Coombs and Franco 2003). (Ringelberg, Foley, and Reynolds 2012)
Beta vulgaris	Firmicutes,	(Dent, Stephen, and Finch-Savage 2004)
Pisum sativum	Firmicutes,	(Sedla, Durnova, and Smı 2018)
Cucurbita pepo	Firmicutes, γ-Proteobacteria	(Fürnkranz et al. 2012)
Arachis hypogaea	Firmicutes, γ-Proteobacteria	(Sobolev, Orner, and Arias 2013)
Brassica oleacea	Firmicutes	(Pleban, Ingel, and Chet 1995)
Lycopersicum esculentum	Firmicutes	(Xu et al. 2014)
Fragaria	γ-Proteobacteria	(Kukkurainen et al. 2005)
Arabidopsis thaliana	Actinobacteria, Firmicutes, α- Proteobacteria, β-Proteobacteria, γ-Proteobacteria	(Truyens et al. 2013)
Lolium multiflorum *Phleum pretense* *Panicum virgatum* *Agrostis capillaris*	Actinobacteria, Firmicutes, γ-Proteobacteria	(Ikeda et al. 2006) (Gagne-Bourgue et al. 2013)
Broadleaf weed species	Bacteroidetes, Firmicutes, β- Proteobacteria, γ-Proteobacteria	(Kremer 1987)
Herbaceous and woody species	Actinobacteria, Bacteroidetes, Firmicutes, β- Proteobacteria, γ-Proteobacteria	(Kremer 1987)

1.2.3 Bacterial communities associated to the hyperaccumulator plant, *Phragmites australis*

Constructed wetlands (CWs) have gained increasingly value as reliable biological technology for wastewater treatment and are characterized by high economic benefits, environmental friendliness and high pollutant removal efficiency. *Phragmites australis*, also known as common reed, is one of the most distributed plant species all over the world (Borruso et al. 2014) and is commonly used in CWs since it is able to flourish in marshy areas and swamps. It has also been described as having high detoxification and phytodepuration potential and is widely used to treat industrial wastewater containing heavy metals (Zhang et al. 2017). One peculiar characteristic of *P. australis* is that its internal environment is characterized by a relatively constant osmotic gradient determined by the downward transportation of Na^+ from stems to roots (Vasquez et al. 2006). For this reason, *P. australis* is also well-adapted to salty ecosystems.

In CWs, vegetation is responsible for only a small amount of pollutants removal (0.02%) (Zhang et al. 2017), while its main function is to provide additional oxygen and organic matter for microorganisms' growth (Zhou et al. 2012). On the contrary, microorganisms have been described as the main actors of pollutants removal in CWs (Zhang et al. 2017). It is noteworthy that vegetation in CWs can influence the structure of microbial communities growing in association with them. In fact, plants have demonstrated to induce and stimulate the growth of specific bacterial taxa creating a well-defined bacterial community around the rhizosphere (Marschner et al. 2001). Different parameters linked to the macrophytes species influence microbial densities and compositions, such as root morphology, growth rate, production of root exudates and oxygen transfer (Philippot et al. 2013). It has also been evidenced that the removal of a specific pollutant in CWs is typically linked to a specific microbial functional group (Faulwetter et al. 2013).

Despite the proved importance of bacterial communities for the CWs functioning and efficiency, only a few studies have concentrated on the bacterial diversity within different *P. australis* tissues. Detailed knowledge of the microbial assemblages involved in phytodepuration processes is essential to better explain CWs functioning and to achieve growing improvement of the process.

Studies performed by Li and colleagues on endophytic bacterial communities from roots of *P. australis* plants growing in a CW in Beijing, China, (Li et al. 2011) showed that the most dominant phylum was Proteobacteria (78.9%), including Alpha, Beta, Gamma and Epsilon classes. The other most represented phyla were Firmicutes (9%), and

Cytophaga/Flexibacter/Bacteroides (CFB) (6.6%). The researchers also evidenced that the most represented bacteria taxa within *P. australis* roots were functionally related to nitrogen fixation, reduction of nitrate to nitrite, denitrification, sulphur cycle, toxic material removal, and utilization of organic phosphorus. These data suggest that endophytic bacteria in reed roots support and enhance phytodepuration, especially in relation to nitrogen and sulfur cycles and removal of organic matter during water purification.

The characterization of bacterial communities associated with the rhizosphere and deep-layer zone of a horizontal flow CW, planted with *P. australis* (Bouali et al. 2014), showed that the predominant portion of bacterial 16S rRNA gene sequences was affiliated to uncultured bacterial clones (79.86%) and that rhizospheric bacterial communities were characterized by higher diversity. In such compartment, Proteobacteria represented the most abundant group (41.57%), followed by Bacteroidetes (11.78%), Planctomycetes (9.36%), Cloroflexi (7.60%), Actinobacteria (5.67%) and Acidobacteria (3.27%). The analyses also focused on the variations of bacterial communities' composition in different periods of the year, showing that the major phylogenetic groups in rhizosphere remained unchanged.

The diversity of bacterial communities associated to *P. australis* was analyzed in a two-stage CW characterized by horizontal flow mode (Calheiros et al. 2009). One stage of the CW was planted with *Typha latifolia*, and the other one with *P. australis*. The different stages were colonized by different bacterial communities, and plant species and stage position (first or second in the series) seemed to have major effects on the dynamics of bacterial communities. Regarding roots of *P. australis*, the most abundant bacterial groups were represented by γ-Proteobacteria, Firmicutes and α-Proteobacteria.

The composition of bacterial and archaeal communities were assayed in different *P. australis* tissues (stem, leaves and roots) in relation to salinity gradient (Ma et al. 2013). The study highlighted that bacterial diversity was significantly higher than archaeal diversity. Furthermore, bacterial diversity was significantly higher in roots than in leaves, but it was similar in different sampling sites along the salinity gradient, suggesting that tissue type was more important than sampling zone in structuring the endophytic bacterial communities.

The structure of bacterial communities associated to *P. australis* plants grown in extreme conditions was assayed by Borruso et al. (Borruso et al. 2014). They examined rhizobacterial communities associated to *P. australis* grown in a hypersaline pond in China. Bacterial communities were dominated by the phylum Proteobacteria, followed by Firmicutes and Bacteroidetes. Interestingly, replicates from different sampling sites were

similar, highlighting the low variability of microbial communities exposed to the same environmental conditions. Salinity was pointed as one of the most important abiotic factors which shape microbial community structure, in comparison to the rhizosphere effect.

The effect of pollutants on the structure of rhizospheric bacterial communities from *P. australis* was evaluated considering *P. australis* grown in vertical flow CWs added with hexachlorobenzene (HCB) (Zhang et al. 2017). The most abundant bacterial populations in *P. australis* rhizosphere, under HCB stress, was Firmicutes and Bacteroidetes. Furthermore, analyses showed that some bacterial taxa were significantly developed following HCB contamination, as *Burkholderia* sp., *Pseudomonas* sp., *Achromobacter xylosoxidans* subsp., *Lysinbacillus fusiformis*, and *Bacillus cereus*. Overall, data from this study suggests that both plant species and contaminants can influence bacterial communities' diversity in the rhizospheric soil.

Table 3 presents a summary of the characterized bacterial communities associated to *P. australis* plants in CWs. It highlights that bacterial communities from *P. australis* have been mainly analyzed in the roots and rhizosphere of the plants. These compartments are characterized by high densities and diversity of bacterial strains and may be widely influenced by the action of plant root exudates.

Table 3. Bacterial communities associated to *P. australis* plants grown in CWs.

CW location	**Plant compartment**	**Dominant associated-bacteria**	**References**
Portugal	Roots	γ-Proteobacteria, Firmicutes	(Calheiros et al. 2009)
China	Roots	α- Proteobacteria, β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria	(Li et al. 2011)
China	Rhizosphere	Proteobacteria, Firmicutes, Bacteroidetes	(Borruso et al. 2014)
Tunisia	Rhizosphere	α- Proteobacteria, β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria	(Bouali et al. 2014)
China	Rhizosphere	Firmicutes, Bacteroidetes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Acidobacteria	(Zhang et al. 2017)

1.2.4 What does influence the structure of the plant-associated microbiome?

The plant microbiome and the plant host have demonstrated to strongly interact, so that the plant physiology is notably influenced by microorganisms, determining effects on plant nutrition, growth, metabolites production, susceptibility to pathogens and secondary metabolites (Maggini, Leo, et al. 2017), (Germaine et al. 2010). Thus, the preservation of beneficial microbial communities within plants microbiome can support plant growth or improve the production of certain molecules, as well as agricultural methods can impact on such aspects. For these reasons, the understanding of plant microbiome taxonomic and functional assemblage is of essential importance. However, our knowledge on the assemblage of bacterial communities associated to plants is very limited (Müller et al. 2016). Soil, especially the rhizosphere, has been pointed out as the main origin of the plant microbiome (Mitter et al. 2013). Furthermore, air-borne microorganisms and seed endophytes may concur to the assemblage of the plant microbiome (Sanchez-Canizares et al. 2017). Biotic and abiotic factors, such as soil type, climate, plant genotype, health and developmental stage have been highlighted as factors influencing the structure of rhizospheric microbial communities. Figure 3 indicates different plant compartments-associated microbiomes which contribute to the assemblage of the total plant microbiome, while figure 4 schematically represents factors shaping rhizospheric communities and mechanisms involved in the selection of microbial communities from soil.

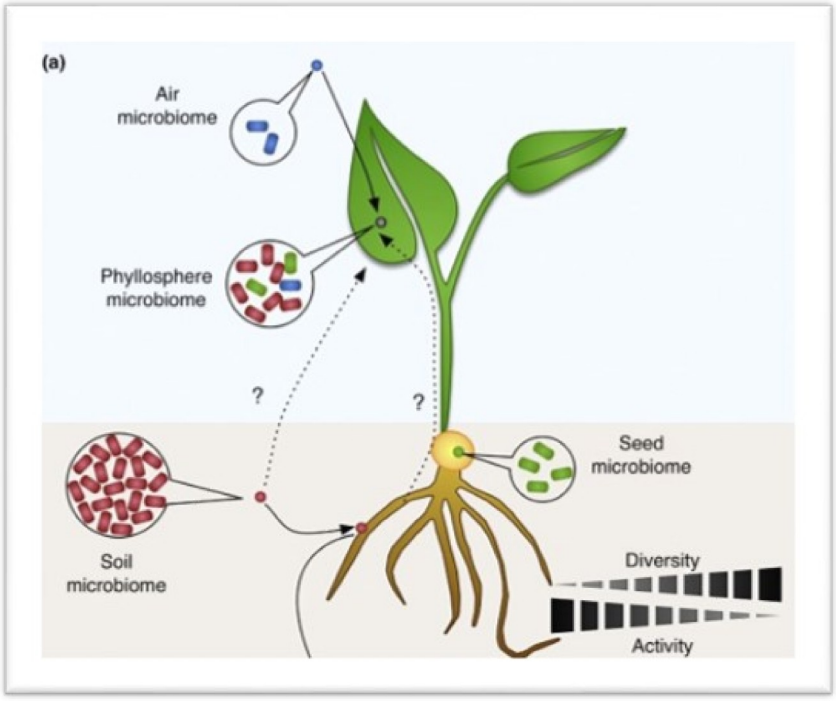

Figure 3. Schematic representation of different sources-associated microbiomes which contribute to the assemblage of the plant microbiome. Soil is the main origin of the plant microbiome, where the microbial diversity is inversely correlated with the activity of its community. The phyllosphere may be colonized by specific air-borne microorganisms and seeds may carry endophytes which will be part of the plant microbiome ((Sanchez-Canizares et al. 2017).

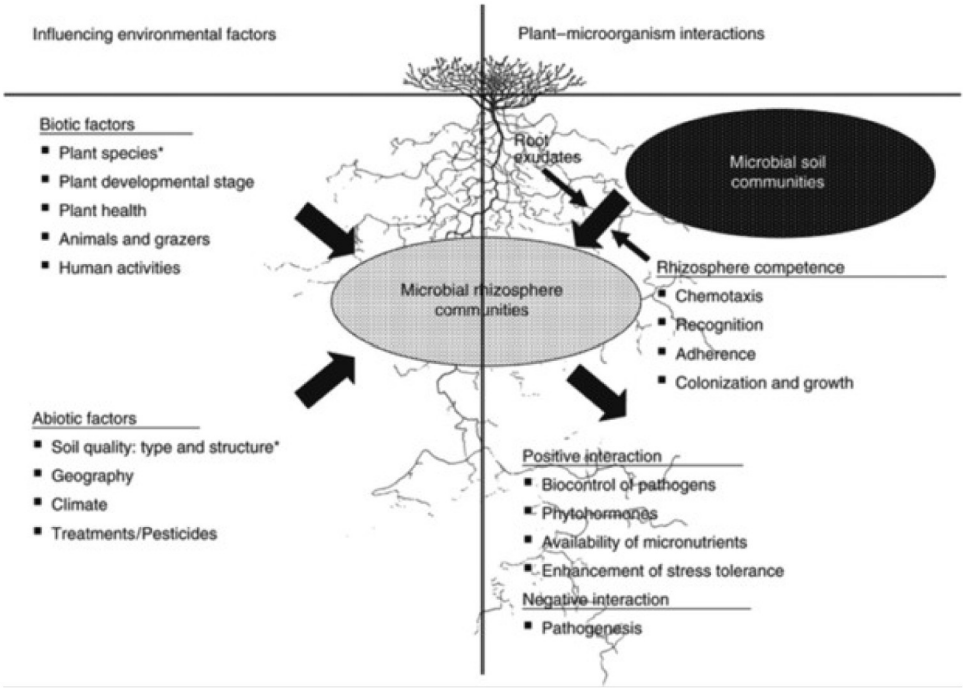

Figure 4. Factors influencing the rhizosphere microbial communities and mechanisms for such microbial communities selection (Berg and Smalla 2009).

The influence of plant species, soil type and root zone location on the composition of bacterial communities in the rhizosphere of three different plants was analyzed by Marschner and colleagues (Marschner et al. 2001). The authors evidenced the clear influence of plants species on the composition of rhizospheric communities of the three plants, chickpea, rape and Sudan grass. The study also showed that the bacterial communities were influenced by soil type and root zone location and by the interaction of these two factors, in different measures. For example, chickpea rhizospheric community was primarily influenced by soil type, while Sudan grass and rape were more influenced by root zone location. The three factors, plant species, soil type and root zone location, presumably interact together creating a complex network that shapes rhizospheric communities.

Growth stage and genotype of the host were also analyzed for their possible influence on the structure of the plant microbiome. Potato-associated bacterial communities (Van Overbeek and Van Elsas 2008) demonstrated to be influenced by the plant growth stage, indicating that such communities are structurally dynamic and change during the development of the plant. Plant genotype also influenced the structure of bacterial communities, in a lower measure in comparison to plant species. The effect of plant genotype on plant-associated communities might be linked to differences in the root exudates. Differently from rhizospheric communities, root-associated communities seem to be more influenced by soil microbial and environmental parameters than by plant species and genotype (Bulgarelli et al. 2012), (Lundberg et al. 2012), (Shakya et al. 2013).

Plants root exudates are one of the factors that are believed to greatly influence plant-associated bacterial communities. Root exudates contain nutrients and organic matter for microorganisms' development and may present unique secondary metabolites that influence the structure of microbial communities as observed for the medicinal plants, chamomile, thyme and eucalyptus (Berg and Smalla 2009). The plant root exudates were shown to promote the accumulation of certain bacteria taxa shaping the rhizospheric community of *Panax notoginseng* and *Fritillaria thunbergii* (Tan 2017), (Shi et al. 2011). Considering that plant endophytes mainly derive from the rhizospheric reservoir of microorganisms, factors acting on the assemblage of rhizospheric community, indirectly but strongly impacts on the structure of all microbial communities associated to plants.

The plant essential oil was observed to determine the selection of the bacterial taxa which colonize the plant tissues. Such evidences were obtained for bacterial endophytes from two phylogenetically close species, *T. vulgaris* and *T. citriodorus* (Checcucci et al. 2017), in

which the microbiota were strongly differentiated especially in relation to their tolerance to the essential oil the host plant produced.

Continuous cropping and plant disease also influence the structure of plant-associated communities, as shown for *P. notoginseng* (Tan 2017) in which bacterial diversity was notably reduced by these factors.

Analysis of rhizospheric and bacterial endophytic communities from *Echinacea* spp. medicinal plants (Chiellini et al. 2014) revealed specific bacterial communities' structure within the different plant tissues, leading to the question for the factors that could determine that high degree of specificity. In order to address this question, further studies have been conducted on these bacterial communities, extracted from *E. purpurea* and *E. angustifolia* plant tissues and rhizosphere. Thus, *E. purpurea* (Mengoni et al. 2014) and *E. angustifolia* (Maggini et al. 2018) associated bacterial communities were assayed on their antibiotic resistance profiles and on their capacity of exhibiting antagonistic interactions (Maida et al. 2016), (Maggini et al. 2018). Both plants associated bacterial communities showed different antibiotic resistance profiles, with higher resistance for rhizospheric and root compartments, while stem/leaves presented more susceptible strains. Considering antagonistic interactions, stem/leaves compartment was much more sensitive to antagonistic effect exerted by endophytic and rhizospheric bacteria than those isolated from the other two compartments. These data suggest that the stem/leaves compartment might be a less competitive environment, in which high antibiotic resistance is not required, thus bacterial taxa with less-antibiotic resistant phenotypes and producing lower antibiotic levels are selected to colonize such compartment. On the other hand, our data suggested that the rhizosphere and root compartment may be more competitive, requiring strong antibiotic-resistant bacteria able to persist in an environment characterized by high levels of antimicrobial molecules. In this scenario, the bacterial communities themselves could be seen as taking part in the shaping of their own communities' structure. Thus, together with many other factors, as those listed in the previous lines, bacteria could themselves determine the structure of plant-associated communities.

1.3 Bacterial endophytes as an untapped source of bioactive molecules

1.3.1 Genetic basis for endophytic bioactivity

Most part of natural secondary metabolites are synthesized by microorganisms and the synthesis of such molecules generally requires complex molecular tools called polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS). NRPSs use amino or

hydroxyl acids as building blocks and catalyze the formation of amide or ester bonds respectively. Figure 5 (Donadio, Monciardini, and Sosio 2007) shows NRPS modules, which present three core domains: an adenylation domain (A), a condensation domain (C) and a T domain. The A domain selects the cognate amino acid, activates it as an amino acyl adenylate and transfers it to the T domain, where a thioester bond is formed. The condensation domain is responsible for peptide bond formation between the amino acid present on the T domain of the same module and the peptidyl intermediate bound to the T domain of the preceding module, and the T domain itself. Furthermore, a loading module and a termination module contains a thioesterase domain (TE) are usually found in NRPS modular systems.

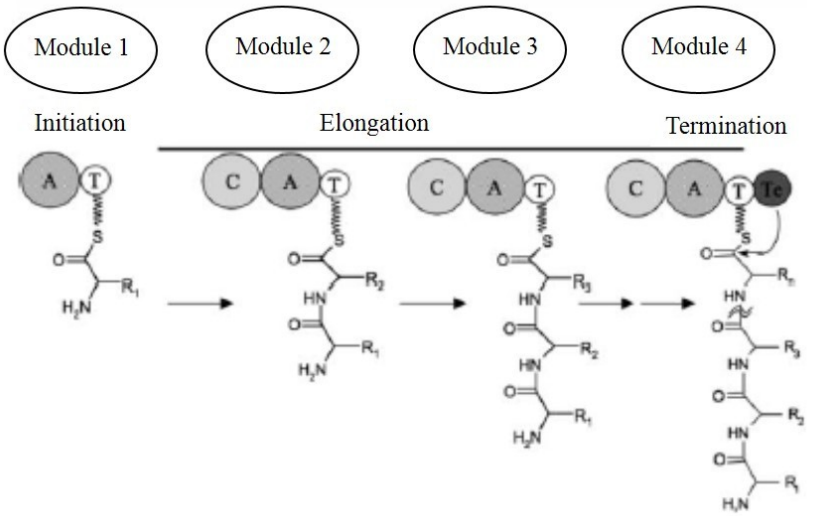

Figure 5. Nonribosomal synthesis of peptides: characteristic domains of the NRPS system and main steps in the peptides' synthesis (Donadio et al. 2007).

PKSs system, represented in figure 6 (Donadio et al. 2007), also consists of three core domains: an acyltransferase (AT) domain, an ACP domain, and a ketosynthase domain (KS). The AT domain selects the appropriate extender unit (usually malonyl-CoA or methylmalonyl-CoA) and transfers it to the ACP domain, where a thioester bond is formed. The KS domain determines the decarboxylative condensation between the extender unit present on the ACP domain of the same module and the polyketide intermediate bound to the ACP domain of the preceding module. The loading module lacks a functional KS domain and the last module contains an additional TE domain, which leads to the release of the finished polyketide from the PKS.

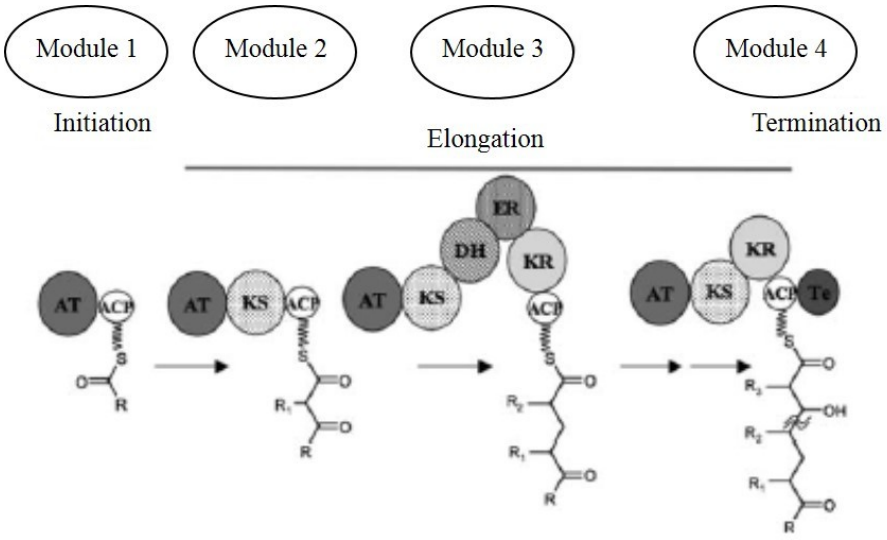

Figure 6. Polyketides synthesis: characteristic domains of the PKS system and main steps in the polyketides synthesis (Donadio et al. 2007).

PKS and NRPS genes are responsible for the synthesis of the most successful antibiotics (i.e. vancomycin, avermectin, erythromycin). About 50% of the completely sequenced bacterial genomes harbor PKS and NRPS genes (Donadio et al. 2007), which accounts for 0.6% of the available genome sequences. In particular, bacterial taxa characterized by the production of natural products, such as *Streptomyces*, *Bacillus* and *Pseudomonas*, present high percentages of PKS and NRPS genes in their genomes. Considering that *Streptomyces*, *Bacillus* and *Pseudomonas* genera are predominant in plant-associated bacterial communities, such communities are very good candidates for the discovery of natural products with biotechnological relevance.

Miller and colleagues (Miller et al. 2012) screened endophytic bacteria isolated from eight different medicinal plants for the presence of PKS and NRPS genes. The detection of KS (PKS) and A domain (NRPS) genes was obtained for 12% and 13% of the analyzed endophytes respectively. The evidenced KS sequences were similar to sequences in database belonging to *Paenibacillus* genus, which are well-known for the production of polymyxin and fusaricidin antibiotics (Choi et al. 2009), (Li and Jensen 2008). Regarding NRPS genes, the obtained sequences shared similarity with sequences from *Pseudomonas* and *Bacillus* strains, known for the synthesis of antimicrobials, siderophores and phytotoxins (Stein, Mikrobiologie, and Goethe- 2005), (Ansari et al. 2013).

The analysis of natural products biosynthesis pathways is of great value since it allows to elucidate the natural synthesis of these compounds and lead to the manipulation of the related gene clusters for the generation of new drugs (Fortman and Sherman 2005).

The strain *Rheinheimera* sp. EpRS3, isolated from the rhizosphere of *E. purpurea* (Chiellini et al. 2014), was reported as capable of inhibition towards human pathogenic bacteria from *Burkholderia cepacia* complex (Chiellini et al. 2017). In order to gain insight into the genetic basis of this antimicrobial activity, its genome sequence was assayed for the presence of genes enconding secondary metabolites (Presta et al. 2017). The analysis revealed the presence of 111 genes associated with the production of antimicrobial molecules. Among these, three biosynthetic clusters involved in the production of non-ribosomal peptides and polyketides, pointing out the important biotechnological relevance of this strain and of plant microbiome in general.

1.3.2 Secondary metabolites from medicinal plants-associated bacteria

The production of secondary metabolites by endophytic bacteria generally derives from the close interactions existing among such endophytes and their plant hosts. Secondary

metabolites deriving from the plant-endophyte interaction may exert important functions in the plant's physiology, such as nutrient uptake. Figure 7 (Brader et al. 2014) provides examples of different interactions among endophytes and the plant host, which lead to the synthesis of metabolites.

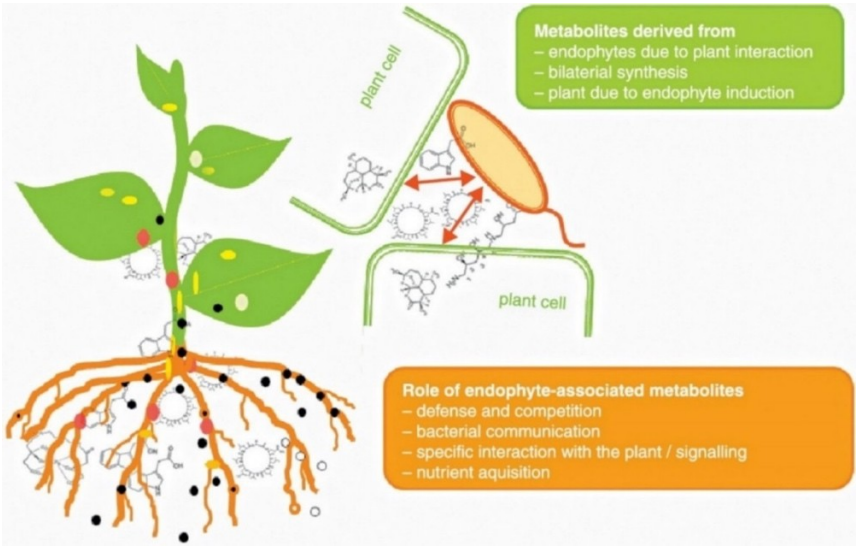

Figure 7. Examples of plant-endophytes interactions leading to the synthesis of metabolites and important functions of such metabolites (Brader et al. 2014).

Since natural products represent a large percentage of pharmaceuticals, accounting for the 50% of new drugs (Alvin, Miller, and Neilan 2014) introduced into the market from 1981 to 2010, bacterial endophytes represent a significant field of research.

Among endophytes, fungi are the most commonly isolated and studied microorganisms. In this context, Taxol is a great example of endophytic bioactive potential (Strobel 2003). Bacteria associated to medicinal plants have been lesser explored but are considered to harbor highly efficient endophytes with biocontrol properties. Also, they have recently been described as able of influencing the synthesis of medicinal plants secondary metabolites with therapeutic properties (Li et al. 2012), (Maggini, De Leo, et al. 2017). Analysis on the bioactivity of medicinal plants-associated bacteria have evidenced many bioactive molecules with different functions, revealing the presence of important antibiotic compounds already characterized and others never described before. *Bacillus* sp. BmB 9 was isolated from stems of *Bacopa monnieri* L., an Indian traditional medicinal plant exhibiting neurologic and gastrointestinal activity (Jasim et al. 2016). Such isolate showed inhibitory effect on the growth of phytopathogenic fungi *Rhizoctonia* sp., *Sclerotium* sp., and *Phytophtora* sp. Also, the isolate was tested for its antibacterial potential towards pathogenic strains, and was able to inhibit *Escherichia coli*, *Salmonella enterica Tiphy*, *B. subtilis*, *S. aureus* and *K. pneumoniae*. Interestingly, important antibiotics as Surfactin, Iturin and Fengycin were found among the bioactive fractions of the strain extracts. A broad-spectrum antimicrobial activity was highlighted for another *Bacillus* endophytic strain, isolated from an ancient oriental medicinal plant, *Andrographis paniculata* Nees (Roy et al. 2016). Such strain was able to inhibit bacterial pathogens such as *B. subtilis*, *B. cereus*, *Vibrio parahaemolyticus*, *Aeromonas caviae*, *Proteus vulgaris*, *P. aeruginosa*. The analysis of the isolate's extracts showed the presence of three different anti-infective metabolites and one of these was an anthracenic derivative. A novel group of bioactive substances, named Munumbicins, were isolated from *Streptomyces* sp. NRRL 30562, extracted from the stem tissue of the medicinal plant *Kennedia nigriscans* (Castillo et al. 2018). Munumbicins are active against plant-pathogenic fungi and human-pathogenic bacteria, comprising antibiotic-resistant strains. Munumbicin B showed to be active against multiple-drug resistant *Mycobacterium tuberculosis*. Very interestingly, each Munumbicin molecule was active against *Plasmodium falciparum*, the most pathogenic plasmodium causing malaria, which causes more than 400 000 deaths per year (World Health Organization 2015). Bacterial isolates from the medicinal plant *E. purpurea* showed inhibitory activity against human opportunistic pathogens of *Burkholderia cepacia* complex (Bcc), which cause severe infections in immunocompromised patients (Chiellini

et al. 2017). More than 97% of the tested strains presented inhibition on the growth of Bcc strains, with environmental and clinical origin. In particular, the root compartment presented the highest levels of inhibition, in comparison to the other plant compartments. The bioactivity of *E. purpurea* associated bacterial strains was also evidenced in the plant's rhizosphere. In fact, the strain *Rheinheimera* sp. EpRS3 exhibited complete inhibition of all the analyzed *B. multivorans* and *B. cenocepacia* strains, and complete inhibition of clinically relevant human pathogens as *Acinetobacter baumannii* N50 and *A. baumannii* YMCR 363 (Presta et al. 2017).

Table 4. Bioactive molecules isolated from bacterial strains associated to medicinal plants.

Microorganism	**Plant host**	**Metabolite**	**Metabolite biological activity**	**References**
Streptomyces sp. NRRL 30562	*Kennedia nigriscans*	Mumbicins A - D	Antifungal, antibacterial, anti Plasmodium falciparum	(Castillo et al. 2018)
Paenibacillus polymyxa EJS-3	*Stemona japonica* (Blume) Miquel	Exopolyssacharide	Scavenging activity on superoxide and hydroxyl radicals	(Liu et al. 2009)
Streptomyces sp LJK109	*Alpinia galanga*	3-methylcarbazole	Suppression of macrophage production of NO, PGE_2, TNFα, IL-1β, IL-6, IL-10	(Taechowisan et al. 2012)
Bacillus sp BmM 9	*Bacopa monnieri*	Surfactin, Iturin, Fengycin	Antifungal, antibacterial	(Jasim et al. 2016)
Streptomyces sp, *Kitasatospora* sp	*Lychnophora ericoides*	Salycilamide, nocardamine, propioveratrone and others	Antifungal, antibacterial, citotoxicity	(Chagas et al. 2016)
Pseudomonas sp, *Paenibacillus* sp, *Staphyloccocus* sp and others	*Echinacea purpurea*	Not determined	Antibacterial	(Chiellini et al. 2017)
Bacillus thuringiensis KL1	*Andrographis paniculate* Nees	Anthracene derivative	Antibacterial	(Roy et al. 2016)
B. subtilis	*Solanum trilobatum*	Not determined	Antibacterial	(Bhuvaneswari 2015)

Table 4 shows that medicinal plants-derived endophytic bacteria produce bioactive molecules with different activities. In fact, actinobacterial endophytic strains, mainly *Streptomyces*, from the Brazilian medicinal plant *Lychnophora ericoides* demonstrated activity not only against bacteria and yeast, but also against human cancer cell lines, showing its cytotoxic potential (Chagas et al. 2016). Very high cytotoxic activity was demonstrated for 39% of the tested extracts versus different cancer cell lines. The antioxidant potential of endophytic bacteria was evidenced by *Paenibacillus polymyxa* EJS-3, isolated from the Chinese medicinal plant *Stemona japonica* (108). The exopolysaccharide (EPS) of the strain was synthesized in vitro and both crude and purified EPS demonstrated strong scavenging activity on superoxide and hydroxyl radicals. The wide spectrum of the activity of endophytes-derived molecules extends to anti-inflammatory field. Carbazole derivatives obtained from the endophytic Streptomyces sp LJK 109 (Taechowisan et al. 2012) suppressed macrophage production of the inflammatory mediators NO, PGE2, TNF-α, IL-1β, IL-6, IL-10 in a dose-dependent manner. The described examples are evidence of the huge potentiality of medicinal plants-associated bacteria to produce bioactive molecules with a wide range of applications and underline that medicinal plant microbiota are good candidates for the isolation of biocontrol agents for both human and plant pathogens.

1.3.3 Endophytic potential to improve phytodepuration

Phytodepuration has proved to effectively remove or neutralize hazardous environmental contaminants and is predicted to have a growing application in the next years. Although, this process presents some limits, as the toxic effects of pollutants (or the end-products of their transformation) on the growth and health of the plants (Glick 2003). In fact, plant biomass is critical for phytodepuration (Germaine et al. 2010) and even hyperaccumulator plants, which can accumulate levels of toxic elements 100-fold higher than other plant species, usually present reduced growth. Also, phytodepuration may determine the accumulation of contaminants in plant tissues, determining ecological and airborne exposure issues (Ho et al. 2012). In this scenario, rhizobacteria and endophytic bacteria can aid plants by supporting their growth (Tesar, Reichenauer, and Sessitsch 2002), (Chaudhry et al. 2005), (Shaw and Burns 2004), reducing phytoxicity effects, increasing pollutants uptake and removal (Glick and Stearns 2011), reducing the release of toxic compounds into the atmosphere (Barac et al. 2004), removing contaminants and accumulating heavy metals (Ho et al. 2012), (Germaine et al. 2010). Many studies have already shown the potential of plant-associated bacterial strains in both enhancing phytodepuration and

reducing phytotoxicity. *Arabidopsis thaliana* plants inoculated with the root endophytic strain *Achromobacter xylosoxidans* F3B (Ho et al. 2012), which is able to metabolize phenol and catechol, showed less phytotoxic effects when exposed to concentration of 0.2 – 0.8 mM of catechol, in comparison to uninoculated plants. Furthermore, plants inoculated with *A. xylosoxidans* F3B were able to completely remove catechol, showing that the endophytic strain is able to increase phytodepuration efficiency. Germaine and colleagues (Germaine et al. 2010) demonstrated that the inoculation of pea plants (*Pisum sativum*) with endophytic strain *Pseudomonas putida* VM1450, able to degrade the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), determined statistically significant increase of plants' biomass. Moreover, the bacterial inoculum provided protection of the plants' root system, avoiding callus formation and root thickening, observed in noninoculated plants. The beneficial effects of the endophytic strain comprised also both no accumulation of 2,4-D in the stem/leaves and soil of inoculated plants. On the contrary, noninoculated plants showed accumulation of 2,4-D in stem/leaves and soil. Bacterial endophytic strains showing capacity to reduce COD and BOD in sewage effluent were chosen to inoculate *Brassica mutica* plants, grown in a floating treatment wetland (Ijaz et al. 2015). Effects on the remediation of sewage effluent were evaluated for *B. mutica* plants treated with the bacterial inoculum and for non-treated plants, showing the reduction of BOD_5 and COD in the inoculated plants. Remediation of heavy metals was also evaluated and, even in this case, bacterial inoculation improved phytodepuration efficiency. Moreover, bacterial inoculation increased iron removal efficiency from 77.4% to 85% in one of the analyzed drains. Interestingly, higher levels of inoculated bacteria were found in the plants roots and shoots, in comparison to wastewater, where the strains were originally inoculated, showing the ability of the endophytic strains to re-colonize the plants tissues.

These studies clearly highlight the usefulness of endophytic bacteria in supporting both the growth of plants in presence of contaminants and the elimination of these toxic compounds. Considering that plant-microbe interactions in the phytodepuration field are still an unexplored area, endophytic bacteria really represent unique untapped tools towards the reduction of many pollutants and their consequences in our environment.

1.4 Bibliography

Abreu-Tarazi, M. F., Navarrete, A. A., Andreote, F. D., Almeida, C. V., Tsai, S. M., & Almeida, M. (2010). Endophytic bacteria in long-term in vitro cultivated "axenic" pineapple microplants revealed by PCR–DGGE. World Journal of Microbiology and Biotechnology, 26(3), 555-560.

Akinsanya, M. A., Goh, J. K., Lim, S. P., & Ting, A. S. Y. (2015). Metagenomics study of endophytic bacteria in *Aloe vera* using next-generation technology. Genomics data, 6, 159-163.

Akinsanya, M. A., Goh, J. K., Lim, S. P., & Ting, A. S. Y. (2015). Diversity, antimicrobial and antioxidant activities of culturable bacterial endophyte communities in *Aloe vera*. FEMS microbiology letters, 362(23).

Alvin, A., Miller, K. I., & Neilan, B. A. (2014). Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiological research, 169(7-8), 483-495.

Andreote, F. D., Gumiere, T., & Durrer, A. (2014). Exploring interactions of plant microbiomes. Scientia agrícola, 71(6), 528-539.

Ansari, M. A., Hussain, S. K., Mudagal, M. P., & Goli, D. (2013). Neuroprotective effect of allopurinol and nimesulide against cerebral ischemic reperfusion injury in diabetic rats. Eur Rev Med Pharmacol Sci, 17(2), 170-178.

Bacilio-Jiménez, M., Aguilar-Flores, S., del Valle, M. V., Pérez, A., Zepeda, A., & Zenteno, E. (2001). Endophytic bacteria in rice seeds inhibit early colonization of roots by *Azospirillum brasilense*. Soil Biology and Biochemistry, 33(2), 167-172.

Bafana, A. (2013). Diversity and metabolic potential of culturable root-associated bacteria from *Origanum vulgare* in sub-Himalayan region. World Journal of Microbiology and Biotechnology, 29(1), 63-74.

Barac, T., Taghavi, S., Borremans, B., Provoost, A., Oeyen, L., Colpaert, J. V., ... & Van Der Lelie, D. (2004). Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nature biotechnology, 22(5), 583.

Bary, A. (1866). Morphologie und physiologie der pilze, flechten und myxomyceten. W. Engelmann.

Bell, C. R., Dickie, G. A., Harvey, W. L. G., & Chan, J. W. Y. F. (1995). Endophytic bacteria in grapevine. Canadian journal of Microbiology, 41(1), 46-53.

Berg, G., & Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS microbiology ecology, 68(1), 1-13.

Bhuvaneswari, S., Madhavan, S., & Panneerselvam, A. (2015). Molecular profiling and bacteriocin production of endophytic bacteria isolated from *Solanum trilobatum* L. leaves. Int J Curr Microbiol App Sci, 4, 539-546.

Böhm, M., Hurek, T., & Reinhold-Hurek, B. (2007). Twitching motility is essential for endophytic rice colonization by the N2-fixing endophyte *Azoarcus* sp. strain BH72. Molecular plant-microbe interactions, 20(5), 526-533.

Borruso, L., Bacci, G., Mengoni, A., De Philippis, R., & Brusetti, L. (2014). Rhizosphere effect and salinity competing to shape microbial communities in *Phragmites australis* (Cav.) Trin. ex-Steud. FEMS microbiology letters, 359(2), 193-200.

Bouali, M., Zrafi, I., Bakhrouf, A., Chaussonnerie, S., & Sghir, A. (2014). Bacterial structure and spatiotemporal distribution in a horizontal subsurface flow constructed wetland. Applied microbiology and biotechnology, 98(7), 3191-3203.

Brader, G., Compant, S., Mitter, B., Trognitz, F., & Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. Current opinion in biotechnology, 27, 30-37.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., ... & Peplies, J. (2012). Revealing structure and assembly cues for Arabidopsis

root-inhabiting bacterial microbiota. Nature, 488(7409), 91.
Buschart, A., Sachs, S., Chen, X., Herglotz, J., Krause, A., & Reinhold-Hurek, B. (2012). Flagella mediate endophytic competence rather than act as MAMPS in rice–*Azoarcus* sp. strain BH72 interactions. Molecular plant-microbe interactions, 25(2), 191-199.
Cakmakçi, R., Dönmez, F., Aydın, A., & Şahin, F. (2006). Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two differe
Calheiros, C. S., Duque, A. F., Moura, A., Henriques, I. S., Correia, A., Rangel, A. O., & Castro, P. M. (2009). Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment. Bioresource Technology, 100(13), 3228-3235.
Castillo, U. F., Strobel, G. A., Ford, E. J., Hess, W. M., Porter, H., Jensen, J. B., ... & Stevens, D. (2002). Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL 30562, endophytic on *Kennedia nigriscans*. Microbiology, 148(9), 2675-2685.
Conti, R., Chagas, F. O., Caraballo-Rodriguez, A. M., Melo, W. G. D. P., do Nascimento, A. M., Cavalcanti, B. C., ... & Andricopulo, A. D. (2016). Endophytic actinobacteria from the Brazilian medicinal plant *Lychnophora ericoides* Mart. and the biological potential of their secondary metabolites. Chemistry & biodiversity, 13(6), 727-736.
Chaudhry, Q., Blom-Zandstra, M., Gupta, S. K., & Joner, E. (2005). Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment (15 pp). Environmental Science and Pollution Research, 12(1), 34-48.
Checcucci, A., Maida, I., Bacci, G., Ninno, C., Bilia, A. R., Biffi, S., ... & Mengoni, A. (2017). Is the plant-associated microbiota of Thymus spp. adapted to plant essential oil?. Research in microbiology, 168(3), 276-282.
Chee-Sanford, J. C., Williams, M. M., Davis, A. S., & Sims, G. K. (2006). Do microorganisms influence seed-bank dynamics?. Weed Science, 54(3), 575-587.
Chiellini, C., Maida, I., Emiliani, G., Mengoni, A., Mocali, S., Fabiani, A., ... & Gallo, E. (2014). Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. Int Microbiol, 17(3), 165-174.
Chiellini, C., Maida, I., Maggini, V., Bosi, E., Mocali, S., Emiliani, G., ... & Fani, R. (2017). Preliminary data on antibacterial activity of *Echinacea purpurea*-associated bacterial communities against *Burkholderia cepacia* complex strains, opportunistic pathogens of cystic fibrosis patients. Microbiological research, 196, 34-43.
Choi, S. K., Park, S. Y., Kim, R., Kim, S. B., Lee, C. H., Kim, J. F., & Park, S. H. (2009). Identification of a polymyxin synthetase gene cluster of P*aenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. Journal of bacteriology, 191(10), 3350-3358.
Chowdhury, E. K., Jeon, J., Rim, S. O., Park, Y. H., Lee, S. K., & Bae, H. (2017). Composition, diversity and bioactivity of culturable bacterial endophytes in mountain-cultivated ginseng in Korea. Scientific reports, 7(1), 10098.
Compant, S., Clément, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, 42(5), 669-678.
Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., & Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial ecology, 62(1), 188-197.
Coombs, J. T., & Franco, C. M. (2003). Isolation and identification of actinobacteria from surface-sterilized wheat roots. Applied and environmental microbiology, 69(9), 5603-5608.

Cottyn, B., Regalado, E., Lanoot, B., De Cleene, M., Mew, T. W., & Swings, J. (2001). Bacterial populations associated with rice seed in the tropical environment. Phytopathology, 91(3), 282-292.

Dent, K. C., Stephen, J. R., & Finch-Savage, W. E. (2004). Molecular profiling of microbial communities associated with seeds of *Beta vulgaris* subsp. vulgaris (sugar beet). Journal of microbiological methods, 56(1), 17-26.

Donadio, S., Monciardini, P., & Sosio, M. (2007). Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. Natural product reports, 24(5), 1073-1109.

Donnarumma, F., Capuana, M., Vettori, C., Petrini, G., Giannini, R., Indorato, C., & Mastromei, G. (2011). Isolation and characterisation of bacterial colonies from seeds and in vitro cultures of *Fraxinus* spp. from Italian sites. Plant Biology, 13(1), 169-176.

Doty, S. L., Oakley, B., Xin, G., Kang, J. W., Singleton, G., Khan, Z., ... & Staley, J. T. (2009). Diazotrophic endophytes of native black cottonwood and willow. Symbiosis, 47(1), 23-33.

Duijff, B. J., Recorbet, G., Bakker, P. A., Loper, J. E., & Lemanceau, P. (1999). Microbial antagonism at the root level is involved in the suppression of *Fusarium* wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and Pseudomonas putida WCS358. Phytopathology, 89(11), 1073-1079.

Dunleavy, J. M. (1989). Curtobacterium plantarum sp. nov. is ubiquitous in plant leaves and is seed transmitted in soybean and corn. International Journal of Systematic and Evolutionary Microbiology, 39(3), 240-249.

Elbeltagy, A., Nishioka, K., Suzuki, H., Sato, T., Sato, Y. I., Morisaki, H., ... & Minamisawa, K. (2000). Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. Soil science and plant nutrition, 46(3), 617-629.

Emiliani, G., Mengoni, A., Maida, I., Perrin, E., Chiellini, C., Fondi, M., ... & Biffi, S. (2014). Linking bacterial endophytic communities to essential oils: Clues from *Lavandula angustifolia* Mill. Evidence-Based Complementary and Alternative Medicine, 2014.

Faulwetter, J. L., Burr, M. D., Parker, A. E., Stein, O. R., & Camper, A. K. (2013). Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. Microbial ecology, 65(1), 111-127.

Ferreira, A., Quecine, M. C., Lacava, P. T., Oda, S., Azevedo, J. L., & Araújo, W. L. (2008). Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by *Pantoea agglomerans*. FEMS microbiology letters, 287(1), 8-14.

Fortman, J. L., & Sherman, D. H. (2005). Utilizing the power of microbial genetics to bridge the gap between the promise and the application of marine natural products. ChemBioChem, 6(6), 960-978.

Fürnkranz, M., Lukesch, B., Müller, H., Huss, H., Grube, M., & Berg, G. (2012). Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. Microbial ecology, 63(2), 418-428.

Gagne-Bourgue, F., Aliferis, K. A., Seguin, P., Rani, M., Samson, R., & Jabaji, S. (2013). Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (P anicum virgatum L.) cultivars. Journal of applied microbiology, 114(3), 836-853.

Galippe, V. (1887). Note sur la présence de micro-organismes dans les tissus végétaux. Comptes Rendus Hebdomadaires des Séances et Mémoires de la Société de Biologie

et des ses Filiales et Associées, 39, 410-416.
Garcia, A., Polonio, J. C., Polli, A. D., Santos, C. M., Rhoden, S. A., Quecine, M., ... & Pamphile, J. A. (2016). Rhizosphere bacteriome of the medicinal plant Sapindus saponaria L. revealed by pyrosequencing. Genetics and molecular research: GMR, 15(4).
Germaine, K. J., Liu, X., Cabellos, G. G., Hogan, J. P., Ryan, D., & Dowling, D. N. (2006). Bacterial endophyte-enhanced phytoremediation of the organochlorine herbicide 2, 4-dichlorophenoxyacetic acid. FEMS microbiology ecology, 57(2), 302-310.
Germaine, K., Keogh, E., Garcia-Cabellos, G., Borremans, B., Van Der Lelie, D., Barac, T., ... & Campbell, C. D. (2004). Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS Microbiology Ecology, 48(1), 109-118.
Glick, B. R. (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnology advances, 21(5), 383-393.
Glick, B. R., & Stearns, J. C. (2011). Making phytoremediation work better: maximizing a plant's growth potential in the midst of adversity. International journal of phytoremediation, 13(sup1), 4-16.
Glick, B. R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., & McConkey, B. (2007). Promotion of plant growth by bacterial ACC deaminase. Critical Reviews in Plant Sciences, 26(5-6), 227-242.
Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., & Rai, M. (2015). Endophytic actinobacteria of medicinal plants: diversity and bioactivity. Antonie Van Leeuwenhoek, 108(2), 267-289.
Granér, G., Persson, P., Meijer, J., & Alström, S. (2003). A study on microbial diversity in different cultivars of Brassica napus in relation to its wilt pathogen, Verticillium longisporum. FEMS microbiology letters, 224(2), 269-276.
Haldar, S., Choudhury, S. R., & Sengupta, S. (2011). Genetic and functional diversities of bacterial communities in the rhizosphere of *Arachis hypogaea*. Antonie Van Leeuwenhoek, 100(1), 161-170.
Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology, 43(10), 895-914.
Hardoim, P. R., Hardoim, C. C., Van Overbeek, L. S., & Van Elsas, J. D. (2012). Dynamics of seed-borne rice endophytes on early plant growth stages. PLoS One, 7(2), e30438.
Hardoim, P. R., van Overbeek, L. S., & van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. Trends in microbiology, 16(10), 463-471.
Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L., & Hedges, S. B. (2001). Molecular evidence for the early colonization of land by fungi and plants. Science, 293(5532), 1129-1133.
Ho, Y. N., Mathew, D. C., Hsiao, S. C., Shih, C. H., Chien, M. F., Chiang, H. M., & Huang, C. C. (2012). Selection and application of endophytic bacterium *Achromobacter xylosoxidans* strain F3B for improving phytoremediation of phenolic pollutants. Journal of hazardous materials, 219, 43-49.
Hurek, T., Reinhold-Hurek, B., Van Montagu, M., & Kellenberger, E. (1994). Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. Journal of bacteriology, 176(7), 1913-1923.
Ijaz, A., Shabir, G., Khan, Q. M., & Afzal, M. (2015). Enhanced remediation of sewage effluent by endophyte-assisted floating treatment wetlands. Ecological Engineering, 84, 58-66.
Ikeda, S., Fuji, S. I., Sato, T., Ytow, N., Ezura, H., Minamisawa, K., & Fujimura, T. (2006). Community analysis of seed-associated microbes in forage crops using

culture-independent methods. Microbes and environments, 21(2), 112-121.
Jasim, B., Sreelakshmi, K. S., Mathew, J., & Radhakrishnan, E. K. (2016). Surfactin, iturin, and fengycin biosynthesis by endophytic *Bacillus* sp. from *Bacopa monnier*i. Microbial ecology, 72(1), 106-119.
Johnston-Monje, D., & Raizada, M. N. (2011). Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. PLoS One, 6(6), e20396.
Kaga, H., Mano, H., Tanaka, F., Watanabe, A., Kaneko, S., & Morisaki, H. (2009). Rice seeds as sources of endophytic bacteria. Microbes and environments, 24(2), 154-162.
Klaedtke, S., Jacques, M. A., Raggi, L., Préveaux, A., Bonneau, S., Negri, V., ... & Barret, M. (2016). Terroir is a key driver of seed-associated microbial assemblages. Environmental microbiology, 18(6), 1792-1804.
Köberl, M., Müller, H., Ramadan, E. M., & Berg, G. (2011). Desert farming benefits from microbial potential in arid soils and promotes diversity and plant health. PLoS One, 6(9), e24452.
Köberl, M., Schmidt, R., Ramadan, E. M., Bauer, R., & Berg, G. (2013). The microbiome of medicinal plants: diversity and importance for plant growth, quality and health. Frontiers in microbiology, 4, 400.
Kremer, R. J. (1987). Identity and properties of bacteria inhabiting seeds of selected broadleaf weed species. Microbial ecology, 14(1), 29-37.
Kukkurainen, S., Leino, A., Vähämiko, S., Kärkkäinen, H. R., Ahanen, K., Sorvari, S., ... & Toldi, O. (2005). Occurrence and location of endophytic bacteria in garden and wild strawberry. HortScience, 40(2), 348-352.
Kumar, A., Singh, R., Yadav, A., Giri, D. D., Singh, P. K., & Pandey, K. D. (2016). Isolation and characterization of bacterial endophytes of *Curcuma longa* L. 3 Biotech, 6(1), 60.
Lautru, S., Deeth, R. J., Bailey, L. M., & Challis, G. L. (2005). Discovery of a new peptide natural product by *Streptomyces coelicolor* genome mining. Nature chemical biology, 1(5), 265-269.
Li, J., Zhao, G. Z., Varma, A., Qin, S., Xiong, Z., Huang, H. Y., ... & Li, W. J. (2012). An endophytic *Pseudonocardia* species induces the production of artemisinin in Artemisia annua. PloS one, 7(12), e51410.
Li, J., & Jensen, S. E. (2008). Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. Chemistry & biology, 15(2), 118-127.
Li, Y. H., Liu, Q. F., Liu, Y., Zhu, J. N., & Zhang, Q. (2011). Endophytic bacterial diversity in roots of *Typha angustifolia* L. in the constructed Beijing Cuihu Wetland (China). Research in microbiology, 162(2), 124-131.
Liu, J., Luo, J., Ye, H., Sun, Y., Lu, Z., & Zeng, X. (2009). Production, characterization and antioxidant activities in vitro of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. Carbohydrate polymers, 78(2), 275-281.
Liu, Y., Zuo, S., Xu, L., Zou, Y., & Song, W. (2012). Study on diversity of endophytic bacterial communities in seeds of hybrid maize and their parental lines. Archives of microbiology, 194(12), 1001-1012.
Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R., Taghavi, S., Mezgeay, M., & der Lelie, D. V. (2002). Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences, 21(6), 583-606.
López-López, A., Rogel, M. A., Ormeno-Orrillo, E., Martínez-Romero, J., & Martínez-Romero, E. (2010). *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as Rhizobium endophyticum sp. nov. Systematic and Applied Microbiology, 33(6), 322-327.
López, J. L., Alvarez, F., Principe, A., Salas, M. E., Lozano, M. J., Draghi, W. O., ... &

Lagares, A. (2018). Isolation, taxonomic analysis, and phenotypic characterization of bacterial endophytes present in alfalfa (*Medicago sativa*) seeds. Journal of biotechnology, 267, 55-62.

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. Annual review of microbiology, 63, 541-556.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., ... & Edgar, R. C. (2012). Defining the core *Arabidopsis thaliana* root microbiome. Nature, 488(7409), 86.

Ma, B., Lv, X., Warren, A., & Gong, J. (2013). Shifts in diversity and community structure of endophytic bacteria and archaea across root, stem and leaf tissues in the common reed, *Phragmites australis*, along a salinity gradient in a marine tidal wetland of northern China. Antonie Van Leeuwenhoek, 104(5), 759-768.

Maggini, V., De Leo, M., Mengoni, A., Gallo, E. R., Miceli, E., Reidel, R. V. B., ... & Bogani, P. (2017). Plant-endophytes interaction influences the secondary metabolism in *Echinacea purpurea* (L.) Moench: an in vitro model. Scientific reports, 7(1), 16924.

Maggini, V., Miceli, E., Fagorzi, C., Maida, I., Fondi, M., Perrin, E., ... & Fabiani, A. (2018). Antagonism and antibiotic resistance drive a species-specific plant microbiota differentiation in Echinacea spp. FEMS microbiology ecology.

Maida, I., Chiellini, C., Mengoni, A., Bosi, E., Firenzuoli, F., Fondi, M., & Fani, R. (2016). Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *Echinacea purpurea*. Environmental microbiology, 18(8), 2357-2365.

Mano, H., Tanaka, F., Nakamura, C., Kaga, H., & Morisaki, H. (2007). Culturable endophytic bacterial flora of the maturing leaves and roots of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes and Environments, 22(2), 175-185.

Marschner, P., Yang, C. H., Lieberei, R., & Crowley, D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil biology and biochemistry, 33(11), 1437-1445.

Mendes, R., Garbeva, P., & Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS microbiology reviews, 37(5), 634-663.

Mengoni, A., Maida, I., Chiellini, C., Emiliani, G., Mocali, S., Fabiani, A., ... & Fani, R. (2014). Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. Research in microbiology, 165(8), 686-694.

Miao, V., & Davies, J. (2010). Actinobacteria: the good, the bad, and the ugly. Antonie Van Leeuwenhoek, 98(2), 143-150.

Miché, L., & Balandreau, J. (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensi*s. Applied and environmental microbiology, 67(7), 3046-3052.

Miller, K. I., Qing, C., Sze, D. M. Y., Roufogalis, B. D., & Neilan, B. A. (2012). Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microbial ecology, 64(2), 431-449.

Mitter, B., Petric, A., Shin, M. W., Chain, P. S., Hauberg-Lotte, L., Reinhold-Hurek, B., ... & Sessitsch, A. (2013). Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Frontiers in plant science, 4, 120.

Mukhopadhyay, K., Garrison, N. K., Hinton, D. M., Bacon, C. W., Khush, G. S., Peck, H. D., & Datta, N. (1996). Identification and characterization of bacterial endophytes of rice. Mycopathologia, 134(3), 151-159.

Müller, D. B., Vogel, C., Bai, Y., & Vorholt, J. A. (2016). The plant microbiota: systems-level insights and perspectives. annual review of genetics, 50, 211-234.

Nalini, M. S., & Prakash, H. S. (2017). Diversity and bioprospecting of actinomycete endophytes from the medicinal plants. Letters in applied microbiology, 64(4), 261-270.

Nelson, E. B. (2004). Microbial dynamics and interactions in the spermosphere. Annu. Rev. Phytopathol., 42, 271-309.

Oehrle, N. W., Karr, D. B., Kremer, R. J., & Emerich, D. W. (2000). Enhanced attachment of Bradyrhizobium japonicum to soybean through reduced root colonization of internally seedborne microorganisms. Canadian journal of microbiology, 46(7), 600-606.

Okunishi, S., Sako, K., Mano, H., Imamura, A., & Morisaki, H. (2005). Bacterial flora of endophytes in the maturing seed of cultivated rice (Oryza sativa). Microbes and Environments, 20(3), 168-177.

Patriquin, D. G., & Döbereiner, J. (1978). Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Canadian Journal of Microbiology, 24(6), 734-742.

Pérez-García, A., Romero, D., & De Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Current opinion in biotechnology, 22(2), 187-193.

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R. A., Del Cerro, P., Espuny, M. R., Jiménez-Guerrero, I., ... & Cubo, T. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiological research, 169(5-6), 325-336.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 11(11), 789.

Pimentel, M. R., Molina, G., Dionísio, A. P., Maróstica Junior, M. R., & Pastore, G. M. (2011). The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnology research international, 2011.

Pleban, S., Ingel, F., & Chet, I. (1995). Control ofRhizoctonia solani andSclerotium rolfsii in the greenhouse using endophytic *Bacillus* spp. European Journal of Plant Pathology, 101(6), 665-672.

Presta, L., Bosi, E., Fondi, M., Maida, I., Perrin, E., Miceli, E., ... & Rossolini, G. M. (2017). Phenotypic and genomic characterization of the antimicrobial producer Rheinheimera sp. EpRS3 isolated from the medicinal plant *Echinacea purpurea*: insights into its biotechnological relevance. Research in microbiology, 168(3), 293-305.

Priti, V., Ramesha, B. T., Singh, S., Ravikanth, G., Ganeshaiah, K. N., Suryanarayanan, T. S., & Uma Shaanker, R. (2009). How promising are endophytic fungi as alternative sources of plant secondary metabolites?. Current Science, 97(4), 477-478.

Puente, M. E., Li, C. Y., & Bashan, Y. (2009). Rock-degrading endophytic bacteria in cacti. Environmental and Experimental Botany, 66(3), 389-401.

Qi, X., Wang, E., Xing, M., Zhao, W., & Chen, X. (2012). Rhizosphere and non-rhizosphere bacterial community composition of the wild medicinal plant *Rumex patientia*. World Journal of Microbiology and Biotechnology, 28(5), 2257-2265.

Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K., & De Ley, J. (1993). *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (Leptochloa fusca (L.) Kunth), and description of two species, Azoarcus indigens sp. nov. and Azoarcus communis sp. nov. International Journal of Systematic and Evolutionary Microbiology, 43(3), 574-584.

Reinhold-Hurek, B., Maes, T., Gemmer, S., Van Montagu, M., & Hurek, T. (2006). An endoglucanase is involved in infection of rice roots by the not-cellulose-metabolizing endophyte *Azoarcus* sp. strain BH72. Molecular plant-microbe interactions, 19(2),

181-188.
Rijavec, T., Lapanje, A., Dermastia, M., & Rupnik, M. (2007). Isolation of bacterial endophytes from germinated maize kernels. Canadian journal of microbiology, 53(6), 802-808.
Ringelberg, D., K. Foley, and C. M. Reynolds. 2012. Bacterial Endophyte Communities of Two Wheatgrass Varieties Following Propagation in Different Growing Media. 80(January):67–80.
Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. Molecular plant-microbe interactions, 19(8), 827-837.
Roy, S., Yasmin, S., Ghosh, S., Bhattacharya, S., & Banerjee, D. (2016). Anti-infective metabolites of a newly isolated *Bacillus thuringiensis* KL1 associated with kalmegh (Andrographis paniculata Nees.), a traditional medicinal herb. Microbiology insights, 9, MBI-S33394.
Ruiza, D., Agaras, B., de Werrab, P., Wall, L. G., & Valverde, C. (2011). Characterization and screening of plant probiotic traits of bacteria isolated from rice seeds cultivated in Argentina. The Journal of Microbiology, 49(6), 902-912.
Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., & Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. FEMS microbiology letters, 278(1), 1-9.
Sanchez-Canizares, C., Jorrín, B., Poole, P. S., & Tkacz, A. (2017). Understanding the holobiont: the interdependence of plants and their microbiome. Current opinion in microbiology, 38, 188-196.
Sauvêtre, A., May, R., Harpaintner, R., Poschenrieder, C., & Schröder, P. (2018). Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from *Phragmites australis*. Journal of hazardous materials, 342, 85-95.
Schlaeppi, K., & Bulgarelli, D. (2015). The plant microbiome at work. Molecular Plant-Microbe Interactions, 28(3), 212-217.
Selosse, M. A., & Le Tacon, F. (1998). The land flora: a phototroph-fungus partnership?. Trends in Ecology & Evolution, 13(1), 15-20.
Šmerda, J., Sedláček, I., Páčová, Z., Durnova, E., Smíšková, A., & Havel, L. (2005). Paenibacillus mendelii sp. nov., from surface-sterilized seeds of *Pisum sativum* L. International journal of systematic and evolutionary microbiology, 55(6), 2351-2354.
Shahzad, R., Khan, A. L., Bilal, S., Asaf, S., & Lee, I. J. (2018). What Is There in Seeds? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth. Frontiers in Plant Science, 9, 24.
Shakya, M., Gottel, N., Castro, H., Yang, Z. K., Gunter, L., Labbé, J., ... & Podar, M. (2013). A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature Populus deltoides trees. PLoS One, 8(10), e76382.
Shaw, L. J., & Burns, R. G. (2004). Enhanced mineralization of [U-14C] 2, 4-dichlorophenoxyacetic acid in soil from the rhizosphere of *Trifolium pratense*. Applied and environmental microbiology, 70(8), 4766-4774.
Shi, J. Y., Yuan, X. F., Lin, H. R., Yang, Y. Q., & Li, Z. Y. (2011). Differences in soil properties and bacterial communities between the rhizosphere and bulk soil and among different production areas of the medicinal plant *Fritillaria thunbergii*. International journal of molecular sciences, 12(6), 3770-3785.
Siciliano, S. D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., ... & Schwab, P. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and environmental microbiology, 67(6), 2469-2475.
Sobolev, V. S., Orner, V. A., & Arias, R. S. (2013). Distribution of bacterial endophytes in peanut seeds obtained from axenic and control plant material under field conditions. Plant and soil, 371(1-2), 367-376.
Spaepen, S., Vanderleyden, J., & Remans, R. (2007). Indole-3-acetic acid in microbial and

microorganism-plant signaling. FEMS microbiology reviews, 31(4), 425-448.
Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular microbiology, 56(4), 845-857.
Strobel, G. A. (2003). Endophytes as sources of bioactive products. Microbes and infection, 5(6), 535-544.
Sturz, A. V., Christie, B. R., & Nowak, J. (2000). Bacterial endophytes: potential role in developing sustainable systems of crop production. Critical reviews in plant sciences, 19(1), 1-30.
Taechowisan, T., Chanaphat, S., Ruensamran, W., & Phutdhawong, W. S. (2012). Anti-inflammatory effect of 3-methylcarbazoles on RAW 264.7 cells stimulated with LPS, polyinosinic-polycytidylic acid and Pam3CSK. Advances in Microbiology, 2(2), 98.
Tan, Y., Cui, Y., Li, H., Kuang, A., Li, X., Wei, Y., & Ji, X. (2017). Diversity and composition of rhizospheric soil and root endogenous bacteria in *Panax notoginseng* during continuous cropping practices. Journal of basic microbiology, 57(4), 337-344.
Tesar, M., Reichenauer, T. G., & Sessitsch, A. (2002). Bacterial rhizosphere populations of black poplar and herbal plants to be used for phytoremediation of diesel fuel. Soil biology and biochemistry, 34(12), 1883-1892.
Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gattinger, A., & Schloter, M. (2006). *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. International journal of systematic and evolutionary microbiology, 56(7), 1677-1680.
Truyens, S., Weyens, N., Cuypers, A., & Vangronsveld, J. (2013). Changes in the population of seed bacteria of transgenerationally Cd-exposed *Arabidopsis thaliana*. Plant Biology, 15(6), 971-981.
Van Overbeek, L., & Van Elsas, J. D. (2008). Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). FEMS microbiology ecology, 64(2), 283-296.
Vasquez, E. A., Glenn, E. P., Guntenspergen, G. R., Brown, J. J., & Nelson, S. G. (2006). Salt tolerance and osmotic adjustment of *Spartina alterniflora* (Poaceae) and the invasive M haplotype of *Phragmites australis* (Poaceae) along a salinity gradient. American Journal of Botany, 93(12), 1784-1790.
Vega, F. E., Pava-Ripoll, M., Posada, F., & Buyer, J. S. (2005). Endophytic bacteria in Coffea arabica L. Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms, 45(5), 371-380.
Wani, Z. A., Ashraf, N., Mohiuddin, T., & Riyaz-Ul-Hassan, S. (2015). Plant-endophyte symbiosis, an ecological perspective. Applied microbiology and biotechnology, 99(7), 2955-2965.
Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology, 97(2), 250-256.
Weyens, N., van der Lelie, D., Taghavi, S., & Vangronsveld, J. (2009). Phytoremediation: plant–endophyte partnerships take the challenge. Current opinion in biotechnology, 20(2), 248-254.
Wilkie, N., Wingrove, P. B., Bilsland, J. G., Young, L., Harper, S. J., Hefti, F., ... & Pollack, S. J. (2001). The non-peptidyl fungal metabolite L-783,281 activates TRK neurotrophin receptors. Journal of neurochemistry, 78(5), 1135-1145.
World Health Organization. 2015. World Malaria Day.
Xu, M., Sheng, J., Chen, L., Men, Y., Gan, L., Guo, S., & Shen, L. (2014). Bacterial community compositions of tomato (*Lycopersicum esculentum* Mill.) seeds and plant growth promoting activity of ACC deaminase producing *Bacillus subtilis* (HYT-12-1) on tomato seedlings. World Journal of Microbiology and Biotechnology, 30(3), 835-845.

Zazopoulos, E., Huang, K., Staffa, A., Liu, W., Bachmann, B. O., Nonaka, K., ... & Farnet, C. M. (2003). A genomics-guided approach for discovering and expressing cryptic metabolic pathways. Nature biotechnology, 21(2), 187.

Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., ... & Durner, J. (2004). Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proceedings of the National Academy of Sciences, 101(44), 15811-15816.

Zhang, C., Wang, B., Dai, X., Li, S., Lu, G., & Zhou, Y. (2017). Structure and function of the bacterial communities during rhizoremediation of hexachlorobenzene in constructed wetlands. Environmental Science and Pollution Research, 24(12), 11483-11492.

Zhou, Y., Tigane, T., Li, X., Truu, M., Truu, J., & Mander, Ü. (2013). Hexachlorobenzene dechlorination in constructed wetland mesocosms. Water research, 47(1), 102-110.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS microbiology reviews, 32(5), 723-735.

2. Aim and presentation of the work

Endophytes are microorganisms characterized by particular traits: they colonize the plant's internal tissues without causing any harm, and in most cases, contribute with some beneficial functions, such as enhancing the uptake of nutrients, promoting the plant's growth, protecting against pathogens and giving resistance under stress conditions. Some of the physiological functions of endophytes could turn into very useful functions from a biotechnological viewpoint. Endophytes have been reported as being able of producing compounds similar to those synthesized by the host plant or modulating the plant's production of secondary metabolites, as well as capable of degrading many toxic compounds. For these reasons, endophytes represent a very promising field of research, representing new sources of bioactive molecules, which could help addressing many issues, such as antibiotic resistance and environmental cleanup of toxic compounds.

Thus, the aim of this work was to study endophytic bacteria isolated from different sources (medicinal plants tissues, medicinal plants seeds, and hyperaccumulator plants), in order to deepen the knowledge on the structure and phenotypic traits of their communities, and to evaluate the possible use of endophytic isolates for biotechnological purposes.

In Chapter 3, the presence of a species-specific composition of plant microbiota was evaluated analyzing two congeneric medicinal plants, *Echinacea purpurea* and *E. angustifolia*, with the aim of investigating on the possible factors shaping the structure of the plant microbiota.

Chapter 4 concentrates on endophytes from a genomic viewpoint, with the aim of deepening their characterization, and also highlighting genes putatively involved in the production of secondary metabolites.

Endophytes associated to plant's seeds were also explored, analyzing the anatomical characteristics of *Echinacea* spp. seeds and the localization of endophytes within them. Cultivable bacterial isolates were also extracted from *E. purpurea* seeds and characterized taxonomically and phenotypically.

More recently, we decided to extend the endophytes investigation to hyperaccumulator plants, being fascinated by the possibility that their extraordinary capabilities of eliminating toxic compounds could be at least indirectly related to the presence of endophytes within their tissues. Thus, we analyzed bacterial communities associated to *Phragmites australis* plants from a constructed wetland, since the knowledge on the composition and phenotypic traits of such communities represent the starting point for a better comprehension of the phytodepuration process and its improvement.

3. Antagonism and antibiotic resistance as driving factors of the structuring of species-specific bacterial communities in *Echinacea* spp.

Medicinal plants from the genus *Echinacea* are among the most commonly used medicinal plants worldwide. *Echinacea purpurea* (L.) Moench and *Echinacea angustifolia* (DC.) Hell are currently used in Europe and the USA to treat the common cold and respiratory infections. The alkylamide, alkaloid, and polyacetylene fractions are considered to have immune-modulatory and anti-inflammatory effects. Interestingly, endophytic bacteria have been highlighted as possible factors determining the effects of medicinal plants on immune system function. In fact, bacterial lipoproteins and lipopolysaccharides were shown to substantially contribute to the *in vitro* macrophage activation properties of immunostimulant botanicals (Pugh *et al.* 2008) and endophytic bacteria appeared as potentially important determinants of such activation. Furthermore, the immune-enhancing effect exerted by *Echinacea* extracts was observed to be strongly related to the total bacterial load within plant samples (Pugh *et al.* 2013) and the influence of *Echinacea* endophytes on macrophage-stimulatory activity was also recorded (Todd *et al.* 2015). Very recently, the influence of the plant-endophyte interaction on the plant secondary metabolism was observed for *Echinacea purpurea* (Maggini *et al.* 2017).

Since the bacterial microbiome of medicinal plants seems to have a key role concerning the therapeutic properties of the plants, it appears very valuable to investigate on the possible factors shaping the assemblages of such communities. Nevertheless, very little is known about the forces driving the functional and taxonomic assemblage of the plant microbiota (Muller *et al.* 2016). A good model to evaluate the presence of species-specific composition of plant microbiota and its relationships is represented by the two congeneric medicinal plants, *E. purpurea* (L.) Moench and *E. angustifolia* (DC.) Heller, presenting distinct medicinal activity in different plant compartments (Karsch-Volk *et al.* 2014). The endophytic and rhizospheric bacterial diversity of these two medicinal plants grown in the same soil has been previously studied, evidencing the presence of distinct bacterial communities in the two species and among compartments of the same plant species (Chiellini *et al.* 2014). In particular, the three compartments, rhizospheric soil (RS), roots (R), and stem/leaves (S/L), harbored different bacterial communities, which could be determined by different antibiotic resistance patterns (Mengoni *et al.* 2014) and antagonistic interactions among the three plant ecological niches (Maida *et al.* 2016).

Thus, the aim of this work was to compare phenotypic traits of bacterial communities associated to the two medicinal plants, *E. purpurea* and *E. angustifolia*. To this purpose, phenotypic tests, such as extracellular enzymatic activity (EEA), siderophore (SPH) and indole-3-acetic acid (IAA) production, and the determination of the antibiotic resistance profiles were performed. Moreover, antagonistic interactions were evaluated among strains from *E. angustifolia*, and between the strains of the two plants species. Data obtained suggest that the bacterial communities themselves could play an important role in shaping their own communities, by means of antimicrobial molecules, which determine the selection of adaptive phenotypes for plant tissue colonization.

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RESEARCH ARTICLE

Antagonism and antibiotic resistance drive a species-specific plant microbiota differentiation in *Echinacea* spp.

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One sentence summary: A deep phenotypic characterization of a collection of cultivable bacterial strains from two plant species suggests that socio-microbiological interactions are the main players in plant-specific differences in the composition of bacterial communities.

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ABSTRACT

A key factor in the study of plant-microbes interactions is the composition of plant microbiota, but little is known about the factors determining its functional and taxonomic organization. Here we investigated the possible forces driving the assemblage of bacterial endophytic and rhizospheric communities, isolated from two congeneric medicinal plants, *Echinacea purpurea* (L.) Moench and *Echinacea angustifolia* (DC) Heller, grown in the same soil, by analysing bacterial strains (isolated from three different compartments, i.e. rhizospheric soil, roots and stem/leaves) for phenotypic features such as antibiotic resistance, extracellular enzymatic activity, siderophore and indole 3-acetic acid production, as well as cross-antagonistic activities. Data obtained highlighted that bacteria from different plant compartments were characterized by specific antibiotic resistance phenotypes and antibiotic production, suggesting that the bacterial communities themselves could be responsible for structuring their own communities by the production of antimicrobial molecules selecting bacterial-adaptive phenotypes for plant tissue colonization.

Keywords: Antibiotic resistance; bacterial antagonism; ecological differentiation; culture collection; plant microbiota; medicinal plants

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INTRODUCTION

Endophytes are microorganisms that internally colonize plant tissues without causing harm to the host(s). They can be seed-borne or recruited from the rhizospheric soil and/or the phyllospheric microbiota (Santhanam *et al.* 2014). Different bacterial communities have been found in different plant tissues and in plants grown in different soils. Some of these endophytes are gaining a reputation as plant growth promoters (PGP) (Ryan *et al.* 2008; Santoyo *et al.* 2016), biocontrol agents (Nair and Padmavathy 2014), bio-fertilizers (nitrogen fixation) (Kumar *et al.* 2016a), environmental chemical reducers (phytoremediation) (Afzal, Khan and Sessitsch 2014; Ma *et al.* 2016), and producers of medicinal, agricultural and/or industrial molecules (Nair and Padmavathy 2014). Specific endophytes are able to enhance the growth of the host plants producing extracellular enzymes (able to control the plant pathogens) and phytohormones (Long, Schmidt and Baldwin 2008; Passari *et al.* 2016) or other molecules. For example, rhizospheric as well as endophytic PGP bacteria (Ryan *et al.* 2008) have been reported acting through indole acetic acid (IAA) (Spaepen, Vanderleyden and Remans 2007) and/or siderophore (SPH) production (Loaces, Ferrando and Scavino 2011). Moreover, we have very recently shown the influence of the plant-endophyte interaction on the plant secondary metabolism of *Echinacea purpurea* (Maggini *et al.* 2017). There is growing evidence that endophytic bacteria may be the main factor determining the enhancing effects of medicinal plants on immune system function. In fact, bacterial lipoproteins and lipopolysaccharides were shown to substantially contribute to the *in vitro* macrophage activation properties of immunostimulant botanicals (Pugh *et al.* 2008) and endophytic bacteria appeared as potentially important determinants of such activation. The influence of *Echinacea* endophytes on macrophage-stimulatory activity was also highlighted (Todd *et al.* 2015). Furthermore, the registered immune-enhancing effect exerted by *Echinacea* extracts was demonstrated to be strongly related to the total bacterial load within plant samples (Pugh *et al.* 2013) and with the bacterial type as well (Haron *et al.* 2016). Thus, since the bacterial community composition seems to be a key factor concerning medicinal plants' properties, it appears to be of essential importance to investigate the possible forces shaping the assemblages of such communities. Nevertheless, very little is known about the forces shaping the functional and taxonomic assemblage of the plant microbiota (Muller *et al.* 2016). In this context, secondary metabolites produced by the plant could exert a selective action in determining the composition of the plant microbiota (Mengoni *et al.* 2014). In fact, antimicrobial molecules inside plant tissues might be responsible for the selection of bacteria exhibiting resistant phenotypes. In this regard, it has been shown that the microbiota of two closely related species of *Thymus* (*T. vulgaris* and *T. citriodorus*) are differentiated in relation to the host plant essential oil composition embedded with antibacterial activity (Checcucci *et al.* 2017). On the other hand, the presence of specific endophytes might contribute to the diversity of plant chemical composition (Long, Schmidt and Baldwin 2008). In fact, specific endophytes could be selected by the plant on the basis of their metabolic abilities allowing plant environment adaptation (Brader *et al.* 2014). In addition, the growth of plant pathogens may be inhibited by antibiotics and extracellular degrading enzymes produced by the endophytes (Berg 2009) that, in turn, might develop antibiotic resistance as a response to similar molecules produced by antagonistic microorganisms within the same ecological niche (Mengoni *et al.* 2014).

A good model to evaluate the presence of species-specific composition of plant microbiota and its relationships is represented by the two congeneric medicinal plants, *Echinacea purpurea* (L.) Moench and *Echinacea angustifolia* (DC.) Heller, widely used in European countries and North America to fight viral and/or bacterial infections and as immunosuppressants (Karsch-Volk, Barrett and Linde 2015). It is known that plant secondary metabolites differ among different *Echinacea* species and their relative plant organs (Barnes *et al.* 2005). For example, in the root compartment of *E. purpurea*, isobutylamides are mainly represented by 2,4-dienoic units, while the same compartment of *E. angustifolia* isobutylamides are characterized by 2-monoene units. Anyway, the alkamides are mainly contained in *E. angustifolia* roots and in the aerial part of *E. purpurea*. Also, echinacoside and cynarin are present only in *E. angustifolia* roots while the chicoric acid is distinctive of *E. purpurea* roots and its aerial part (Barnes *et al.* 2005). Consequently, the medicinal activity of the two plants is distinct in different plant compartments (Karsch-Volk *et al.* 2014). The endophytic and rhizospheric bacterial diversity of these two medicinal plants grown in the same soil has been previously studied, highlighting the presence of distinct bacterial communities in the two species and among compartments of the same plant species (Chiellini *et al.* 2014). In particular, the three compartments, rhizospheric soil (RS), roots (R), and stem/leaves (S/L), harboured different bacterial communities, which could be determined by different antibiotic resistance patterns (Mengoni *et al.* 2014) and antagonistic interactions among the three plant ecological niches (Maida *et al.* 2016).

Hence, the aim of this work was to compare phenotypic features of bacterial microbiota belonging to two medicinal plants, *E. purpurea* and *E. angustifolia*, exhibiting different therapeutic properties. To this purpose, a combination of phenotypic tests, such as extracellular enzymatic activity (EEA) assays, SPH and IAA production, and the determination of the antibiotic resistance profiles were carried out. Moreover, antagonistic interactions assays have been performed among strains from each *E. angustifolia*, and between the two plant strains.

MATERIALS AND METHODS

Bacterial strains

The bacterial strain panel used in this work (Supplementary Table S1) comprised 140 bacterial strains from the medicinal plant *E. purpurea* and 117 bacterial strains from the medicinal plant *E. angustifolia*. These strains were representative of a collection previously described (Chiellini *et al.* 2014; Mengoni *et al.* 2014) and set up from two pools of five *E. purpurea* and five *E. angustifolia* plants grown in a common garden at the "Giardino delle Erbe", Casola Valsenio (Italy). The R and S/L of the plants were separated and RS was also collected. Bacteria were isolated on Tryptic Soy Agar (TSA) plates after two days' incubation at 30°C, as previously described (Chiellini *et al.* 2014). Strains were characterized by Rapid Amplification of polymorphic DNA (RAPD) fingerprinting and then a strain from each RAPD profile (Mori *et al.* 1999) was taxonomically identified via 16S rRNA gene sequencing (Chiellini *et al.* 2014).

Preparation of the cell suspensions

Each strain was grown on a TSA medium at 30°C for 48 h; then one colony for each strain was suspended in 100 µl saline solution (0.9% NaCl) and the cell suspension was streaked on the specific solid medium for each test.

Extracellular enzymatic activity

Extracellular amylolytic, proteolytic, phospholipase, and lipasic activities were assessed (Atlas 1994) on all of the 257 bacterial strains as follows: a cell suspension prepared as described above was streaked on a 10% TSA medium supplemented with the appropriate substrate. The amylolytic activity was evaluated by streaking the strains in the presence of 1% potato starch (Fluka Analytical). After incubation at 30°C for 48 h, 2 ml of Lugol's iodine solution was added to the plate. In the case of a positive reaction, a pale yellow zone around the colony could be observed, indicating starch degradation. Proteolytic activity was evaluated in the presence of 1% skim milk powder (Fluka Analytical). After incubation at 30°C for 48 h, a clear halo around the colony indicated milk degradation and thus a positive reaction. Phospholipase activity was evaluated in the presence of 2% egg yolk emulsion (Fluka Analytical). The cleavage of the phosphate ester bonds formed water-insoluble lipids. After incubation for 48 h at 30°C, the enzyme activity could be detected as a halo of opalescence in the opaque medium around the colony. The presence of extracellular lipase was evaluated either in the presence of 1% (v/v) tributyrin (Sigma Aldrich) or 1% (v/v) Tween 80 (Thermo Scientific). After incubation at 30°C for 48 h, a positive reaction was indicated by a clear halo around the colony in opaque tributyrin agar medium and by a precipitate surrounding the colony in the Tween 80 agar medium, respectively.

IAA production

Three ml of 1:10 dilution of a TSB (tryptone soya broth; Biorad, CA, USA) solution, supplemented with 1 mg/ml L-tryptophan, were inoculated with 200 µl of each strain liquid culture as described previously (Gordon and Weber 1951). After incubation over night at 30°C, 50 µl of Salkowsky reagent (50 ml, 35% perchloric acid and 1 ml 0.5M $FeCl_3$) were added to 50 µl of medium (single strain cultures). Absorbance (Abs) was measured after 30 min at 530 nm (Gordon and Weber 1951). Active IAA production was considered for Abs values [Abs Unit (AU)] equal or higher than the positive control (8-hydroxyquinoline) value (0.37 AU). Abs values for negative control (medium only) were also evaluated (0.04 AU).

Siderophore production

Siderophore production by bacterial strains was assayed using the Blue Agar chrome azurol S (CAS) Assay method (Louden, Haarmann and Lynne 2011). Each strain was grown on TSB medium at 30°C for 24 h. Then 10 µl of each culture was spotted on CAS agar medium (Louden, Haarmann and Lynne 2011). Siderophore production was identified by an orange colour around the bacterial colony. The characteristic blue colour of the medium changes to orange if iron is removed from the CAS/HDTMA (hexadecyltrimethylammonium bromide) complex by a siderophore.

Antibiotic resistance

Antibiotic resistance of *E. angustifolia*-associated strains was assayed by evaluating their growth on TSA medium supplemented with one of the six antibiotics listed below and possessing different mechanism(s) of action: Chloramphenicol inhibits translation by binding the 50S ribosomal subunit; Ciprofloxacin blocks DNA replication through the inhibition of DNA gyrase; Rifampicin blocks transcription by binding the β-subunit of RNA polymerase; Streptomycin, Kanamycin and Tetracycline alter translation by inhibiting the translocation of the peptidyl-RNA from the A-site to the P-site. Briefly, each strain was grown on TSA medium for 48 h at 30°C, then a colony of each strain was suspended in 100 µl saline solution (0.9% NaCl), streaked on TSA medium supplemented with different antibiotic concentrations and afterwards incubated at 30°C for 48 h. The following antibiotic concentrations (in µg/ml) were tested: Chloramphenicol (1-2.5-5-10-25-50); Ciprofloxacin (0.5-1-2.5-5-10-50); Rifampicin (5-10-25-50-100); Streptomycin and Kanamycin (0.5-1-2.5-5-10-50); Tetracycline (0.5-1.25-2.5-5-12.5-25).

Antagonistic interactions among bacterial strains

Bacterial strains equally selected from each compartment of the two plant species were screened for antagonistic interactions by the cross-streak method (Maida *et al.* 2016). Antagonistic interactions were assayed: (i) among strains (38 for each compartment) isolated from the three different *E. angustifolia* compartments and (ii) between *E. purpurea* and *E. angustifolia* strains (10 strains for each compartment of each plant). Bacterial strains tested for inhibitory activity were termed 'tester' strains, whereas those used as targets were called 'target' strains. Tester strains were streaked across one half of a TSA plate and grown at 30°C for 48 h in order to promote the possible production of antimicrobial compounds.

Target strains were then streaked perpendicularly to tester strains and the plates were further incubated at 30°C for 48 h. Additionally, target strains were grown at 30°C for 48 h in the absence of the tester strain, in order to control their growth and to compare it with that of the same strain in the cross-streaking plate. The antagonistic effect was indicated by the absence or reduction of the target strain growth. Each interaction was tested twice. The different inhibition levels have been indicated with numbers from 0 to 3 as follows: complete (3), strong (2), weak (1), and absence of inhibition (0). Next, inhibition and sensitivity scores (defined as I.S. and S.S.) were computed for tester and target strains to better evaluate their inhibition potential and their susceptibility towards inhibition. Inhibition scores of strains belonging to the same plant compartment were summed to obtain an overall evaluation of the inhibitory power and sensitivity level of a particular organ of the plant. The degree of self-inhibition (SI), referring to the ability of a given strain to inhibit its own growth, was also calculated (Maida *et al.* 2016).

Statistical analysis

Analysis of variance (ANOVA) was carried out on a binary matrix (1/0, growth/no growth) derived from the antibiotic resistance and EAA profiles and from IAA and SPH (1/0, presence/absence of activity/production). Principal Component Analysis (PCA) and ANOVA were performed by using the modules present in PAST3 software (Hammer, Harper and Ryan 2001). Variance was analysed by running an AMOVA (Analysis of Molecular Variance) using Arlequin package 3.0 (Excoffier, Laval and Schneider 2007).

MEGA7 (Molecular Evolutionary Genetics Analysis, version 7.0) was used to perform a cluster analysis on pairwise differences of antibiotic resistance profiles among compartments (Kumar, Stecher and Tamura 2016b); scale bar reported Slatkin's similarity after AMOVA computation (Slatkin 1995).

RESULTS

Phenotypic characterization of cultivable bacterial communities associated to *E. purpurea* and *E. angustifolia* plants

Bacterial communities were characterized by testing: (i) physiological features, possibly related to adaptation to different plant compartments; and (ii) antagonistic interactions among the whole strain collection.

Extracellular enzymatic activity (EEA)

Bacteria isolated from *E. purpurea* and *E. angustifolia* plants were analysed for the presence of proteolytic, phospholipase, lipasic, and amylolytic activities. The entire set of data obtained is reported in Supplementary Tables S2 and S3 and illustrated in Figure 1.

Concerning *E. purpurea* strains, R and/or RS compartments registered high levels of EEA. In particular, the R compartment registered the highest percentages of proteolytic (p_{anova} value $<$ 0.001) and phospholipase activities (p_{anova} value $<$ 0.01): 55.10% of strains from the R compartment showed proteolytic activity and 46.93% of strains showed phospholipase activity. The RS compartment registered the highest level of amylolytic activity (11.62%; p_{anova} value $<$ 0.05). Moreover, the lipasic activity towards Tween 80 (TW80) was exhibited by bacteria from the S/L and RS compartments.

However, among *E. angustifolia* strains, the R compartment registered the highest levels of lipasic TW80 (68.29%; p_{anova} value $<$ 0.001) and Tributyrin (TBT; 78.04%; p_{anova} value $<$ 0.001). Interestingly, in general *E. angustifolia* differed from *E. purpurea* for EEA, most likely because *E. angustifolia* was mainly characterized by proteolytic (p_{anova} value $<$ 0.001) and TBT activities (p_{anova} value $<$ 0.01) whereas *E. purpurea* was mostly represented by phospholipase ones (p_{anova} value $<$ 0.001).

Indole-acetic acid production

In order to check whether bacteria associated to *Echinacea* plants were able to produce IAA, and that there was a distinctive production in the two plants and among the different compartments of a single plant species, the entire panel of bacteria from the two plants were tested for IAA production. Data obtained (Supplementary Tables S2 and S3) revealed that all the tested strains exhibited capability for IAA production, independently from different plant species or compartments.

Siderophores production

Siderophores allow microorganisms to exclude competitors (by iron sequestration) from the same niche, and within plant tissues may offer a competitive advantage in colonization (Loaces, Ferrando and Scavino 2011). Thus, endophytic and rhizospheric strains from *E. purpurea* and *E. angustifolia* were also assayed for siderophore production. As shown in Supplementary Tables S2 and S3, the R compartment from both *E. purpurea* and *E. angustifolia* exhibited the highest number of siderophore-producing strains (77.55% in *E. purpurea* and 86.84% in *E. angustifolia*). The two plants showed the same trend with the siderophore production decreasing from R strains to S/L strains, and *E. angustifolia*-associated strains showed a production of siderophores higher than *E. purpurea*-associated strains ($p_{anova} = 0.01$).

Antibiotic resistance differentiates *Echinacea* plant strains

Antibiotic resistance might be one of the driving factors in shaping the structure of bacterial communities inside plants since the presence of antimicrobial molecules [synthesized by the plant itself and/or by endophytic microorganisms as signal molecules and biotic control agents (Yim, Wang and Davies 2007)] in the plant tissues could select for resistant phenotypes. *E. purpurea*-associated bacterial communities have already been investigated for their resistance to antibiotics (Mengoni *et al.* 2014) and, from this viewpoint, wide variable profiles were revealed by analysing strains from different compartments of the plants. Interestingly, at least for *Pseudomonas* strains, antibiotic resistance turned out to be more influenced by the plant compartment (R, RS or S/L) than by their genomic relatedness (Mengoni *et al.* 2014). Thus, for *E. purpurea*, the overall data suggested that the antibiotic resistance of plant-associated bacteria may play an important role in the differential colonization of plant tissues. Here, endophytic and rhizospheric strains from *E. angustifolia* were screened for their antibiotic resistance against six compounds. The data obtained, reported in Supplementary Table S4, showed that the R and RS compartments harboured the highest number of resistant strains, especially at the highest antibiotic concentrations, while bacterial strains from the S/L compartment showed resistance limited to low and intermediate antibiotic concentrations. These data are illustrated in Fig. 2, where the heatmap obtained for the antibiotic resistance of *E. angustifolia* strains showed that strains from all compartments are generally able to resist a low concentration of antibiotics (represented by red spots in Fig. 2). On the other hand, strains from the S/L compartment were more sensitive to intermediate antibiotic concentrations and high concentrations of Chloramphenicol (Fig. 2) and Streptomycin than those isolated from R or RS compartments. Finally, strains from the three plant compartments were sensitive to high concentrations of Rifampicin, Ciprofloxacin and Tetracycline.

The different antibiotic profiles for *E. angustifolia* strains obtained from R and RS compartments, compared to those from the S/L one, were also highlighted by PCA (Supplementary Fig. S1), which showed a higher similarity between strains from R and RS in comparison with those from the S/L. PCA analysis performed on the antibiotic resistance profiles of the entire set of bacterial strains also revealed that *E. purpurea* and *E. angustifolia* strains exhibited similar resistance profiles (Fig. 3).

The variation in antibiotic resistance among plant species and compartments was evaluated using the non-parametric statistics implemented in AMOVA (Excoffier, Smouse and Quattro 1992). Data obtained are shown in Table 1 and indicate that about 25% of total variance can significantly separate strains belonging to the different compartments of the plants, while no significant differences in antibiotic resistance profiles were found for the two plant species. In order to evaluate the similarity of antibiotic resistance profiles of strains belonging to the different compartments, cluster analysis was performed on pairwise differences among compartments (Slatkin's distance after AMOVA computation; Fig.

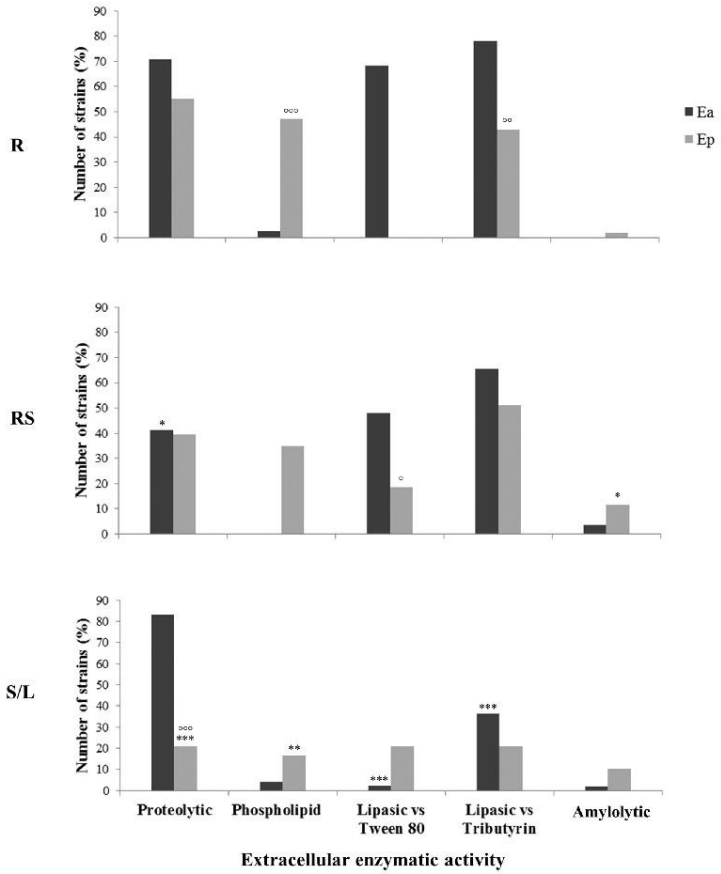

Figure 1. Enzymatic activity analyses performed on strains from R, RS and S/L compartments of *E. angustifolia* (Ea) and *E. purpurea* (Ep). Significant differences between plant compartments are indicated with respect to the R compartment: *** = p_{anova} value < 0.001; ** = p_{anova} value < 0.01; * = p_{anova} value < 0.05. Significant differences between plants are indicated with respect to *E. purpurea*: °°° = p_{anova} value < 0.001; °° = p_{anova} value < 0.01; ° = p_{anova} value < 0.05.

Table 1. Hierarchical analysis[a] of differentiation of antibiotic resistance profiles.

Variation source	df	Sum of squared deviation	Variance component	Variation (%)	*P* value
Among compartments	3	181.41	1.40	26.85	< 0.0001
Within compartments	240	936.70	3.90	74.96	
Total	243	1226.28	5.21		

[a]AMOVA was performed with antibiotic resistance profiles from strains of the three environments (RS, R and S/L compartments). Data show the degrees of freedom (df), the sum of squared deviation, the variance component estimate, the percentage of total variance contributed by each component, and the probability (*p* value) of obtaining a more extreme component estimate by chance alone, estimated by computing 10 100 permutations.

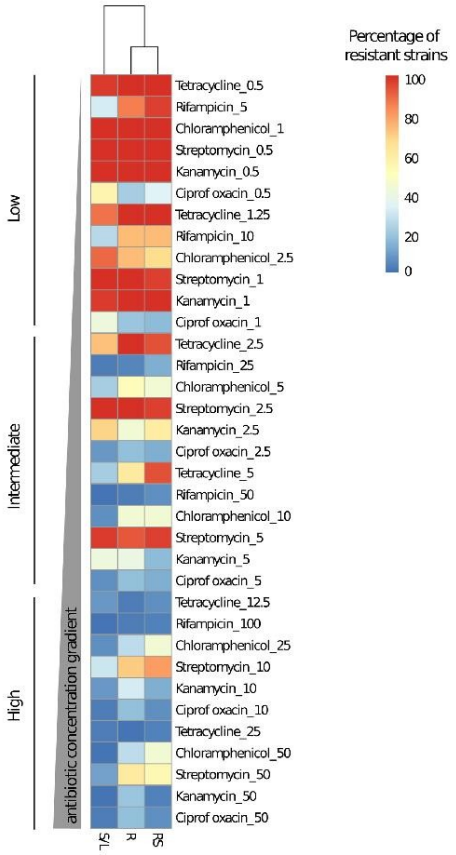

Figure 2. Antibiotic resistance phenotypes for *E. angustifolia*- associated bacterial strains. The heatmap reports antibiotic names, displayed on the right, and tested concentration (μg/ml). The heatmap colour code indicates the percentage of resistant strains retrieved in the corresponding compartment. Abbreviations: RS, rhizospheric soil; R, roots; S/L, stem/leaves.

4). Bacteria associated to roots of both plants clustered together and separated from the other compartments. On the contrary, RS were highly dissimilar. S/L compartments showed higher differences than R ones.

Antagonistic interactions acting within *E. angustifolia* compartments

The inhibition of certain bacterial strains and the growth of others exhibiting antibiotic resistance could determine differential colonization by bacterial communities inside *Echinacea* plant organs. Antagonistic interactions were already observed among rhizospheric and endophytic strains from *E. purpurea* (Maida *et al.* 2016), which highlighted an actual scenario of an arms race within and among the compartments of the plant, since its strains were indeed able to synthesize antimicrobial molecules, and the S/L compartment harbours the most susceptible strains to such molecules. In order to check whether such interactions can also act among bacterial components of *E. angustifolia* microbiota, antagonistic assays were performed for these strains using the cross-streak method. Antagonistic assays for *E. angustifolia* strains were performed considering strains from the three compartments, which were tested both as tester and target ones. Results (Fig. 5) revealed that strains unable to inhibit any of the target strains belonged mainly to the S/L compartment, which also harboured the most susceptible strains to the antagonistic effect of rhizospheric soil and endophytic strains from the R compartment and from the S/L compartment itself; the latter also showed the highest degree of self-inhibition. Moreover, RS and R strains exhibited high and similar degrees of inhibitory activity versus the S/L bacteria; lastly, RS and R were much less sensitive than S/L strains to the antimicrobial activity exhibited by all the *E. angustifolia*-associated bacteria. Hence, the main traits of antagonistic interactions among *E. purpurea* strains (i.e. higher sensibility of S/L strains and higher inhibition by RS and R strains) were also exhibited by *E. angustifolia* strains.

High level of antagonistic interactions between *E. angustifolia* and *E. purpurea* strains

Antagonistic assays were also performed between 30 strains (10 for each compartment) of the two plants, *E. purpurea* and *E. angustifolia*. Data obtained by cross-streaking experiments are shown in Table 2 and expressed as described in Materials and Methods as I.S. The analysis of data obtained revealed that bacteria exhibiting the highest degree of inhibition belong to the *E. angustifolia* RS and, to a lesser extent, R compartment. Bacteria isolated from the S/L compartment were the less active versus the *E. purpurea* strains. On the other side, the *E. purpurea*-associated bacteria were less active versus their *E. angustifolia* counterparts. Indeed, the total inhibition scores of the three compartments of the single plants showed that *E. angustifolia* strains had an overall inhibition power of 352, whereas *E. purpurea* bacterial communities presented an inhibition score of 116.

DISCUSSION

As previously reported by Chiellini *et al.* (2014), *E. purpurea*- and *E. angustifolia*-associated bacteria were isolated and characterized from the three different plant compartments (RS, R and S/L) revealing that the two plant species harboured very diverse bacterial communities in all the three analysed compartments (Supplementary Table S1), even although the two plants were phylogenetically very close, and that they germinated and grew in the same soil within a few cm of each other. At least three different and not mutually exclusive scenarios can be depicted to explain these findings.

(a) It is possible that plant anatomical and/or phytochemical features might create specific ecological niches for endophytes (Turner, James and Poole 2013); also, different root exudates may create different strong rhizospheric effects which allow them to select different bacterial communities (Kowalchuk *et al.* 2002). Additionally, it is known that different compartments of the *Echinacea* plants are characterized by different profiles of secondary metabolites (Barnes *et al.* 2005), thus the specific environment created by the metabolites produced by the plant might determine, or at

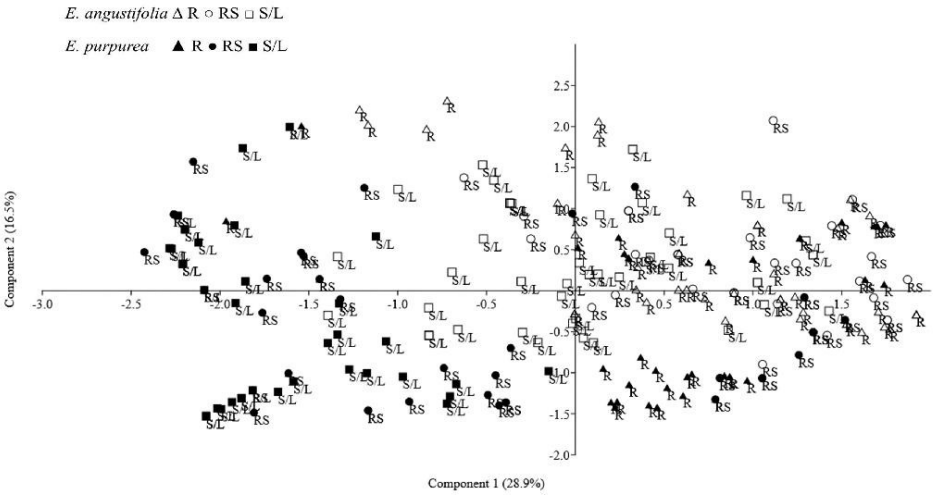

Figure 3. Plant-specificity of antibiotic resistance profiles of bacterial strains. Principal Component Analysis based on the antibiotic resistance profiles of the strains isolated from the three compartments of *E. purpurea* (Mengoni *et al.* 2014) and *E. angustifolia*. Abbreviations and symbols: *E. purpurea* (filled symbols) and *E. angustifolia* (empty symbols); RS (dot), rhizospheric soil; R (triangle), roots; S/L (square), stem/leaves.

Table 2. Inhibition scores (I.S.) calculated for inhibitory interactions exerted by *E. angustifolia* strains versus *E. purpurea* ones and *vice versa*.

			Tester strains			*Total I.S.*
			E. angustifolia			
Target strains	***E. purpurea***		**RS**	**R**	**S/L**	
		RS	58	42	18	118
		R	0	0	0	0
		S/L	144	79	11	234
	Total I.S.		*202*	121	29	352
				E. purpurea		
	E. angustifolia		**RS**	**R**	**S/L**	
		RS	8	0	10	18
		R	9	7	12	28
		S/L	29	31	10	70
	Total I.S.		46	38	32	116
EaRS >	EaR >	EpRS >	EpR >	EpS/L >	EaS/L	
202	**121**	**46**	**38**	**32**	**29**	

least influence, the composition of the bacterial communities that thrive best in such an environment.

(b) The second scenario predicts that the bacterial communities themselves can shape the microenvironment of the plant compartment determining, or partly influencing, a different profile of plant-produced secondary metabolites.

(c) Finally, a third scenario related to socio-microbiology can be suggested, in which only a few bacterial strains are initially favoured by the plant environment and largely determine, by antagonistic and synergistic interaction with the other bacteria, the major differences in the bacterial community assemblage. This hypothesis could be supported by the evidence of different antibiotic resistance profiles already observed for bacteria associated to *Echinacea* plants (Mengoni *et al.* 2014), as well as antagonistic interactions that have been observed within *E. purpurea* endophytes in different compartments (Maida *et al.* 2016). In this scenario, bacterial communities would themselves be (one of) the determinants of specific endophytic and rhizospheric communities.

Next, this work aimed to investigate the possible factor(s) responsible for the structuring of bacterial communities associated to plants. For this purpose, a large panel of cultivable endophytic and rhizospheric bacteria isolated from two congeneric medicinal plants, *E. purpurea* (L.) Moench and *E. angustifolia* (DC.), was analysed through a combination of phenotypic tests.

The first phenotypic analysis concerned the extracellular enzymatic activity of each bacterium. These tests were performed since EEAs are essential for bacteria to exploit nutrient sources, colonize habitats, and therefore to thrive within

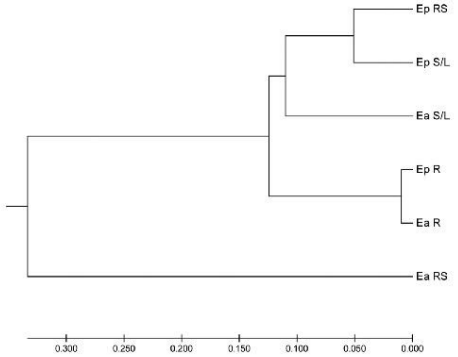

Figure 4. Unweighted pair group method with arithmetic mean (UPGMA) clustering of antibiotic resistance profiles of *E. purpurea* (Ep) and *E. angustifolia* (Ea) strains from R, RS and S/L compartments. Scale bar reports Slatkin's distance after AMOVA computation. Abbreviations: RS, rhizospheric soil; R, roots; S/L, stem/leaves.

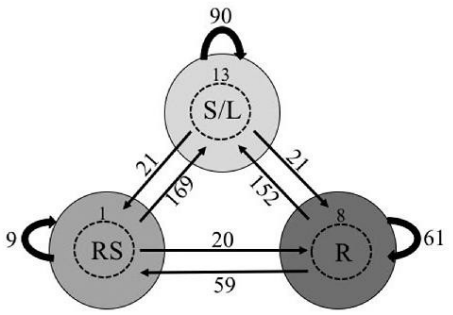

Figure 5. Schematic representation of the inhibiting activity of bacterial strains isolated from *E. angustifolia* rhizospheric soil (RS), roots (R), and stem/leaves (S/L). Each node represents a plant compartment and numbers represent the inhibiting scores of the bacteria isolated from those compartments. Direct links represent inhibition patterns. Dashed links indicate the occurrence and the extent of self-inhibition.

an environment (Hibbing *et al.* 2010). For example, enzymatic activity allows pathogenic bacteria to invade the host tissues (Ribet and Cossart 2015). The overall body of data revealed that in the two *Echinacea* species, bacteria from the same compartment did not show consistent EEA profiles, suggesting that different plant species (within their compartments) could possess peculiar features putatively involved in shaping plant bacterial communities. Repetition of this common garden experimental setting in different sites/growing conditions could allow confirmation of these preliminary findings, and enable general conclusions regarding the effect host plant species may have on the EEA activities of their associated bacterial communities. Moreover, the environment might have a greater impact on the bacterial distribution within the plants than the bacterial taxonomy since strains belonging to the same bacterial genus (e.g. *Pseudomonas*) showed different EAA profiles (Supplementary Fig. S2), mirroring those of entire communities. Even although the two *Echinacea* plants are phylogenetically very close, their different EEA profiles are in line with other data showing that EEA of bacterial endophytes may differ among plant species. For example, bacterial microbiota from the medicinal plant *Jacaranda decurrens*, showed high levels of amylolytic and proteolytic activities (60% of strains), followed by esterasic (40% of strains) and lipasic activities (40% of strains) (Carrim, Barbosa and Gonçalves Vieira 2006). Moreover, considerable amylolytic, lipasic, proteasic and cellulosic activities were described for different endophytic bacteria isolated from *Plectranthus tenuiflorus* (El-Deeb, Fayez and Gherbawy 2013). Conversely, enzymatic activities were estimated in very low quantities for bacterial endophytes from the three medicinal plants *Mangifera indica*, *Hibiscus rosasinensis*, and *Calotropis gigantea* (Vijayalakshmi 2016).

We also checked the ability of bacterial strains to synthesize IAA, which is considered to be involved in many aspects of plant growth and development (Woodward and Bartel 2005; Teale, Paponov and Palme 2006). IAA is the main natural auxin that initiated the formation of new leaves accumulating in the apical meristem. Subsequently, IAA is basipetally transported throughout the plant, from the apex to the base (Friml *et al.* 2004). In fact, it has been reported to increase root surface and lateral roots formation (Overvoorde, Fukaki and Beeckman 2010) and, in its presence, plants inoculated with PGP bacteria showed an improved uptake of nutrients. The finding that all strains from both plants were able to produce IAA suggests also that the endophytes of the R compartments might contribute to the plant's IAA content.

Concerning the siderophores production, it was not unexpected that the highest percentage of siderophore-producing strains was detected in RS and R compartments compared with the S/L compartment of both plants. Indeed, the latter compartment may constitute a less competitive compartment compared to the RS one, inhabited by a high density of microorganisms. This could justify higher percentages of siderophore-producing strains in RS and R compartments of both plants, since such compartments are highly competitive. Moreover, siderophores could be predominant in root tissues in respect to the S/L compartment as they may transport iron from soil to plant tissues improving plant development and rendering the iron unavailable to plant pathogens (Beneduzi, Ambrosini and Passaglia 2012).

The analysis of the antibiotic resistance profiles of *E. angustifolia*-associated bacteria revealed that different plant compartments were colonized by bacterial strains with dissimilar antibiotic resistance patterns in agreement with our previous data on *E. purpurea* strains (Mengoni *et al.* 2014). Thus, in both plants, R and RS compartments were characterized by higher antibiotic resistance while S/L showed higher susceptibility. This scenario gives insights into the interactions that may occur among bacterial isolates within plant compartments and how these interactions may influence the structure of bacterial communities. On the other hand, we cannot exclude a role of potentially present fungal endophytes, known to be present in upper plant tissues (Fisher, Petrini and Lappin Scott 1992). Indeed, fungal antibiotics were not used for the antibiotic resistance experiments. Thus, we can hypothesize the presence also of bacteria (i.e. those inhabiting the S/L compartment) resistant to antibiotics of fungal origin (Martinez-Klimova, Rodríguez-Peña and Sánchez 2017), which would reinforce that biotic interactions into the overall plant microbiota are one of the key players of phenotypic differentiation among strains. However, data obtained seemed to confirm that the biochemical environment of each plant organ might influence the relative bacterial adaptation (Emiliani *et al.* 2014). Moreover, comparing medicinal plants with different antibacterial activity, plant-selective

effects on physiological properties of endophytes were also recently reported (Egamberdieva *et al.* 2017). The finding that the RS bacterial communities from the two plants exhibited highly dissimilar antibiotic resistance phenotypes might be due to a different rhizospheric priming effect (decomposition of labile soil organic matter) and/or root exudates production (Fontaine, Mariotti and Abbadie 2003). Indeed, the plant species has been reported as being an important determinant in the level of rhizospheric priming (Cheng 2005; Dijkstra and Cheng 2007; Cheng 2009). Thus, it could be hypothesized that the two *Echinacea* plants select different microbial communities by means of their different rhizospheric priming effects (or, in general, root exudates composition).

Interestingly, the antibiotic resistance profiles of R- and S/L-associated bacteria were similar between the two plant species. This might suggest a common selective pressure towards antibiotic resistance profiles, which could be related to common environmental features of such tissues (i.e. the presence of metabolites in both plant species). However, a higher resistance to antibiotics of *E. angustifolia* strains with respect to *E. purpurea* ones was observed (Supplementary Table S5). This may suggest the existence of a different amount of antibiotic molecules within the plant's tissues, which could be produced by the plant itself or by the bacteria inhabiting its tissues.

To shed some light on this hypothesis, analysis of antagonistic interactions possibly existing between strains isolated from the two different plant species, and also between strains isolated from the different compartments of *E. angustifolia*, was performed. Data from the latter experiments were in line with those obtained in the antagonistic analysis between strains isolated from the three different *E. purpurea* compartments (Maida *et al.* 2016), showing that *E. angustifolia* RS and R strains had a high antimicrobial potential versus the *E. angustifolia* S/L strains. Hence, the overall body of data indicated the existence of antagonistic interactions between strains belonging to different compartments of *E. angustifolia*, in full agreement with previous data obtained on *E. purpurea* bacterial communities, which, in turn, might suggest that in the two plants species the shaping of bacterial communities was mainly due to the antagonistic interactions between them. Moreover, these data are also in agreement with the antibiotic resistance profiles exhibited by *E. angustifolia* strains.

The relative degree of inhibitory effects of strains belonging to different compartments is the same in the two plants (EaRS > EaR > EpRS > EpR > EpS/L > EaS/L; Table 2) and could be related to the presence of certain bacterial genera within compartments, which could themselves be intrinsically characterized by inhibitory potential or sensibility. However, the presence of bacterial genera with high inhibitory power and resistance in the rhizospheric soil could derive from the ecological niche conditions; therefore, highly inhibiting bacterial genera could be recruited because of the high level of competitiveness within the rhizosphere. In fact, soil microorganisms are particularly defined as active competitors that have evolved strategies to increase resource acquisition (Hibbing *et al.* 2010). For example, *Pseudomonas* strains enhancing competitive ability have been previously observed to be selected within the rhizosphere (Essarioui 2014). Plant community richness and plant host have been also demonstrated to significantly influence *Streptomyces* competitive phenotypes (Essarioui 2016). Strains inhabiting the rhizosphere, characterized by high microbial densities, would obtain an advantage from the production of antimicrobial molecules. In the same way, the presence of high susceptible and low inhibiting bacterial genera in plant S/L could be related to the lower level of competitiveness within such a compartment, which does not require intensively inhibiting strains or abundant resistance to antimicrobial molecules. A lower level of competitiveness could be figured for S/L than in RS since the bacterial abundance is significantly lower in the phyllosphere in comparison with that in the rhizospheric soil (Bodenhausen, Horton and Bergelson 2013). Indeed, low population sizes create lower levels of competitiveness since nutritional resources are a central point in microbial competition (Hibbing *et al.* 2010). According to this interpretation, the ecological niche would also constitute an important factor in shaping bacterial communities within the compartments of the analysed medicinal plants. We cannot then exclude an additional role of volatile compounds since the sensitivity of bacteria in the S/L compartment was tested only for water-soluble antibiotics.

Finally, the much higher I.S. exhibited by *E. angustifolia* strains (I.S. 352) versus *E. purpurea* strains than *vice versa* (I.S. 116) mirrors the antibiotic resistance profiles exhibited by strains belonging to the two medicinal plants, suggesting, as stated above, the existence of a different amount of antibiotic molecules within the tissues of the two plants, which could be synthesized by the plant itself or by the bacteria inhabiting its tissues.

CONCLUSIONS

The whole body of data reported in this work may predict the following steps:

(i) The two medicinal plants, even though grown very close to each other, select from the soil very different bacterial communities (at both the species and strain level). This could be due to the production of different root exudates and/or different priming effects. However, it still needs to be shown if these differences are also found in other environments or soils, and how far other factors such as seeds or plants let borne microorganisms play a role in the colonization process.

(ii) Some of the bacteria inhabiting the rhizospheric soil (RS) colonize the plant roots, giving rise to different bacterial communities in the R compartment of the two plants where they exhibit a high degree of both siderophore and IAA production.

(iii) Once inside the plant roots, bacteria may undergo selective pressures (such as the activity of secondary metabolites produced by the plant and/or by the bacterial communities themselves), which may lead to the differentiation of bacterial communities inhabiting different compartments.

(iv) Antibiotic molecules within plants' tissues could determine selective pressures and consequently influence the assemblage of bacterial communities. Antibiotics in plants' compartments may derive from bacteria, fungi or from the plant itself. Considering non-volatile bacteria-derived molecules, the S/L compartment appears to select bacteria which are both less prone to interfere with the growth of other bacteria and are less antibiotic-resistant. From this point of view, S/L represents a less competitive environment.

(v) Moreover, it is well known that different *Echinacea* species synthesize different bioactive molecules with different biological activity (Barnes *et al.* 2005), and this may result in the selection of more (or less) resistant bacterial communities. This may explain why *E. angustifolia* strains are more resistant to antibiotics and possess a higher antagonistic effect than *E. purpurea*- associated bacteria.

(vi) Our data show that bacterial antagonism and antibiotic resistance contribute to drive species-specific microbiota differentiation in *Echinacea* spp.

Thus, plant-bacteria and bacteria-bacteria interactions, and especially the interplay between plant and bacterial metabolites, as shown by our recent results (Maggini *et al.* 2017) might drive the differentiation of bacterial communities inhabiting different compartments of the same plant.

Finally, from a biotechnological and/or pharmacological viewpoint, the screening for antimicrobial molecules in plant microbiome could also unveil an unexplored source for new antibiotics. Bioactive molecules with clinical relevance have already been isolated from endophytes (Shweta *et al.* 2013). In this regard, *Rheinheimera* EpRS3, isolated from the rhizosphere of *E. purpurea*, showed capacity for inhibiting important human pathogens resistant to the commonly used antibiotics (Presta *et al.* 2017). Moreover, very recently it has been demonstrated that the endophytes associated with *E. purpurea* roots (and to a much lesser extent, *E. purpurea* stem/leaves) used in this work are able to inhibit the growth of bacteria belonging to the *Burkholderia cepacia* complex (Chiellini *et al.* 2017).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

REFERENCES

Afzal M, Khan QM, Sessitsch A. Endophytic bacteria: prospects and applications for the phytoremediation of organic pollutants. *Chemosphere* 2014;**117**:232–42.

Atlas RM. Handbook of biological media. *Parks CRC Press* 1994;**22**:1–1953.

Barnes J, Anderson LA, Gibbons S *et al.* Echinacea species (Echinacea angustifolia (DC.) Hell., Echinacea pallida (Nutt.) Nutt.,Echinacea purpurea (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *J Pharm Pharmacol* 2005;**57**:929–54.

Beneduzi A, Ambrosini A, Passaglia LM. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genet Mol Biol* 2012;**35**:1044–51.

Berg G. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 2009;**84**:11–18.

Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. *PLoS One* 2013;**8**:e56329.

Brader G, Compant S, Mitter B *et al.* Metabolic potential of endophytic bacteria. *Curr Opin Biotechnol* 2014;**27**:30–37.

Carrim AJI, Barbosa EC, Gonçalves Vieira JD. Enzymatic Activity of Endophytic Bacterial Isolates of Jacaranda decurrens Cham. (Carobinha-do-campo). *Brazilian Archives of Biology and Technology* 2006;**49**:353–9.

Checcucci A, Maida I, Bacci G *et al.* Is the plant-associated microbiota of Thymus spp. adapted to plant essential oil? *Res Microbiol* 2017;**168**:276–82.

Cheng W. Rhizosphere priming effect: Its functional relationships with microbial turnover, evapotranspiration, and C-N budgets. *Soil Biology and Biochemistry* 2009;**41**:1795–1801.

Cheng W, Kuzyakov Y. Root effects on soil organic matter decomposition. In: Zobel RW, Wright S (eds). *Roots and Soil Management: Interactions between Roots and the Soil*. Madison, Wisconsin: American Society of Agronomy, 2005, 119–43.

Chiellini C, Maida I, Maggini V *et al.* Preliminary data on antibacterial activity of Echinacea purpurea-associated bacterial communities against Burkholderia cepacia complex strains, opportunistic pathogens of Cystic Fibrosis patients. *Microbiol Res* 2017;**196**:34–43.

Chiellini C, Maida I, Emiliani G *et al.* Endophytic and rhizospheric bacterial communities isolated from the medicinal plants Echinacea purpurea and Echinacea angustifolia. *Int Microbiol* 2014;**17**:165–74.

Dijkstra FA, Cheng W. Interactions between soil and tree roots accelerate long-term soil carbon decomposition. *Ecol Lett* 2007;**10**:1046–53.

Egamberdieva D, Wirth S, Behrendt U *et al.* Antimicrobial Activity of Medicinal Plants Correlates with the Proportion of Antagonistic Endophytes. *Front Microbiol* 2017;**8**:199.

El-Deeb B, Fayez K, Gherbawy Y. Isolation and characterization of endophytic bacteria from Plectranthus tenuiflorus medicinal plant in Saudi Arabia desert and their antimicrobial activities. *J Plant Interactions* 2013;**8**:56–64.

Emiliani, G, Mengoni A, Maida I *et al.* Linking bacterial endophytic communities to essential oils: clues from lavandula angustifolia mill. *Evid Based Complement Alternat Med* 2014;**2014**:650905.

Essarioui A, Kistler HC, Kinkel LL. Nutrient use preferences among soil Streptomyces suggest greater resource competition in monoculture than polyculture plant communities. *Plant Soil* 2016;**409**:329–43.

Essarioui A, LeBlanc N, Kistler HC *et al.* Plant host and community diversity impact the dynamics of resource use by soil Streptomyces. *Phytopathology* 2014;**104**:38.

Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 1992;**131**:479–91.

Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online* 2007;**1**:47–50.

Fisher PJ, Petrini O, Lappin Scott HM. The distribution of some fungal and bacterial endophytes in maize (Zea mays L.). *New Phytologist* 1992;**122**:299–305.

Fontaine S, Mariotti A, Abbadie L. The priming effect of organic matter: a question of microbial competition? *Soil Biology & Biochemistry* 2003;**35**:837–43.

Friml J, Yang X, Michniewicz M *et al.* A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* 2004;**306**:862–65.

Gordon SA, Weber RP. Colorimetric Estimation of Indoleacetic Acid. *Plant Physiol* 1951;**26**:192–5.

Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 2001;**4**:9.

Haron MH, Tyler HL, Pugh ND *et al.* Activities and prevalence of Proteobacteria members colonizing Echinacea purpurea fully account for macrophage activation exhibited by extracts of this botanical. *Planta medica*. 2016;**82**:1258.

Hibbing ME, Fuqua C, Parsek MR *et al.* Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* 2010;**8**:15–25.

Karsch-Volk M, Barrett B, Linde K. Echinacea for preventing and treating the common cold. *JAMA* 2015;**313**:618–9.

Karsch-Volk M, Barrett B, Kiefer D *et al.* Echinacea for preventing and treating the common cold. *Cochrane Database Syst Rev* 2014:CD000530.

Kowalchuk GA, Buma DS, de Boer W *et al.* Effects of aboveground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek* 2002;**81**:509–20.

Kumar M, Tomar RS, Lade H *et al.* Methylotrophic bacteria in sustainable agriculture. *World J Microbiol Biotechnol* 2016;**32**:120.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* 2016;**33**:1870–74.

Loaces I, Ferrando L, Scavino AF. Dynamics, diversity and function of endophytic siderophore-producing bacteria in rice. *Microb Ecol* 2011;**61**:606–18.

Long HH, Schmidt DD, Baldwin IT. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One* 2008;**3**:e2702.

Louden BC, Haarmann D, Lynne AM. Use of Blue Agar CAS Assay for Siderophore Detection. *J Microbiol Biol Educ* 2011;**12**:51–3.

Ma Y, Rajkumar M, Zhang C *et al.* Beneficial role of bacterial endophytes in heavy metal phytoremediation. *J Environ Manage* 2016;**174**:14–25.

Maggini V, De Leo M, Mengoni A *et al.* Plant-endophytes interaction influences the secondary metabolism in Echinacea purpurea (L.) Moench: an in vitro model. *Sci Rep* 2017;**7**:16924.

Maida I, Chiellini C, Mengoni A *et al.* Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant Echinacea purpurea. *Environ Microbiol* 2016;**18**:2357–65.

Martinez-Klimova E, Rodríguez-Peña K, Sánchez S. Endophytes as sources of antibiotics. *Biochem Pharmacol*. 2017;**134**:1–17.

Mengoni A, Maida I, Chiellini C *et al.* Antibiotic resistance differentiates Echinacea purpurea endophytic bacterial communities with respect to plant organs. *Res Microbiol* 2014;**165**:686–94.

Mori E, Lio P, Daly S *et al.* Molecular nature of markers from Haemophilus influenzae Rd genome. *Res Microbiol* 1999;**150**:83–93.

Muller DB, Vogel C, Bai Y *et al.* The Plant Microbiota: Systems-Level Insights and Perspectives. *Annu Rev Genet* 2016;**50**:211–34.

Nair DN, Padmavathy S. Impact of endophytic microorganisms on plants, environment and humans. *ScientificWorldJournal* 2014;**2014**:250693.

Overvoorde P, Fukaki H, Beeckman T. Auxin control of root development. *Cold Spring Harb Perspect Biol* 2010;**2**:a001537.

Passari AK, Mishra VK, Leo VV *et al.* Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from Clerodendrum colebrookianum Walp. *Microbiol Res* 2016;**193**:57–73.

Presta L, Bosi E, Fondi M *et al.* Phenotypic and genomic characterization of the antimicrobial producer Rheinheimera sp. EpRS3 isolated from the medicinal plant Echinacea purpurea: insights into its biotechnological relevance. *Res Microbiol* 2017;**168**:293–305.

Pugh ND, Tamta H, Balachandran P *et al.* The majority of in vitro macrophage activation exhibited by extracts of some immune enhancing botanicals is due to bacterial lipoproteins and lipopolysaccharides. *International immunopharmacology* 2008;**8**:1023–32.

Pugh ND, Jackson CR, Pasco DS. Total bacterial load within Echinacea purpurea, determined using a new PCR-based quantification method, is correlated with LPS levels and in vitro macrophage activity. *Planta medica* 2013;**79**:9.

Ribet D, Cossart P. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect* 2015;**17**:173–83.

Ryan RP, Germaine K, Franks A *et al.* Bacterial endophytes: recent developments and applications. *FEMS Microbiol Lett* 2008;**278**:1–9.

Santhanam R, Groten K, Meldau DG *et al.* Analysis of plant-bacteria interactions in their native habitat: bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages. *PLoS One* 2014;**9**:e94710.

Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda Mdel C *et al.* Plant growth-promoting bacterial endophytes. *Microbiol Res* 2016;**183**:92–9.

Shweta S, Bindu JH, Raghu J *et al.* Isolation of endophytic bacteria producing the anti-cancer alkaloid camptothecine from Miquelia dentata Bedd. (Icacinaceae). *Phytomedicine* 2013;**20**:913–7.

Slatkin M. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 1995;**139**:457–62.

Spaepen S, Vanderleyden J, Remans R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol Rev* 2007;**31**:425–48.

Teale WD, Paponov IA, Palme K. Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol* 2006;**7**:847–59.

Todd DA, Gulledge TV, Britton ER *et al.* Ethanolic Echinacea purpurea extracts contain a mixture of cytokine-suppressive and cytokine-inducing compounds, including some that originate from endophytic bacteria. *PloS one* 2015;**10**:e0124276.

Turner TR, James EK, Poole PS. The plant microbiome. *Genome Biol* 2013;**14**:209.

Vijayalakshmi R, Kairunnisa K, Sivvaswamy Narender S *et al.* Enzyme Production and Antimicrobial Activity of Endophytic Bacteria Isolated from Medicinal Plants. *Indian Journal of Science and Technology* 2016;**9**:1–8.

Woodward AW, Bartel B. Auxin: regulation, action, and interaction. *Ann Bot* 2005;**95**:707–35.

Yim G, Wang HH, Davies J. Antibiotics as signalling molecules. *Philos Trans R Soc Lond B Biol Sci* 2007;**362**:1195–1200.

4. Genomics of endophytic bacteria

The analysis of the genomic features of bacterial endophytes might offer valuable insights into the mechanisms involved in successful plant colonization and interaction, as well as secondary metabolites production.

The characterization of *Echinacea* spp. medicinal plants-associated bacteria performed in the last years in our laboratory, has led to the selection of potentially relevant isolates from a biotechnological point of view. Strain *Pseudomonas* sp. Ep S/L25 was highlighted as presenting important ability to inhibit human opportunistic pathogens belonging to the *Burkholderia cepacia* complex (Bcc). Another strain from *E. purpurea* stem/leaves, *Arthrobacter* sp. Ep S/L 27 was evidenced with high resistance to oxidative stress and degradation of diesel fuel, as well as inhibition of Bcc strains. Inhibition of Bcc strains was also evidenced for *Pseudomonas* sp. Ep R 1. Moreover, *Rheinheimera* sp. Ep RS 3 exhibited not only inhibition of growth of Bcc strains, but also against multidrug resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Considering the important phenotypes observed for such strains, we decided to deepen their characterization, analyzing their genomes, which can also identify putative genes for production of bioactive compounds.

Data obtained concerning this issue have been published in the below attached papers.

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Arthrobacter sp. EpRS66 and *Arthrobacter* sp. EpRS71: Draft Genome Sequences from Two Bacteria Isolated from *Echinacea purpurea* Rhizospheric Soil

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INTRODUCTION

One of the most promising, but still overlooked fields of microbiological research is represented by endophytic microorganisms, i.e., those organisms living in the tissues of host plants and/or in their rhizosphere (Rosenblueth and Martínez-Romero, 2006; Reinhold-Hurek and Hurek, 2011). These microbes are emerging as a new potential source of secondary metabolites and products, for exploitation in medicine, agriculture, and industry.

From a biotechnological perspective, a controlled (engineered) colonization of plant's tissues by some bacteria may be desirable because of their ability to produce a variety of plant growth promoting (PGP) molecules, spanning from siderophores, nitrogenases, hormones, and so on. In addition, host-microbe interaction confers indirect advantage to the plant, resulting from the inhibition activity exerted by the associated microbial community toward potential pathogens.

Moreover, in the field of medicine, there are examples of very well-known molecules derived by endophytes like antibiotics, antimycotics, and anticancer drugs. Also, it is still unknown if plant-associated bacteria may enhance (or be responsible for) some of the effects exerted by the extracts of medicinal plants (essential oils) (Kloepper and Ryu, 2006; Hardoim et al., 2008).

In this regard, in October 2012, in Casola Valsenio (Italy), a collection of microorganisms was isolated from both internal tissues and the rhizospheric soil of the medicinal plant *Echinacea purpurea*, as reported in Chiellini et al. (2014). Among others, two strains sampled from the rhizosphere and belonging to *Arthrobacter* species were identified, i.e., *Arthrobacter* sp. EpRS66 and *Arthrobacter* sp. EpRS71. Based on their antibiotic resistance profile, reported in Mengoni et al. (2014), and on further tests performed on these two strains, they were selected as good candidates for genome sequencing analysis. The last, will constitute a resource to deeply investigate their genomic features and to perform comparative genomics analysis. Moreover, in the aim of new drugs discovery, the genome sequence will facilitate the identification of putative genes responsible for the production of bioactive compounds.

MATERIALS AND METHODS

DNA Extraction and Sequencing

Arthrobacter sp. EpRS66 and *Arthrobacter* sp. EpRS71 strains were inoculated overnight on TSB medium at 30°C. Their genomic DNA was then extracted using the CTAB method (Perrin et al., 2015). Furthermore, the authenticity of the genomic DNA was confirmed by 16S RNA gene sequencing.

MiSeq sequencing system (Illumina Inc., San Diego, CA) was used to perform the whole genome shot-gun of the two organisms. The method used a 2 × 300 bp paired-end approach, which produced a genome coverage of 246.0 x for *Arthrobacter* sp. EpRS66 and 91x for *Arthrobacter* sp. EpRS71.

Genome Assembly and Annotation

The quality of the obtained read pairs was evaluated by inspecting them with FastQC software package v. 0.52 (Kunde-Ramamoorthy et al., 2014). Poor quality bases were removed with StreamingTrim (Bacci et al., 2014). *De novo* assembly was performed by using SPAdes 3.5 software (Bankevich et al., 2012) with a k-mer length of 21, 33, and 55. After, those contigs with length inferior to 2000 bp were trimmed and the remaining (6 and 24 for *Arthrobacter* sp. EPRS66 and *Arthrobacter* sp. EPRS71, respectively) were launched in a multi-draft based analysis through MeDuSa scaffolder (Bosi et al., 2015), by using as references 5 *Arthrobacter* genomes retrieved at NCBI database (*Arthrobacter arilaitensis* Re117, *Arthrobacter* FB24, *Arthrobacter* Rue61a, *Arthrobacter aurescens* TC1, *Arthrobacter chlorophenolicus* A6).

Automated annotation of the two draft genome sequences has then been performed with NCBI Prokaryotic Genome Annotation Pipeline.

RESULTS

The last version of *Arthrobacter* sp. EpRS66 genome has a total length of 3,707,708 bp and embeds only 2 scaffolds (L50 equal to 1), with a mean G+C content of 59.27%. The annotation analysis identified a total of 3485 genes, of which 3383 have been annotated as coding DNA sequences (CDS), 29 as pseudogenes, 4 as rRNAs, 68 as tRNAs, and 1 as ncRNA.

The draft genome sequence of *Arthrobacter* sp. EpRS71 24 is 4,849,450 bp long and its contigs are set-up in 10 scaffolds (L50 equal to 1). The G+C content is 61.60%, a value slightly higher than the previous but still perfectly comparable with that of other *Arthrobacter* genomes sequenced so far. The annotation of *Arthrobacter* sp. EpRS71 genome revealed the presence of 4515 genes. This total amount includes 4379 proteins coding sequences, 71 pseudogenes, and 62 RNA (6 rRNAs, 55 tRNAs, 1 ncRNA) coding sequences.

Both genome sequences have been deposited at NCBI database and are available in both fasta and GenBank format; the GenBank accession number of *Arthrobacter* sp. EPRS66 is LNUU00000000 and the version reported in this work was named LNUU01000000; the GenBank accession number of *Arthrobacter* sp. EPRS71 is LNUV00000000 and the version reported in this work is LNUV01000000.

AUTHOR CONTRIBUTIONS

This project was planned by RF and AM. The DNA extraction was performed by IM and EP. The DNA sequencing has been performed by GR and VD. The data processing has been performed by LP and MF. CC, VM, PB, and EM assisted substantially on the technical part of this work. All author contributed to writing and editing the present manuscript.

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REFERENCES

Bacci, G., Bazzicalupo, M., Benedetti, A., and Mengoni, A. (2014). StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. *Mol. Ecol. Resour.* 14, 426–434. doi: 10.1111/1755-0998.12187

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bosi, E., Donati, B., Galardini, M., Brunetti, S., Sagot, M.-F., Lió, P., et al. (2015). MeDuSa: a multi-draft based scaffolder. *Bioinformatics* 31, 2443–2451. doi: 10.1093/bioinformatics/btv171

Chiellini, C., Maida, I., Emiliani, G., Mengoni, A., Stefano, M., Fabiani, A., et al. (2014), Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. *Int. Microbiol.* 17, 165–174. doi: 10.2436/20.1501.01.219

Hardoim, P. R., van Overbeek, L. S., and Elsas van, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* 16, 463–471. doi: 10.1016/j.tim.2008.07.008

Kloepper, J. W., and Ryu, C.-M. (2006). "Bacterial endophytes as elicitors of induced systemic resistance," in *Microbial Root Endophytes*, eds B. Schulz, C. Boyle, and T. N. Sieber (Berlin; Heidelberg: Springer), 33–52. doi: 10.1007/3-540-33526-9_3

Kunde-Ramamoorthy, G., Coarfa, C., Laritsky, E., Kessler, N. J., Harris, R. A., Xu, M., et al. (2014). Comparison and quantitative verification of mapping algorithms for whole-genome bisulfite sequencing. *Nucleic Acids Res.* 42:e43. doi: 10.1093/nar/gkt1325

Mengoni, A., Maida, I., Chiellini, C., Emiliani, G., Mocali, S., Fabiani, A., et al. (2014). Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. *Res. Microbiol.* 165, 686–694. doi: 10.1016/j.resmic.2014.09.008

Perrin, E., Fondi, M., Maida, I., Mengoni, A., Chiellini, C., Mocali, S., et al. (2015). Genomes analysis and bacteria identification: the use of overlapping genes as molecular markers. *J. Microbiol. Methods* 117, 108–112. doi: 10.1016/j.mimet.2015.07.025

Reinhold-Hurek, B., and Hurek, T. (2011). Living inside plants: bacterial endophytes. *Curr. Opin. Plant Biol.* 14, 435–443. doi: 10.1016/j.pbi.2011.04.004

Rosenblueth, M., and Martínez-Romero, E. (200
their interactions with hosts. *Mol. Plant. Micro*
10.1094/MPMI-19-0827

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Original Article

Phenotypic and genomic characterization of the antimicrobial producer *Rheinheimera* sp. EpRS3 isolated from the medicinal plant *Echinacea purpurea*: insights into its biotechnological relevance

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Abstract

In recent years, there has been increasing interest in plant microbiota; however, despite medicinal plant relevance, very little is known about their highly complex endophytic communities. In this work, we report on the genomic and phenotypic characterization of the antimicrobial compound producer *Rheinheimera* sp. EpRS3, a bacterial strain isolated from the rhizospheric soil of the medicinal plant *Echinacea purpurea*. In particular, EpRS3 is able to inhibit growth of different bacterial pathogens (Bcc, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*) which might be related to the presence of gene clusters involved in the biosynthesis of different types of secondary metabolites. The outcomes presented in this work highlight the fact that the strain possesses huge biotechnological potential; indeed, it also shows antimicrobial effects upon well-described multidrug-resistant (MDR) human pathogens, and it affects plant root elongation and morphology, mimicking indole acetic acid (IAA) action.

Keywords: Endophytes; Antibiotic resistance; Antibiotic production; Biotechnological applications; Genome analysis

1. Introduction

A diverse range of bacteria, including parasites, commensals and mutualists, can colonize the rhizosphere of plants, growing around their roots [1] and eventually colonizing plant internal tissues and surfaces (as endophytes or epiphytes). Despite medicinal plant relevance, very little is known about their plant-associated bacteria. In recent years, there has been increasing interest in medicinal plant microbiota; numerous efforts have been made to explore both endophytic and rhizospheric diversity [2–9]. Those studies showed that medicinal plants harbor highly complex bacterial communities, whose structure is possibly influenced by the plant organ colonized and secondary compounds with medicinal properties (e.g. plant essential oils) [7,8]. In particular, in a recent work on *Echinacea purpurea* [7], different antibiotic resistance phenotypes have been observed for rhizospheric and

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endophytic bacterial communities, suggesting the presence of a plethora of defense mechanisms and differential production of antimicrobial compounds by members of these bacterial communities.

Rheinheimera sp. EpRS3, a Gammaproteobacterium, belonging to the family of *Chromatiales*, is one such member. It was isolated from a sample of rhizospheric soil of *E. purpurea* [7,8], a medicinal plant with documented antimicrobial activity [6]. *Rheinheimera* sp. EpRS3 showed resistance to several antibiotic compounds and was able to inhibit growth of various bacteria isolated from both *E. purpurea* rhizospheric soil and plant tissues [7–9].

Therefore, the aim of this work was to characterize the *Rheinheimera* sp. EpRS3 strain from different viewpoints so as to evaluate its possible biotechnological potential, in terms of both the ability to affect plant growth and to interfere with growth of human bacterial pathogens. For this purpose, it was characterized at a phenotypic level by testing different traits (especially those related to production of antimicrobials and those involved in plant growth promotion); moreover, the genome sequence was determined and analyzed in order to correlate phenotypic data with the genetic repertoire.

2. Materials and methods

2.1. Bacterial strains, isolation and growth conditions

Rheinheimera sp. EpRS3 was isolated from the medicinal plant *E. purpurea* as described by Chiellini et al. [5]. This strain belongs to a collection of isolates from a pool of five *E. purpurea* plants that were grown in a common garden at the "Giardino delle Erbe", Casola Valsenio (Italy).

Rheinheimera sp. EpRS3 was grown either in tryptic soy agar (TSA) or in minimal medium Davis (MMD) (1.0 g l^{-1} of $(NH_4)_2SO_4$, 7.0 g l^{-1} of K_2HPO_4, 2.0 g l^{-1} of KH_2PO_4, 0.5 g l^{-1} of Na_3-citrate $2H_2O$, 0.1 g l^{-1} of $MgSO_4 \cdot 7H_2O$) and in minimal medium Venetia (MMV) (1.0 g l^{-1} of $MgSO_4 \cdot 7H_2O$, 0.7 g l^{-1} of KCl, 2.0 g l^{-1} of KH_2PO_4, 3.0 g l^{-1} of Na_2HPO_4, 1.0 g l^{-1} of NH_4NO_3, and 24.0 g l^{-1} of NaCl in deionized water) containing 0.4% diesel fuel or 1% glucose as the sole carbon and energy source. Diesel fuel (Esso Italiana) was previously filtered through a 0.2 μm-pore-size filter (Sartorius) for sterilization and particle removal. Bacterial cultures were incubated at 30 °C.

Burkholderia strains were grown in TSA medium at 37 °C.

2.2. Response to oxidative stresses and heavy-metals resistance

Resistance to oxidative stress was tested by evaluating *Rheinheimera* sp. EpRS3 growth on TSA medium supplemented with hydrogen peroxide, zinc, copper, cadmium or nickel (heavy metals). The strain was grown for 48 h (or until satisfactory growth) in each of the supplemented TSA media at 30 °C, and an isolated colony was then suspended in 100 μl of saline solution (0.85% NaCl). The cell suspension was then streaked onto TSA plates supplemented with different concentrations of: hydrogen peroxide (1-5-10-20 mM); zinc ($ZnSO_4$ 5-10-15-25 mM); copper ($CuSO_4$ 0.25-0.5-1-2.5-5-10-15-25 mM); cadmium ($Cd(NO_3)2$ 5-10-15-25 mM); nickel ($NiCl_2$ 5-10-15-25 mM). Strain growth was rated as absence of growth (i.e. sensitivity to exposure) or growth (i.e. tolerance at the tested oxidative stress). No quantitative assessment of the amount of growth was evaluated.

2.3. Extracellular enzymatic activity (EEA)

Extracellular amylolytic, proteolytic, phospholipase and lipase enzyme activities were assessed as follows. Amylolytic activity was evaluated by streaking *Rheinheimera* sp. EpRS3 onto 10% TSA medium enriched with 1% starch from potatoes (Fluka Analytical). After incubation at 30 °C for 48 h, 2 ml of Lugol's iodine solution was added to the plate. A positive reaction was defined as the observation of a pale yellow zone around the colony, indicating starch degradation [7].

Proteolytic activity was evaluated in 10% TSA supplemented with 1% skim milk powder (Fluka Analytical). After incubation for 48 h at 30 °C, a clear halo around the colony indicated milk degradation and thus a positive reaction [7].

Phospholipase activity was evaluated in 10% TSA supplemented with 2% egg yolk emulsion (Fluka Analytical). The cleavage of the phosphate ester bonds forms water-insoluble lipids. After incubation for 48 h at 37 °C, enzyme activity can be observed as a halo of opalescence in the opaque medium around the colony.

The presence of extracellular lipase was evaluated either in 10% TSA enriched with 1% (v/v) tributyrin (Sigma Aldrich) or in 10% TSA enriched with 1% (v/v) Tween 80 (Thermo Scientific). After incubation at 37 °C for 48 h, a positive reaction is indicated by a clear halo around the colony in opaque tributyrin agar medium, and by a precipitate around the colony in Tween 80 agar medium, respectively. Each experiment was performed in triplicate.

2.4. Antimicrobial activity assay *vs.* Burkholderia cepacia *complex,* Acinetobacter baumannii *and* Klebsiella pneumoniae *strains*

Antibacterial activity of *Rheinheimera* sp. EpRS3 (defined as a "tester strain") was tested using the cross-streak method as described by Papaleo et al. [8] against six *A. baumannii* (two of which, RUH 875 and RUH 134, are well-described MDR strains of the respective international clones II and I [9],) eleven *K. pneumoniae* and thirty *Burkholderia* strains, listed in Table 1 and referred to as "target strains". Moreover, in susceptibility testing, some *A. baumannii* and *K. pneumoniae* strains used in this work showed a colistin resistance phenotype (see Table 1).

In the assay against *Burkholderia* strains, the tester strain was streaked across two different half Petri dishes containing TSA medium either with or without a central septum separating the tester strain from target strains. Such an expedient has been used to test whether any antimicrobial effect occurring is detected only when molecules can reach the target by

Table 1
List of bacterial strains belonging to the *Burkh*
Fibrosis; AI, Animal Infection; E, Environment;

Position on plate		
1		
2		
3		
4		
5		
6		
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Position on plate	Taxonomy	Strain
1	*A. baumannii*	RHU
2	*A. baumannii*	RHU
3	*A. baumannii*	ATC
4	*A. baumannii*	OSC
5	*A. baumannii*	N5O
6	*A. baumannii*	YMC
1	*K. pneumoniae*	BO 1
2	*K. pneumoniae*	BO 4
3	*K. pneumoniae*	B1
4	*K. pneumoniae*	B2
5	*K. pneumoniae*	ATC
6	*K. pneumoniae*	KP39
7	*K. pneumoniae*	KP47
8	*K. pneumoniae*	KPC
9	*K. pneumoniae*	KPC
10	*K. pneumoniae*	KPC
11	*K. pneumoniae*	NTU

diffusing into the medium, or if the antimicrobial effect is carried by a volatile compound that hence can also exert its effect when the tester and target are separated by a barrier. In tests to probe antimicrobial activity against *A. baumannii* and *K. pneumoniae*, we used the plates without septum.

All plates were then incubated at 30 °C for 48 h. Next, target strains were streaked perpendicularly to the initial streak in the empty half of the plate; plates with *Burkholderia* strains were incubated at 30 °C and 37 °C for an additional 48 h, respectively, while the other strains were incubated at 37 °C for 24 h. The antagonistic effect was indicated by growth inhibition of target strains in the confluence area. All experiments were performed in triplicate and in parallel with a positive control to verify viability of target strains.

2.5. Determination of fosfomycin minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of fosfomycin was determined by the agar dilution reference method according to CLSI guidelines [10]. Mueller-Hinton agar plates, supplemented with 25 μg/ml of glucose-6-phosphate, were added with the following concentrations of the antibiotic: 0.064 μg/ml, 0.125 μg/ml, 0.256 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml, 128 μg/ml, 256 μg/ml and 512 μg/ml. Starting from a 0.5 McFarland suspension of *Rheinehimera* EpRS3, a 1:10 dilution in saline solution was prepared and agar plates were inoculated with 5 μl spots from the diluted McFarland inoculum. Plates were then incubated at 37 °C overnight. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains were used as quality control strains.

2.6. Colorimetric estimation of 3-indole acetic acid production

Three ml of tryptic soy broth (TSB), Biorad solution 1:10, supplemented with 1 mg/ml L-tryptophan, were inoculated with 200 μl of *Rheinheimera* sp. EpRS3 liquid culture. According to the assay described in [20], after incubation overnight at 30 °C, 50 μl of $FeCl_3$-$HClO_4$ reagent were added to 50 μl of medium. Absorbance was measured after 30 min at 530 mμ.

2.7. Plant growth conditions

Nicotiana tabacum cv. Xanthi seeds were surface-sterilized for 20 min in 5% NaOCl solution, followed by three washes with sterile distilled water. Sterilized seeds were then transferred onto Petri dishes containing Lindsmaier and Skoog medium (LS, Lab Associates BV, the Netherlands) supplemented with 1% sucrose and 0.7% plant agar. Plates were incubated in a growth chamber at 24 ± 1 °C, 80% humidity and in the dark until root differentiation. Seeds with primary roots were then placed in the light at 1500 lux and with a 16 h light/8 dark photoperiod regimen. Ten days from germination, *N. tabacum* seedlings were used for a vertical agar plate (VAP) assay [11].

2.8. Dual culture vertical agar plate assay

The endophytic *Rheinheimera* sp. EpRS3 strain and *E. coli* DH5α (used as control) were removed from −80 °C storage, streaked onto plates containing TSA and incubated at 30 °C for 48 h. Single colonies were then inoculated into tryptic soy broth (TSB) and grown in a shaker at the same temperature overnight. The bacterial suspensions were then adjusted to 10^8 cfu/ml ($OD_{600} = 1$). Twenty *N. tabacum* seedlings of the same age and dimension were grown on 15 cm Petri dishes containing LS basal medium; 100 μl of each of the OD_{600} suspension cultures or 100 μl culture filtrates were inoculated onto a sterilized filter paper disc placed 1 cm below the root tips of the seedlings, approximately at the center of the line of plants. Control treatments were made with 100 μl of TSB culture medium. Plates were incubated vertically in the growth chamber at 24 ± 1 °C and scored for root growth and morphology after 7 and 14 days from treatments. Root growth was reported as root length fold increase (mm) measured as (fl−il)/il where fl was the length of primary root after 7 or 14 days of culture and il was the initial length of primary roots. Each experiment was performed in duplicate.

2.9. Statistical analysis

All statistical analyses were performed using the PAST program, version 3.12 [12]. The analysis of variance between treated and untreated *N. tabacum* plants was conducted using One-way ANOVA ($P < 0.05$). Mean separations were performed using the method of Tukey. Student's *t* test was used for comparing two averages.

2.10. DNA isolation and whole genome sequencing

The *Rheinheimera* sp. EpRS3 strain was grown at 30 °C on TSB medium; genomic DNA was extracted using the CTAB method [13]. Authenticity of genomic DNA was confirmed by 16S rRNA gene sequencing. Whole genome shotgun sequencing was performed with a 2 × 300 bp paired-end approach using the MiSeq sequencing system (Illumina Inc., San Diego, CA), which produced a total of 1,245,634 high-quality reads.

2.11. Phylogenetic affiliation

Eighteen 16S rRNA gene sequences belonging to *Rheinheimera* species (representing all the species available for this genus) were selected from the Ribosomal DataBase Project (RDP) (http://rdp.cme.msu.edu/) [14] and from NCBI ftp site. Moreover, the 16S rRNA gene sequence from *Gallaecimonas pentaromativorans* CEE 131 was included as an outgroup in

phylogenetic analysis. The program Muscle v3.8.31 [15] was used to align 16S rRNA coding sequences; poorly aligned positions and divergent regions were deleted using Gblocks software v.0.91b [16]. The phylogenetic tree was inferred by using PhyML software v.3.0 [17], which computes genetic distances using a maximum likelihood method, with a GTR substitution model, 4 substitution rate categories and an estimated gamma shape parameter.

2.12. Genome assembly and annotation

FastQC software package v. 0.52 [18] was used to evaluate the quality of the obtained read pairs; poor quality bases were then removed using StreamingTrim [19]. Assembly was performed using SPAdes 3.5 software [20] with a k-mer length of 21, 33 and 55. The resulting contigs were annotated using the NCBI Automated Genome Annotation Pipeline.

The whole-genome shotgun project has been deposited at NCBI WGS database under accession number LNQS00000000; the version reported in this work was named LNQS01000000.

2.13. Comparative genomics

All *Rheinheimera* representative genomes sequenced thus far (i.e. *Rheinheimera* A13L, *Rheinheimera nanhaiensis* E407-8, *Rheinheimera perlucida* DSM 18276, *Rheinheimera baltica* DSM 14885, *Rheinheimera texasensis* DSM 17496, *R.* sp. KL1, *Rheinheimera* sp. F8, *Rheinheimera* sp. IITR-13) were collected from the NCBI ftp site (ftp://ftp.ncbi.nlm.nih.gov/assembly/) and, alongside the *Rheinheimera* sp. EpRS3 genome, were analyzed using the dgenome module of the Ductape suite [21], to identify shared orthologous and strain-specific genes. Furthermore, the COG database [22] was consulted to classify them.

2.14. Secondary metabolite search parameters

The stand-alone version of antiSMASH software [23] for genome-wide identification, annotation and analysis of secondary metabolite biosynthetic gene clusters was used to scan the *Rheinheimera* sp. EpRS3 genome sequence. The homology relationship between clusters of the same families were inferred via First Best Hit (FBH) BLAST analysis [24] using a threshold e-value of $1e^{-20}$.

Table 2
Rheinheimera sp. EpRS3 genome features.

Attribute	Value
Genome size (bp)	4,396,207
DNA G+C (%)	49.3
DNA scaffolds	14
Total genes	3961
Protein coding genes	3868
RNA genes	62
Pseudo genes	31
ncRNAs	1
Genome coverage	82.0×

3. Results and discussion

3.1. The genome of Rheinheimera sp. EpRS3

After assembling the reads, a *Rheinheimera* sp. EpRS3 draft genome embedding 14 contigs with an overall length of 4,396,207 bp was obtained. The EpRS3 genome was annotated to identify the functional elements and their putative biological role, revealing the presence of 3961 genes, 3868 of which are protein-coding genes, 62 are RNA encoding genes, one is an ncRNA (non-coding RNA), and 30 are pseudogenes. The main features of the *Rheinheimera* sp. EpRS3 genome are reported in Table 2.

The phylogenetic tree (Fig. 1) inferred from the 16S rRNA gene sequence of the strain shows strain *Rheinheimera* sp. EpRS3 within the other members of the genus *Rheinheimera*. In particular, the *Rheinheimera* sp. EpRS3 strain proved to be close to *R. perlucida* DSM 18276 (99% of sequences identity).

Comparative genomics analysis was then performed between *Rheinheimera* sp. EpRS3 and *Rheinheimera* genomes available in NCBI databases (i.e. *Rheinheimera* A13L, *R. nanhaiensis* E407-8, *R. perlucida* DSM 18276, *R. baltica* DSM 14885, *R. texasensis* DSM 17496, *R.* sp. KL1, *Rheinheimera* sp. F8, *Rheinheimera* sp. IITR-13). Fig. 2 shows the size of unique, accessory and core genomes possessed by these organisms as a framework to estimate the genomic diversity of the dataset. The core genome of the genus *Rheinheimera* proved to be relatively small (13.8%, 1413 genes), while the ensemble of unique and accessory genome fraction of *Rheinheimera* was large, 51.2% (5237 genes) and 35.0% (3587 genes), respectively. Such large dispensable genomes could be related to the different ecological niches colonized by members of this genus, including water, soil and plant tissues [24–26], as seen for other bacterial genera [27]. Additionally, as shown in Fig. 2, the pangenome is open, a feature very common when bacteria belonging to the same taxonomic group live with many partners in very diverse environments, since gene gain events are common and lead to a heterogeneous gene pool among the members of the same genus.

In order to obtain functional characterization of the *Rheinheimera* genus, the open-reading frames (ORFs) were mapped to the COG [22] database; the resulting categories assigned to each gene are reported in Table 3. The abundance of core, accessory and unique genes was plotted and is compared in Fig. 3. As expected, the core genome displayed predominance in COG categories generally associated with housekeeping functions, such as categories C (energy production and conversion, 719 genes), E (amino acid transport and metabolism, 1119 genes), T (signal transduction mechanisms, 777 genes) and J (translation ribosomal structure and biogenesis, 856 genes) (Table 3). However, the core genome also contains a large number of genes with unknown function. Conversely, the accessory genome was enriched for the COG categories M (cell wall/membrane/envelope biogenesis, 960 genes), T (signal transduction mechanisms, 871 genes) and K (transcription, 897 genes) (Table 3). However, the largest part

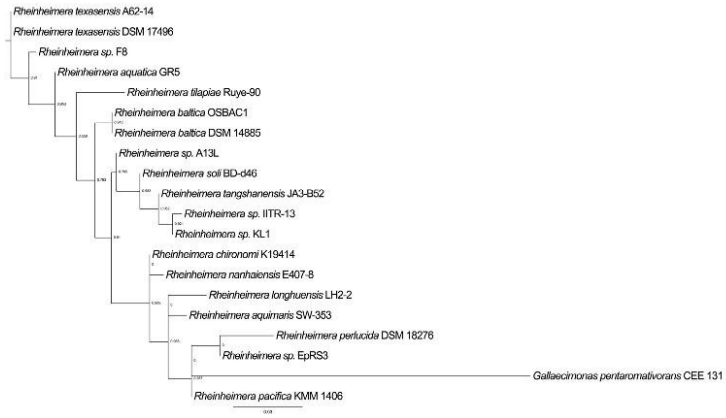

Fig. 1. Phylogenetic tree of the genus *Rheinheimera* based on 16S RNA sequences.

of both the accessory and unique genome was constituted by genes without a characterized functional class (X).

3.2. Antibiotics resistance

In a previous work [4], *Rheinheimera* sp. EpRS3 was demonstrated to be unable to grow in the presence of tetracycline (MIC 0.5 mg/l), rifampicin (MIC 5 mg/l), chloramphenicol (MIC 1 mg/l) or ciprofloxacin (MIC 0.5 mg/l). On the other hand, it showed resistance to some aminoglycoside antibiotics, including streptomycin and kanamycin at concentrations $\geq$10 mg/l and 5 mg/l, respectively. In an effort to identify the genetic determinants responsible for such resistance profiles, the genome of *Rheinheimera* sp. EpRS3 was annotated using the Resistance Gene Identifier (RGI) [28] tool of the Comprehensive Antibiotic Resistance Database (CARD). This led to identification of several elements usually associated with antibiotic resistance (original output obtained from CARD database inquiry are reported in Supplementary materials). The majority of these genes encode only minor components of efflux pump systems (like AcrB (belonging to the AcrAB/TolC system), MexI (belonging to MexGHI-OpmD complex), or MdtB and MdtC (that forms a heteromultimer complex, a subunit of MdtABC-TolC efflux pump)), but others encode complete RND-types systems (such as the complete set of genes encoding for the multidrug transporter MexEF-OprN complex and its transcriptional regulator *mexT*, an LysR-type transcriptional activator that positively regulates expression). Some of these efflux systems might be involved in resistance to kanamycin and streptomycin [29], which has been observed in this strain [5]. Additionally, such resistance could be due to another genetic element found in this investigation, i.e. a gene homologous to *acrD*, which is known to encode an aminoglycoside efflux pump in *E. coli* [30].

Also, inspection of the *Rheinheimera* sp. EpRS3 genome through the CARD database led to identification of unexpected genetic features that could potentially confer resistance to other drugs. For example, we found a gene coding for the Cfr 23S ribosomal RNA methyltransferase, which catalyzes methylation of the 23S rRNA subunit at A2503, suggesting its potential involvement in resistance to many drugs that target protein synthesis [36]. Moreover, the *Rheinheimera* sp. EpRS3 genome harbors genes coding for FosC2 and for MurA, two enzymes that usually confer resistance to fosfomycin [31,32,39]. Given the latter hint, we investigated the capacity of *Rheinheimera* sp. EpRS3 to grow in the presence of fosfomycin by MIC testing (as described in Section 2). Data obtained revealed that *Rheinheimera* sp. EpRS3 was able to grow up to 16 μg/ml, suggesting that the FosC2 and the MurA gene products could be involved in poor susceptibility to fosfomycin. Interestingly, such complex resistance profiles, joined with its ability to inhibit other strains inhabiting host-tissues [4], highlights the importance of *Rheinheimera* sp. EpRS3 from an ecological perspective.

3.3. Genome mining and inhibition of human pathogen strains

It has been previously reported that the *Rheinheimera* sp. EpRS3 strain is able to produce antibacterial compounds capable of inhibiting growth of many other bacteria isolated from the same plant [5] (although the molecules responsible for such inhibition have not yet been identified). Therefore, to gain insight into the genetic basis of the observed antimicrobial activity spectrum, the genome of *Rheinheimera* sp. EpRS3 was analyzed using the antiSMASH suite, allowing for identification and annotation of genes encoding secondary metabolites. As reported in Table 5, this approach revealed that the *Rheinheimera* sp. EpRS3 genome harbors a considerable number of genes with putative inhibitory activity. Indeed, we found a total of 111 genes split in 8 clusters, associated with synthesis of different molecules exhibiting antimicrobial

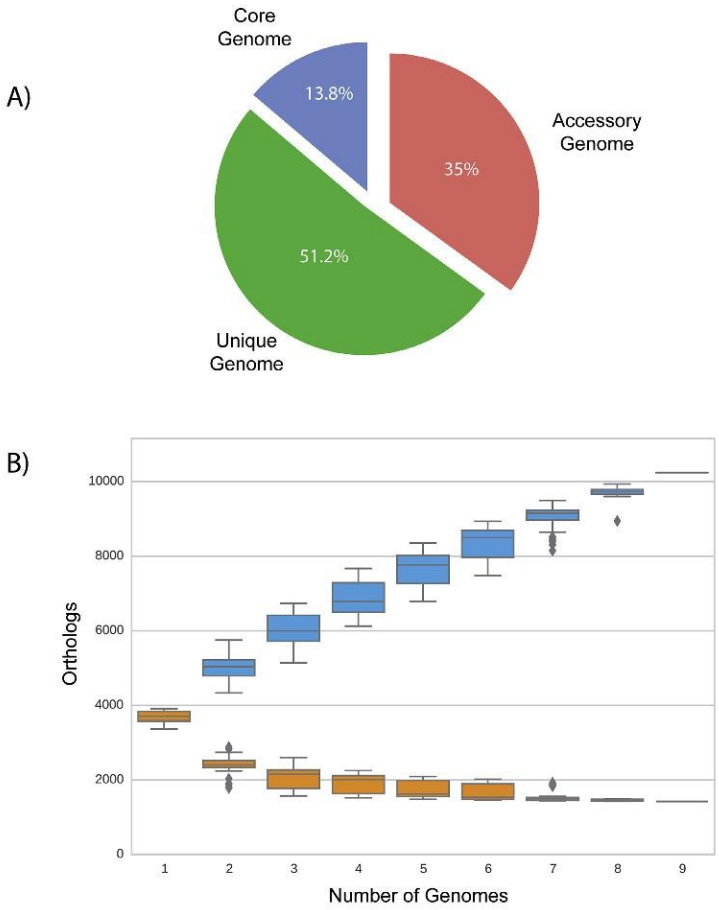

Fig. 2. The *Rheinheimera* genus PanGenome. 1 A: Pangenome shape. B: number of orthologs genes vs. number of genomes orange and blue represent core genome genes and pangenome size, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity. In particular, we found two different putative bacteriocin clusters (a class of extracellular small peptides exhibiting bactericidal activity) with a comparable number of genes (6 and 7). Two clusters were found to be associated with the synthesis of lantipeptides (harboring 11 and 12 genes each), a class of polycyclic peptides characterized by the presence of the thioether-cross-linked amino acids *meso*-lanthionine (Lan) and (2*S*, 3*S*, 6*R*)-3-methyllanthionine (MeLan; [33]). The latter three biosynthetic clusters are involved in production of different compound classes (resorcinol, non-ribosomal peptides, polyketides). In particular, the presence of polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) have a topical relevance for future biotechnological exploitation of this strain, in that some of the most successful antibiotics (i.e. vancomycin, avermectin, erythromycin) are produced by these enzymes.

The finding that the *Rheinheimera* sp. EpRS3 strain was able to inhibit growth of other bacteria, especially Gram-negative ones, stimulated us to evaluate its inhibitory effects against human pathogens. Hence, we tested *Rheinheimera* sp. EpRS3 through the cross-streaking method against a panel of 30 (target) strains belonging to the *B. cepacia* complex (Bcc), a group of opportunistic pathogens able to cause severe infections in immune-compromised subjects, such as cystic fibrosis (CF) patients [34]. As shown in Table 1, Bcc strains were of different origin (environmental, animal infection or clinical). Data from cross-streaking experiments are shown in Fig. 4A and Table 4. As reported, the assay revealed that *Rheinheimera* sp. EpRS3 is able to interfere with growth of most target Bcc strains. In particular, we observed that it is strongly effective against all tested *Burkholderia multivorans* and *Burkholderia cenocepacia* strains. Moreover, since it has

Table 3
Distribution of *Rheinheimera* sp EpRS3 genes in COG functional category.

COG functional category	Description	No. of genes	%
J	Translation ribosomal structure and biogenesis	175	4.52
A	RNA processing and modification	1	0.03
K	Transcription	203	5.24
L	Replication recombination and repair	133	3.43
B	Chromatin structure and dynamics	3	0.08
D	Cell cycle control cell division chromosome partitioning	33	0.85
Y	Nuclear structure	0	0
V	Defense mechanisms	69	1.78
T	Signal transduction mechanisms	206	5.32
M	Cell wall/membrane/envelope biogenesis	209	5.39
N	Cell motility	134	3.46
Z	Cytoskeleton	0	0
W	Extracellular structures	0	0
U	Intracellular trafficking secretion and vesicular transport	43	1.11
O	Posttranslational modification protein turnover chaperones	138	3.56
C	Energy production and conversion	165	4.26
G	Carbohydrate transport and metabolism	163	4.21
E	Amino acid transport and metabolism	222	5.73
F	Nucleotide transport and metabolism	62	1.6
H	Coenzyme transport and metabolism	114	2.94
I	Lipid transport and metabolism	97	2.5
P	Inorganic ion transport and metabolism	157	4.05
Q	Secondary metabolites biosynthesis transport and catabolism	45	1.16
R	General function prediction only	335	8.65
S	Function unknown	296	7.64
X	No functional class found	865	22.5

been previously demonstrated that growth of Bcc strains is inhibited by microbial volatile organic compounds (mVOCs) synthesized by Antarctic bacteria [8], we performed the same cross-streaking experiments on Petri dishes with a central septum separating the tester strain from the target strains. Data obtained revealed that *Rheinheimera* sp. EpRS3 is still able to inhibit Bcc strains, though to a lesser extent. The latter finding suggests that at least some of the molecules exhibiting antimicrobial activity vs. Bcc strains might be volatile organic compounds (VOCs). Thus, it is quite possible that the inhibitory activity exhibited by the *Rheinheimera* sp. EpRS3 strain relies on a combination of both volatile and diffusible compounds, reminiscent of the inhibitory activity that many Antarctic bacteria possess vs. Bcc strains [8]. Chemical profiling is currently ongoing to characterize such molecules.

In order to check whether the antimicrobial compounds synthesized by *Rheinheimera* sp. EpRS3 might also be effective against other human clinically relevant pathogens, we performed cross-streaking experiments using a panel of MDR strains belonging to *A. baumannii* and *K. pneumoniae* species as targets (Table 1). Data obtained are shown in Fig. 4 and demonstrate that *Rheinheimera* sp. EpRS3 completely inhibits *A. baumanni* N50 and *A. baumannii* YMCR363 strains (Table 1, Fig. 4B) and partially inhibits *K. pneumoniae* KPC284, *K. pneumoniae* B04, *K. pneumoniae* B2 (Table 1, Fig. 4C). Interestingly, some of these strains are resistant to a specific class of antibiotics, i.e. polymyxins, that are encoded by NRPSs. More in detail, in *K. pneumoniae* B04 and B2 strains, such resistance depends on modifications of the lipopolysaccharide (LPS), while in *K. pneumoniae* KPC284 and *A. baumanni* N50, the precise mechanism responsible for such a phenotype is currently unknown. Notably, colistin resistance in *A. baumannii* may occur due to mutations in the PmrAB two-component system or in *lpxA*, *lpxC*, and *lpxD* genes (as in *K. pneumoniae* strains); the two systems, respectively, lead to the modification and loss of LPS (as in *K. pneumoniae* strains) [35]. Thus, we can argue that *Rheinheimera* sp. EpRS3 is probably strongly effective against the colistin-resistant organisms tested since, due to their (known or putative) LPS-associated differences, they may be the only ones in which the antimicrobial molecules produced by the tester are able to enter into the target cells. We are completely aware that, at this stage, this scenario represents a hypothesis that will require more in-depth investigation. Nevertheless, it could represent an important resource in the treatment of infections sustained by MDR pathogens, particularly those resistant to last-line treatment with colistin.

3.4. *Identification of genetic elements in* Rheinheimera *sp. EpRS3 associated with its ecological role in rhizospheric soil*

Different bacterial genera are involved in a plethora of biotic activities of the soil ecosystem, such as biocontrol of microbial communities and/or control of nutrient dynamic turn-over [39],[40]. Several mechanisms come into play when dealing with plant growth promotion, both directly and indirectly. Among the most well-known, we can cite indole acetic acid (IAA) production (that increases the root surface) [36], nodulation and nitrogen-fixing ability, siderophore production

Table 4
Inhibition level of the *Rheinheimera* sp RS3 (tester) versus a panel of 30 Bcc members (target), 6 A. baumannii and 11 K. pneumoniae. The numbers indicate the different inhibition level from 0 to 3 as: complete (3), strong (2), weak (1), and absent (0). Abbreviations: E, Environmental; A, Animal Infection; CF, Cystic Fibrosis, C, Clinical. Plates with and without septum.

Target			Tester strain	Inhibition level	
Position on plate	Species	Strain		Without Septum	With septum
1	*B.ambifaria*	LMG 19182		2	2
2	*B. anthina*	LMG 20980		2	2
3	*B. arboris*	LMG 24066		1	0
4	*B. cenocepacia*	LMG 16656		3	3
5	*B. cepacia*	LMG 1222		0	0
6	*B. contaminans*	LMG 23361		1	1
7	*B. diffusa*	LMG 24065		1	0
8	*B. dolosa*	LMG 18943		1	1
9	*B. lata*	LMG 22485		1	1
10	*B. latens*	LMG 24064		2	3
11	*B. metallica*	LMG 24068		0	0
12	*B. multivorans*	LMG 13010		1	1
13	*B. pseudomultivorans*	LMG 26883		0	0
14	*B. pyrrocinia*	LMG 14191		0	0
15	*B. seminalis*	LMG 24067	*Rheinheimera* sp. EpRS3	0	0
16	*B. stabilis*	LMG 14294		1	1
17	*B. ubonensis*	LMG 20358		0	0
18	*B. vietnamiensis*	LMG 10929		1	1
19	*B. cenocepacia*	FCF 12		3	3
20	*B. cenocepacia*	FCF 13		3	3
21	*B. cenocepacia*	FCF 14		3	3
22	*B. cenocepacia*	FCF 15		3	3
23	*B. cenocepacia*	J2315		3	2
24	*B. cenocepacia*	FCF 18		3	2
25	*B. cenocepacia*	FCF 19		3	2
26	*B. multivorans*	FCF 5		3	1
27	*B. multivorans*	FCF 6		3	1
28	*B. multivorans*	FCF 7		3	0
29	*B. multivorans*	FCF 8		3	0
30	*B. multivorans*	FCF 9		3	0
1	*A. baumannii*	RHU 134	*Rheinheimera* sp. EpRS3	0	–
2	*A. baumannii*	RHU 875		0	–
3	*A. baumannii*	ATCC17978		0	–
4	*A. baumannii*	O8C29		0	–
5	*A. baumannii*	N5O		3	–
6	*A. baumannii*	YMCR363		3	–
1	*K. pneumoniae*	BO 1	*Rheinheimera* sp. EpRS3	0	–
2	*K. pneumoniae*	BO 4		1	–
3	*K. pneumoniae*	B1		0	–
4	*K. pneumoniae*	B2		1	–
5	*K. pneumoniae*	ATCC 700603		0	–
6	*K. pneumoniae*	KP397		0	–
7	*K. pneumoniae*	KP477		0	–
8	*K. pneumoniae*	KPC249		0	–
9	*K. Pneumoniae*	KPC261		0	–
10	*K. pneumoniae*	KPC284		1	–
11	*K. pneumoniae*	NTUHK2044		0	–

and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Therefore, the *Rheinheimera* sp. EpRS3 genome was investigated for the presence of genes involved in such metabolic abilities. Nodulation-related genes (*nod)* and several siderophore encoding genes have been detected. Also, data obtained revealed the absence of genetic elements related to ACC deaminase biosynthesis; moreover, the *Rheinheimera* sp. EpRS3 genome did not encounter the in silico criteria necessary to be considered a diazotroph (i.e. the co-presence of *nifHDKENB* genes in its genetic makeup [37]). On the other hand, we detected genetic traits responsible for production of enzymes involved in IAA biosynthesis (i.e. indole-3-acetaldehyde dehydrogenase (IpyA/TAM), indole-3-pyruvate decarboxylase (IpyA), tryptophan monooxydase (IAM),

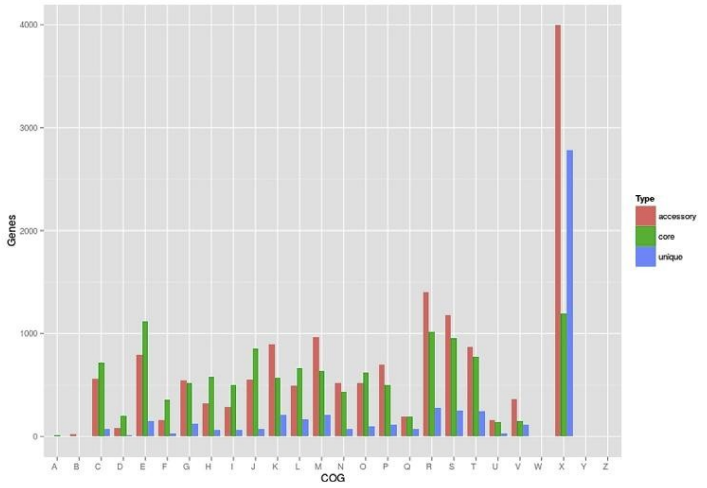

Fig. 3. Distribution of *Rheinheimera* genus, core, 1 accessory and unique gene plot according to COG functional category. Abbreviations: A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control cell division chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, co-enzyme transport and metabolism; I, lipid transport and metabolism; J, translation ribosomal structure and biogenesis; K, transcription; L, replication recombination and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification protein turnover chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking secretion and vesicular transport; V, defense mechanisms; W, extracellular structures; X, no functional class found; Y, nuclear structure; Z, cytoskeleton.

tryptophanase). Colorimetric assay to estimate production of IAA was performed as described in Section 2 to confirm in silico data; tests gave positive results ($OD_{530} = 0.225$). On this track, we decided to investigate whether IAA production by the *Rheinheimera* sp. EpRS3 strain could affect plant growth, as already reported for several endophytic strains in different plant systems [36,38], or whether, inside the plant rhizosphere, such ability is not expressed. Hence, we monitored elongation of primary roots of a test plant, namely *N. tabacum* cv. Xanthi, after inoculation with *Rheinheimera* sp. EpRS3 cells. As described in Section 2, we used the dual culture vertical agar plate approach previously used in other systems by other authors [38]. Results on the effect of different concentrations of indole acetic acid (IAA) on inhibition of the length of primary roots in *N. tabacum* seedlings after 7 days of treatment are shown in Supplementary Material SM1. Data obtained revealed that different concentrations of IAA induce a significant decrease ($p < 0.001$), compared to the control in primary root length of *N. tabacum* seedlings in a dose-dependent manner. In particular, inhibition of primary root length seemed to be strongly affected by the distance of the inoculated paper disc from the seedlings. In fact, seedlings grown at a distance of more than 2 cm (>2 cm) from the paper disc showed a lower decrease in root elongation in contrast to seedlings placed within 2 cm (<2 cm) of the disc. In detail, statistical analysis showed p values, respectively, of <0.01 for treatment of plants with 200 μM IAA and $p < 0.05$ for plants subjected to 20 μM IAA (SM1). The same trend was observed after 14 days of treatment, in this case differences between the two groups of plants (>2 cm or <2 cm) were not significant (data not shown). The effect of bacterial inoculations of tobacco seedlings with either *E. coli*, both DH5α (known IAA overproducer used as control) or *Rheinheimera* sp. EpRS3 strains, was to inhibit primary root elongation, with *E. coli* DH5α having a higher activity than *Rheinheimera* sp. EpRS3 (see Supplementary Material SM2, SM3). In particular, the inoculation with DH5α cells or culture filtrate registered a significant (respectively $p < 0.01$ and $p < 0.001$) decrease of root elongation compared to the control. The same was true only in the case of culture filtrate ($p < 0.01$) of *Rheinheimera* sp. EpRS3 strain. For both the strains we have not observed

Table 5
Gene clusters involved in secondary metabolites biosynthesis in the *Rheinheimera* sp. RS3 genome; the type of secondary metabolite produced and the number of genes embedded in the clusters are reported.

Biosynthetic cluster	Type	No. of genes
Cluster 1	Resorcinol	22
Cluster 2	Lantipeptide	12
Cluster 3	Lantipeptide	11
Cluster 4	Hserlactone	9
Cluster 5	Bacteriocin	6
Cluster 6	Nrps	7
Cluster 7	Hserlactone	11
Cluster 8	Bacteriocin	7

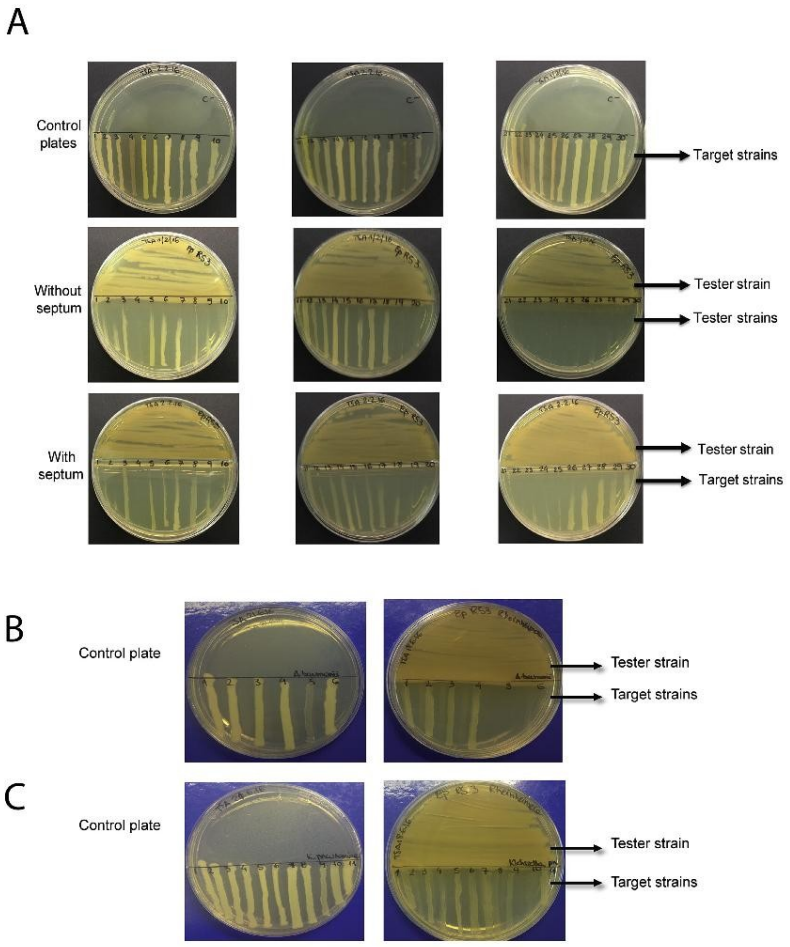

Fig. 4. Petri dishes of cross-streaking experiments against: A) BCC strains (numbers refer to different organisms from 1 to 10, 11–20, 21–30); B) *A. baumannii* strains (numbers refer to different organisms from 1 to 6); C) *K. pneumoniae* strains (numbers refer to different organisms from 1 to 11).

any significant difference between groups of plants classified as >2 cm or <2 cm (Fig. SM2). Moreover, the treatments of plants with bacterial suspensions induced changes of primary root morphology concerning either the increase in root hairs formations and lateral root induction compared to the negative control (SM3).

Results concerning both the inhibition of the primary root elongation and morphological changes of roots of tobacco seedlings suggested a possible relationship between the IAA production by *Rheinheimera* sp. EpRS3 strain and these growth modifications. Experiments are currently ongoing to demonstrate a possible role of the strain as plant growth promoting bacteria (PGPB).

3.5. Other phenotypic traits of Rheinheimera sp. EpRS3

Since the ability of some soil bacteria to degrade organic contaminants is promising for the development of biotreatment systems to counteract soil pollution, we analyzed whether *Rheinheimera* sp. EpRS3 is able to degrade complex hydrocarbon molecules or to resist to oxidative stresses, like those occurring in presence of heavy metals. To this purpose the *Rheinheimera* sp. EpRS3 was tested for its ability to grow on two different minimal media (MMV and MMD), differing in salt concentration, supplemented with either glucose or diesel fuel as the sole carbon source. Data obtained revealed that *Rheinheimera* sp. EpRS3 was not able to grow on diesel

fuel as carbon source. The only condition supporting growth was MMD supplemented with glucose as the sole carbon and energy source, suggesting the inability to degrade hydrocarbon compounds. The absence of genes involved *n*-alkanes degradation (i.e. *alk* genes) in *Rheinheimera* sp. EpRS3 genome was in agreement with the experimental data.

Moreover, since plant root tissues can produce reactive oxygen species (ROS) in response to different *stimuli*, we tested the resistance of the strain to oxidative stress by using hydrogen peroxide or a heavy metal (i.e. zinc, copper, cadmium and nickel), revealing that *Rheinheimera* sp. EpRS3 is sensitive to zinc, cadmium, nickel and hydrogen peroxide, while it exhibits a tolerance to low concentrations of $CuCl_2$ (0.25, 0.5 and 1 mM). The genome analysis results, however, were in disagreement with this conclusion, in that we found a number of genes (*katA*, *B*, *C* and *sodB*) involved in the response to oxidative stress. Indeed, the disagreement between experiments and genomic analyses is puzzling and prompts for further investigation. A possible explanation is that heavy metals may somehow impair the proper expression of these genes or the functionality of their products. On the other hand, we have no data regarding the expression of these genes, which might even be not functional.

Lastly, we performed experimental assays to test whether EpRS3 is able to secrete enzymes into the extracellular space in order to hydrolyze macromolecules into constituents that can be imported for microbial nutrition. Data obtained suggest that the *Rheinheimera* sp. EpRS3 strain possesses extracellular lipases, phospholipase and proteolytic activities, while no amylolytic activity towards starch has been detected (data not shown).

In conclusion, the aim of this work was to characterize newly isolated rhizospheric strain *Rheinheimera* sp. EpRS3, isolated from rhizospheric soil of the medicinal plant *E. purpurea*, to test its ability to influence plant growth and to synthesize antimicrobial compounds that might be effective against bacterial human pathogens. For this purpose, a set of phenotypic parameters was tested and data obtained were correlated with the gene repertoire of the *Rheinheimera* sp. EpRS3 genome. Cross-streaking experiments revealed that *Rheinheimera* sp. EpRS3 is able to inhibit growth of different bacterial pathogens (Bcc, *A. baumanni*, and *K. pneumoniae*), most of which exhibit a multi-drug-resistance phenotype. This ability was very likely due to the presence of gene clusters involved in different types of secondary metabolites. However, it cannot be excluded that some of these molecule are VOCs, as shown by cross-streaking experiments performed with Petri dishes harboring a central septum. It is particularly intriguing that the *Rheinheimera* sp. EpRS3 strain synthesize molecules able to "bypass" the resistance of *K. pneumoniae* and *A. baumanni* strains to colistin.

Moreover, in silico analysis indicated the presence in the genome of various components involved in multidrug-efflux transporter and cation efflux system biosynthesis, alongside traits usually responsible for specific resistance.

These data underline how endophytic and/or rhizospheric bacteria may play an important ecological role inside the host plant; it is possible that both antibiotic resistance and synthesis of antimicrobial compounds might help in shaping the structure of bacterial communities living near or inside the plant, as recently suggested [4].

If this is so, the possibility that such bacterial strains might also influence the growth of the host plant should be taken into account. Data obtained in this work (i.e. the presence of a gene encoding triptophanase, involved in IAA production, as well as results from IAA production and effects on plant growth) are in agreement with this idea. Lastly, it is worth mentioning that these bacteria may directly synthesize or influence host synthesis of some bioactive molecules identified in extracts/essential oils of medicinal plants, as recently suggested by a work performed on rhizospheric and endophytic bacteria isolated from the medicinal plant *Lavandula angustifolia* [39].

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.11.001.

References

[1] Danhorn T, Fuqua C. Biofilm formation by plant-associated bacteria. Annu Rev Microbiol 2007;61:401–22. http://dx.doi.org/10.1146/annurev.micro.61.080706.093316.

[2] Taghavi S, van der Lelie D. Genome sequence of the plant growth-promoting endophytic bacterium *Enterobacter* sp. 638. Mol Microb Ecol Rhizosph 2013;2:899–908. http://dx.doi.org/10.1002/9781118297674.ch84.

[3] Maida I, Chiellini C, Mengoni A, Bosi E, Firenzuoli F, Fondi M, et al. Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *E chinacea purpurea*. Environ Microbiol 2015;18:2357–65. http://dx.doi.org/10.1111/1462-2920.12911. n/a-n/a, Thematic Issue: Thematic Issue on Microbial Mutualism and Symbiosis.

[4] Mengoni A, Maida I, Chiellini C, Emiliani G, Mocali S, Fabiani A, et al. Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. Res Microbiol 2014;165:686–94. http://dx.doi.org/10.1016/j.resmic.2014.09.008.

[5] Chiellini C, Maida I, Emiliani G, Mengoni A, Stefano M, Fabiani A, et al. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia* n.d. http://revistes.iec.cat/index.php/IM/article/viewFile/136531/135273. [Accessed 17 March 17 2016].

[6] Hudson JB. Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. J Biomed Biotechnol 2012; 2012. http://dx.doi.org/10.1155/2012/769896.
[7] Corry JEL. Handbook of biological media, vol. 22; 1994. http://dx.doi.org/10.1016/0168-1605(94)90011-6.
[8] Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice A, Michaud L, et al. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv 2012;30:272–93. http://dx.doi.org/10.1016/j.biotechadv.2011.06.011.
[9] Diancourt L, Passet V, Nemec A, Dijkshoorn L, Brisse S. The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. PLoS One 2010;5. http://dx.doi.org/10.1371/journal.pone.0010034.
[10] Clsi. Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement, vol. 32; 2013.
[11] Zhang H, Forde BG. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 1998;279:407–9. http://dx.doi.org/10.1126/science.279.5349.407.
[12] Hammer Ø, Harper DAT, Ryan PD. Paleontological statistics software package for education and data analysis. Palaeontol Electron 2001;4: 9–18. http://dx.doi.org/10.1016/j.bcp.2008.05.025.
[13] Perrin E, Fondi M, Maida I, Mengoni A, Chiellini C, Mocali S, et al. Genomes analysis and bacteria identification: the use of overlapping genes as molecular markers. J Microbiol Methods 2015;117:108–12. http://dx.doi.org/10.1016/j.mimet.2015.07.025.
[14] Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37. http://dx.doi.org/10.1093/nar/gkn879.
[15] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–7. http://dx.doi.org/10.1093/nar/gkh340.
[16] Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 2000;17:540–52. http://dx.doi.org/10.1093/oxfordjournals.molbev.a026334.
[17] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 2010;59: 307–21. http://dx.doi.org/10.1093/sysbio/syq010.
[18] Kunde-Ramamoorthy G, Coarfa C, Laritsky E, Kessler NJ, Harris RA, Xu M, et al. Comparison and quantitative verification of mapping algorithms for whole-genome bisulfite sequencing. Nucleic Acids Res 2014; 42. http://dx.doi.org/10.1093/nar/gkt1325.
[19] Bacci G, Bazzicalupo M, Benedetti A, Mengoni A. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. Mol Ecol Resour 2014;14:426–34. http://dx.doi.org/10.1111/1755-0998.12187.
[20] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77. http://dx.doi.org/10.1089/cmb.2012.0021.
[21] Galardini M, Mengoni A, Biondi EG, Semeraro R, Florio A, Bazzicalupo M, et al. DuctApe: a suite for the analysis and correlation of genomic and OmniLog™ phenotype microarray data. Genomics 2014; 103:1–10. http://dx.doi.org/10.1016/j.ygeno.2013.11.005.
[22] Tatusov RL, Galperin MY, Natale DA, Koonin EV. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 2000;28:33–6. http://dx.doi.org/10.1093/nar/28.1.33.
[23] Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, et al. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 2011;39:W339–46. http://dx.doi.org/10.1093/nar/gkr466.
[24] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10. http://dx.doi.org/10.1016/S0022-2836(05)80360-2.
[25] Brettar I, Christen R, Manfred GH. *Rheinheimera baltica* gen. nov., sp. nov., a bluecoloured bacterium isolated from the central Baltic Sea. Int J Syst Evol Microbiol 2002;52:1851–7. http://dx.doi.org/10.1099/ijs.0.02151-0.
[26] Zhang X, Sun L, Qiu F, McLean RJC, Jiang R, Song W. *Rheinheimera tangshanensis* sp. nov., a rice root-associated bacterium. Int J Syst Evol Microbiol 2008;58:2420–4. http://dx.doi.org/10.1099/ijs.0.65605-0.
[27] Tettelin H, Riley D, Cattuto C, Medini D. Comparative genomics: the bacterial pan-genome. Curr Opin Microbiol 2008;11:472–7. http://dx.doi.org/10.1016/j.mib.2008.09.006.
[28] McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 2013;57:3348–57. http://dx.doi.org/10.1128/AAC.00419-13.
[29] Moore RA, Deshazer D, Reckseidler S, Weissman A, Woods DE. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob Agents Chemother 1999;43:465–70.
[30] Rosenberg EY, Ma D, Nikaido H. AcrD of Escherichia coli is an aminoglycoside efflux pump. J Bacteriol 2000;182:1754–6. http://dx.doi.org/10.1128/JB.182.6.1754-1756.2000.
[31] Lee SY, Park YJ, Yu JK, Jung S, Kim Y, Jeong SH, et al. Prevalence of acquired fosfomycin resistance among extended-spectrum??-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in korea and IS26-composite transposon surrounding fosA3. J Antimicrob Chemother 2012;67:2843–7. http://dx.doi.org/10.1093/jac/dks319.
[32] Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, et al. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*. Int J Antimicrob Agents 2010;35:333–7. http://dx.doi.org/10.1016/j.ijantimicag.2009.11.011.
[33] Knerr PJ, van der Donk WA. Discovery, biosynthesis, and engineering of Lantipeptides. 2012.
[34] Döring G, Flume P, Heijerman H, Elborn JS. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros 2012;11:461–79. http://dx.doi.org/10.1016/j.jcf.2012.10.004.
[35] López-Rojas R, McConnell MJ, Jiménez-Mejías ME, Domínguez-Herrera J, Fernández-Cuenca F, Pachón J. Colistin resistance in a clinical *Acinetobacter baumannii* strain appearing after colistin treatment: effect on virulence and bacterial fitness. Antimicrob Agents Chemother 2013; 57:4587–9. http://dx.doi.org/10.1128/AAC.00543-13.
[36] Spaepen S, Vanderleyden J, Remans R. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 2007;31: 425–48. http://dx.doi.org/10.1111/j.1574-6976.2007.00072.x.
[37] Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 2012;13:162. http://dx.doi.org/10.1186/1471-2164-13-162.
[38] Shi C-L, Park H-B, Lee JS, Ryu S, Ryu C-M. Inhibition of primary roots and stimulation of lateral root development in *Arabidopsis thaliana* by the rhizobacterium Serratia marcescens 90–166 is through both auxin-dependent and -independent signaling pathways. Mol Cells 2010;29: 251–8. http://dx.doi.org/10.1007/s10059-010-0032-0.
[39] Emiliani G, Mengoni A, Maida I, Perrin E, Chiellini C, Fondi M, et al. Linking bacterial endophytic communities to essential oils: clues from *Lavandula angustifolia* mill. Evidence-Based Complement Altern Med 2014;2014. http://dx.doi.org/10.1155/2014/650905.

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Draft Genome Sequence of *Pseudomonas* sp. EpS/L25, Isolated from the Medicinal Plant *Echinacea purpurea* and Able To Synthesize Antimicrobial Compounds

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We announce here the draft genome sequence of *Pseudomonas* sp. strain EpS/L25, isolated from the stem/leaves of the medicinal plant *Echinacea purpurea*. This genome will allow for comparative genomics in order to identify genes associated with the production of bioactive compounds and antibiotic resistance.

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The genus *Pseudomonas* consists of a group of bacteria particularly relevant from both medical and biotechnological viewpoints (1). Thanks to their metabolic versatility, they successfully colonized several different niches, including water, soil, plants, and animals. Here, we present the draft genome sequence of *Pseudomonas* sp. EpS/L25, a strain close to *Pseudomonas oleovorans*, isolated from the stem/leaves of *Echinacea purpurea*, a medicinal plant whose essential oil possesses antimicrobial activity (2). The *E. purpurea* plants were collected in October 2012 (3) at the "Giardino delle Erbe," Casola Valsenio. Medicinal plants are known for their beneficial effects for humans (including their antibacterial activity), but, in spite of their high relevance, endophytic bacterial communities inhabiting their rhizosphere or internal tissues are almost totally unknown. Thus, it is still unknown if they contribute to the antimicrobial activity exerted by *E. purpurea* extracts.

Previous characterization of *Pseudomonas* sp. EpS/L25 revealed the ability of this strain to inhibit the growth of other *E. purpurea*-associated bacteria (4) and, more interestingly, some opportunistic bacterial pathogens belonging to the *Burkholderia cepacia* complex. Furthermore, it showed resistance to several antibiotic compounds (5). Due to these properties, it represents a good candidate for further molecular investigations on the genetic basis of such features, prompting for sequencing of its genome.

The genome sequence of *Pseudomonas* sp. EpS/L25 was determined by a 2 × 300-bp paired-end approach using the MiSeq sequencing system (Illumina Inc., San Diego, CA, USA). A total of 3,020,786 paired-end reads were obtained, representing approximately 158× coverage of the whole genome. *De novo* assembly was performed using SPAdes version 3.5 (6), which generated 300 contigs. Contigs with length less than 2,000 bp were discarded and the remaining ones used for a multi-draft-based analysis using 16 *Pseudomonas* genomes retrieved from the NCBI database (*Pseudomonas* ND6, *Pseudomonas* TKP, *Pseudomonas* VLB120, *P. aeruginosa* B136 33, *P. aeruginosa* UCBPP PA14, *P. brassicacearum* NFM421, *P. denitrificans* ATCC 13867, *P. entomophila* L48, *P. fluorescens* R124, *P. mendocina* NK 01, *P. poae* RE 1 1 14, *P. putida* BIRD 1, *P. putida* KT2440, *P. stutzeri* CCUG 29243, *P. syringae* B728a) through MeDuSa scaffolder (7). The final version of the genome embeds 18 scaffolds, the longest of which is 1,664,566 bp long. The draft genome assembly of *Pseudomonas* sp. EpS/L25 has a total length of 5,435,234 bp. The G+C content is 65.5%, similar to that of other *Pseudomonas* genomes. Automated annotation of the *Pseudomonas* sp. EpS/L25 draft genome sequence using NCBI Prokaryotic Genome Annotation Pipeline detected 4,690 protein coding genes, 76 RNA coding genes (5 complete rRNAs, 57 tRNAs, 14 ncRNAs), and 105 pseudogenes. Three CRISPR arrays were also identified.

Comparative genomics analysis confirmed the presence of antibiotic efflux pumps, some conferring specific resistance to beta-lactams (*pdc*), florfenicol (*cfrA*), and polymyxins (*arnA* and *pmrF*). Moreover, genes involved in the production of secondary metabolites with antimicrobial activity have also been detected (terpene, aryl-polyene, and two nonribosomal peptides).

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at GenBank under the accession number LNUP00000000. The version described in this paper is the first version, LNUP01000000.

REFERENCES:

1. Nikel PI, Martínez-García E, de Lorenzo V. 2014. Biotechnological domestication of pseudomonads using synthetic biology. Nat Rev Microbiol 12:368–379. http://dx.doi.org/10.1038/nrmicro3253.

2. **Hudson JB**. 2012. Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. BioMed Res Int **2012:** http://dx.doi.org/10.1155/2012/769896.
3. **Chiellini C, Maida I, Emiliani G, Mengoni A, Mocali S, Fabiani A, Biffi S, Maggini V, Gori L, Vannacci A, Gallo E, Firenzuoli F, Fani R**. 2014. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. Int Microbiol **17:**165–174. http://dx.doi.org/10.2436/20.1501.01.219.
4. **Maida I, Chiellini C, Mengoni A, Bosi E, Firenzuoli F, Fondi M, Fani R**. 22 July 2015. Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *Echinacea purpurea*. Environ Microbiol [Epub ahead of print.] http://dx.doi.org/10.1111/1462-2920.12911.
5. **Mengoni A, Maida I, Chiellini C, Emiliani G, Mocali S, Fabiani A, Fondi M, Firenzuoli F, Fani R**. 2014. Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. Res Microbiol **165:**686–694. http://dx.doi.org/10.1016/j.resmic.2014.09.008.
6. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA**. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol **19:**455–477. http://dx.doi.org/10.1089/cmb.2012.0021.
7. **Bosi E, Donati B, Galardini M, Brunetti S, Sagot MF, Lió P, Crescenzi P, Fani R, Fondi M**. 2015. MeDuSa: a multi-draft based scaffolder. Bioinformatics **31:**2443–2451. http://dx.doi.org/10.1093/bioinformatics/btv171.

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New Genome Sequence of an *Echinacea purpurea* Endophyte, *Arthrobacter* sp. Strain EpSL27, Able To Inhibit Human-Opportunistic Pathogens

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ABSTRACT We announce here the draft genome sequence of *Arthrobacter* sp. strain EpSL27, isolated from the stem and leaves of the medicinal plant *Echinacea purpurea* and able to inhibit human-pathogenic bacterial strains. The genome sequencing of this strain may lead to the identification of genes involved in the production of antimicrobial molecules.

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Medicinal plants are well known and have been largely explored for centuries for their therapeutic properties (1). What is little known is that their therapeutic potential could be related to endophytic microorganisms inhabiting their tissues (2). Many bioactive molecules have been already extracted from endophytic bacteria (3). The promising potential of such organisms has led to the characterization of endophytic bacterial communities from medicinal plants, which are poorly known. Endophytic and rhizospheric bacterial communities from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia* have been characterized, highlighting the specific composition of such communities within plants' compartments (4). *Arthrobacter* sp. strain EpSL27, extracted from the stem and leaves of *E. purpurea*, has been evidenced as being resistant to a high level of oxidative stress (20 mM H_2O_2) and is able to degrade diesel fuel. Among such notable biotechnological potentialities, *Arthrobacter* sp. EpSL27 has also been found to show strong inhibition activity toward human-pathogenic bacteria from the *Burkholderia cepacia* complex (5), which are multidrug-resistant organisms able to induce serious infections in immunocompromised patients.

The intriguing information obtained by the above-cited analyses led to whole sequencing of the strain genome.

Arthrobacter sp. EpSL27 genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (6), and its authenticity has been confirmed by 16S rRNA gene sequencing. Whole-genome shotgun sequencing was performed with a 2 × 300-bp paired-end approach using the MiSeq sequencing system (Illumina, Inc., San Diego, CA). The FastQC software package version 0.52 (7) was used to evaluate the quality of the obtained read pairs, and poor-quality bases were removed using StreamingTrim (8). Assembly was performed using the SPAdes 3.5 software (9), with k-mer lengths of 21, 33, and 55, generating 21 contigs. Those having a length shorter than 200 nucleotides were removed and the others launched for scaffolding through Medusa software (10), using the following genomes as references: *Arthrobacter arilaitensis* Re117 (11), *Arthrobacter* Rue61a (12), *Arthrobacter* sp. strain FB24 (13), *Arthrobacter*

aurescens TC1 (14), and *Arthrobacter chlorophenolicus* A6. The resulting scaffolds were then annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAAP) (15). The final version of the *Arthrobacter* sp. EpSL27 draft genome consists of 8 scaffolds, and its total length is 4,176,054 bp, with a coverage of 215.0×. The G+C content is about 67.8%, which reflects the characteristic high G+C content of the genus. The *Arthrobacter* sp. EpSL27 genome harbors 3,758 genes, 3,610 of which are protein-coding genes, 66 are RNA-coding genes (5 5S rRNA, 1 23S rRNA, 1 16S rRNA, 50 tRNAs, and 9 noncoding RNA [ncRNA]), and 91 are pseudogenes.

The EpSL27 genome was analyzed using CARD (16) for the presence of genes conferring antibiotic resistance. The analysis has evidenced genes putatively involved in specific antibiotic resistance to isoniazid (*Mycobacterium tuberculosis kasA* mutant), fluoroquinolones (*mfd*), amynocoumarin (*Streptomyces rishiriensis parY* mutant), rifamycin (*rphB*), mupirocin (*Bifidobacterium* intrinsic *ileS*), and fosfomycin (*Chlamydia trachlomatis* intrinsic *murA*). antiSMASH (17) analysis for secondary metabolites with antimicrobial activities was also performed, revealing the presence of 5 clusters, with one cluster encoding nonribosomal peptide synthetase (NRPS), one cluster encoding type 3 polyketide synthase (T3pks), and another three clusters with an unspecified reference.

Accession number(s). The whole-genome shotgun project has been deposited at NCBI whole-genome sequencing (WGS) database under accession number LNUT00000000, and the version reported in this work is version LNUT00000000.1.

REFERENCES

1. Staub PO, Casu L, Leonti M. 2016. Back to the roots: a quantitative survey of herbal drugs in Dioscorides' de materia medica (ex Matthioli, 1568). Phytomedicine 23:1043–1052. https://doi.org/10.1016/j.phymed.2016.06.016.
2. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1–9. https://doi.org/10.1111/j.1574-6968.2007.00918.x.
3. Shweta S, Bindu JH, Raghu J, Suma HK, Manjunatha BL, Kumara PM, Ravikanth G, Nataraja KN, Ganeshaiah KN, Uma Shaanker R. 2013. Isolation of endophytic bacteria producing the anti-cancer alkaloid camptothecin from *Miquelia dentata* Bedd. (Icacinaceae). Phytomedicine 20:913–917. https://doi.org/10.1016/j.phymed.2013.04.004.
4. Chiellini C, Maida I, Emiliani G, Mengoni A, Mocali S, Fabiani A, Biffi S, Maggini V, Gori L, Vannacci A, Gallo E, Firenzuoli F, Fani R. 2014. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. Int Microbiol 17:165–174. https://doi.org/10.2436/20.1501.01.219.
5. Chiellini C, Maida I, Maggini V, Bosi E, Mocali S, Emiliani G, Perrin E, Firenzuoli F, Mengoni A, Fani R. 2017. Preliminary data on antibacterial activity of *Echinacea purpurea*-associated bacterial communities against *Burkholderia cepacia* complex strains, opportunistic pathogens of cystic fibrosis patients. Microbiol Res 196:34–43. https://doi.org/10.1016/j.micres.2016.12.001.
6. Perrin E, Fondi M, Maida I, Mengoni A, Chiellini C, Mocali S, Cocchi P, Campana S, Taccetti G, Vaneechoutte M, Fani R. 2015. Genomes analysis and bacteria identification: the use of overlapping genes as molecular markers. J Microbiol Methods 117:108–112. https://doi.org/10.1016/j.mimet.2015.07.025.
7. Kunde-Ramamoorthy G, Coarfa C, Laritsky E, Kessler NJ, Harris RA, Xu M, Chen R, Shen L, Milosavljevic A, Waterland RA. 2014. Comparison and quantitative verification of mapping algorithms for whole-genome bisulfite sequencing. Nucleic Acids Res 42:e43. https://doi.org/10.1093/nar/gkt1325.
8. Bacci G, Bazzicalupo M, Benedetti A, Mengoni A. 2014. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. Mol Ecol Resour 14:426–434. https://doi.org/10.1111/1755-0998.12187.
9. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
10. Bosi E, Donati B, Galardini M, Brunetti S, Sagot MF, Lió P, Crescenzi P, Fani R, Fondi M. 2015. Medusa: a multi-draft based scaffolder. Bioinformatics 31:2443–2451. https://doi.org/10.1093/bioinformatics/btv171.
11. Monnet C, Loux V, Gibrat JF, Spinnler E, Barbe V, Vacherie B, Gavory F, Gourbeyre E, Siguier P, Chandler M, Elleuch R, Irlinger F, Vallaeys T. 2010. The *Arthrobacter arilaitensis* Re117 genome sequence reveals its genetic adaptation to the surface of cheese. PLoS One 5:e15489. https://doi.org/10.1371/journal.pone.0015489.
12. Niewerth H, Schuldes J, Parschat K, Kiefer P, Vorholt JA, Daniel R, Fetzner S. 2012. Complete genome sequence and metabolic potential of the quinaldine-degrading bacterium *Arthrobacter* sp. Rue61a. BMC Genomics 13:534. https://doi.org/10.1186/1471-2164-13-534.
13. Nakatsu CH, Barabote R, Thompson S, Bruce D, Detter C, Brettin T, Han C, Beasley F, Chen W, Konopka A, Xie G. 2013. Complete genome sequence of *Arthrobacter* sp. strain FB24. Stand Genomic Sci 9:106–116. https://doi.org/10.4056/sigs.4438185.
14. Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn A, Radune D, Vamathevan J, Riggs F, Grinberg V, Khouri H, Wackett LP, Nelson KE, Sadowsky MJ. 2006. Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. PLoS Genet 2:e214. https://doi.org/10.1371/journal.pgen.0020214.
15. Angiuoli SV, Gussman A, Klimke W, Cochrane G, Field D, Garrity G, Kodira CD, Kyrpides N, Madupu R, Markowitz V, Tatusova T, Thomson N, White O. 2008. Toward an online repository of Standard Operating Procedures (SOPs) for (meta)genomic annotation. OMICS 12:137–141. https://doi.org/10.1089/omi.2008.0017.
16. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJV, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T, Wright GD. 2013. The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother 57:3348–3357. https://doi.org/10.1128/AAC.00419-13.
17. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0–a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. https://doi.org/10.1093/nar/gkv437.

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Draft Genome Sequence of *Pseudomonas* sp. Strain Ep R1 Isolated from *Echinacea purpurea* Roots and Effective in the Growth Inhibition of Human Opportunistic Pathogens Belonging to the *Burkholderia cepacia* Complex

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ABSTRACT In this announcement, we detail the draft genome sequence of the *Pseudomonas* sp. strain Ep R1, isolated from the roots of the medicinal plant *Echinacea purpurea*. The elucidation of this genome sequence may allow the identification of genes associated with the production of antimicrobial compounds.

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Endophytic bacterial communities inhabiting the rhizosphere or internal tissues of the medicinal plants (MPs) may contribute to the therapeutic properties of these plants (1). Here we report on the draft genome sequence of *Pseudomonas* sp. strain Ep R1, a strain isolated from the roots of *Echinacea purpurea*, an MP with immunomodulant, antiviral, and antimicrobial activity (2). The *E. purpurea* bacterial endophytes were isolated and molecular and phenotypic characterizations were conducted (3). In particular, *Pseudomonas* sp. Ep R1 showed the ability to inhibit the growth of other *E. purpurea* endophytes (4) and of cystic fibrosis bacterial pathogens belonging to the *Burkholderia cepacia* complex (5). Moreover, it has been demonstrated to be highly (50 μg/ml) resistant to chloramphenicol and streptomycin (6).

The genome sequence of *Pseudomonas* sp. Ep R1 was determined by a 2- × 300-bp paired-end approach using the MiSeq sequencing system (Illumina Inc., San Diego, CA). A total of 1,148,852 paired-end reads were obtained, representing approximately 100× coverage of the whole genome. *De novo* assembly was performed using SPAdes 2.3 (7), which generated 363 contigs. Contigs with length less than 2,000 bp were discarded. The remaining contigs were used for a multidraft-based analysis using genome sequences of 13 *Pseudomonas* strains retrieved from the NCBI database (*P. aeruginosa* PAO1, *P. alkylphenolia* KL28, *P. denitrificans* ATCC 13867, *P. entomophila* L48, *P. fluorescens* F113, *P. fulva* 12-X, *P. knackmussii* B13, *P. mendocina* ymp, *P. protegens* CHA0, *P. putida* KT2440, *P. resinovorans* NBRC, *P. stutzeri* CGMCC, and *P. syringae* pv. tomato DC3000) through MeDuSa scaffolder (8). The final version of the draft genome assembly of *Pseudomonas* sp. Ep R1 is 6,797,087 bp long and embeds 158 contigs (the longest of which is 1,954,067 bp long). The G+C content is 65.5%, similar to that of other *Pseudomonas* genomes sequenced so far. Automated annotation of the *Pseudomonas* sp. Ep R1 draft genome sequence using the NCBI Prokaryotic Genome Annotation

Pipeline detected 6,001 protein-coding genes, 67 RNA-coding genes (7 complete rRNAs, 56 tRNAs, 4 noncoding RNAs [ncRNAs]), and 173 pseudogenes.

Genes involved in the biosynthesis of secondary metabolites with antimicrobial activity were searched. The analysis was performed within an antiSMASH shell (9), which revealed that the *Pseudomonas* Ep R1 genome harbors 6 clusters involved in the biosynthesis of streptomycin, stenothricin, pimaricin, type 3 polyketide synthase (T3PKS), siderophore (desferrioxamine B), and nonribosomal peptide synthetase (NRPS) (amychelin). Moreover, the genome sequence was analyzed through CARD (10), which led to the identification of several genes (*mexABEJKMNW*, *omrMN*, *katG*, *triC*, *mfd*, and *mdtC*) putatively involved in antibiotic resistance, some conferring specific resistance to fluoroquinolone, mupirocin, beta-lactam, aminocoumarin molecules, and others involved in regulatory or inactivating systems and efflux pumps.

Accession number(s). This whole-genome shotgun project has been deposited in GenBank under the accession no. MWTQ00000000. The version described in this paper is the version MWTQ00000000.1.

REFERENCES

1. Strobel GA. 2003. Endophytes as sources of bioactive products. Microbes Infect 5:535–544. https://doi.org/10.1016/S1286-4579(03)00073-X.
2. Hudson JB. 2012. Applications of the phytomedicine *Echinacea purpurea* (Purple Coneflower) in infectious diseases. J Biomed Biotechnol 2012: 769896. https://doi.org/10.1155/2012/769896.
3. Chiellini C, Maida I, Emiliani G, Mengoni A, Mocali S, Fabiani A, Biffi S, Maggini V, Gori L, Vannacci A, Gallo E, Firenzuoli F, Fani R. 2014. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. Int Microbiol 17:165–174. https://doi.org/10.2436/20.1501.01.219.
4. Maida I, Chiellini C, Mengoni A, Bosi E, Firenzuoli F, Fondi M, Fani R. 2016. Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *Echinacea purpurea*. Environ Microbiol 18:2357–2365. https://doi.org/10.1111/1462-2920.12911.
5. Chiellini C, Maida I, Maggini V, Bosi E, Mocali S, Emiliani G, Perrin E, Firenzuoli F, Mengoni A, Fani R. 2017. Preliminary data on antibacterial activity of *Echinacea purpurea*-associated bacterial communities against *Burkholderia cepacia* complex strains, opportunistic pathogens of cystic fibrosis patients. Microbiol Res 196:34–43. https://doi.org/10.1016/j.micres.2016.12.001.
6. Mengoni A, Maida I, Chiellini C, Emiliani G, Mocali S, Fabiani A, Fondi M, Firenzuoli F, Fani R. 2014. Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. Res Microbiol 165:686–694. https://doi.org/10.1016/j.resmic.2014.09.008.
7. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
8. Bosi E, Donati B, Galardini M, Brunetti S, Sagot MF, Lió P, Crescenzi P, Fani R, Fondi M. 2015. Medusa: a multidraft based scaffolder. Bioinformatics 31:2443–2451. https://doi.org/10.1093/bioinformatics/btv171.
9. Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39:W339–W346. https://doi.org/10.1093/nar/gkr466.
10. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJV, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T, Wright GD. 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 57:3348–3357. https://doi.org/10.1128/AAC.00419-13.

5. An insight on seed-borne endophytes: endophytic bacteria associated to *E. purpurea* seeds

5.1 Introduction

Echinacea Moench is a north American genus of tribe Heliantheae (Asteraceae). This tribe is characterized by a modified achene, named cypsela, and, within it, an internal phytomelanin layer. Despite their Northamerican origin, three species of *Echinacea*, i.e. *E. angustifolia* DC., *E. purpurea* (L.) Moench, and *E. pallida* (Nutt.) Nutt., are widely cultivated worldwide for their pharmaceutical properties. These three *Echinacea* species were used as medicines by American indigenous people in the north of Mexico for the ailment of various diseases, mainly sore mouth and throat, colic, stomach cramps and toothache (Shemluck 1982). Currently, the properties attributed to the cultivated *Echinacea* species are mainly related to the stimulation of the immune system for the treatment of respiratory infections (Stuart and Wills 2003); however, also analgesic, anti-inflammatory and antibiotic activities have been proposed (Parsons et al. in press2018). The medicinal effects are attributed to phytochemical compounds: such as alkylamides, polysaccharides and various phenolics, such as echinacoside, cichoric acid, caftaric, and chlorogenic acid (Parsons et al. 2018, Sharifi-Rad et al. 2018).

Mcgregor assigned 9 species to the genus *Echinacea*, while after a later revision by Binns et al. (2002), the total number of species belonging to genus *Echinacea* amounted to 4, divided into two subgenera: subg. *Echinacea*, comprising the only *E. purpurea*, and subg. *Pallida* containing *E. atrorubens* (Nutt.) Nutt., *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake and *E. pallida*, with *E. angustifolia* classified as *E. pallida* var. *angustifolia* (DC.) Cronq.

The fruits of *Echinacea* are called cypselas, defined as similar to the achenes, but derived from an inferior ovary (Simpson 2006), while the achenes derive from superior ovaries (Marzinek et al., 2008). For this reason, the cypselas, externally to the pericarp present a further structure, the perianth, derived from the flower corolla (Spjut, 1994). A large variation in the germination capability is known for *Echinacea* seeds, mainly attributed to seed dormancy. Quite surprisingly, the removal of perianth and pericarp from the cypsela resulted in a decreased germination in the soil but in improved germination in a sterile agar medium (Parsons et al. 2018).

In various *Echinacea* species , the investigation about the presence of endophytes has shown that the bacterial communities vary between the compartments of the same species

and between different species (Chiellini et al. 2014) and that different compartments of the same plant did not share strains suggesting the existence of a selective pressure responsible for structuring the microbial communities (Maida et al, 2016, Mengoni et al, 2014). Endophytes can be defined as microorganisms living within the plant tissues with no pathogenic effects (Wilson, 1995) and they are widely distributed in plants (Malfanova et al., 2013; Ryan et al., 2008). The presence of endophytes is considered useful for the plants promoting the host growth by interaction with nitrogen and phosphorous metabolism (Molina-Favero et al. 2008; Rodriguez et al., 2006). In *E. purpurea* the presence of endophytes has recently been related to the increase of alkilamides content and to the higher expression level of the valine decarboxylase (VDC) gene (Maggini et al. 2017) involved in the biosynthesis of the amine moieties of alkylamides (Rizhsky et al. 2016). These compounds with other phenolics, have been found at high levels in seeds of the three commercial *Echinacea* species (Parsons et al. 2018) while no data concerning the presence, biodiversity and localization of *Echinacea* seed-borne endophytes are known.

The aim of the present work is, therefore, to observe the cypselas of the most frequently cultivated *Echinacea* species in order to evaluate the possible presence of fungi and bacteria in the different components of the seed i.e. perianth, pericarp and cotyledons. Additionally, this work aims at exploring the biodiversity of seed-borne endophytes by extracting and characterizing from a taxonomic and phenotypic point of view bacterial endophytes from *E. purpurea* seeds.

Since seed-borne endophytes could be related to seed germination capability of different *Echinacea* spp., seed germinability will also be evaluated.

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Plant Material

Seeds of the three *Echinacea* spp. were provided by the “Il Giardino delle Erbe”, Casola Valsenio, Italy.

5.2.1.2 Bacterial isolates used in this work

Table 5. List of the endophytic bacterial isolates from *E. purpurea* seeds analyzed in this work.

Isolate code	Accession Number	Genus affiliation
1		*Paenibacillus*
2	MH670938	*Paenibacillus*
3		*Paenibacillus*
4	MH670939	*Pantoea*
5		*Pantoea*
6		*Pantoea*
7	MH670940	*Paenibacillus*
8		*Paenibacillus*
9	MH670941	*Paenibacillus*
12	MH670942	*Paenibacillus*
13	MH670943	*Sanguibacter*
14	MH670944	*Sanguibacter*
15	MH670945	*Pantoea*
16	MH670946	*Paenibacillus*
17		*Paenibacillus*
18	MH670947	*Paenibacillus*
21	MH670948	*Paenibacillus*
22		*Paenibacillus*
23		*Paenibacillus*
24		*Paenibacillus*
25		*Pantoea*
26		*Pantoea*
27		*Paenibacillus*
28	MH670949	*Pantoea*
29		*Pantoea*
32		*Paenibacillus*
36	MH670937	*Pantoea*
38		*Pantoea*
39	MH670950	*Pantoea*
40		*Pantoea*
41		*Pantoea*
45		*Pantoea*
46		*Pantoea*
47		*Pantoea*
51	MH670951	*Paenibacillus*
52		*Paenibacillus*
53		*Paenibacillus*

5.2.2 Methods

5.2.2.1 Fixation and embedding

This part of the work has been performed in collaboration with Prof. Alessio Papini, Department of Biology, University of Florence.

Some developing seeds were prefixed overnight in 1.25% glutaraldehyde at 4° C in 0.1 M phosphate buffer (pH 6.8), and then fixed in 1% OsO4 in the same buffer for 1 hr. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr's epoxy resin (Spurr, 1969).

5.2.2.2 Sectioning and Staining for Light and Fluorescence Microscopy

This part of the work has been performed in collaboration with Prof. Alessio Papini, Department of Biology, University of Florence.

Seeds embedded in Spurr's epoxy resin were transversely sectioned with glass knives to obtain semi thin sections (1-5µm), which were stained with Toluidine blue, 0.1%, then observed and photographed with a Leitz DM RB light microscope. Seeds that were not embedded were instead sectioned with a Cryostat to generate sections of 10-20µm of thickness. Some of these seed sections were stained with 1% phloroglucinol (w/v) in 12% HCl for 5 min and observed with a brightfield light microscope for detecting lignin. Another set of cryostat sections were stained with Sudan III for the detection and localization of lipids under brightfield microscopy (Brundrett et al., 1991). The remainder of the Cryostat sections were stained with Fluorol Yellow 088 and viewed with a fluorescent microscope Leica DM RB Fluo in the range of 515-565 nm (green) to detect lipids (Brundrett et al., 1991). Any series of images with differential staining were treated with the python program ALLAMODA 2.0 (Papini, 2012) to reduce noise.

5.2.2.3 Transmission electron microscopy (TEM)

This part of the work has been performed in collaboration with Prof. Alessio Papini, Department of Biology, University of Florence.

Seeds embedded in Spurr's epoxy resin (Spurr, 1969) were also cut with a diamond knife to generate sections that were approximately 80 nm thick. These ones were stained with uranyl acetate and lead citrate, and then examined with a Philips EM300 TEM operating at 80 kV.

5.2.2.4 Extraction of bacterial endophytic strains from *E. purpurea* seeds

This part of the work has been performed in collaboration with Prof. Anna Maria Puglia, University of Palermo.

E. purpurea seeds were surface sterilized by treating them with sterile water for 3 min; ethanol 70% for 1 min; HClO 2.5% for 2 min; ethanol 70% for 1 min. Then they were washed twice with sterile water. One ml of the water used for washing seeds is plated in LA, R2YED, SFM and PDA media in order to verify its sterility. Following, seeds were idrated by incubation in water for 1h at room temperature. Seeds were then grounded using sterile mortar and pestle. Fifty ml of phosphate-buffered saline was added to each 7.5g of seeds. Seeds are then incubated under shaking at 150 rpm for 1h at 30°C. Aliquots of 100µl are then platen on LA, R2YED, SFM and PDA media.

5.2.2.5 Random amplified polymorphic DNA (RAPD) analysis

Cell lysates of the endophytic bacterial isolates were obtained by thermal lysis by incubating an isolated bacterial colony for each isolate at 95°C for 10 min, and cooling on ice for 5 min. Amplification of DNA (Weller DM, 2007) was performed on 2 µl of cell lysate in a 25-µl total volume reaction composed by 1× reaction buffer, 300 µM $MgCl_2$, deoxynucleoside triphosphate (200 µM each), 0.5 U of PolyTaq DNA polymerase (all reagents were from Polymed, Florence, Italy), 500 ng of primer 1253 [5′-GTTTCCGCCC-3′] (Mocali et al., 2003). Amplification conditions were the following: 90°C for 1 min, and 95°C for 90 s followed by 45 cycles at 95°C for 30 s, 36°C for 1 min, and 75°C for 2 min. Finally, the reaction mixtures were incubated at 75°C for 10 min, 60°C for 10 min, and 5°C for 10 min. Reaction products were analyzed by agarose (2% w/v) gel electrophoresis in Tris-acetate EDTA buffer (TAE) containing 0.5 µg ethidium bromide/ml. Bacterial isolates showing the same RAPD fingerprinting were grouped together into an haplotype. For each RAPD haplotype, a single bacterial strain was randomly chosen for 16S rRNA gene amplification and taxonomic attribution.

5.2.2.6 PCR amplification and sequencing of 16S rRNA coding genes

PCR amplification of 16S rRNA genes was carried out in 20-µl reactions using DreamTaq DNA Polymerase reagents (Thermofisher Scientific) at the concentrations suggested by the company, and 0.5 µM of primers P0 (5′-GAGAGTTTGATCCTGGCTCAG) and P6 (5′-CTACGGCTACCTTGTTACGA) (Di Cello and Fani, 1996); 1 µl of cell lysate was used as template. Amplification conditions were the following: 90-s denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C, followed by a final extension of 10

min at 72°C. Direct sequencing of the amplified 16S rRNA genes was performed with primer P0 by an external company (IGA Technology Services-Udine-Italy). Each 16S rRNA gene sequence was submitted to GenBank and assigned an accession number from MH670937 to MH670951.Taxonomic affiliation of the 16S rRNA gene sequences were attributed using the "classifier" tool of the Ribosomal Database Project –RDP (Cole et al. 2014).

5.2.2.7 Antibiotic resistance

Endophytic bacterial strains were assayed for their antibiotic resistance on Tryptic Soy Agar medium (TSA) supplemented with one of the following antibiotics, showing different mechanisms of action: Chloramphenicol inhibits translation by binding the 50S ribosomal subunit; Ciprofloxacin blocks DNA replication through the inhibition of DNA gyrase; Rifampicin blocks transcription by binding the β subunit of RNA polymerase; Streptomycin, Kanamycin and Tetracycline alter translation by inhibiting the translocation of the peptidyl-RNA from the A-site to the P-site. Briefly, each strain was grown on TSA medium for 48h at 30°C, then a colony of each strain was suspended in 100 µl saline solution (0.9% NaCl), streaked on TSA medium supplemented with different antibiotic concentrations and afterwards incubated at 30°C for 48h. Isolates were also streaked on TSA plates without antibiotics in order to evaluate their growth in presence of the only medium. Results were obtained by comparing the growth of an isolate on TSA supplemented with one of the antibiotics to the growth registered in only TSA medium. Levels of growth were defined as complete growth, weak growth or absent growth corresponding respectively to resistance, partial resistance and sensibility to the antibiotic. Moreover, in order to obtain an easier visualization of results, these were associated to colors as follows: white for complete growth, salmon for weak growth and red for absent growth.

The following antibiotic concentrations (in µg/ml) were tested: Chloramphenicol (1-2.5-5-10-25-50); Ciprofloxacin (0.5-1-2.5-5-10-50); Rifampicin (5-10-25-50-100); Streptomycin and Kanamycin (0.5-1-2.5-5-10-50); Tetracycline (0.5-1.25-2.5-5-12.5-25).

5.2.2.8 Inhibition of bacterial endophytes from *E. purpurea* seeds by *E. purpurea* rhizosphere-associated strain EpRS3 *Rheinheimera*

Inhibitory activity of EpRS3 *Rheinheimera* towards endophytic strains from *E. purpurea* seeds was assayed using the Cross-Streak method (Maida et al. 2015). EpRS3

Rheinheimera was termed *tester* strain, while seed-endophytic strains were referred to as *target* strains. Tester strain was streaked across one-half of a TSA plate and grown at 30°C for 48h to promote the production of antimicrobial compounds. Then, target strains were streaked perpendicularly to tester strain and plates were further incubated at 30°C for 48h. Additionally, target strains were grown at 30°C for 48h, in order to control their proper growth in absence of the tester strain. The antagonistic effect was indicated by the absence or reduction of the target strain growth. Each interaction was tested twice.

5.3 Results

5.3.1 Anatomical observations and symbionts localization

We followed here the nomenclature and the general description of the *Echinacea* cypsela by Parsons et al. (2018) and Schultes et al. (1991). For the identification of the most frequent components of the parenchyma cotyledon cell (oil bodies and protein bodies), we followed Evert (2006), specifically page 54.

The cypselas in the three species showed a similar general aspect (see Figure 8). A more external layer (perianth) was of variable thickness and appeared porous and lignified (Figure 8a and 8b). Fungal hyphae were observed inside the cells forming the perianth (Figure 8b, 10b) and the cell walls of the perianth were PAS positive (Figure 9a). Outside of this layer, clusters of microorganisms appeared to adhere strongly to the external boundary of the perianth (Figure 8c), since they were observed even after the fixation and inclusion procedure (no previous fruit washing was done in this case).

Inside the perianth layer, a space opened, lined by a bicellular layer of sclereids (pericarp) showing a dark material (phytomelanin) in the intercellular spaces outside the internal tangential walls towards the perianth. The phytomelanin was found in both *E. purpurea* (Figure 8d) and *E. angustifolia* (data not shown) and on both sides of the sclereids layer in *E. pallida* (Figure 9a). In *E. purpurea*, the sclereids layer contained SUDAN III positive droplets (Figure 9b). The space between the pericarp and the seed coat contained secretory canals, constituted by an external suberized (Sudan III positive, data not shown) monocellular layer and an internal layer of living cells surrounding a central space (Figure 9c). Inside the pericarp, a flattened endosperm layer surrounded the rest of the seed (Figure 9d) where the cotyledon cells appeared to contain apparently two types of large bodies with a different degree of positivity to toluidine blue (Figure 9d).

The TEM images confirmed the presence of microorganisms outside the perianth, adhering to the external tegument in all the three species. An example is shown in Figure 10a. A

layer with a low level of electron density was observed outside the last outer perianth cells. Some microorganisms were observed included in this layer (Figure 10a). Within the perianth, septate hyphae were able to occupy almost the entire volume of some cells that appeared empty (Figure 9b).

Inside the perianth layer, a double layer of sclereids was observed, normally empty of cytoplasm or containing only dark residuals (Figure 10c). On the perianth side of this layer, an electron dense material (phytomelanin), apparently formed by lamellae was observed outside the sclereid walls in the intercellular spaces (Figure 10d). Some of the sclereid cytoplasms appeared condensed and electron dense (Figure 10d).

Inside the seed, the cotyledon cells appeared occupied by large oil bodies and protein bodies (Figure 11a). In *E. purpurea*, between some lipid bodies, endophytic bacteria occupied a narrow space with only a few nm between the external bacterial wall and the lipid bodies (Figure 11b). In *E. pallida* the cotyledon cell nucleus showed often a very condensed chromatin (Figure 10c and 11c). In these cells, we observed smaller endophytic bacteria in comparison with those observed in *E. purpurea*, close to the wall of the cotyledon parenchyma cells, with a larger space between the bacterial wall and the surrounding plant cell membrane, while other bodies of more complex identification were apparently surrounded by an electron transparent wall (Figure 11d). Some of the endophytic microorganisms observed *in E. angustifolia* were of larger dimension than those observed in *E. purpurea* and apparently showed a cell wall (Figure 12a and Figure 12b).

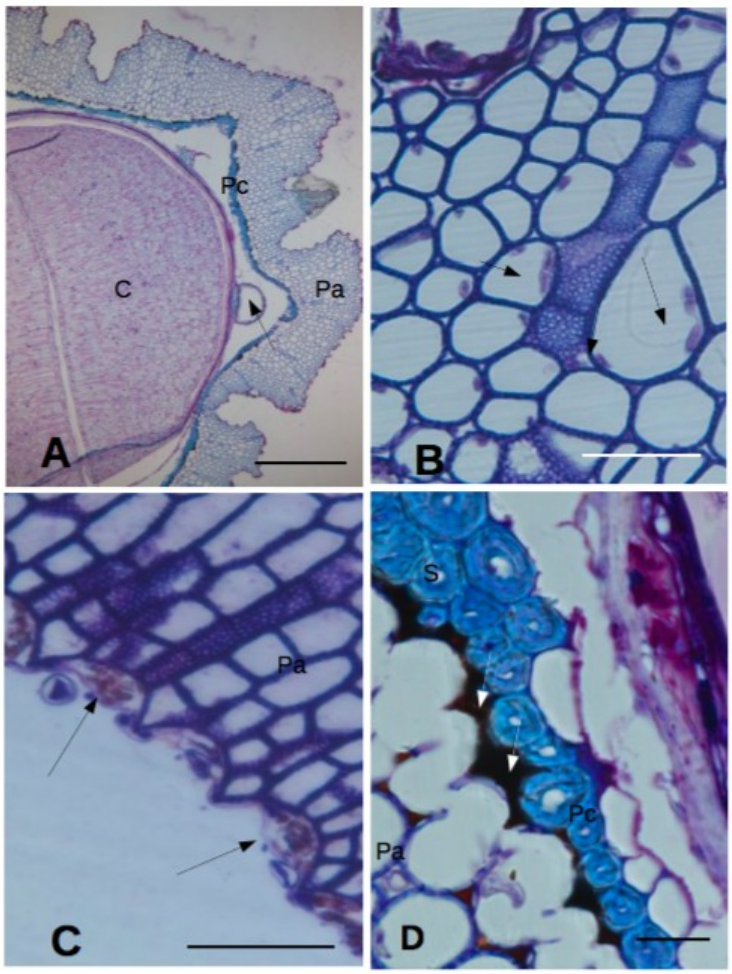

Figure 8. General cypsela anatomy with detail of the perianth in *E. purpurea*. A: *E. purpurea* cypsela. The perianth has a contorted profile outside. A secretory canal is shown (arrow). Bar = 250 µm. B: *E. purpurea* perianth. Hyphae (arrows) are visible inside the perianth cells. Bar = 25 µm. C: *E. angustifolia.* Bacterial colonies (arrows) are visible on the external side of the perianth. Bar = 50 µm. D: *E. purpurea* pericarp with phytomelanin (white arrows) on the side of the perianth. Bar = 10 µm.

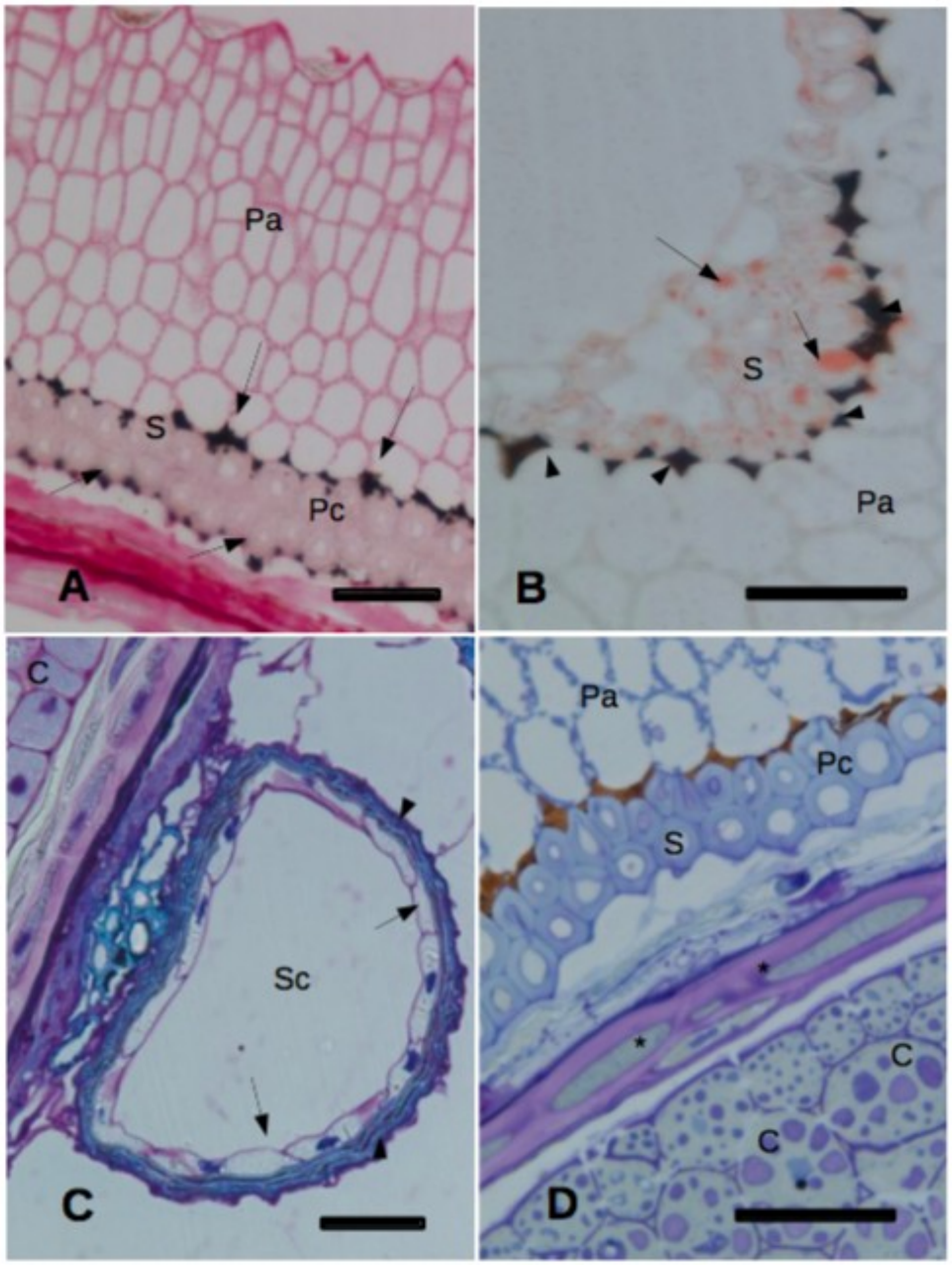

Figure 9. Histochemistry reactions on the cypsela of Echinacea spp.. A: *E. pallida*. PAS reaction. Perianth and pericarp layer. Phytomelanin (arrows) is present on both sides of the pericarp, constituted by two layers of sclereids. Bar = 25 µm;. bB: *E. purpurea* . Sudan III reaction. Lipid droplets (arrows) in the pericarp layer underneath the phytomelanin layer. (arrowheads) 219/2. Bar = 25 µm;. cC: *E. purpurea 210/7* secretory canal. The arrows indicate the living cells inside the canal. The arrowheads indicate the suberified external cells of the canal. Bar = 25 µm;. dD: *E. purpurea*. Zone of transition from fruit to seed. The asterisks indicate the endoderm. 220/34. Bar = 25 µm.

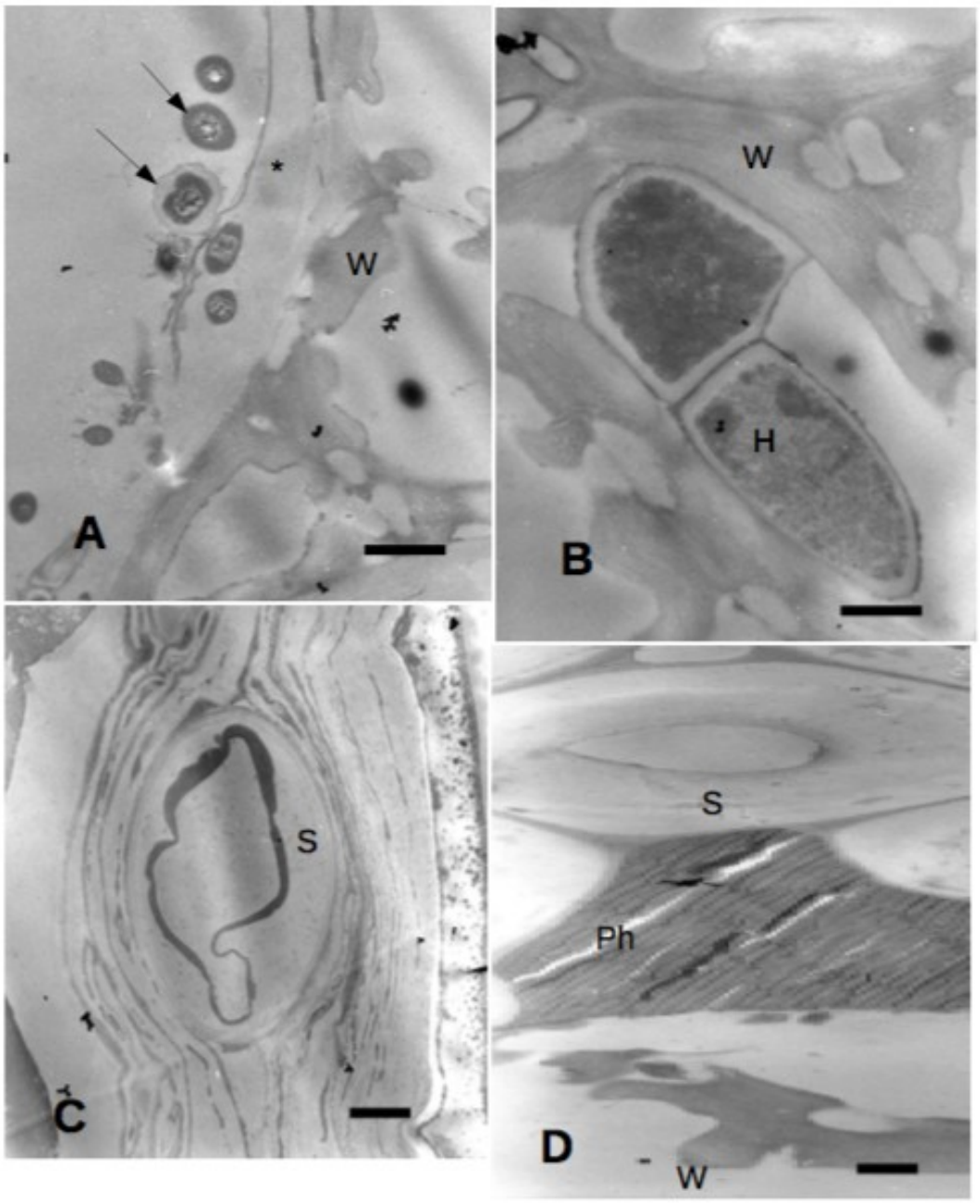

Figure 10. Transmission Electron Microscope images of the perianth and the pericarp. A: *E. purpurea.* External side of the perianth. Microorganisms (arrows) are adhering on the external surface of the perianth. Lowly electron dense layer (asterisk) outside the last outer perianth cells. Bar = 2 µm. B: *E. purpurea.* Hyphae inside the perianth cells. Bar = 2 µm. C: *E. purpurea.* Sclereid belonging to the pericarp. Bar = 2 µm. D: *E. angustifolia.* Pericarp sclereid with phytomelanin deposition. Bar = 5 µm.

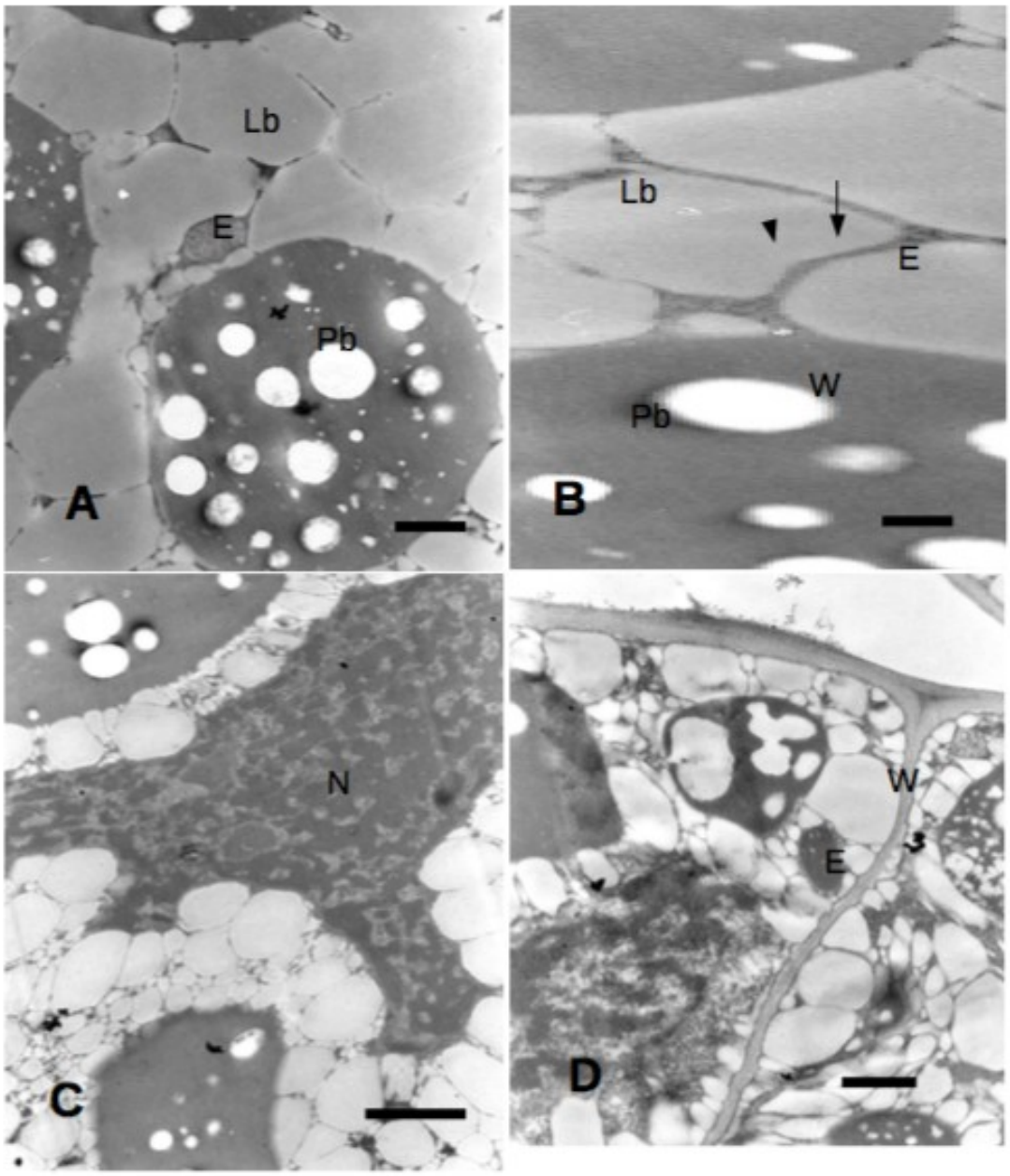

Figure 11. Transmission Electron Microscope images of the cotyledons in *E. purpurea* and *E. pallida*. A: *E. purpurea*. Cotyledon. Endophyte between lipid bodies. Bar = 1 µm. B: *E. purpurea*. Cotyledon. Large endophyte between lipid bodies. A small endophyte (arrowhead) is enclosed in a larger space close to the plasma membrane. Another endophyte (arrow) with a relatively thick wall is adjacent to the cell wall. Bar = 1 µm. C: *E. pallida.* Cotyledon. A large multilobate nucleus shows condensed chromatin. Bar = 2 µm. D: *E. pallida.* Cotyledon. Endophyte between lipid bodies. Bar = 1 µm.

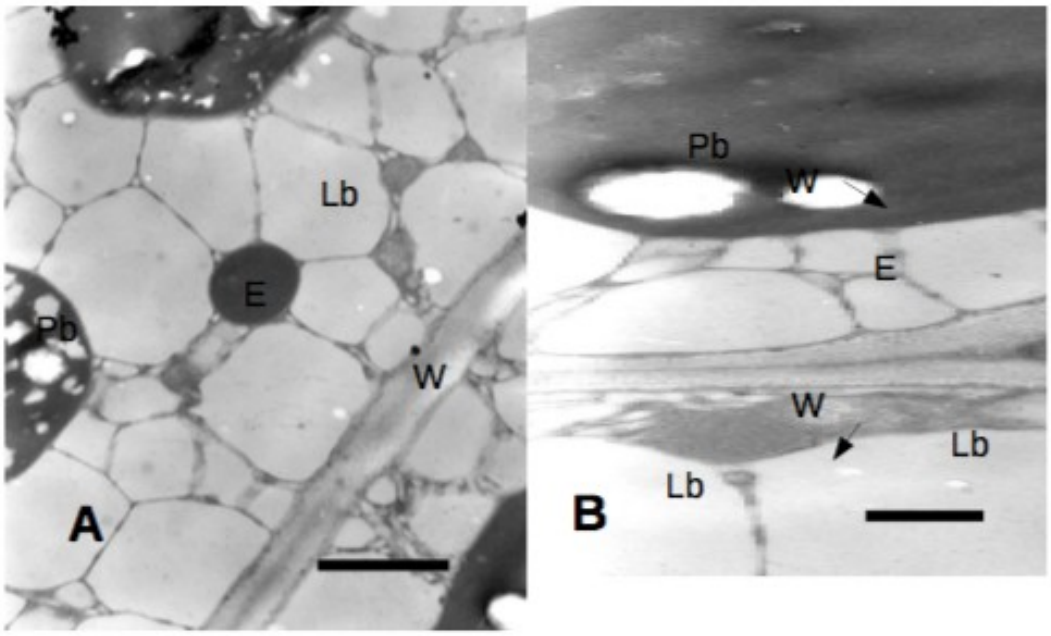

Figure 12. Transmission Electron Microscope images of the cotyledons in *E. angustifolia*. A: Endophyte between lipid bodies with a thick wall and electron dense cytoplasm. Bar = 1 µm. B: Endophyte between lipid bodies. Smaller endophytes are indicated by arrows. Bar = µm.

Abbreviations:
C, cotyledon; E, endophyte; H, hypha; Lb, lipid body; N, nucleus; Pa, Perianth; Pb, protein body; Pc, Pericarp; Ph, phytomelanin; S, sclereid; Sc, secretory canal; W, cell wall.

5.3.2 Structure of endophytic bacterial community isolated from *E. purpurea* seeds

Bacterial isolates extracted from superficially sterilized *E. purpurea* seeds were submitted to RAPD fingerprinting analysis in order to determine the isolates' variability at the strain level and to analyze the community structure. DNA from each single bacterial isolate was obtained by thermal lysis and was randomly amplified with a 10-mer oligonucleotide, as described in Materials and methods. Amplicons were then analyzed by agarose gel electrophoresis. All RAPD profiles were compared to each other and isolates showing the same RAPD profile were grouped together into an haplotype. As shown in Table 6, 15 RAPD haplotypes were identified out of the 37 analyzed bacterial isolates. The 15 observed RAPD haplotypes correspond at least to 15 bacterial strains. Among the haplotypes, 7 were composed by only one bacterial strain, 1 was composed by 2 isolates, 3 haplotypes were composed by 3 isolates, two haplotypes comprised 4 isolates, two haplotypes showed 5 and 6 isolates each.

Table 6. RAPD analysis and genus affiliation for *E. purpurea* seed associated bacterial endophytes.

RAPD Haplotype	Isolate code	Accession Number	Genus affiliation
1	16	MH670946	*Paenibacillus*
	24		*Paenibacillus*
	27		*Paenibacillus*
2	5		*Pantoea*
	6		*Pantoea*
	26		*Pantoea*
	36	MH670937	*Pantoea*
	38		*Pantoea*
3	12	MH670942	*Paenibacillus*
4	13	MH670943	*Sanguibacter*
5	14	MH670944	*Sanguibacter*
6	15	MH670945	*Pantoea*
7	7	MH670940	*Paenibacillus*
	8		*Paenibacillus*
8	9	MH670941	*Paenibacillus*
9	1		*Paenibacillus*
	3		*Paenibacillus*
	17		*Paenibacillus*
	18	MH670947	*Paenibacillus*
10	21	MH670948	*Paenibacillus*
	22		*Paenibacillus*
	23		*Paenibacillus*
	32		*Paenibacillus*
11	51	MH670951	*Paenibacillus*
	52		*Paenibacillus*
	53		*Paenibacillus*
12	39	MH670950	*Pantoea*
	40		*Pantoea*
	41		*Pantoea*
	45		*Pantoea*
	46		*Pantoea*
	47		*Pantoea*
13	25		*Pantoea*
	28	MH670949	*Pantoea*
	29		*Pantoea*
14	4	MH670939	*Pantoea*
15	2	MH670938	*Paenibacillus*

5.3.3 Composition of endophytic bacterial community isolated from *E. purpurea* seeds

Amplification and sequencing of 16S rRNA coding genes were performed for one bacterial isolate from each RAPD haplotype, assuming that isolates sharing the same haplotype represented the same or closely related strains. Thus, one strain was randomly chosen from each RAPD haplotype as a haplotype representative strain. 16S rRNA amplicons were obtained and sequenced from each of the 15 representative strains. Taxonomic affiliation of the 16S rRNA gene sequences were obtained as described in Material and methods. Table 6 shows that from the 15 obtained sequences, with accession numbers from MH670937 to MH670951, 8 were affiliated to *Paenibacillus* genus, 5 to *Pantoea* and 2 to *Sanguibacter* genus, respectively 53.4%, 33.3% and 13.3% of RAPD haplotypes. Bacterial strains belonging to the same haplotype were affiliated to the same genus.

5.3.4 Antibiotic resistance profiles of bacterial endophytes from *E. purpurea* seeds

Isolates from *E. purpurea* seeds were analyzed for their resistance to six different antibiotics and concentrations, as described in Materials and methods. Results were associated to colors, for a better visualization, as described in Materials and methods. Figure 13 shows that among all the tested antibiotics, Rifampicin and Ciprofloxacin appeared to be the most effective ones. None of the isolates were able to grow at Rifampicin maximum tested concentration (100 µg/ml) and most isolates (45.9%) were able to grow only at the minimum antibiotic tested concentration (5 µg/ml). No isolate was able to grow on Ciprofloxacin maximum tested concentration (50 µg/ml) and the majority of isolates (40.5%) was able to only at a concentration of 0.5 µg/ml, the minimum tested concentration.

The registered antibiotic resistance profiles vary within a single bacterial genus since isolates sharing the same genus show different resistance patterns.

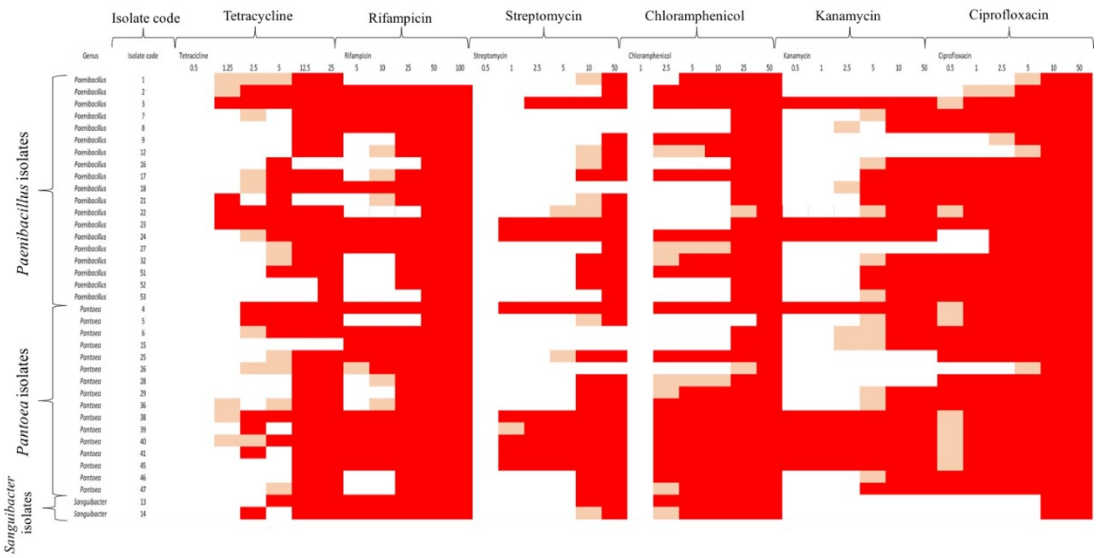

Figure 13. Heat map showing the antibiotic resistance patterns for endophytic bacterial isolates extracted from *E. purpurea* seeds. Each isolate is shown in rows on the left, divided into genera, while the different antibiotics and their concentrations are shown in columns. Red spots represent cases of total inhibition of isolates, salmon spots represent cases of weak growth of isolates (partial inhibition), and white spots show cases of growth of isolates (resistance).

5.3.5 Antagonistic interactions between *E. purpurea* seed-borne endophytes and *E. purpurea* rhizosphere-associated strain EpRS3 *Rheinheimera*

The EpRS3 *Rheinheimera* strain, isolated from the rhizosphere of *E. purpurea* plants, as described by Chiellini and colleagues (Chiellini et al. 2014) and exhibiting notable antimicrobial effects (Chiellini et al. 2017, Presta et al. 2017), was tested for its ability to inhibit the growth of *E. purpurea* seeds endophytic strains, following the Cross-streak method illustrated in Material and methods. Tests showed that all the analyzed target strains were able to grow properly in presence of the tester strain EpRS3 *Rheinheimera*, showing that such strain does not produce effective antimicrobial molecules towards the seed-borne endophytes.

5.4 Discussion

Echinacea spp. are a group of widely used plants for their therapeutic properties. Recently, growing evidence has been found on the influence of bacterial endophytes on the medicinal properties of plants (Maggini et al., 2017), (Li et al., 2012). Despite their importance, few studies have focused on the medicinal plants-associated microbiome, and even less on the seed-associated microbiome. Microorganisms associated to plant seeds are of particular interest, since they might be transmitted through generations and can persist within seeds for a long time. For these reasons, we have analyzed *Echinacea* spp. seeds, in order to investigate its anatomical features and the presence of associated microorganisms. Furthermore, bacterial endophytes have been extracted from *E. purpurea* seeds and characterized from a taxonomical and phenotypic point of view.

We observed that the perianth in all the three investigated species contained a remarkable presence of fungi that appeared to occupy the interior of the particular cell types present in this fruit organ, apparently dead and lignified at maturity and empty of cytoplasmic remnants. This observation may be considered an indirect evidence of the importance of the fungal component at least for seed germination in the soil, where it may play a role in collecting nutrients at the beginning of germination, thus explaining the reduced germination rate in perianth-less cypselas (Parsons et al. (2018). Unfortunately, the quality of the ultrastructure fine detail of the fungal component did not permit a better clue about the fungal identity. This is in agreement with the well-known difficulty in fungi ultrastructural investigation due to poor fixation as a consequence of the presence of the chitin wall (Osumi 1998). The presence of fungal endophytes in *Echinacea* was previously

recorded by Rosa et al. (2012) who attributed to this presence the property to protect the plant from phytopathogenic fungi by production of specific compounds.

The presence of endophytic bacteria in *Echinacea* spp. was already recorded by PCR amplification by Chiellini et al. (2014) in the root, stem and leaves, these two last compartments considered altogether. These authors observed species specificity of endophytic bacteria, i.e. those of *E. angustifolia* were in large part different from those in *E. purpurea*, both in the shoot/leaf system and in the root. Miller et al. (2012) and Chiellini et al. (2014) proposed that at least part of the medicinal properties of the plants may depend on the bacterial endophytes and the recent findings obtained in one of our laboratories suggest the bacterial endophytes could really affect the therapeutic features of these important medicinal plants (Maggini et al. 2017). Maggini et al. (2018) observed also that bacteria from different plant compartment showed specific antibiotic resistance and antibiotic production, suggesting that the bacterial communities may actively select their neighbors in the different plant compartment (Maggini et al., 2018).

In the seed, the endophytic bacteria appear to be localized in the cotyledon cells and to be at least of three different types: large with few space between bacterial wall and plant cell surrounding membrane, normally among lipid bodies; a second type of smaller dimension, apparently with a large wall and a larger space between wall and a surrounding plant cell membrane and a third type large and with a very electron dense cytoplasm. These endophytes were endocellular, whereas no endophyte was observed neither in the intercellular spaces nor in the walls. The bacteria were enclosed in a membrane structure similarly to the situation observed for other endocellular bacteria such as Mollicutes, as those find in the fungus *Geosiphon pyriformis* by Schuessler and Kluge (2001). Mollicutes however do not have a wall and assume an ameboid shape. No clear evidence of the bacterial wall was observed here, but the shape of the bacterium was maintained, suggesting that a bacterial wall is present. The endophytic bacteria in *Echinacea* were enclosed within the host membrane, apparently leaving a very narrow space between this last and the bacterial membrane. This is a difference with respect to pathogenic bacteria such as *Burkholderia pseudomallei* (Gong et al., 2011) and *Staphylococcus aureus* (Gresham et al., 2018), were a larger space is observed between the autophagosomal vacuole and the bacteria as a prelude to their escape from macrophages. Tabsence of a large space between the bacterial wall and the host membrane may suggest a high interchange of substances between the bacterium and his host.

The bacteria present on the outer side of the perianth apparently did not cross the perianth barrier that appeared to be occupied only by fungi, while the phytomelanin barrier

apparently arrested the penetration of fungal hyphae towards the seed. Phytomelanin is found in 8 tribes of Heliantheae (Phytomelanin cypsela clade, sensu Panero and Funk, 2008) and is chemically considered to be a compound derived from carbohydrates (Pandey et al., 2014) or from "phytoacetylen" (Hegnauer 1977; Tadesse and Crawford, 2014) while other authors attributed to it a catechin-like or flavonoid-like nature (Graven et al., 1998). The function has not yet been clarified, being attributed to this layer the property of providing resistance against dessiccation and predator insects (Pandey et al. 2014). A key role for a possible reduction in extinction rate during the species radiation in Heliantheae evolution is attributed to the phytomelanization (Panero and Crozier 2016). Our data suggest that the phytomelanin could play a role in blocking the fungi present in the perianth.

The analysis of bacterial endophytes extracted from *E. purpurea* seeds highlighted the predominance of *Paenibacillus* and *Pantoea* genera. These genera were also the most represented among the studied bacterial communities associated to seeds of different plants such as *Oryza sativa* (Liu et al. 2007, Verma et al. 2017, Hardoim et al. 2012, Ruiza et al. 2011, Kaga et al. 2009, Mano et al. 2006), *Phragmites australis* (White et al. 2017), *Triticum aestivum* (Diaz Herrera et al. 2016), *Tylosema esculentum* (Chimwamurombe et al. 2016), *Zea mays* (Liu et al. 2013, Rijavec et al. 2007), *Arachis hypogaea* (Sobolev et al. 2013), *Phaseolus vulgaris* (Rosenblueth et al. 2012), *Curcubita pepo* (Fumkranz et al. 2012), *Vitis vinifera* (Compant et al. 2011), *Fraxinus* (Donnarumma et al. 2011), *Nicotiana tabacum* (Mastretta et al. 2009), *Eucalyptus* (Ferreira et al. 2008), *Coffee Arabica* (Vega et a.l 2005). The wide diffused presence of such genera among seed-associated microbiomes may be linked to the dominance of these genera in water and soil ecosystems (Fierer et al. 2012, Shafi et al. 2017). The genus *Sanguibacter* was evidenced among the bacterial endophytes from *E. purpurea* seeds in a smaller percentage of isolates (13.3%). Bacterial endophytic isolates associated to different plant seeds also appear to be affiliated to *Sanguibacter* in a small measure. Such genus was observed among the microbiome associated to *Nicotiana tabacum* seeds by Mastretta and colleagues. (Mastretta et al. 2009). Antibiotic resistance analysis showed that many of the analyzed isolates were able to grow at different concentrations of the tested antibiotics, and to resist in some cases to high concentrations. Antibiotic resistance could be an important phenotype for seed-borne endophytes since it could preserve them from many adverse conditions and allow them to persist up to germination and plant development. In fact, seed-associated endophytes have been described as capable of performing different functions which could be essential for the plant-to-be, such as phytohormone production (Shazad et al 2016), seedling and plant

growth promotion (Oehrle et al. 2000, Chimwaumurombe et al. 2016, White et al. 2017, Xu et al. 2014, Hardoim et al. 2012) siderophore production (Diaz Herrera et al. 2016), as well as antifungal property and antibiotic production (Verma et al. 2017. Fumkranz et al. 2012, Donnarumma et al. 2011). Antibiotic resistance has been evaluated for *E. purpurea* and *E. angustifolia* associated bacterial endophytes (Mengoni et al, 2014, Maggini et al. 2018) and it has been hypothesized to be one of the factors shaping the plant-associated communities. Among seed-endophytes, antibiotic resistance could hypothetically be implied in determining communities leading to the selection of those strains exhibiting higher probability of persistence and of transmission of important properties to the future plant.

Antagonistic interactions showed that the rhizospheric strain EpRS3 *Rheinheimera* was not able to influence the grow of the bacterial endophytes associated to *E. purpurea* seeds and this might suggest that these are important for the plant germination and development, so that they are not sensible to antimicrobial effects that might take place in the rhizosphere.

5.5 Conclusions

Our results suggest that an endophytic bacterial community of *Echinacea* spp. is already present at the seed stage, hosted by the cotyledons, in addition to being in roots and stem/leaves. In seeds, the endophytic bacteria are localized inside the cells and not in the intercellular spaces. A further microbial fungal component may be transported together with the seed in the perianth of the cypsela and may influence the capability of the seed to germinate in the soil. The cypsela of *Echinacea* may be considered an adapted envelope to transport microbial components together with the seed in order to improve germinability. Extraction and characterization of bacterial strains from *E. purpurea* seeds has shown that such strains are mainly represented by *Paenibacillus* and *Pantoea* genera, and that some of these may show high antibiotic resistant profiles. Altogether, our data provide information of the presence and characteristics of endophytic bacteria within medicinal plants seeds.

5.6 Bibliography

Binns S. E., Baum, B. R., & Arnason, J. T. (2002). A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). *Systematic Botany*, 610-632.

Chiellini C, Maida I, Maggini V, Bosi E, Mocali S, Emiliani G, Perrin E, Firenzuoli F, Fani R. (2017). Preliminary data on antibacterial activity of *Echinacea purpurea*-associated bacterial communities against *Burkholderia cepacia* complex strains, opportunistic pathogens of cystic fibrosis patients. Microbiological research, 196, 34-43.

Chiellini C., Maida I., Emiliani G., Mengoni A., Mocali S., Fabiani A., Biffi S., Maggini V., Gori L., Vannacci A., Gallo E., Firenzuoli F., Fani R. (2014). Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. Int Microbiol, 17(3), 165-174.

Chimwamurombe P. M., Grönemeyer, J. L., & Reinhold-Hurek, B. (2016). Isolation and characterization of culturable seed-associated bacterial endophytes from gnotobiotically grown Marama bean seedlings. *FEMS microbiology ecology*, *92*(6).

Chimwamurombe P.M., Grönemeyer J.L., Reinhold-Hurek B. (2016). Clades of the Asteraceae revealed. Mol Phylogenet Evol 47: 757–782.

Cole J.R., Q. Wang J.A., Fish B., Chai B., McGarrell D., Sun Y., Brown C.T., Porras-Alfaro A., Kuske C.R., Tiedje J.M. (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis *Nucl Acids Res* , D633-D642.

Compant S., Mitter B., Colli-Mull J.G., Gangl H., Sessitsch A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microb. Ecol. 62, 188–197.

Di Cello F., Fani R. A molecular strategy for the study of natural bacterial communities by PCR-based techniques. *Minerva Biotecnol* 1996; 8: 126–134.

Díaz Herrera S., Grossi C., Zawoznik M., Groppa M.D. (2016). Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of Fusarium graminearum. Microbiol. Res. 186, 37–43.

Donnarumma F., Capuana M., Vettori C., Petrini G., Giannini R., Indorato C., et al. (2011). Isolation and characterisation of bacterial colonies from seeds and in vitro cultures of Fraxinus spp. from Italian sites. Plant Biol. 13, 169–176.

Evert, R.F. (2006) Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development. Hoboken, NJ: John Wiley & Sons, Inc.

Ferreira A., Quecine M.C., Lacava P.T., Oda S., Azevedo J.L., Araújo W.L. (2008). Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by Pantoea agglomerans. FEMS Microbiol. Lett. 287, 8–14.

Fürnkranz M., Lukesch B., Müller H., Huss H., Grube M., Berg G. (2012). Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. Microb. Ecol. 63, 418–428.

Gong L., Cullinane M., Treerat P., Ramm G., Prescott M., Adler B., Boyce J.D., Devenish R.J. (2011) The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis**.** PLoS ONE 6: e17852.

Graven P., Wittich, P.E., Bouman, F., Nachtegaal, G. & Kentgens, A.P.M. 1998. Characterization of phytomelan in the seed coat of *Gasteria verrucosa* (Mill.) H. Duval. – In: Wittich, P.E. (ed.), Seed Development and Carbohydrates. 121–147. – Ph.D. Thesis, Landbouwuniversiteit, Wageningen.

Gresham H.D., Lowrance J.H., Caver T.E., Wilson B.S., Cheung A.L., Lindberg F.P. (2018) Survival of *Staphylococcus aureus* Inside Neutrophils Contributes to Infection. The Journal of Immunology

Hall T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT In: *Nucleic acids symposium series*. 1999; 41: 95-98.

Hammer O., Harper D., Ryan P.D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontol Electron 4: 1–9.

Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD (2012). Dynamics of seed-borne rice endophytes on early plant growth stages. PLoS ONE 7:e30438.

Hegnauer R. 1977. Th e chemistry of the Compositae. – In: Heywood, V. H. et al. (eds), Th e biology and chemistry of the Compositae, Academic Press, pp. 283 – 335.

Kaga H., Mano H, Tanaka F, Watanabe A, Kaneko S, Morisaki H. (2009). Rice seeds as sources of endophytic bacteria. Microbes Environ. 24, 154–162.

Liu Y., Bai F., Li N., Wang W., Cheng C. (2017). Identification of endophytic bacterial strain RSE1 fromseeds of super hybrid rice Shenliangyou 5814 (*Oryza sativa* L.,) and evaluation of its antagonistic activity. Plant Growth Regul. 82, 403–408.

Liu Y., Zuo S., Zou Y., Wang J., Song W. (2013). Investigation on diversity and population succession dynamics of endophytic bacteria from seeds of maize (Zea mays L., Nongda108) at different growth stages. Ann. Microbiol. 63, 71–79.

Maggini V., De Leo M., Mengoni A., Gallo E.R., Miceli E., Reidel R.V.B., Biffi S., Pistelli L., Fani R., Firenzuoli F., Bogani P. (2017) Plant-endophytes interaction influences the secondary metabolism in *Echinacea purpurea* (L.) Moench: an in vitro model. Sci Rep. 7(1):16924.

Maggini V., Miceli E., Fagorzi C., Maida I., Fondi M., Perrin E., Mengoni A., Bogani P., Chiellini C., Mocali S., Fabiani A., Decorosi F., Giovannetti L., Firenzuoli F., Fani R. (2018) Antagonism and antibiotic resistance drive a species-specific plant microbiota differentiation in *Echinacea* spp. *FEMS Microbiology Ecology,* 94(8): fiy118.

Maida I., Chiellini C., Mengoni A., Bosi E., Firenzuoli F., Fondi M., Fani R. (2016) Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *Echinacea purpurea*. Environ Microbiol. 18(8):2357-65.

Malfanova N., Lugtenberg B.J.J. Berg, G. (2013) Bacterial Endophytes: Who and Where, and What are they doing there? In: de Bruijn J (ed) Molecular microbial ecology of the rhizosphere. John Wiley, Hoboken, NJ, USA, (vol 1) pp 393-403.

Mano H., Tanaka F., Watanabe A., Kaga H., Okunishi S., Morisaki H. (2006). Culturable surface and endophytic bacterial flora of the maturing seeds of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes Environ. 21, 86–100.

Marzinek J., De-Paula O.C. & Oliveira D.M.T. 2008. Cypsela or achene? Refining terminology by considering anatomical and historical factors. Revista Brasileira de Botânica 31: 549–553.

Mastretta C., Taghavi S., van der Lelie D., Mengoni A., Galardi F., Gonnelli C., et al. (2009). Enodphytic bacteria from seeds of *Nicotiana Tabacum* can reduce cadmium phytotoxicity. Int. J. Phytoremed. 11, 251–267.

McGregor R.L. 1968. The taxonomy of the genus *Echinacea* (Compositae). University of Kansas Science Bulletin, 48(4): 113-142.

Mengoni A., Maida I., Chiellini C., Emiliani G., Mocali S., Fabiani A., Fondi M., Firenzuoli F., Fani R. (2014) Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. Research in Microbiology 165: 686-694.

Miller K.I., Qing C., Sze D.M., Roufogalis B.D., Neilan B.A. (2012) Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microb Ecol 64:431-449.

Mocali S., Bertelli E., Di Cello F., Mengoni A., Sfalanga A., Tegli S., Viliani F., Surico G., Caciotti A., Fani R. (2003) Fluctuations of endophytic bacterial communities isolated from tissues of elm plants. Res Microbiol 154:105-114.

Osumi M. (1998) The ultrastructure of yeast: cell wall structure and formation. Micron 29(2-3): 207-233.

Pandey A.K., Stuessy T.F., Mathur R.R. Phytomelanin and systematics of the Heliantheae alliance. Plant Div. Evol., 131 (3) (2014), pp. 145-165.

Panero J.L. & Funk V.A. 2008: The value of sampling anomalous taxa in phylogenetic studies: major. Molecular phylogenetics and evolution 47.2: 757-782

Panero J.L., Crozier B.S. (2016) Macroevolutionary dynamics in the early diversification of Asteraceae. Molecular phylogenetics and evolution 99:116-132.

Papini A (2012) A new algorithm to reduce noise in microscopy images implemented with a simple program in Python. Microscopy and Research Technique 75(3): 334-342.

Parsons J.L., Liu R., Smith M.L., Harris C.S. (2018) Echinacea fruits: Phytochemical localization and germination in four Echinacea species. Botany.

Presta L., Bosi E., Fondi M., Maida I., Perrin E., Miceli E., Maggini V., Bogani P., Firenzuoli F., Di Pilato V., Rossolini G.M., Mengoni A., Fani R. (2017). Phenotypic and genomic characterization of the antimicrobial producer *Rheinheimera* sp. EpRS3 isolated from the medicinal plant Echinacea purpurea: insights into its biotechnological relevance. Research in microbiology, 168(3), 293-305.

Rijavec T., Lapanje A., Dermastia M., Rupnik M. (2007). Isolation of bacterial endophytes from germinated maize kernels. Can. J. Microbiol. 53, 802–808.

Rosa L.H., Tabanca N., Techen N., Wedge D.E., Pan Z., Bernier U.R., Becnel J.J., Agramonte N.M., Walker L.A., Moraes R.M. (2012) Diversity and biological activities of endophytic fungi associated with micropropagated medicinal plant *Echinacea purpurea* (L.) Moench. Am J Plant Sci 3:1105–1114

Rosenblueth M., López-López A., Martínez J., Rogel M.A., Toledo I., Martínez-Romero E. (2012). Seed bacterial endophytes: common genera, seedto-seed variability and their possible role in plants. Acta Hortic. 938, 39–48.

Ruiza D., Agaras B., de Werrab P., Wall L.G., Valverde C. (2011). Characterization and screening of plant probiotic traits of bacteria isolated from rice seeds cultivated in Argentina. J. Microbiol. 49, 902–912.

Ryan R.P., Germaine K., Franks A., Ryan D.J., Dowling D.N. (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1-9

Schultess B.H., Giger A., Baumann T.W. (1991) *Echinacea*: anatomy, phytochemical pattern and germination of the achene. Planta Medica 57: 384-388.

Schüßler A., Kluge M. (2001) *Geosiphon pyriforme*, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research. The Mycota IX, Hock B (ed), Springer Verlag, Berlin Heidelberg New York, pp. 151-161

Shahzad R., Waqas M., Khan A.L., Al-Hosni K., Kang S.M., Seo C.W., et al. (2017). Indoleacetic acid production and plant growth promoting potential of bacterial endophytes isolated fromrice (Oryza sativa L.) seeds. Acta Biol. Hung. 68, 175–186. doi: 10.1556/018.68.2017.2.5

Sharifi-Rad M., Mnayer D., Morais-Braga M.F.B., et al. Echinacea plants as antioxidantand antibacterial agents: From traditional medicine to biotech-nological applications. Phytotherapy Research. 2018;1–11.

Simpson, M.G. (2006) Chapter 8. Diversity and classification of flowering plants: Eudicots. In Simpson MG (Ed.) Plant systematics. Elsevier, Burlington, MA.

Sobolev V.S., Orner V.A., Arias R.S. (2013). Distribution of bacterial endophytes in peanut seeds obtained from axenic and control plant material under field conditions. Plant Soil 371, 367–376. doi: 10.1007/s11104-013-1692-2

Spjut R.W. (1994) A systematic treatment of fruit types. Memoirs of the New York Botanical Garden 70: 1-182.

Tadesse M., Crawford D.J. 2014. The phytomelanin layer in traditional members of Bidens and Coreopsis and phylogeny of the Coreopsideae (Compositae). Nordic Journal of Botany 32: 80–91

Tamura K., Peterson D., Peterson N. *et al.* MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* 2011; 28: 2731-2739

Vega F.E., Pava-Ripoll M., Posada F., Buyer J.S. (2005). Endophytic bacteria in Coffea arabica L. J. Basic Microbiol. 45, 371–380.

Verma S.K., Kingsley K., Irizarry I., Bergen M., Kharwar R.N., White J.F. (2017). Seed-vectored endophytic bacteria modulate development of rice seedlings. J. Appl. Microbiol. 122, 1680–1691.

Weller D.M. (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250-256

White J.F., Kingsley K.I., Kowalski K.P., Irizarry I., Micci A., Soares M.A., et al. (2017). Disease protection and allelopathic interactions of seed-transmitted endophytic pseudomonads of invasive reed grass (*Phragmites australis*). Plant Soil.

Wilson D. (1995) Endophyte: The evolution of a term, and clarifi cation of its use and definition. Oikos 73:274-276

6. Endophytes and phytodepuration: evaluating the influence of bacterial endophytic communities associated to *P. australis* plants on the phytodepuration process

6.1 Introduction

The term phytodepuration indicates different techniques that utilize living plants and their associated microorganisms to remove or transform hazardous contaminants from soil, sediments, water and air (He et al., 2017). Constructed wetlands (CWs) are artificial intermediate environments between terrestrial and aquatic ecosystems, where the natural properties of plants, soil, and microorganisms are applied to the treatment of wastewater. In the last decades, CWs have efficiently helped addressing the need for alternative wastewater treatment methods, demonstrating to eliminate diffuse pollutants from urban, rural, and industrial emissions (Vymazal, 2011). The cooperative growth between plants and the associated microorganisms has been pointed out as the driving force in the treatment of wastewater (Calheiros et al., 2010). The main active zone for reactions in constructed wetlands is the root zone (the rhizosphere), where physiochemical and biological processes take place (Stottmeister et al., 2003).

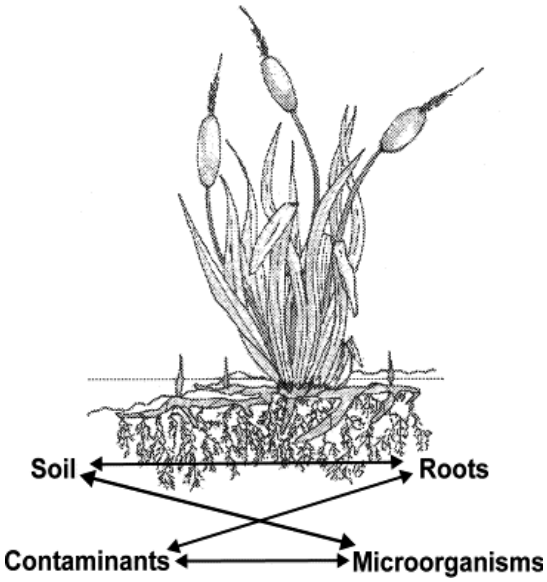

Figure 14. Possible interactions in the root zone of wetlands for wastewater treatment (Stottmeister et al., 2003)

Within this close interaction between plants and microorganisms, the latter have been observed as the main protagonists acting in pollutants removal from wastewater (Zhang et al., 2017). However, such evidence does not suggest that vegetation aspects are not important in the process. In fact, the choice of plant species is very important, since they have to survive under possible phytotoxicity and changing environmental conditions determined by the wastewater variability (Carvalho, Basto, & Almeida, 2012). Furthermore, different macrophytes influence the microbial density and composition by means of their growth rates, root morphology, production of root exudates, and oxygen transfer (Philippot, Raaijmakers, Lemanceau, & Van Der Putten, 2013). In this context, one of the most used plant is *Phragmites australis* (Common reed), a perennial grass, wide-spread in every continent (Soares et al., 2016). It is very commonly used in CWs, since it is fast-growing, cost-effective and highly productive also in environments characterized by limiting conditions, such as high salt concentrations and low nutrients.

Recently, there has been growing evidence of endophytes potential of degrading important xenobiotics (Sauvêtre, May, Harpaintner, Poschenrieder, & Schröder, 2018), such as petroleum derivatives, polycyclic aromatic hydrocarbons (PAHs), trichloroethylene (TCE), organochlorines, naphthalene, pyrene, or phenolic compounds (Siciliano et al., 2001), (Germaine et al., 2006), (Germaine, Keogh, Ryan, & Dowling, 2009), (Yousaf, Afzal, Reichenauer, Brady, & Sessitsch, 2011), (Weyens et al., 2009), (Kang, Khan, & Doty, 2012). In this regard, the effective use of an endophytic strain in phytodepuration was demonstrated by Ho and colleagues (Ho et al., 2012), who explored the effects of *Acinetobacter xylosoxidans* F3B inoculation in *Arabdopsis thaliana* plants. To this purpose, inoculated and uninoculated plants were immersed in a flask containing medium added with 0,4mM catechol and the compound's concentration in the medium was then examined by High performance liquid chromatography (HPLC). The analyses highlighted that plants inoculated with the endophytic *A. xylosoxidans* strain were able to completely remove catechol (100% of removal), while uninoculated plants registered much lower levels of removal (41% of removal). Figure 15 clearly shows the decrease of catechol's concentration registered for the combination of plants and *Acinetobacter* F3B strain.

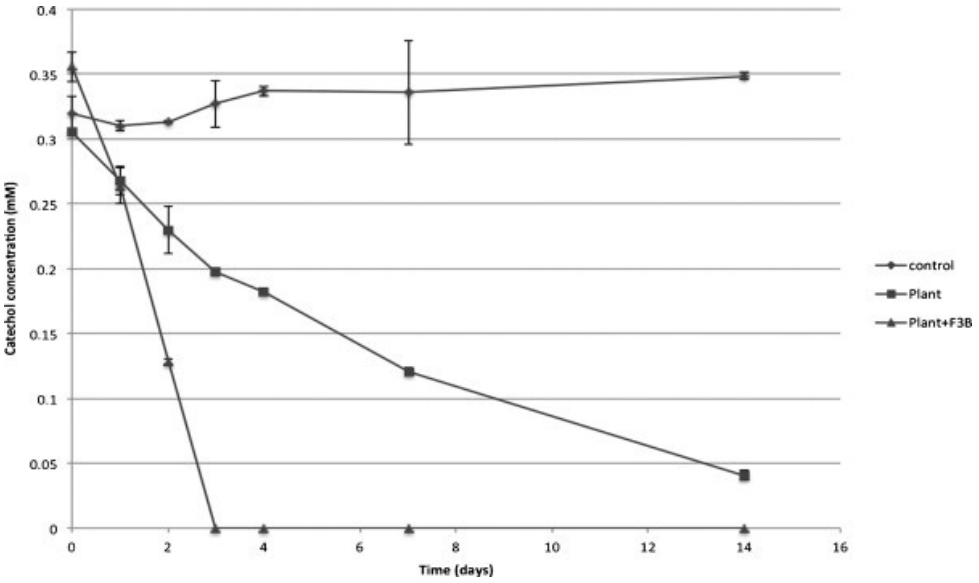

Figure 15. Effects on catechol concentrations in MS medium, determined by control medium without plants (*control*), uninoculated plants (*Plants*), and plants inoculated with *A. xylosoxidans* F3B (*Plant+F3B*). (Ho et al., 2012).

Furthermore, pollutants removal is not the only useful property of microorganisms in the phytodepuration process. The well-described plant-growth promoting activity of endophytes (Pérez-Montaño et al., 2014) can be applied to phytodepuration, in order to aid plants coping with stress determined by exposure to xenobiotics. In fact, hyperaccumulator plants often register scarce growth due to toxic effects of contaminants in wastewater (Glick, 2003). The ability of endophytic strains to support plants growth in toxic conditions was evidenced by Germaine and coworkers (Germaine et al., 2006), who observed the effect of the endophyte *Pseudomonas putida* POPHV6 on pea plants exposed to 2,4-dichlorophenoxyacetic acid. Plants inoculated with the endophytic strain registered a biomass increase from 1.5% to 16%. The endophytic inoculum showed to protect the plants' root system, which did not face callus formation and thickening. The protection the plants' root system in essential for phytodepuration since the root apparatus, and root exudates in particular, influence the accumulation and uptake of pollutants (Germaine et al., 2006).

Taken together, all these evidences suggest the remarkable potential of microorganisms in supporting the phytodepuration process. In order to better understand mechanisms of pollutants removal and pave the way to a continuous increasing efficiency of the process, it is necessary to gain deep knowledge on the microbial communities associated to vegetation in CWs. So far, very few works have investigated on the bacterial communities associated to *P. australis* in CWs. Also, to the best of our knowledge, such communities have never been analyzed before the activation of the CW.

6.2 Aim of the work

The aim of this work was to evaluate whether bacterial communities associated to *P. australis* plants and surrounding soil in Calice CW were able to influence the phytodepuration process. To this purpose, the following aspects were assayed:

i. Composition of cultivable bacterial communities associated to different compartments of *P. australis* plants (aerial part, stem, roots) and soil (rhizospheric soil and bulk soil) *before* the activation of the plant
ii. Composition of total bacterial communities associated to different compartments of *P. australis* plants (aerial part, stem, roots) and soil (rhizospheric soil and bulk soil) *before* the activation of the plant
iii. Composition of cultivable bacterial communities associated to *P. australis* plants roots *after* the activation of the plant, from different periodic samplings

iv. Composition of total bacterial communities associated to different compartments of *P. australis* plants (aerial part, stem, roots) and soil (rhizospheric soil and bulk soil) *after* the activation of the plant, from different periodic samplings

v. Growth in presence of Iron, Boron, Selenium and Sodium Chloride of root bacterial isolates from the different sampling

6.3 Materials and Methods

6.3.1 Materials

6.3.1.1 Calice Constructed Wetland Pilot Plant

Phragmites australis plants, as well as rhizospheric soil and bulk soil used in this work were obtained from Calice constructed wetland (CW) pilot plant, which is managed by G.I.D.A. S.p.A. and is located at Calice Wastewater Treatment Plant (WWTP) in Prato. Figure 16 shows the localization of the plant inside the Calice WWTP.

Figure 16. Calice WWTP in Prato. A / B indicate the vertical flow CW flow CW.

Calice CW was designed for the tertiary treatment of landfill leachate (LFL). The CW is located downstream of a membrane bio-reactor (MBR) designed to pretreat a mixture of LFLs prior to being discharged in the main line of a full-scale wastewater treatment plant (WWTP) treating urban and industrial wastewater (Calice WWTP in Prato). The experimental plant studied is a hybrid multistage.

Fine gravel was used as substrate for the macrophyte *Phragmites australis* (common reed) grown in the CW medium. CW implant was designed with two parallel lines, line A and line B, with a total surface area of 1680 m^2. Each line is a two-stage subsurface flow system, a horizontal one followed by a vertical one. Line A SFS-v is subdivided into four parallel separated tanks (SFS-v 1, SFS-v 2, SFS-v 3 e SFS-v 4) and Line B SFS-v is composed by 2 tanks (SFS-v 5 e SFS-v 6). Furthermore, both SFS-h lines are composed by 3 tanks, each one receiving the same hydraulic load. The maximum hydraulic load supplied to the entire system was 95 m^3/day that corresponds to a 1.9 day Hydraulic Retention Time for the horizontal stage.

A schematic presentation of the subsurface flow constructed wetland system is shown in Figure 1.

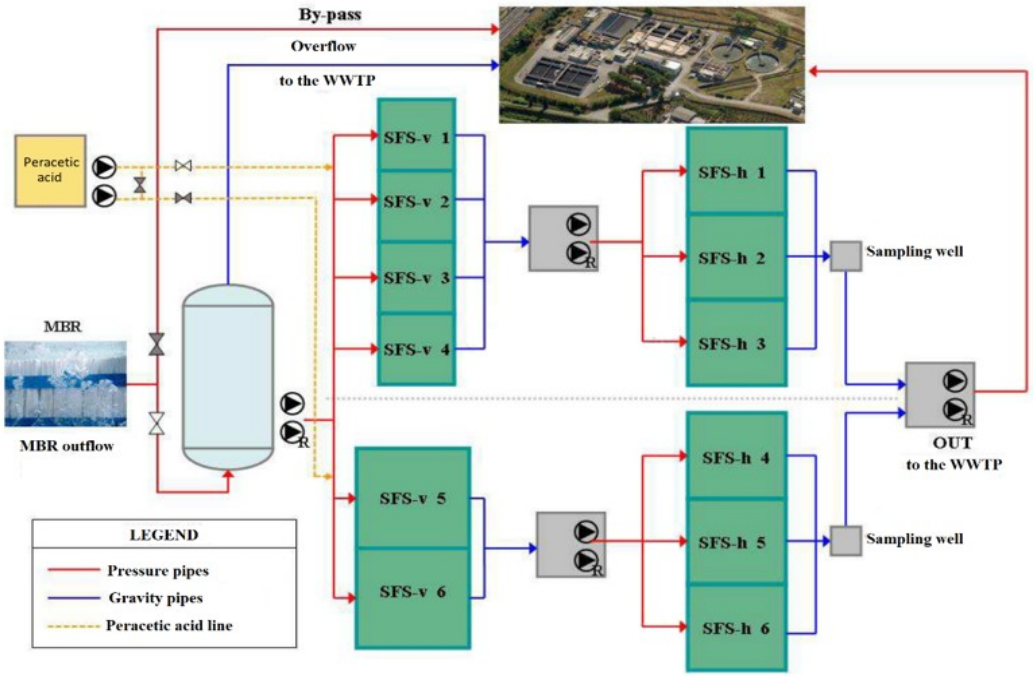

Figure 17. Schematic overview of the subsurface flow constructed wetland system.

6.3.1.2 *Phragmites australis* compartments analyzed in this work

The plants compartments analyzed in this work are listed and presented in figure 18. Stem was distinguished from the whole aerial part since, at the time of the first sampling, the portion of stem close to roots was the only green portion of aerial parts. Thus, this portion of the plant was named *green* stem, which became simply stem.

Figure 18. Plant compartments analyzed in this work.

6.3.1.3 Permeate sample sites analyzed in this work

Figure 19 illustrates the sampling sites for permeate, located upstream the vertical flow CW, between the vertical and horizontal flow CW, and downstream the horizontal flow CW.

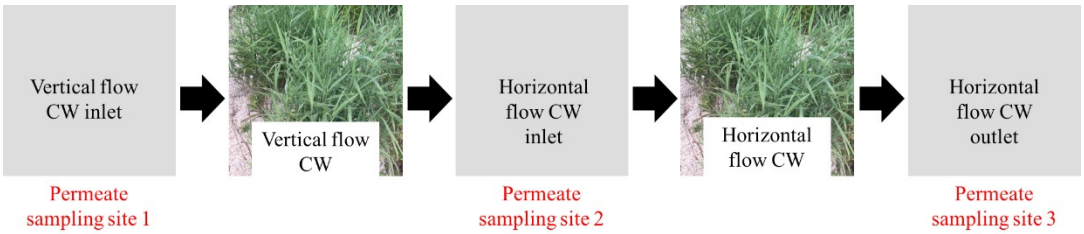

Figure 19. Representation of permeate sampling sites with respect to vertical and horizontal CWs.

6.3.1.4 Bacterial isolates used in this work

Table 7. Bacterial isolates extracted from the aerial part compartment of *P. australis* plants before the pilot plant activation. The A letter in the isolate name stands for *aerial* part.

Isolates	Genus	Accession number
A_10	*Acinetobacter*	MK156534
A_336	*Acinetobacter*	MK156537
A_11_	*Acinetobacter*	MK156538
A_12	*Acinetobacter*	MK156541
A_1	*Acinetobacter*	MK156569
A_85	*Arthrobacter*	MK156560
A_422	*Curtobacterium*	MK156520
A_408	*Curtobacterium*	MK156550
A_404	*Curtobacterium*	MK156551
A_405	*Curtobacterium*	MK156552
A_31	*Curtobacterium*	MK156553
A_81	*Curtobacterium*	MK156561
A_83	*Curtobacterium*	MK156571
A_49	*Curtobacterium*	MK156572
A_407	*Frigoribacterium*	MK156548
A_73	*Frigoribacterium*	MK156559
A_60	*Massilia*	MK156521
A_63	*Massilia*	MK156526
A_62	*Massilia*	MK156554
A_299	*Microbacterium*	MK156545
A_7	*Paenibacillus*	MK156566
A_301	*Pantoea*	MK156518
A_303	*Pantoea*	MK156523
A_306	*Pantoea*	M K156527
A_294	*Pantoea*	MK156531
A_295	*Pantoea*	MK156535
A_296	*Pantoea*	MK156539
A_298	*Pantoea*	MK156542
A_67	*Pseudomonas*	MK156515
A_39	*Pseudomonas*	MK156516
A_59	*Pseudomonas*	MK156517
A_318	*Pseudomonas*	MK156519
A_292	*Pseudomonas*	MK156522
A_319	*Pseudomonas*	MK156524
A_423	*Pseudomonas*	MK156525
A_320	*Pseudomonas*	MK156528
A_424	*Pseudomonas*	MK156529
A_65	*Pseudomonas*	MK156530
A_313	*Pseudomonas*	MK156532
A_335	*Pseudomonas*	MK156533
A_314	*Pseudomonas*	MK156536
A_315	*Pseudomonas*	MK156540

A_316	*Pseudomonas*	MK156543
A_420	*Pseudomonas*	MK156544
A_317	*Pseudomonas*	MK156546
A_421	*Pseudomonas*	MK156547
A_69	*Pseudomonas*	MK156549
A_50	*Pseudomonas*	MK156555
A_24	*Pseudomonas*	MK156562
A_22	*Pseudomonas*	MK156564
A_28	*Pseudomonas*	MK156567
A_91	*Pseudomonas*	MK156568
A_87	*Pseudomonas*	MK156570
A_32	*Rhizobium*	MK156557
A_297	*Rhizobium*	MK156558
A_79	*Rhizobium*	MK156563
A_76	*Rhizobium*	MK156565
A_25	*Staphylococcus*	MK156556

Table 8. Bacterial isolates extracted from the stem compartments of *P. australis* plants before the pilot plant activation. The S letter in the isolate name stands for *stem*.

Isolates	Genus	Accession number
S_160	*Acinetobacter*	MK179173
S_468	*Flavobacterium*	MK179188
S_470	*Flavobacterium*	MK179193
S_471	*Flavobacterium*	MK179195
S_127	*Frigoribacterium*	MK179166
S_359	*Janthinobacterium*	MK179169
S_445	*Janthinobacterium*	MK179199
S_469	*Pantoea*	MK179191
S_357	*Pseudomonas*	MK179159
S_436	*Pseudomonas*	MK179160
S_462	*Pseudomonas*	MK179161
S_358	*Pseudomonas*	MK179162
S_442	*Pseudomonas*	MK179163
S_463	*Pseudomonas*	MK179164
S_360	*Pseudomonas*	MK179165
S_368	*Pseudomonas*	MK179167
S_191	*Pseudomonas*	MK179168
S_129	*Pseudomonas*	MK179170
S_152	*Pseudomonas*	MK179171
S_118	*Pseudomonas*	MK179172
S_119	*Pseudomonas*	MK179174
S_165	*Pseudomonas*	MK179175
S_340	*Pseudomonas*	MK179176
S_120	*Pseudomonas*	MK179177
S_184	*Pseudomonas*	MK179178
S_124	*Pseudomonas*	MK179179
S_356	*Pseudomonas*	MK179180
S_434	*Pseudomonas*	MK179181
S_461	*Pseudomonas*	MK179182
S_464	*Pseudomonas*	MK179183
S_361	*Pseudomonas*	MK179184
S_457	*Pseudomonas*	MK179185
S_458	*Pseudomonas*	MK179186
S_466	*Pseudomonas*	MK179187
S_460	*Pseudomonas*	MK179189
S_448	*Pseudomonas*	MK179190
S_366	*Pseudomonas*	MK179192
S_367	*Pseudomonas*	MK179194
S_384	*Pseudomonas*	MK179196
S_369	*Pseudomonas*	MK179197
S_472	*Pseudomonas*	MK179198
S_446	*Pseudomonas*	MK179200
S_476	*Pseudomonas*	MK179201
S_121	*Pseudomonas*	MK179202

S_101	*Pseudomonas*	MK179203
S_183	*Pseudomonas*	MK179204
S_179	*Pseudomonas*	MK179205
S_185	*Pseudomonas*	MK179206
S_116	*Pseudomonas*	MK179207
S_348	*Pseudomonas*	MK179208

Table 9. Bacterial isolates extracted from the root compartment of *P. australis* plants before the pilot plant activation.

Isolates	Genus	Accession number
194	*Pantoea*	MK110895
195	*Pseudomonas*	MK110946
196	*Pantoea*	MK110896
197	*Pantoea*	MK110920
198	*Pantoea*	MK110921
200	*Pseudomonas*	MK110947
203	*Flavobacterium*	MK110948
204	*Pseudomonas*	MK110922
208	*Pseudomonas*	MK110897
209	*Pseudomonas*	MK110949
211	*Pseudomonas*	MK110898
218	*Pseudomonas*	MK110950
219	*Lelliottia*	MK110899
221	*Pantoea*	MK110959
222	*Pseudomonas*	MK110923
226	*Pseudomonas*	MK110925
228	*Pseudomonas*	MK110924
229	*Pseudomonas*	MK110960
230	*Pseudomonas*	MK110926
231	*Janthinobacterium*	MK110945
234	*Pantoea*	MK110957
238	*Pseudomonas*	MK110927
239	*Pantoea*	MK110928
250	*Bacillus*	MK110929
251	*Bacillus*	MK110930
252	*Staphylococcus*	MK110931
253	*Bacillus*	MK110932
254	*Pseudomonas*	MK110900
255	*Bacillus*	MK110901
263	*Pseudomonas*	MK110902
265	*Pseudomonas*	MK110933
266	*Pseudomonas*	MK110934
267	*Bacillus*	MK110935
268	*Bacillus*	MK110958
271	*Bacillus*	MK110936
272	*Bacillus*	MK110937
276	*Pseudomonas*	MK110938
279	*Pseudomonas*	MK110939
281	*Pseudomonas*	MK110940
286	*Pseudomonas*	MK110941
287	*Buttiauxella*	MK110942
288	*Pseudomonas*	MK110943
482	*Pseudomonas*	MK110903
483	*Pseudomonas*	MK110904

484	*Pseudomonas*	MK110905
485	*Pseudomonas*	MK110906
491	*Pseudomonas*	MK110907
493	*Pseudomonas*	MK110908
495	*Pseudomonas*	MK110909
501	*Pseudomonas*	MK110910
505	*Pseudomonas*	MK110911
506	*Pseudomonas*	MK110912
507	*Pseudomonas*	MK110951
509	*Pectobacterium*	MK110913
510	*Pseudomonas*	MK110914
511	*Pseudomonas*	MK110915
512	*Pseudomonas*	MK110952
514	*Pseudomonas*	MK110916
515	*Lelliottia*	MK110953
516	*Pseudomonas*	MK110917
517	*Pseudomonas*	MK110954
535	*Pseudomonas*	MK110918
539	*Pseudomonas*	MK110944
540	*Pseudomonas*	MK110961
566	*Pseudomonas*	MK110919
568	*Pseudomonas*	MK110955
572	*Stenotrophomonas*	MK110956

Table 10. Bacterial isolates extracted from the rhizosphere compartment of *P. australis* plants before the pilot plant activation. The RS letters in the isolate name stand for *rhizosphere.*

Isolates	Genus	Accession number
RS_577	*Acinetobacter*	MK156608
RS_590	*Acinetobacter*	MK156609
RS_697	*Acinetobacter*	MK156613
RS_695	*Acinetobacter*	MK156614
RS_598	*Acinetobacter*	MK156617
RS_597	*Acinetobacter*	MK156619
RS_693	*Arthrobacter*	MK156612
RS_702	*Bacillus*	MK156575
RS_711	*Bacillus*	MK156577
RS_700	*Bacillus*	MK156588
RS_602	*Bacillus*	MK156594
RS_701	*Bacillus*	MK156622
RS_726	*Flavobacterium*	MK156576
RS_669	*Flavobacterium*	MK156618
RS_641	*Pseudomonas*	MK156573
RS_722	*Pseudomonas*	MK156574
RS_729	*Pseudomonas*	MK156578
RS_706	*Pseudomonas*	MK156581
RS_712	*Pseudomonas*	MK156583
RS_733	*Pseudomonas*	MK156584
RS_708	*Pseudomonas*	MK156585
RS_734	*Pseudomonas*	MK156586
RS_709	*Pseudomonas*	MK156587
RS_735	*Pseudomonas*	MK156589
RS_607	*Pseudomonas*	MK156590
RS_601	*Pseudomonas*	MK156591
RS_609	*Pseudomonas*	MK156592
RS_631	*Pseudomonas*	MK156593
RS_610	*Pseudomonas*	MK156595
RS_633	*Pseudomonas*	MK156596
RS_612	*Pseudomonas*	MK156597
RS_634	*Pseudomonas*	MK156598
RS_626	*Pseudomonas*	MK156599
RS_605	*Pseudomonas*	MK156600
RS_627	*Pseudomonas*	MK156601
RS_713	*Pseudomonas*	MK156602
RS_628	*Pseudomonas*	MK156603
RS_715	*Pseudomonas*	MK156604
RS_613	*Pseudomonas*	MK156605
RS_614	*Pseudomonas*	MK156606
RS_676	*Pseudomonas*	MK156607
RS_666	*Pseudomonas*	MK156610

RS_684	*Pseudomonas*	MK156611
RS_657	*Pseudomonas*	MK156615
RS_658	*Pseudomonas*	MK156616
RS_653	*Pseudomonas*	MK156620
RS_716	*Pseudomonas*	MK156621
RS_611	*Pseudomonas*	MK156623
RS_714	*Pseudomonas*	MK156624
RS_615	*Pseudomonas*	MK156625
RS_667	*Pseudomonas*	MK156626
RS_686	*Pseudomonas*	MK156627
RS_732	*Rahnella*	MK156582

Table 11. Bacterial isolates extracted from the bulk soil before the pilot plant activation. The BS letters in the isolate name stand for *bulk soil.*

Isolates	*Genus*	Accession number
BS_782	*Arthrobacter*	MK156469
BS_783	*Arthrobacter*	MK156471
BS_775	*Arthrobacter*	MK156474
BS_786	*Arthrobacter*	MK156477
BS_787	*Arthrobacter*	MK156479
BS_790	*Arthrobacter*	MK156482
BS_796	*Arthrobacter*	MK156505
BS_780	*Arthrobacter*	MK156508
BS_785	*Bacillus*	MK156475
BS_750	*Bacillus*	MK156483
BS_764	*Duganella*	MK156499
BS_774	*Flavobacterium*	MK156472
BS_784	*Flavobacterium*	MK156473
BS_777	*Flavobacterium*	MK156476
BS_778	*Flavobacterium*	MK156478
BS_781	*Flavobacterium*	MK156480
BS_789	*Flavobacterium*	MK156481
BS_792	*Flavobacterium*	MK156488
BS_797	*Flavobacterium*	MK156511
BS_793	*Plantibacter*	MK156492
BS_779	*Pseudomonas*	MK156468
BS_773	*Pseudomonas*	MK156470
BS_767	*Pseudomonas*	MK156484
BS_752	*Pseudomonas*	MK156485
BS_760	*Pseudomonas*	MK156486
BS_769	*Pseudomonas*	MK156487
BS_753	*Pseudomonas*	MK156489
BS_761	*Pseudomonas*	MK156490
BS_770	*Pseudomonas*	MK156491
BS_754	*Pseudomonas*	MK156493
BS_762	*Pseudomonas*	MK156494
BS_771	*Pseudomonas*	MK156495
BS_755	*Pseudomonas*	MK156496
BS_763	*Pseudomonas*	MK156497
BS_756	*Pseudomonas*	MK156498
BS_748	*Pseudomonas*	MK156500
BS_757	*Pseudomonas*	MK156501
BS_765	*Pseudomonas*	MK156502
BS_766	*Pseudomonas*	MK156503
BS_758	*Pseudomonas*	MK156504
BS_819	*Pseudomonas*	MK156506
BS_814	*Pseudomonas*	MK156507
BS_788	*Pseudomonas*	MK156509
BS_818	*Pseudomonas*	MK156510

BS_802	*Pseudomonas*	MK156512
BS_800	*Pseudomonas*	MK156513
BS_768	*Pseudomonas*	MK156514

Table 12. Bacterial isolates extracted from the roots of *P. australis* plants after the pilot plant activation, at the second sampling. H stands for horizontal CW and V stands for vertical CW. R stands for root compartment.

Isolates	Genus	Accession number
V3 R3	*Achromobacter*	MK134509
V4 R15	*Acinetobacter*	MK134489
V4 R17	*Acinetobacter*	MK134488
V4 R18	*Acinetobacter*	MK134487
V4 R20	*Acinetobacter*	MK134486
H3 R12	*Agrobacterium*	MK134554
V3 R5	*Agrobacterium*	MK134508
H4 R8	*Bacillus*	MK134547
V4 R1	*Bacillus*	MK134496
H4 R3	*Comamonas*	MK138850
V3 R13	*Halomonas*	MK134502
V3 R1	*Idiomarina*	MK134511
H3 R19	*Microbacterium*	MK134551
H3 R9	*Microbacterium*	MK134555
H3 R2	*Ochrobactrum*	MK134559
H3 R3	*Ochrobactrum*	MK134558
H4 R1	*Ochrobactrum*	MK134549
V4 R21	*Ochrobactrum*	MK138851
H3 R14	*Pannonibacter*	MK134553
H4 R22	*Paracoccus*	MK134542
H3 R4	*Pseudomonas*	MK134557
H4 R13	*Pseudomonas*	MK134546
H4 R19	*Pseudomonas*	MK134544
H4 R21	*Pseudomonas*	MK134543
H4 R23	*Pseudomonas*	MK134541
H4 R24	*Pseudomonas*	MK134540
V3 R2	*Pseudomonas*	MK134510
V3 R23	*Pseudomonas*	MK134497
V4 R13	*Pseudomonas*	MK134490
V4 R2	*Pseudomonas*	MK134495
V4 R3	*Pseudomonas*	MK134494
V4 R4	*Pseudomonas*	MK134493
V3 R16	*Pseudoxanthomonas*	MK134499
V3 R9	*Pseudoxanthomonas*	MK134505
H3 R17	*Rheinheimera*	MK134552
H3 R6	*Rheinheimera*	MK134556
H4 R18	*Rheinheimera*	MK134545
H4 R7	*Rheinheimera*	MK134548
V3 R15	*Rheinheimera*	MK134500
V3 R4	*Rheinheimera*	*Under submission*
V3 R7	*Rheinheimera*	MK134507
V3 R11	*Staphylococcus*	MK134504

V3 R12	*Staphylococcus*	MK134503
H3 R24	*Thalassospira*	MK134550
V3 R14	*Thalassospira*	MK134501
V3 R19	*Thalassospira*	MK134498
V3 R8	*Thalassospira*	MK134506
V4 R5	*Pseudomonas*	MK134492
V4 R7	*Pseudomonas*	MK134491

Table 13. Bacterial isolates extracted from the roots of *P. australis* plants after the pilot plant activation, at the third sampling. H stands for horizontal CW and V stands for vertical CW. R stands for root compartment.

Isolates	Genus	Accession number
H6 R17	*Achromobacter*	MK134518
V6 R16	*Achromobacter*	MK130938
V6 R5	*Achromobacter*	MK130934
V6 R6	*Achromobacter*	MK130935
H6 R10	*Agrobacterium*	MK134524
H5 R1	*Bacillus*	MK134539
H5 R2	*Bacillus*	MK134538
H6 R20	*Bacillus*	MK134515
H6 R21	*Bacillus*	MK138852
H6 R8	*Bacillus*	MK134526
V6 R1	*Bacillus*	MK130907
V6 R2	*Bacillus*	MK130906
V6 R8	*Bacillus*	MK130937
H5 R6	*Enterobacter*	MK134534
H5 R7	*Enterobacter*	MK134533
V5 R10	*Halomonas*	MK138853
V5 R13	*Halomonas*	MK130915
V5 R15	*Halomonas*	MK130913
V5 R20	*Halomonas*	MK130910
V5 R5	*Halomonas*	MK130921
V5 R9	*Halomonas*	MK130917
H6 R22	*Isoptericola*	MK134514
V5 R14	*Microbacterium*	MK130914
H6 R19	*Pannonibacter*	MK134516
V5 R18	*Pannonibacter*	MK130911
H5 R8	*Pseudomonas*	MK134532
H6 R1	*Pseudomonas*	MK134531
H6 R2	*Pseudomonas*	MK134530
H6 R4	*Pseudomonas*	MK134528
V5 R1	*Pseudomonas*	MK134485
V5 R11	*Pseudomonas*	MK138854
V5 R17	*Pseudomonas*	MK130912
V5 R22	*Pseudomonas*	MK130908
V5 R6	*Pseudomonas*	MK130920
V6 R3	*Pseudomonas*	MK130932
V6 R4	*Pseudomonas*	MK130933
V6 R7	*Pseudomonas*	MK130936
H6 R23	*Pseudoxanthomonas*	MK134513
V5 R4	*Rheinheimera*	MK130922
V5 R7	*Rheinheimera*	MK130919
H6 R14	*Rhizobium*	MK134521
H6 R24	*Rhizobium*	MK134512

H5 R3	*Stenotrophomonas*	MK134537
H5 R4	*Stenotrophomonas*	MK134536
H5 R5	*Stenotrophomonas*	MK134535
H6 R12	*Stenotrophomonas*	MK134522
H6 R16	*Stenotrophomonas*	MK134519
H6 R18	*Stenotrophomonas*	MK134517
H6 R3	*Stenotrophomonas*	MK134529
H6 R9	*Stenotrophomonas*	MK134525
H6 R11	*Thalassospira*	MK134523
H6 R15	*Thalassospira*	MK134520
H6 R7	*Thalassospira*	MK134527
V5 R12	*Thalassospira*	MK130916
V5 R2	*Thalassospira*	MK130931
V5 R21	*Thalassospira*	MK130909
V5 R3	*Thalassospira*	MK130923
V5 R8	*Thalassospira*	MK130918

Table 14. Bacterial isolates extracted from the roots of *P. australis* plants after the pilot plant activation, at the fourth sampling. H stands for horizontal CW and V stands for vertical CW. R stands for root compartment.

Isolates	Genus	Accession number
V8 R2	*Achromobacter*	MK130945
V8 R21	*Achromobacter*	MK130957
V8 R23	*Achromobacter*	MK130901
V8 R3	*Achromobacter*	MK130905
H8 R5	*Aeromonas*	MK133358
H7 R23	*Agrobacterium*	MK138868
H7 R3	*Agrobacterium*	MK138869
H7 R5	*Agrobacterium*	MK138870
H7 R7	*Agrobacterium*	MK138872
H7 R9	*Agrobacterium*	MK138874
H8 R1	*Agrobacterium*	MK138875
H8 R16	*Agrobacterium*	MK130924
H8 R18	*Agrobacterium*	MK130928
H8 R3	*Agrobacterium*	MK138881
H7 R16	*Arthrobacter*	MK138862
V8R7	*Devosia*	MK130903
H7 R22	*Flavobacterium*	MK138867
H8 R12	*Flavobacterium*	MK138878
H7 R15	*Lysobacter*	MK138861
V7 R8	*Microbacterium*	MK130939
H7 R19	*Micrococcus*	MK138863
V8 R12	*Pannonibacter*	MK130949
V8 R16	*Pannonibacter*	MK130953
V8 R4	*Pannonibacter*	MK130904
H8 R10	*Pseudomonas*	MK138876
H8 R13	*Pseudomonas*	MK138879
H8 R14	*Pseudomonas*	MK130926
H8 R17	*Pseudomonas*	MK130927
H8 R19	*Pseudomonas*	MK130929
H8 R2	*Pseudomonas*	MK138880
H8 R20	*Pseudomonas*	MK130930
H8 R4	*Pseudomonas*	MK138882
H8 R6	*Pseudomonas*	MK138883
H8 R7	*Pseudomonas*	MK138884
H8 R8	*Pseudomonas*	MK138885
H8 R9	*Pseudomonas*	MK138886
V7 R13	*Pseudomonas*	MK138887
V7 R19	*Pseudomonas*	MK130940
V7 R21	*Pseudomonas*	MK130941
V7 R23	*Pseudomonas*	MK130943
V7 R9	*Pseudomonas*	MK138889
V8 R1	*Pseudomonas*	MK130944

V8 R22	*Pseudomonas*	MK130902
V8 R24	*Pseudomonas*	MK130900
V8 R8	*Pseudomonas*	MK130946
H8 R11	*Rheinheimera*	MK138877
H7 R1	*Rhizobium*	MK138855
H7 R10	*Rhizobium*	MK138856
H7 R11	*Rhizobium*	MK138857
H7 R12	*Rhizobium*	MK138858
H7 R13	*Rhizobium*	MK138859
H7 R14	*Rhizobium*	MK138860
H7 R2	*Rhizobium*	MK138864
H7 R20	*Rhizobium*	MK138865
H7 R21	*Rhizobium*	MK138866
H7 R6	*Rhizobium*	MK138871
H7 R8	*Rhizobium*	MK138873
H8 R15	*Rhizobium*	MK130925
V7 R22	*Rhizobium*	MK130942
V8 R11	*Shinella*	MK130948
V8 R13	*Shinella*	MK130950
V8 R18	*Sphingobium*	MK130955
V7 R24	*Stenotrophomonas*	MK138888
V8 R14	*Thalassospira*	MK130951
V8 R15	*Thalassospira*	MK130952
V8 R17	*Thalassospira*	MK130954
V8 R19	*Thalassospira*	MK130956
V8 R9	*Thalassospira*	MK130947

6.3.2 Methods

6.3.2.1 Sampling, extraction and isolation of bacterial strains from *P. australis* compartments and soils samples

Plants collected at Calice CW were immediately taken to the laboratory. The anatomical parts of the plants, i.e. aerial parts, stem, roots were separated and considered as independent samples throughout the experiment.

Aerial parts from three different *P. australis* plants were grouped and pooled. The same was done for stem and roots. This procedure was followed for the samples from both horizontal and vertical CW. Then, one gram of fresh tissue from each pool was surface-sterilized with 1% HClO solution at room temperature and washed three times with sterile water to eliminate HClO residues. The surface sterilizations remove epiphytic bacteria. Aliquots of 100 µl of the last water wash were plated in triplicate as sterility controls, which by the end of the experiment had not become contaminated. Subsequently, the samples were homogenously pottered in a sterile mortar with the addition of 2 ml of 0.9% NaCl solution. One hundred µl samples of tissues extracts and their different dilutions were plated in triplicate.

Following an analogous procedure, one gram of rhizospheric soil from three different *P. australis* plants and one gram of bulk soil were as well analyzed and treated separetely at room temperature for 1h with 20 ml of 10 mM Mg_2SO_4 in 50-ml sterile Falcon tubes, in order to obtain sedimentation of soil particles. After sedimentation, 100 µl samples of the supernatant and different dilutions were plated in triplicate.

Endophytic and soil-associated bacteria were grown on solid trytone soya broth (TSB) medium (Biorad, CA, USA) at 30°C for 48h. The total number of aerobic heterotrophic fast-growing bacteria was expressed as colony-forming-units (CFU), which were determined for each sample based on an average number obtained for the replicates. From each compartment of the plant and soil samples, about 50 colonies were selected and individually plated onto solid TSB Petri dishes, in order to obtain the single isolates.

6.3.2.2 PCR amplification and sequencing of 16S rRNA coding genes

PCR amplification of 16S rRNA genes was carried out in 20-µl reactions using DreamTaq DNA Polymerase reagents (Thermofisher Scientific) at the concentrations suggested by the company, and 0.5 µM of primers P0 (5′-GAGAGTTTGATCCTGGCTCAG) and P6 (5′-CTACGGCTACCTTGTTACGA) (Di Cello and Fani, 1996); 1 µl of cell lysate was used as template. Amplification conditions were the following: 90-s denaturation at 95°C, 30

cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. Direct sequencing of the amplified 16S rRNA genes was performed with primer P0 by an external company (IGA Technology Services-Udine-Italy). Each 16S rRNA gene sequence was submitted to GenBank and assigned an accession number from MH670937 to MH670951.Taxonomic affiliation of the 16S rRNA gene sequences were attributed using the "classifier" tool of the Ribosomal Database Project –RDP (Cole et al. 2014).

6.3.2.3 Extraction of genomic DNA

Total DNA extraction was performed from each sample by using PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc., Carlsbad, California, USA) following the manufacturer's instruction. Concentration and purity of extracted DNA were checked by 0.8% agarose gel electrophoresis. Extracted DNAs were delivered to an external company (IGA Technology Services, Udine, Italy) for library construction and sequencing on MiSeq Illumina platform.

6.3.2.4 Growth in presence of Synthetic Wastewater (SWW)

The growth of *P. australis* root isolates in presence of Boron, Iron, Selenium and Sodium Chloride was assayed through the broth microdilution methods using TSB medium.

The bacterial inoculum for the experiment was prepared by dissolving in liquid TSB the isolated bacterial colony after 24h growth at 30°C in solid medium; the inoculum was incubated overnight at 30°C under shaking. After incubation optical densities of cultures at 600 nm wavelength were measured and adjusted to 0.1.

The experiment was performed in 96-well plates. Each well contained 10 µl of bacterial inoculum, 80µl of TSB medium and 10 µl of SWW 1X or SWW 2X or SWW 3X. The final tested concentrations of Boron, Iron, Selenium and Sodium Chloride in SWW are shown in table 15.

Table 15. Concentrations (mg/l) of Boron, Iron, Selenium and Sodium Chloride used in SWW.

Element/Compound	SWW 1X	SWW 2X	SWW 3X
Boron	20	40	60
Iron	15	30	45
Selenium	0.03	0.06	0.09
Sodium Chloride	5000	10000	15000

The growth of bacterial isolates in presence of only TSB medium was also tested. Sterility of the medium and SWW used was verified by adding only TSB, SWW 1X, SWW 2X, SWW 3X separately in each well in triplicate.

The growth of each isolate in each of the conditions was performed in duplicate.

Results were measured by using TECAN microplate reader (Tecan, Durham, USA) at 600 nm wavelength, after 24h incubation at 30°C. For each growing condition, an average value of optical density was obtained from the two performed measures.

6.4 Results

6.4.1 Experimental Strategy

The experimental strategy used in this work is schematically represented in the flow-chart in Figure 20. The analyses performed included:

i. Separation of plants' compartments (aerial part, stem, roots)
ii. Extraction of endophytic bacteria from plants' compartments and associated bacteria to rhizospheric and bulk soils
iii. Determination of bacterial counts
iv. Amplification and sequencing of 16S rRNA gene for all plants' compartments isolates from the first sampling and for root isolates from second to fourth sampling
v. Growth of root isolates from all samplings in presence of Synthetic Wastewater (SWW)
vi. Extraction of total DNA from all plants' compartments for all samplings

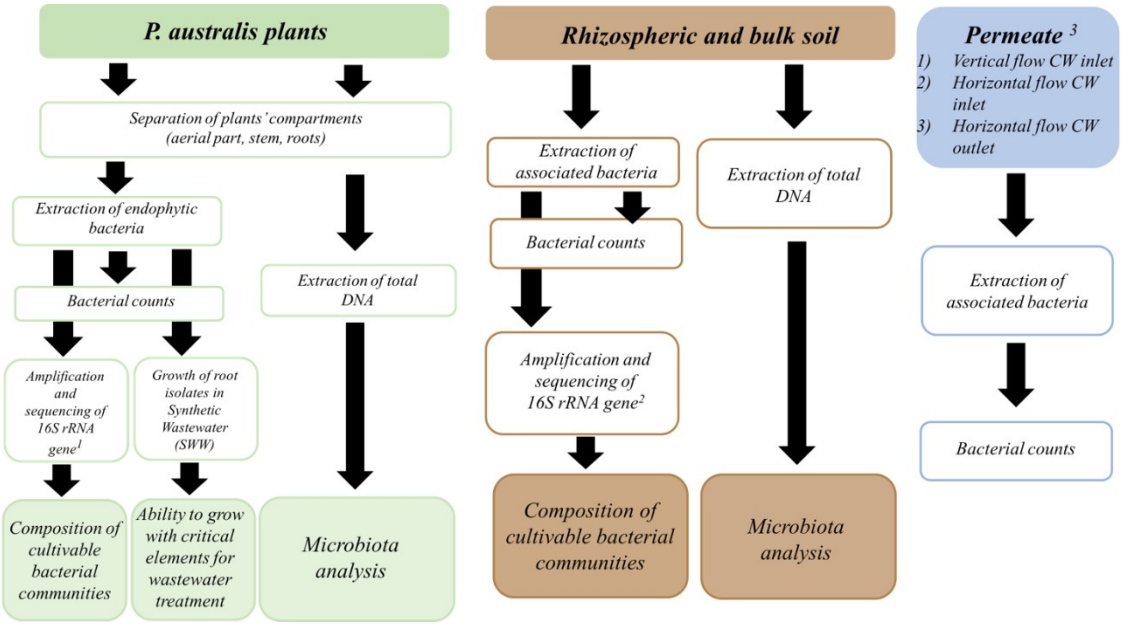

Figure 20. Experimental strategy of the work.
[1] Performed for isolates from all plants' compartments for the first sampling, and for root isolates from second to fourth samplings.
[2] Performed for isolates from the first sampling.
[3] Performed for the samplings after the plant's activation.

6.4.2 Samplings at Calice Constructed Wetland

Periodic samplings were performed at Calice CW in order to evaluate the composition of bacterial communities associated to *P. australis* plants and soil, and to evidence differences

in such communities along different periods of time. Four samplings were performed so far at Calice CW, as shown in Figure 21. The next sampling is scheduled for December 2018.

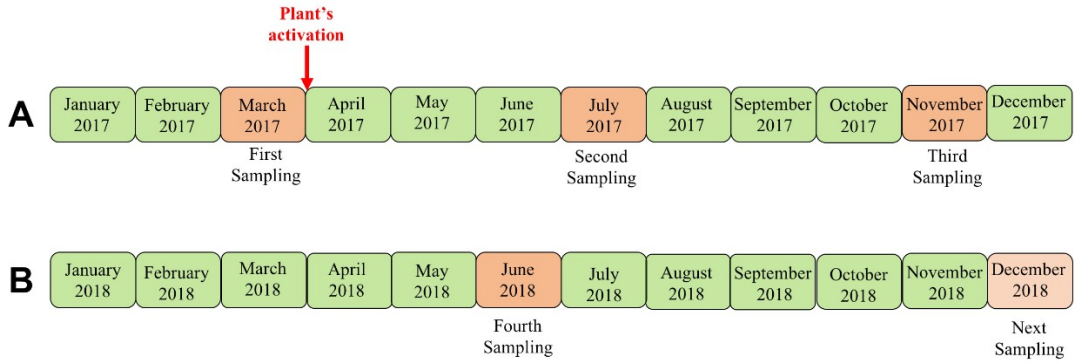

Figure 21. Temporal representation of the samplings performed at Calice CW. Line A represents samplings performed in 2017, and line B represents samplings performed in 2018.

6.4.3 Bacterial counts

In order to characterize bacterial communities associated to *P. australis* plants and soil at Calice pilot plant, cultivable bacteria were extracted from the different compartments of the plants and soil samples, then they were diluted in saline solution and plated in TSA medium, as described in Materials and methods. Bacterial counts were obtained as CFU (Colony Forming Unit) per gram of plant tissue or soil. Table 16 shows values for bacterial counts for each plant compartment and soil for each of the performed samplings.

Table 16. Bacterial counts expressed as CFU/g obtained in each sampling.

		Samplings			
	Plant compartment	**I (March 2017)**	**II (July 2017)**	**III (November 2017)**	**IV (June 2018)**
Vertical Flow CW	Aerial part	$4x10^4$	$1x10^6$	$4x10^4$	$5x10^3$
	Stem	$8x10^5$	$3x10^6$	$1x10^5$	$4.5x10^4$
	Roots	$4x10^6$	$1x10^7$	$1x10^6$	$3x10^6$
	Rhizosphere	$1x10^5$	$5x10^6$	$6x10^5$	$1x10^6$
	Bulk soil	$5x10^3$	$1x10^4$	$2x10^5$	$2x10^4$
Horizontal Flow CW	Aerial part	$6x10^4$	$9.4x10^4$	$9x10^4$	$2x10^3$
	Stem	$5x10^6$	$3x10^6$	$5x10^6$	$3.5x10^4$
	Roots	$5x10^6$	$1.5x10^7$	$5.5x10^6$	$2.5x10^7$
	Rhizosphere	$1.7x10^5$	$5x10^6$	$1x10^6$	$5.5x10^6$
	Bulk soil	$2x10^4$	$7x10^3$	$5x10^4$	$1x10^4$

Concerning the first sampling performed at Calice pilot plant, before the plant's activation, the lowest bacterial counts were registered for the bulk soil ($5x10^3$ in vertical flow CW and

$2x10^4$ in horizontal flow CW), followed by the aerial part of the plant ($4x10^4$ and $6x10^4$ for vertical and horizontal flow CW respectively). On the other hand, stem ($8x10^5$ in vertical flow CW, and $5x10^6$ in horizontal flow CW) and roots ($4x10^6$ in vertical flow CW, and $5x10^6$ in horizontal flow CW) compartments registered the highest bacterial counts. At the time of the second sampling, the compartment with lowest bacterial counts was the bulk soil, both in the vertical ($1x10^4$) and the in horizontal flow CW ($7x10^3$). The highest bacterial counts in the second sampling were registered for the roots compartment ($1x10^7$ in vertical flow CW and $1.5x10^7$ in horizontal flow CW). In the third sampling, the compartment with the lowest bacterial counts in the vertical flow CW was the aerial part ($4x10^4$), while in the horizontal flow CW the bulk soil ($5x10^4$) showed the lowest bacterial titers. Also, in the third sampling, the root compartment ($1x10^6$ in vertical flow CW and $5.5x10^6$ in horizontal flow CW) showed the highest bacterial counts. Finally, at the time of the fourth sampling, the lowest bacterial counts were presented by the aerial part of the plants, both in the vertical ($5x10^3$) and in the horizontal flow CW ($2x10^3$). The roots compartments showed, also in the fourth sampling, the highest bacterial counts ($3x10^6$ in vertical flow CW and $2.5x10^7$ in horizontal flow CW). Overall, the bulk soil and aerial part were the compartments with the lowest bacterial counts over the four samplings, while the roots compartments showed the highest bacterial titers over the four samplings. The lowest bacterial counts registered for the bulk soil might be due to its composition, which may not contribute to bacterial adherence and storage of nutrients, as it might be hypothesized for rhizospheric soil. On the other hand, the roots compartment could be expected to be highly populated by bacteria, since it might be rich of nutrients obtained from soil and may constitute the first plant compartment colonized by rhizobacteria, which switch to an endophytic lifestyle.

Cultivable bacteria were also extracted from permeate samples from the second, third and fourth samplings at different sampling sites. Permeate was only sampled from the second to the fourth sampling since it was only present in the plant after its activaction. Table 17 shows bacterial counts computed for permeate samples expressed in CFU per ml of sample.{Formatting Citation}

Table 17. Bacterial counts expressed as CFU/ml obtained in each sampling for permeate samples in the different sampling sites.

	Samplings		
	II (July 2017)	**III (November 2017)**	**IV (June 2018)**
Inward Vertical Flow CW	$2.74x10^5$	$1.32x10^5$	$2.1x10^6$
Between Vertical and	$4x10^3$	$5.15x10^4$	$3.5x10^4$

Horizontal Flow CW			
Outward Horizontal Flow CW	$8x10^4$	$2.3x10^5$	$4.5x10^5$

For the second sampling, the permeate between vertical and horizontal flow CW ($4x10^3$) registered the lowest bacterial counts, while permeate entering the vertical flow CW ($2.74x10^5$) showed the highest bacterial counts. Concerning the third sampling, similar bacterial counts were registered for permeate entering the vertical flow CW ($1.32x10^5$) and for permeate leaving the horizontal flow CW ($2.3x10^5$). At the time of the fourth sampling, the highest bacterial counts were present in the permeate entering the vertical flow CW ($2.1x10^6$) and the lowest values of bacterial counts were encountered in permeate leaving the horizontal flow CW ($4.5x10^5$). Considering each permeate sample, over the different samplings, a growing trend in bacterial titers can be evidenced for inward vertical flow permeate ($2.74x10^5 \rightarrow 1.32x10^5 \rightarrow 2.1x10^6$) and for permeate leaving horizontal flow CW ($8x10^4 \rightarrow 2.3x10^5 \rightarrow 4.5x10^5$). Permeate between vertical and horizontal flow CWs registers higher bacterial counts from second to third sampling, and similar values from third to fourth sampling. Bacterial loads in permeate samples represent a very variable parameter, and for this reason it might be a difficult factor to predict or rationalize. Bacterial colonization and growth within permeate may be related to the wastewater composition, to the frequency of rainings, and other factors, such as seasonal ones. For example, the composition of wastewater may favor or not bacterial growth, since it may be rich of nutrients or may present toxic compounds. Then, the composition of wastewater is also subjected to the seasonal and climatic changes, as for example the frequency of rainings, and range of temperatures.

6.4.4 Bacterial communities associated to *P. australis* plants and soil from Calice CW pilot plant *before* its activation

The analysis of the composition of bacterial communities associated to vegetation in a phytodepuration plant is essential to better understand the phytodepuration process itself, to gain insight into the level of bacterial role in such process, and to (possibly) ameliorate the efficiency of the process, thus enhancing the quality of the final treated wastewater. The analysis of the composition of such communities before the activation of the pilot plant offers a picture of the bacterial communities before the presence of wastewater in the plant, allowing to evidence changes in such communities possibly resulting from the phytodepuration process and the presence of contaminants. For these reasons, we decided

to analyze both the total and the cultivable bacterial communities associated to different compartments of *P. australis* plants and to the soil samples (bulk soil and rhizospheric soil) before the activation of Calice CW pilot plant. These data offer a complete picture of the bacterial communities assemblage prior to the CW activation. Here we present data obtained for cultivable bacterial communities associated to the different compartments of *P. australis* and soil samples. Data emerging from microbiota analysis will be compared all together after the last sampling, which will be performed in December 2018. In order to analyze the composition of cultivable bacterial communities from the different plants' compartments and soil samples, cell lysates were obtained from the bacterial isolates shown in Tables 7 to 11. Afterwards, the 16S rRNA gene was amplified from each bacterial isolate, and the obtained amplicons were sequenced. The nucleotide sequences were compared to those available in databases. Microbiota analysis was performed by extracting total DNA from each of the plants compartments and soil samples, as described in Materials and methods.

Figure 22 shows the percentages of the different bacterial genera observed in the different analyzed compartments of *P. australis* and soils before the plant's activation.

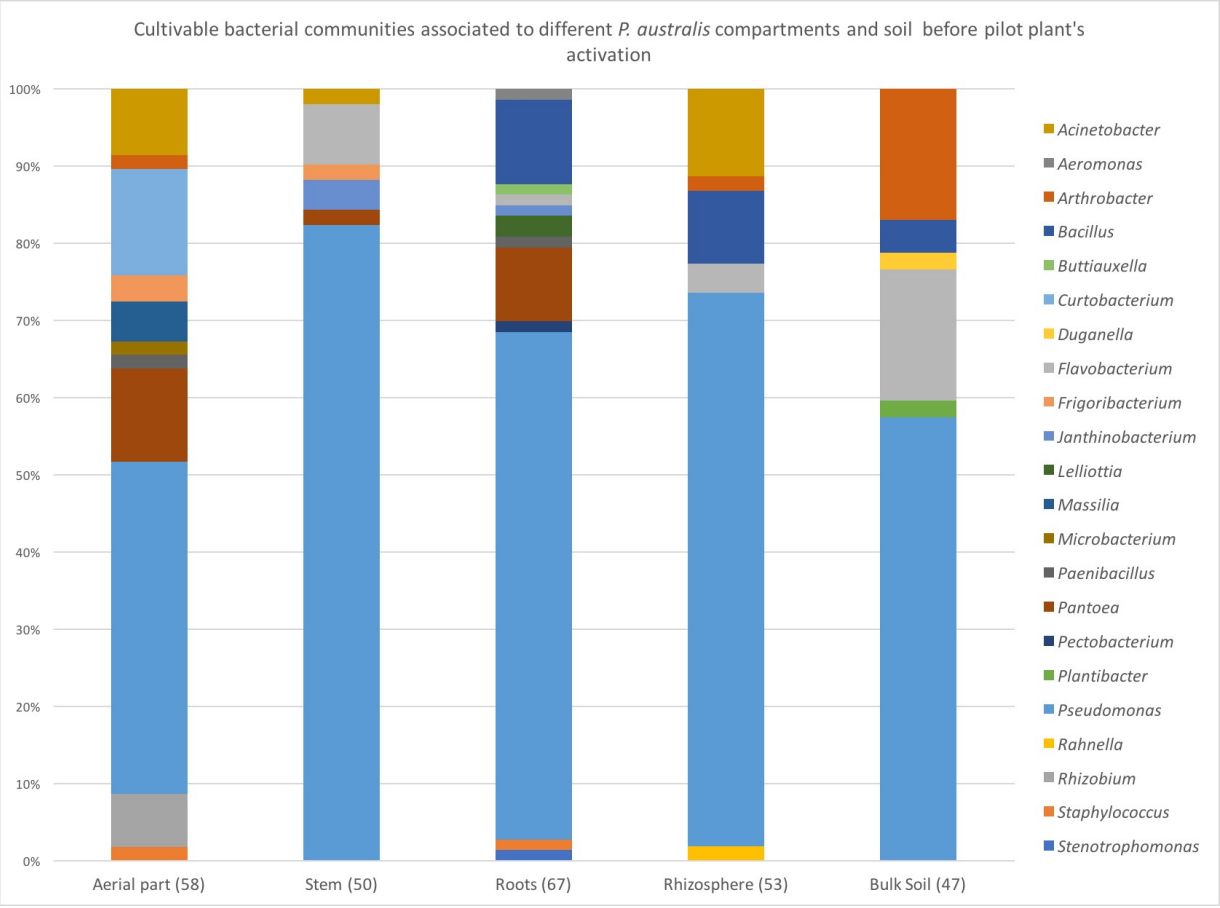

Figure 22. Composition of bacterial communities from different plants compartments and soil from the first sampling performed at Calice pilot plant, before its activation. The number of analyzed isolates is indicated for each compartment into brackets.

Cultivable bacterial isolates obtained for the first sampling at Calice pilot plant were affiliated to 22 different bacterial genera. In each of the analyzed plant compartments the predominant bacterial genus was *Pseudomonas*, which registered 43.1% for the aerial part, 82.35% for the stem, 66.21% for roots, and 71.69% for rhizosphere and 55.1% for bulk soil. Within the aerial part of the plants, 11 different bacterial genera were highlighted. The second most represented genus was *Curtobacterium* with a percentage of 13.79%, followed by *Pantoea* with 12.06%. *Acinetobacter* and *Rhizobium* genera were also well-represented within aerial part, showing respectively percentages of 8.62% and 6.89%. The genus *Massilia* accounted for 5.17% of isolates within aerial part. *Frigoribacterium* was represented by 3.44% of the isolates, while other four genera (*Arthrobacter*, *Microbacterium*, *Paenibacillus*, *Staphylococcus*) shared the same percentage among isolates (1.72%). The stem compartment indicates the portion of stem very next to the roots, as described in Materials and methods, and was distinguished from the rest of the aerial part since, at the moment of the first sampling, it was the only green part of the aerial part of the plant. In this green portion of stem, six different bacterial genera were highlighted among cultivable communities, and the second most represented genus was *Flavobacterium* (7.84%), followed by *Janthinobacterium* (3.92%). Other three genera (*Acinetobacter*, *Frigoribacterium*, *Pantoea*) registered the same percentages each, 1.96%. Bacterial isolates from roots were affiliated with 12 different genera. After *Pseudomonas*, the most represented genus was *Bacillus* (10.81%), followed by *Pantoea* (9.45%) and *Lelliottia* (2.7%). Other 8 genera (*Aeromonas, Buttiauxella*, *Flavobacterium*, *Janthinobacterium*, *Paenibacillus*, *Pectobacterium*, *Staphylococcus*, *Stenotrophomonas*) shared the same percentage of 1.35% each. The compartment of the rhizosphere harbored 6 different genera, showing the predominance of *Acinetobacter* (11.32%) and *Bacillus* (9.43%) genera, after *Pseudomonas*. *Flavobacterium* showed a percentage of 3.77% and two genera, *Arthrobacter* and *Rahnella,* shared the same percentage of 1.88%. The bulk soil which was represented by the fine gravel filling the plant's tanks, as described in Materials and methods, showed a cultivable bacterial community represented by 6 different bacterial genera. Following *Pseudomonas*, the most represented genera were *Arthrobacter* and *Flavobacterium* with percentages of 16.32% each. *Bacillus* genus was represented by

4.08% of isolates, while 2.04% of them were affiliated to *Duganella* genus and another 2.04% with *Plantibacter* genus.

6.4.5 Bacterial communities associated to *P. australis* plants roots from Calice pilot plant

The composition of total bacterial communities and root cultivable bacterial communities associated to *P. australis* plants and soil samples were evaluated after the activation of the CW, and performed for three times, in different periods of the year, in occasion of the second, third and fourth samplings, respectively on July 2017, November 2017 and June 2018, as described in Materials and methods. A further sampling, scheduled for December 2018, will complete the whole body of samplings. As previously highlighted, microbiota data will be altogether analyzed after the final sampling.

After the plant's activation, analysis of cultivable bacterial communities was focused on the root compartment since it emerges as a key compartment in the phytodepuration processes and could lead to the isolation of bacteria with outstanding potential in biotechnological applications.

Total and cultivable bacterial communities were assayed as described in Materials and methods, and the amplification and sequencing of gene 16S rRNA was performed for isolates listed in Tables 9, and 12 to 14. We here present the results for cultivable bacterial communities composition associated to roots of *P. australis* plants.

As previously observed, *Pseudomonas* genus was the most abundant genus in root cultivable bacterial communities from *P. australis* plants at the first sampling. Figure 23 shows the composition of cultivable bacterial communities from roots of *P. australis* plants observed at the different samplings performed at Calice CW pilot plant.

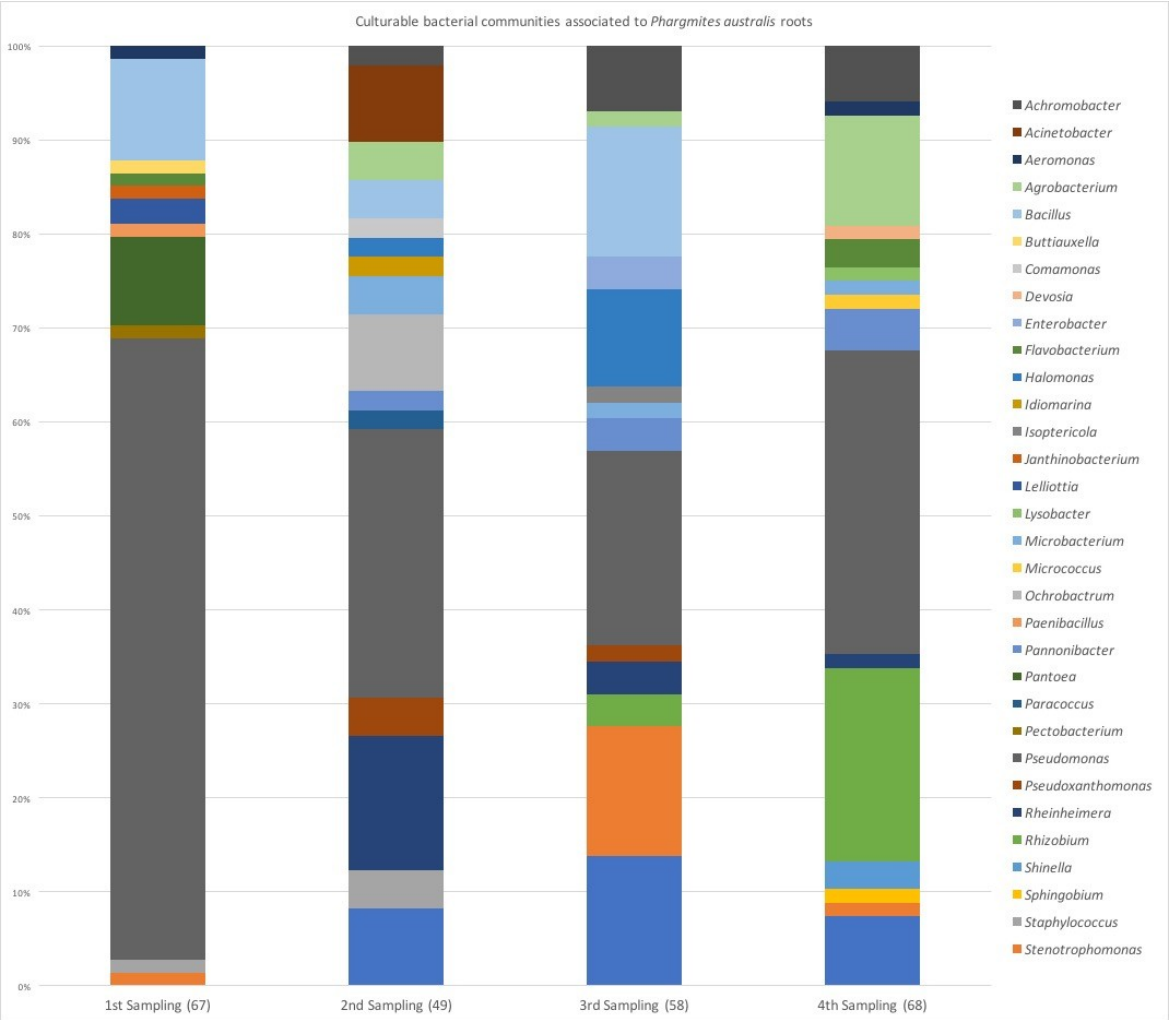

Figure 23. Composition of cultivable bacterial endophytic communities from roots of *P. australis* plants observed at the different samplings performed at Calice CW pilot plant. The number of analyzed isolates is indicated into brackets for each sampling.

Interestingly, *Pseudomonas* genus was also the most predominant in root cultivable bacterial communities of all the performed samplings, and registered percentages of 28.57%, 20.68%, 32.35%, respectively for the second, third and fourth sampling. For the second sampling, bacterial root community was represented by 16 different genera, while for the third and fourth samplings, 14 and 16 bacterial genera were registered respectively. Regarding the second sampling, the root bacterial community was represented by *Rheinheimera* genus for 14.28% of the isolates. Other 3 genera (*Acinetobacter*, *Ochrobactrum*, *Thalassospira*) shared the same percentage of 8.16% each. Each of the following genera, *Agrobacterium*, *Bacillus*, *Microbacterium*, *Pseudoxanthomonas* and *Staphylococcus*, were each represented by 4.08% of the isolates. Six bacterial genera

represented each 2.04% of the community, namely *Achromobacter*, *Comamonas*, *Halomonas*, *Idiomarina*, *Pannonibacter* and *Paracoccus*.

The bacterial communities from the third sampling were mostly represented by *Bacillus*, *Stenotrophomonas*, and *Thalassospira* genera, after *Pseudomonas*. Each of those bacterial genera accounted for accounted for 13.79% of root bacterial community. Another well-represented genus was *Halomonas* which was affiliated to the 10.34% of the isolates. *Achromobacter* genus showed a percentage of 6.89% among the bacterial community. Four genera were each represented by 3.44% of the isolates, and these genera were *Enterobacter*, *Pannonibacter*, *Rheinheimera* and *Rhizobium*. The other 4 bacterial genera present in roots in third sampling were *Agrobacterium*, *Isoptericola*, *Microbacterium* and *Pseudoxanthomonas*, which showed 1.72% each.

Overall, results show that *Pseudomonas* genus was the predominant genus in the four samplings and that the communities from first and third samplings showed also abundance of the genus *Bacillus*.

6.4.6 *P. australis* root cultivable bacterial endophytes growth in presence of Boron, Iron, Selenium and Sodium Chloride

The isolates obtained from the roots of *P. australis* plants in the four performed samplings at Calice pilot plant CW were grown in presence of Boron, Iron, Selenium, or Sodium Chloride. Such elements were chosen since they represent critical elements for the wastewater treatment, and it would be desiderable to select bacteria capable of reducing the concentration of such molecules in the final treated wastewater. Moreover, such analyses were performed on the root isolates since there is evidence that the important events implied in the phytodepuration process may take place in such compartment of the plant (Stottmeister et al., 2003), thus such isolates could be the main interesting with regard to their potential of metabolizing certain molecules. As described in Materials and methods, the solution containing B, Fe, Se and NaCl, which was added to the bacterial growth medium (TSB), was called *Synthetic Wastewater* (SWW). Thus, bacterial isolates were assayed for their growth in TSB medium added with SWW, in which B, Fe, Se and NaCl concentrations were the maximum concentrations allowed for sewer emission, and this was called SWW 1X. Also, bacterial isolates were tested for their growth in SWW 2X and SWW 3X, in which TSB medium was added with, respectively, two-fold and three-fold higher concentrations of B, Fe, Se and NaCl with respect to SWW 1X. The growth of bacterial isolates in the different SWWs was evaluated by registering the culture optical density at 600 nm (OD_{600}) after incubation at 30°C for 24h, as described in Materials and

methods. The OD_{600} was also evaluated for bacterial isolates growing in the culture medium (TSB), which represented the positive control (C+). Optical densities were associated to different colors (blue, yellow, red) indicating growing values of optical densities, as shown in Figure 24. Thus, lower levels of growth were associated to blue colors, while higher levels of growth were associated to orange-red colors.

Figure 24. Color code for the optical densities represented in the following Tables.

Table 18 shows the registered optical densities for each root bacterial isolate from the first sampling at each growing condition (C+, SWW 1X, SWW 2X, SWW 3X).

Table 18. Growth in presence of SWW for root isolates from the **first sampling**.

Isolate	Plant	Compartment	Genus	Accession number	C+	SWW 1X	SWW 2X	SWW 3X
250	V	Roots	*Bacillus*	MK110929	0.94	0.87	0.77	0.81
251	V	Roots	*Bacillus*	MK110930	0.83	0.69	0.51	0.49
253	V	Roots	*Bacillus*	MK110932	1.00	0.94	0.93	0.92
255	V	Roots	*Bacillus*	MK110901	0.91	0.84	0.76	0.78
267	V	Roots	*Bacillus*	MK110935	0.77	0.6	0.46	0.44
268	V	Roots	*Bacillus*	MK110958	0.93	0.82	0.7	0.66
271	V	Roots	*Bacillus*	MK110936	1.49	1.375	0.51	0.58
272	V	Roots	*Bacillus*	MK110937	0.87	0.82	0.69	0.72
287	V	Roots	*Buttiauxella*	MK110942	0.79	0.77	0.69	0.75
203	V	Roots	*Flavobacterium*	MK110948	0.45	0,3	0,27	0,145
231	V	Roots	*Janthinobacterium*	MK110945	1.08	1.04	1.01	1.02
219	V	Roots	*Lelliottia*	MK110899	1.02	0.98	0.93	0,85
515	H	Roots	*Lelliottia*	MK110953	1.22	1.28	1.23	1.27
194	V	Roots	*Pantoea*	MK110895	0.71	0.64	0.63	0.62
196	V	Roots	*Pantoea*	MK110896	0.84	0.95	0.9	0.93
197	V	Roots	*Pantoea*	MK110920	0.74	0.68	0.63	0.59
198	V	Roots	*Pantoea*	MK110921	0.73	0.835	0.775	0.78
221	V	Roots	*Pantoea*	MK110959	0.65	0.69	0.76	0.76
234	V	Roots	*Pantoea*	MK110957	0.75	0.75	0.68	0.76
239	V	Roots	*Pantoea*	MK110928	0.76	0.80	0.75	0.80
509	H	Roots	*Pectobacterium*	MK110913	0.78	0.66	0.67	0.69
195	H	Roots	*Pseudomonas*	MK110946	0.51	0.52	0.51	0.38
200	V	Roots	*Pseudomonas*	MK110947	0.62	0.51	0.53	0.67
204	V	Roots	*Pseudomonas*	MK110922	0.42	0.51	0.55	0.56
208	V	Roots	*Pseudomonas*	MK110897	0.8	0.92	0.90	0.85
209	V	Roots	*Pseudomonas*	MK110949	1.04	0.94	0.79	0.61
211	V	Roots	*Pseudomonas*	MK110898	0.60	0.53	0.55	0.39

218	V	Roots	*Pseudomonas*	MK110950	0.49	0.92	0.56	0.45
222	V	Roots	*Pseudomonas*	MK110923	0.77	1.03	0.95	0.76
226	V	Roots	*Pseudomonas*	MK110925	0.92	1.1	0.69	0.59
228	V	Roots	*Pseudomonas*	MK110924	0.81	0.82	1.06	0.80
229	V	Roots	*Pseudomonas*	MK110960	0.67	0.61	0.43	0.57
230	V	Roots	*Pseudomonas*	MK110926	0.535	0.45	0.445	0.59
238	V	Roots	*Pseudomonas*	MK110927	0.985	1.27	1.24	1.14
254	V	Roots	*Pseudomonas*	MK110900	0.95	0.93	0.77	0.75
263	V	Roots	*Pseudomonas*	MK110902	0.79	1.07	0.86	0.67
265	V	Roots	*Pseudomonas*	MK110933	1.00	1.34	1.24	1.18
266	H	Roots	*Pseudomonas*	MK110934	0.41	0.35	0.24	0.12
276	V	Roots	*Pseudomonas*	MK110938	1.09	1.30	1.31	1.33
279	V	Roots	*Pseudomonas*	MK110939	1.37	1.40	1.38	1.24
281	V	Roots	*Pseudomonas*	MK110940	1.22	1.33	1.34	1.33
286	V	Roots	*Pseudomonas*	MK110941	0.90	0.94	0.85	0.77
288	V	Roots	*Pseudomonas*	MK110943	0.83	0.97	0.89	0.69
482	H	Roots	*Pseudomonas*	MK110903	0.91	0.95	0.74	0.68
483	H	Roots	*Pseudomonas*	MK110904	0.90	0.78	0.76	0.78
484	H	Roots	*Pseudomonas*	MK110905	0.55	0.92	0.75	0.56
485	H	Roots	*Pseudomonas*	MK110906	0.76	0.75	0.66	0.74
491	H	Roots	*Pseudomonas*	MK110907	0.71	0.68	0.63	0.49
493	H	Roots	*Pseudomonas*	MK110908	0.65	0.59	0.37	0.38
495	H	Roots	*Pseudomonas*	MK110909	1.07	1.02	1.06	0.96
501	H	Roots	*Pseudomonas*	MK110910	1.36	1.27	1.14	0.97
505	H	Roots	*Pseudomonas*	MK110911	0.95	0.94	0.92	0.91
506	H	Roots	*Pseudomonas*	MK110912	0.91	1.14	0.99	1.01
507	H	Roots	*Pseudomonas*	MK110951	0.56	0.44	0.55	0.61
510	H	Roots	*Pseudomonas*	MK110914	0.75	0.89	0.82	0.52
511	H	Roots	*Pseudomonas*	MK110915	0.65	0.76	0.71	0.53
512	H	Roots	*Pseudomonas*	MK110952	0.77	0.955	0.485	0.89
514	H	Roots	*Pseudomonas*	MK110916	1.275	1.33	1.215	1.18
516	H	Roots	*Pseudomonas*	MK110917	0.67	0.97	0.83	0.54
517	H	Roots	*Pseudomonas*	MK110954	1.39	1.35	1.25	1.12
535	H	Roots	*Pseudomonas*	MK110918	1.04	1	0.85	1.08
539	H	Roots	*Pseudomonas*	MK110944	1.36	1.40	0.48	0.23
540	H	Roots	*Pseudomonas*	MK110961	0.88	0.93	0.92	0.87
566	H	Roots	*Pseudomonas*	MK110919	0.86	1.27	1.3	1.21
568	H	Roots	*Pseudomonas*	MK110955	1.17	0.86	0.75	0.75
252	V	Roots	*Staphylococcus*	MK110931	0.74	073	0.62	0.74
572	H	Roots	*Stenotrophomonas*	MK110956	0.73	0.82	0.82	0.8

The main aspect emerging from the analysis of Table 18 is that most of the isolates are able to grow in SWW, in fact a great percentage of spots in Table 18 are orange colored, which indicates optical densities levels referring to presence of growth. In SWW 1X, therefore at concentrations of B, Fe, Se and NaCl of 20, 15, 0.03 and 5000 mg/l respectively, the lowest levels of OD_{600} were represented by isolates 203, 230, 266, and

507, belonging to *Flavobacterium* and *Pseudomonas* genera, with optical densities levels from 0.3 to 0.45. Thus, 94.11% of the isolates are able to grow in SWW 1X.

In presence of SWW 2X, six isolates show the lowest levels of OD_{600}, ranging from 0.24 to 0.43. These isolates are 203, 229, 230, 266, and 493, belonging to *Flavobacterium* and *Pseudomonas* genera. The other isolates (91.17%) show levels of growth in SWW 2X similar to the growth in only medium (C+). At concentrations of B, Fe, Se and NaCl of 60, 45, 0.09 and 15000 mg/l (SWW 3X), 7 isolates (195, 203, 211, 266, 493, 539) show low levels of growth raging from OD_{600} levels from 0.12 to 0.39, while the remaining 89.7% are able to grow in such condition.

Table 19 presents the growth of root isolates from **second sampling** in presence of SWW in the different concentrations.

Table 19. Growth in presence of SWW for root isolates from the **second sampling**.

Isolate	Plant	Compartment	Genus	Accession number	C+	SWW 1X	SWW 2X	SWW 3X
V3 R3	V	Roots	*Achromobacter*	MK134509	0.83	0.66	0.47	0.33
V4 R15	V	Roots	*Acinetobacter*	MK134489	1.01	0.81	0.71	0.59
V4 R17	V	Roots	*Acinetobacter*	MK134488	1.08	1.18	1.09	0.50
V4 R18	V	Roots	*Acinetobacter*	MK134487	1.39	1.37	1.27	0.84
V4 R20	V	Roots	*Acinetobacter*	MK134486	1.22	1.31	0.97	0.725
H3 R12	H	Roots	*Agrobacterium*	MK134554	0.36	0.31	0.16	0.07
V3 R5	V	Roots	*Agrobacterium*	MK134508	0.33	0.28	0.17	0.09
H4 R8	H	Roots	*Bacillus*	MK134547	0.9	0.87	0.815	0.74
V4 R1	V	Roots	*Bacillus*	MK134496	0.86	0.94	0.7	0.49
H4 R3	H	Roots	*Comamonas*	MK138850	1.03	0.77	0.32	0.07
V3 R13	V	Roots	*Halomonas*	MK134502	1.17	1.3	1.29	1.13
V3 R1	V	Roots	*Idiomarina*	MK134511	1.08	0.76	0.26	0.05
H3 R19	H	Roots	*Microbacterium*	MK134551	0.51	0.46	0.35	0.30
H3 R9	H	Roots	*Microbacterium*	MK134555	0.49	0.31	0.2	0.27
H3 R2	H	Roots	*Ochrobactrum*	MK134559	0.7	0.62	0.58	0.48
H3 R3	H	Roots	*Ochrobactrum*	MK134558	0.51	0.41	0.43	0.28
H4 R1	H	Roots	*Ochrobactrum*	MK134549	0.88	0.85	0.75	0.64
V4 R21	V	Roots	*Ochrobactrum*	MK138851	1.13	1.08	0,75	0.76
H3 R14	H	Roots	*Pannonibacter*	MK134553	0.76	0.91	0.50	0.09
H4 R22	H	Roots	*Paracoccus*	MK134542	0.63	0.76	0.7	0.32
V3 R9	V	Roots	*Pseudo xanthomonas*	MK134505	0.39	0.12	0.03	0.06
H3 R4	H	Roots	*Pseudomonas*	MK134557	0.93	1.03	1.01	0.63
H4 R13	H	Roots	*Pseudomonas*	MK134546	0.94	0.87	0.75	0.33
H4 R19	H	Roots	*Pseudomonas*	MK134544	0.99	1.09	1.06	0.88
H4 R21	H	Roots	*Pseudomonas*	MK134543	0.67	0.72	0.665	0.53
H4 R23	H	Roots	*Pseudomonas*	MK134541	1.07	1.19	1.3	1.02
H4 R24	H	Roots	*Pseudomonas*	MK134540	1.07	1.17	1.04	0.95
V3 R2	V	Roots	*Pseudomonas*	MK134510	0.91	1.01	1.06	0.97

V3 R23	V	Roots	*Pseudomonas*	MK134497	0.51	0.67	0.69	0.62
V4 R13	V	Roots	*Pseudomonas*	MK134490	1.17	1.08	1.01	1.02
V4 R2	V	Roots	*Pseudomonas*	MK134495	1.19	1.19	1.08	0.81
V4 R3	V	Roots	*Pseudomonas*	MK134494	1.12	1.13	0.96	0.69
V4 R4	V	Roots	*Pseudomonas*	MK134493	1.16	1.27	1.11	0.89
V4 R5	V	Roots	*Pseudomonas*	MK134492	1.10	1.07	1.14	1.23
V4 R7	V	Roots	*Pseudomonas*	MK134491	1.14	1.20	1.26	1.26
V3 R16	V	Roots	*Pseudo xanthomonas*	MK134499	0.36	0.27	0.23	0.09
H3 R17	H	Roots	*Rheinheimera*	MK134552	0.36	0.5	0.62	0.77
H3 R6	H	Roots	*Rheinheimera*	MK134556	0.45	0.52	0.59	0.82
H4 R18	H	Roots	*Rheinheimera*	MK134545	0.84	0.94	1.16	1.45
H4 R7	H	Roots	*Rheinheimera*	MK134548	0.78	0.76	0.06	0.01
V3 R15	V	Roots	*Rheinheimera*	MK134500	0.83	0.89	1.08	0.99
V3 R4	V	Roots	*Rheinheimera*		0.65	0.78	1.05	0.92
V3 R7	V	Roots	*Rheinheimera*	MK134507	1.11	0.91	1.10	0.98
V3 R11	V	Roots	*Staphylococcus*	MK134504	0.55	0.41	0.33	0.14
V3 R12	V	Roots	*Staphylococcus*	MK134503	0.79	0.74	0.66	0,8
H3 R24	H	Roots	*Thalassospira*	MK134550	0.86	0.92	0.60	0.08
V3 R14	V	Roots	*Thalassospira*	MK134501	0.72	0.67	0.42	0.27
V3 R19	V	Roots	*Thalassospira*	MK134498	0.63	0.69	0.47	0.30
V3 R8	V	Roots	*Thalassospira*	MK134506	0.67	0.62	0.37	0.16

For second sampling, in SWW 1X, the lowest levels of growth were registered for 6 isolates, namely H3R.12, H3R.9, V3R.11, V3R.16, V3R.5 and V3R.9, belonging to the genera *Agrobacterium*, *Microbacterium*, *Staphylococcus* and *Pseudoxanthomonas*, showing levels of optical densities ranging from 0.41 to 0.12. The remaining 87.6% of isolates showed levels of growth in SWW 1X similar to their growth in only TSB (C+) or higher. In SWW 2X, 10 isolates showed the lowest OD_{600} levels, ranging from 0.07 to 0.35. Such isolates were H3R.12, H3R.19, H3R.9, H4R.3, H4R.7, V3R.1, V3R.11, V3R.16, V3R.5 and V3R.9, belonging to *Agrobacterium*, *Micrococcus*, *Comamonas*, *Rheinheimera*, *Idiomarina*, *Staphylococcus* and *Pseudoxanthomonas*. The other isolates (79.59%) showed levels of OD_{600} which were indicatives of growth. At SWW 3X, 19 isolates present the lowest levels of growth, with OD_{600} ranging from 0.01 to 0.33. The other isolates (61.22%) show ability to grow in SWW 3X.

In table 20 the growth in SWW of root isolates from third sampling is presented.

Table 20. Growth in presence of SWW for root isolates from the **third sampling**.

Isolate	Plant	Compartment	Genus	Accession number	C+	SWW 1X	SWW 2X	SWW3 X
H6 R17	H	Roots	*Achromobacter*	MK134518	0.81	0.66	0.53	0.27
V6 R16	V	Roots	*Achromobacter*	MK130938	0.79	1.01	0.53	0.07

V6 R5	V	Roots	*Achromobacter*	MK130934	0.8	0.57	0.37	0.19
V6 R6	V	Roots	*Achromobacter*	MK130935	0.70	0.66	0.49	0.26
H6 R10	H	Roots	*Agrobacterium*	MK134524	0.67	0.3	0.24	0.20
H5 R1	H	Roots	*Bacillus*	MK134539	0.54	0.36	0.265	0.18
H5 R2	H	Roots	*Bacillus*	MK134538	0.50	0.64	0.54	0.25
H6 R20	H	Roots	*Bacillus*	MK134515	0.52	0.36	0.27	0.25
H6 R21	H	Roots	*Bacillus*	MK138852	0.52	0.45	0.57	0.71
H6 R8	H	Roots	*Bacillus*	MK134526	1.01	0.80	0.19	1.03
V6 R1	V	Roots	*Bacillus*	MK130907	0.43	0.51	0.61	0.69
V6 R2	V	Roots	*Bacillus*	MK130906	1.28	1.35	1.22	1.1
V6 R8	V	Roots	*Bacillus*	MK130937	1.04	1.05	0.76	0.72
H5 R6	H	Roots	*Enterobacter*	MK134534	0.71	0.75	0.67	0.53
H5 R7	H	Roots	*Enterobacter*	MK134533	1.25	1.31	1.19	1.09
V5 R10	V	Roots	*Halomonas*	MK138853	1.00	1.06	1.09	0.25
V5 R13	V	Roots	*Halomonas*	MK130915	1.04	1.19	1.12	0.45
V5 R15	V	Roots	*Halomonas*	MK130913	1.03	1.09	1.07	0.24
V5 R20	V	Roots	*Halomonas*	MK130910	0.85	1.03	1.15	1.11
V5 R5	V	Roots	*Halomonas*	MK130921	0.99	1.21	1.20	0.44
V5 R9	V	Roots	*Halomonas*	MK130917	0.96	1.10	1.07	0.95
H6 R22	H	Roots	*Isoptericola*	MK134514	0.58	0.41	0.19	0.19
V5 R14	V	Roots	*Microbacterium*	MK130914	0.69	0.28	0.25	0.23
H6 R19	H	Roots	*Pannonibacter*	MK134516	0.75	0.81	0.69	0.14
V5 R18	V	Roots	*Pannonibacter*	MK130911	0.77	0.89	0.42	0.11
H5 R8	H	Roots	*Pseudomonas*	MK134532	0.61	0.61	0.52	0.46
H6 R1	H	Roots	*Pseudomonas*	MK134531	1.32	1.41	1.24	1.31
H6 R2	H	Roots	*Pseudomonas*	MK134530	1.36	1.37	1.30	1.31
H6 R4	H	Roots	*Pseudomonas*	MK134528	1.39	1.42	1.23	1.37
V5 R1	V	Roots	*Pseudomonas*	MK134485	0.56	0.67	0.77	1.06
V5 R11	V	Roots	*Pseudomonas*	MK138854	0.51	0.68	0.71	0.22
V5 R17	V	Roots	*Pseudomonas*	MK130912	0.48	0.69	0.93	0.27
V5 R22	V	Roots	*Pseudomonas*	MK130908	1,.4	1.32	1.2	0.54
V5 R6	V	Roots	*Pseudomonas*	MK130920	0.55	0.58	0.7	0.91
V6 R3	V	Roots	*Pseudomonas*	MK130932	1.09	1.11	1.28	0.51
V6 R4	V	Roots	*Pseudomonas*	MK130933	1	1.13	0.83	0.29
V6 R7	V	Roots	*Pseudomonas*	MK130936	1	1.16	0.89	0.32
H6 R23	H	Roots	*Pseudoxanthomonas*	MK134513	0.41	0.25	0.08	0.03
V5 R4	V	Roots	*Rheinheimera*	MK130922	0.78	0.89	0.99	0.15
V5 R7	V	Roots	*Rheinheimera*	MK130919	0.8	0.81	0.62	0.23
H6 R14	H	Roots	*Rhizobium*	MK134521	0.44	0.42	0.30	0.08
H6 R24	H	Roots	*Rhizobium*	MK134512	0.43	0.36	0.3	0.29
H5 R3	H	Roots	*Stenotrophomonas*	MK134537	0.81	0.74	0.66	0.45
H5 R4	H	Roots	*Stenotrophomonas*	MK134536	0.89	0.85	0.75	0.51
H5 R5	H	Roots	*Stenotrophomonas*	MK134535	0.87	0.79	0.60	0.52
H6 R12	H	Roots	*Stenotrophomonas*	MK134522	1.32	1.47	1.38	1.25
H6 R16	H	Roots	*Stenotrophomonas*	MK134519	1.31	1.46	1.33	0.99
H6 R18	H	Roots	*Stenotrophomonas*	MK134517	1.22	1..33	1.44	0.79
H6 R3	H	Roots	*Stenotrophomonas*	MK134529	1.25	1.53	1.41	1.32

H6 R9	H	Roots	*Stenotrophomonas*	MK134525	1.30	1.48	1.3	1.27
H6 R11	H	Roots	*Thalassospira*	MK134523	0.56	0.51	0.30	0.25
H6 R15	H	Roots	*Thalassospira*	MK134520	0.98	0.81	0.75	0.47
H6 R7	H	Roots	*Thalassospira*	MK134527	1.32	1.47	1.32	1.27
V5 R12	V	Roots	*Thalassospira*	MK130916	0.58	0.41	0.36	0.13
V5 R2	V	Roots	*Thalassospira*	MK130931	1.05	0.93	0.58	0.45
V5 R21	V	Roots	*Thalassospira*	MK130909	0.7	0.7	0.43	0.29
V5 R3	V	Roots	*Thalassospira*	MK130923	0.54	0.59	0.34	0.22
V5 R8	V	Roots	*Thalassospira*	MK130918	0.57	0.49	0.52	0.36

From third sampling, a percentage of 87.93% of isolates are able to grow in SWW 1X. The isolates registering the lowest levels of OD_{600} were H5R.1, H6R.10, H6R.20, H6R.23, H6R.24, V5R.12, V5R.14, belonging to genera *Bacillus*, *Agrobacterium*, *Pseudoxanthomonas*, *Rhizobium* and *Microbacterium*, whose OD_{600} ranged from 0.28 to 0.41. At concentrations of B, Fe, Se and NaCl of 40, 30, 0.06, 10000 mg/l respectively (SWW 2X), 77.58% of the isolates showed ability to grow. Thirteen isolates (H5R.1, H6R.10, H6R.11, H6R.14, H6R.20, H6R.22, H6R.23, H6R.24, H6R.8, V5R.12, V5R.14, V5R.3, V6R.5, belonging to *Bacillus*, *Agrobacterium*, *Thalassospira*, *Rhizobium*, *Isoptericola*, *Pseudoxanthomonas*, *Microbacterium* and *Achromobacter* registered lowest levels of optical densities, ranging from 0.19 to 0.37. Concerning the growth of root isolates of third sampling in SWW 3X, more than half of isolates (53.44%) grow at such concentrations of B, Fe, Se and NaCl.

Table 21 shows growth of root isolates from fourth sampling in presence of SWW.

Table 21. Growth in presence of SWW for root isolates from the **fourth sampling**.

Isolate	Plant	Compartment	Genus	Accession number	C+	SWW 1X	SWW 2X	SWW 3X
V8 R2	V	Roots	*Achromobacter*	MK130945	1.02	0.82	0.725	0.69
V8 R21	V	Roots	*Achromobacter*	MK130957	0.72	1.21	0.82	0.25
V8 R23	V	Roots	*Achromobacter*	MK130901	0.93	0.85	0.8	0.60
V8 R3	V	Roots	*Achromobacter*	MK130905	0.99	1.09	0.93	0.88
H8 R5	H	Roots	*Aeromonas*	MK133358	1.27	1.56	0.875	0.51
H7 R23	H	Roots	*Agrobacterium*	MK138868	0.5	0.40	0.33	0.2
H7 R3	H	Roots	*Agrobacterium*	MK138869	0.81	0.53	0.4	0.18
H7 R5	H	Roots	*Agrobacterium*	MK138870	0.67	0.71	0.53	0.29
H7 R7	H	Roots	*Agrobacterium*	MK138872	1.42	1.30	0.47	0.21
H7 R9	H	Roots	*Agrobacterium*	MK138874	0.54	0.4	0.16	0.08
H8 R1	H	Roots	*Agrobacterium*	MK138875	0.47	0.38	0.04	0.02
H8 R16	H	Roots	*Agrobacterium*	MK130924	0.8	0.96	0.48	0.10
H8 R18	H	Roots	*Agrobacterium*	MK130928	0.99	0.63	0.25	0.11
H8 R3	H	Roots	*Agrobacterium*	MK138881	1.36	1.44	0.78	0.39
H7 R16	H	Roots	*Arthrobacter*	MK138862	1.15	0.63	0.15	0.11

V8R7	V	Roots	*Devosia*	MK130903	0.93	1.23	0.795	0.47
H7 R22	H	Roots	*Flavobacterium*	MK138867	0.48	0.2	0.01	0.01
H8 R12	H	Roots	*Flavobacterium*	MK138878	1.43	1.39	0.68	0.28
H7 R15	H	Roots	*Lysobacter*	MK138861	0.42	0.22	0.10	0.11
V7 R8	V	Roots	*Microbacterium*	MK130939	0.35	0.27	0.16	0.22
H7 R19	H	Roots	*Micrococcus*	MK138863	0.66	0.45	0.3	0.18
V8 R12	V	Roots	*Pannonibacter*	MK130949	0.78	0.66	0.72	0.59
V8 R16	V	Roots	*Pannonibacter*	MK130953	0.75	0.82	0.8	0.47
V8 R4	V	Roots	*Pannonibacter*	MK130904	0.8	1.01	0.61	0.18
H8 R10	H	Roots	*Pseudomonas*	MK138876	1.36	1.32	1.22	0.96
H8 R13	H	Roots	*Pseudomonas*	MK138879	1.33	1.355	1.34	1.13
H8 R14	H	Roots	*Pseudomonas*	MK130926	1.28	1.33	1.305	1.195
H8 R17	H	Roots	*Pseudomonas*	MK130927	1.33	1.39	1.28	1.28
H8 R19	H	Roots	*Pseudomonas*	MK130929	0.42	0.21	0.11	0.10
H8 R2	H	Roots	*Pseudomonas*	MK138880	1.3	1.39	1.37	1.21
H8 R20	H	Roots	*Pseudomonas*	MK130930	1.32	1.35	1.24	1.2
H8 R4	H	Roots	*Pseudomonas*	MK138882	1.31	1.28	1.28	0.99
H8 R6	H	Roots	*Pseudomonas*	MK138883	1.38	1.39	1.43	1.18
H8 R7	H	Roots	*Pseudomonas*	MK138884	1.32	1.39	1.34	1.3
H8 R8	H	Roots	*Pseudomonas*	MK138885	1.35	1.38	1.33	1.29
H8 R9	H	Roots	*Pseudomonas*	MK138886	1.39	1.43	1.36	1.33
V7 R13	V	Roots	*Pseudomonas*	MK138887	1.21	1.50	1.45	1.17
V7 R19	V	Roots	*Pseudomonas*	MK130940	1.22	1.36	1.30.	0.79
V7 R21	V	Roots	*Pseudomonas*	MK130941	0.88	0.45	0.45	0.66
V7 R23	V	Roots	*Pseudomonas*	MK130943	1.12	1.39	1.29	0.85
V7 R9	V	Roots	*Pseudomonas*	MK138889	1.17	1,.40	1.28	0.70
V8 R1	V	Roots	*Pseudomonas*	MK130944	0.61	0.65	0.52	0.21
V8 R22	V	Roots	*Pseudomonas*	MK130902	0.86	0.46	0.29	0,.18
V8 R24	V	Roots	*Pseudomonas*	MK130900	0.52	0.59	0.73	0,.97
V8 R8	V	Roots	*Pseudomonas*	MK130946	0.82	1.15	1.2	1.1
H8 R11	H	Roots	*Rheinheimera*	MK138877	0.35	0.22	0.11	0.07
H7 R1	H	Roots	*Rhizobium*	MK138855	0.39	0.33	0.17	0.08
H7 R10	H	Roots	*Rhizobium*	MK138856	0.39	0.31	0.22	0.22
H7 R11	H	Roots	*Rhizobium*	MK138857	0.72	0.46	0.46	0.26
H7 R12	H	Roots	*Rhizobium*	MK138858	0.61	0.51	0.29	0.33
H7 R13	H	Roots	*Rhizobium*	MK138859	0.4	0.31	0.22	0.11
H7 R14	H	Roots	*Rhizobium*	MK138860	0.46	0.32	0.23	0.18
H7 R2	H	Roots	*Rhizobium*	MK138864	0.37	0.30	0.16	0.1
H7 R20	H	Roots	*Rhizobium*	MK138865	0.36	0.28	0.16	0,18
H7 R21	H	Roots	*Rhizobium*	MK138866	0.71	0.50	0.54	0.34
H7 R6	H	Roots	*Rhizobium*	MK138871	0.38	0,3	0.22	0.14
H7 R8	H	Roots	*Rhizobium*	MK138873	0.41	0.29	0.17	0.16
H8 R15	H	Roots	*Rhizobium*	MK130925	0.36	0.25	0.1	0.06
V7 R22	V	Roots	*Rhizobium*	MK130942	0.78	0.37	0.47	0.61
V8 R11	V	Roots	*Shinella*	MK130948	0.77	0.72	0.62	0.34
V8 R13	V	Roots	*Shinella*	MK130950	0.66	0.735	0.43	0.11
V8 R18	V	Roots	*Sphingobium*	MK130955	0.81	1.11	0.81	0.69

V7 R24	V	Roots	*Stenotrophomonas*	MK138888	1.28	1.4	1.34	0.99
V8 R14	V	Roots	*Thalassospira*	MK130951	0.98	1.2	0.87	0.3
V8 R15	V	Roots	*Thalassospira*	MK130952	0.79	0,.61	0.38	0.06
V8 R17	V	Roots	*Thalassospira*	MK130954	0.89	0.76	0.58	0.31
V8 R19	V	Roots	*Thalassospira*	MK130956	0.99	1.11	0.86	0.62
V8 R9	V	Roots	*Thalassospira*	MK130947	0.83	0.78	0.83	0.92

Root isolates from fourth sampling were able to grow in SWW 1X in a percentage of 80.88%. The lowest values of optical densities were highlighted for 13 isolates with values ranging from 0.2 – 0.33. In SWW 2X, a percentage of 67.64% of isolates are able to grow, and 22 isolates show reduced ability to grow in such conditions. These are affiliated to the following genera, *Rhizobium*, *Lysobacter*, *Arthrobacter*, *Microbacterium*, *Flavobacterium*, *Agrobacterium*, *Pseudomonas* and *Thalassospira*.

At the highest tested concentrations of SWW, 50% of isolates were able to grow, while the other half registered low optical densities ranging from 0.1 to 0.34.

It is remarkable that many isolates show high levels of growth in each of the tested conditions. In the first sampling, 15 isolates associated with the genera *Pseudomonas*, *Janthinobacterium*, *Bacillus*, *Lelliottia* registered high levels of optical densities in all of the tested concentrations of SWW. Such isolates were 231, 238, 253, 265, 276, 279, 281, 495, 501, 505, 506, 514, 515 and 517 and accounted for 22.05% of the total analyzed isolates from the first sampling. From the second sampling, 20.40% of isolates were able to grow with high levels in the three concentrations of SWW. Such isolates were affiliated to the genera *Rheinheimera*, *Pseudomonas*, *Halomonas* and *Pseudoxanthomonas*, and were H4R.18, H4R.23, H4R.24, V3R.13, V3R.15, V3R.2, V3R.7, V4R.13, V4R.5 and V4R.7. In the third sampling 18.96% of the isolates showed good levels of growth in all the tested SWW. Such isolates were H5R.7, H6R.1, H6R.12, H6R.16, H6R.2, H6R.3, H6R.4, H6R.7, H6R.9, V5R.20 and V6R.2. These isolates were affiliated to the following genera: *Enterobacter*, *Pseudomonas*, *Stenotrophomonas*, *Thalassospira*, *Bacillus* and *Halomonas*. At fourth sampling, 20.58% of the isolates showed notable levels of growth in all concentrations of SWW. These isolates were H8R.10, H8R.13, H8R.14, H8R.17, H8R.2, H8R.20, H8R.4, H8R.6, H8R.7, H8R.8, H8R.9, V7R.13, V7R.24, V8R.8, affiliated to the genera *Pseudomonas* and *Stenotrophomonas*. Thus, around 20% of isolates in each of the samplings were able to register important levels of growth in the three concentrations of SWW tested.

6.5 Discussion

Phytodepuration represents a green valuable solution for environmental cleanup, in particular for wastewater treatment. The selection of the vegetation species in a phytodepuration plant is very important; however the main players of pollutants removal have been pointed out to be the microorganisms growing in association with such vegetation (Zhang et al., 2017). Microorganisms in phytodepuration are not only implied in pollutants removal but they also support the plant's growth under toxic conditions, since endophytes are generally characterized by plant growth-promoting activity (Jasim, Joseph, John, Mathew, & Radhakrishnan, 2014). Despite the great relevance of microorganisms in the phytodepuration process (Germaine et al., 2010), the microbiome associated to aquatic plants have so far been poorly explored (Li, Liu, Liu, Zhu, & Zhang, 2011). For this reason, the role of microorganisms in the phytodepuration process remains still quite obscure. The efficiency of phytodepuration in CWs could be strongly improved if the relationship between host plant and microbiome was clearly understood.

Thus, the aim of this work was to investigate on the influence of bacterial communities associated to *P. australis* plants on the phytodepuration process. Since the first step towards a better understanding of the role of microorganisms in phytodepuration is represented by the characterization of microbial communities structure associated to vegetation in CWs, the experimental strategy in this work included the extraction of bacterial cultivable isolates associated to *P. australis* plants in a CW and to rhizospheric and bulk soil samples from the CW. Then, the composition of bacterial cultivable communities associated to different compartments of the plants and soil samples was evaluated, and total DNA from each compartment and soil sample was extracted in order to assay the total communities associated to the plants compartments and soil. Together with plants and soil samples, permeate samples from the CW was also analyzed for their bacterial counts and total bacterial communities composition. At this time, four samplings were performed at Calice CW, in different periods of the last and the ongoing year. The first of such samplings was performed before the activation of the CW, therefore before the presence of wastewater in the CW tanks. Cultivable bacterial communities associated to the *P. australis* roots and total bacterial communities were characterized at this point. To the best of our knowledge, this is the first work to compare microbiotas associated to plants in a CW before and after the activation of the plant. In order to deepen the characterization of such bacterial communities and also to explore their potential to help increasing the

efficiency of phytodepuration, we decided to characterize plant root isolates for their ability to grow in presence of Boron, Iron, Selenium and Sodium Chloride, which represent critical molecules in the treatment of wastewater. Bacterial isolates showing remarkable ability to grow in such conditions are good candidates to potentially enhance the quality of the treated wastewater. Taken together, these analyses allow to better understand the bacterial communities associated to *P. australis* plants and soil in the analyzed CW and represent the starting point for the evaluation of the influence of such communities on the phytodepuration process and pave the way to a potential application of such bacterial isolates for the enhancing of phytodepuration process.

The CW analyzed in this work is located in Prato, within the Calice WWTP, managed by G.I.D.A. S.p.A, as described in Materials and methods.

The yield of cultivable heterotrophic bacteria in the different samples (plant compartments and soils) through the different samplings ranged from 10^3 CFU/g to 10^7 CFU/g. The lowest bacterial numbers were highlighted for bulk soil samples and plant aerial parts. This evidence may derive from the fact that bulk soil was represented by fine gravel, which is probably not very suitable for bacterial growth, in comparison to, for example, classical cultivation soil. The aerial part also showed the lowest bacterial counts throughout the samplings. At the moment of the first sampling, the plant's aerial part, was not green and, probably, for this reason not populated by many bacteria. On the other hand, the roots presented the highest bacterial counts. Roots might represent a well-populated compartment because of the high concentrations of nutrients that might be present in it, as a result of the plant's assumption of rich substances from the soil, representing a prosperous environment for bacteria to thrive. Our data are in agreement with previous studies highlighting greater abundances of plant-associated bacterial populations in the roots compartments than in aerial parts (Lamb, Tonkyn, & Kluepfel, 1996). A specific comparison with bacterial loads from *P. australis* plants tissues is difficult to perform, since most of the studies focusing on *P. australis* associated bacterial communities developed a culture-indipendent approach. A study performed by Calheiros and colleagues (Calheiros et al., 2009) analyzed bacterial communities from two series of two-stage CWs treating tannery wastewater. In such study, cultivable bacterial communities were analyzed and bacterial yield within *P. australis* roots ranged from 10^6 to 10^8 CFU ml^{-1}. Also, in such study, bacterial loads were evaluated for the CWs inlet and outlet, showing respectively bacterial loads of 10^4 and 10^5.

At the time of the first sampling, before the pilot plant activation, the different compartments of the plants were inhabited by cultivable bacteria belonging mainly to

Pseudomonas, *Pantoea*, *Flavobacterium*, *Bacillus*, *Arthrobacter*, *Acinetobacter*, and *Curtobacterium*. Such genera are typically found in environments as water and soil. Thus, their presence in the bacterial communities associated to *P. australis* and surrounding soil indicate microorganisms typically found in such environments. On the other hand, these genera could also suggest the presence of valuable isolates of biotechnological importance. In this concern, many *Pseudomonas* strains have been evidenced as capable of degrading toxic compounds, as polycyclic and organic aromatic compounds, toluene, carbazole, and carbon tetrachloride (O'Mahony, Dobson, Barnes, & Singleton, 2006) (Yen et al., 1991) (Nojiri et al., 2002) (Nam, Chang, Hong, & Lee, 2003) (Onaca, Kieninger, Engesser, & Altenbuchner, 2007) (Del C. Sepúlveda-Torres, Rajendran, Dybas, & Criddle, 1999). Similar evidences have been highlighted for *Arthrobacter* strains, which are able to exert important functions in bioremediation (Camargo, Bento, Okeke, & Frankenberger, 2004) (Westerberg, Elvang, Stackebrandt, & Jansson, 2000) (O'loughlin, Sims, & Traina, 1999). Also, *Bacillus* strains have been highlighted with antimicrobial activity able to inhibit plant pathogens, which could be an important function for enhancing the plant growth (Jeong et al., 2012).

Pseudomonas genus has also characterized bacterial root cultivable communities in all the performed samplings. At the first sampling, before the plant's activation, *Pseudomonas* genus showed the highest percentages among the isolates (66.21%), in comparison to the other samplings. Moving on to the other samplings, the percentages of *Pseudomonas* genus in root bacterial population varies, but remains around 20 and 32%, without reaching high levels as seen at the first sampling. Apparently from the first to the other samplings, *Pseudomonas* population leaves space for other bacterial taxa, which maybe are more suitable for the presence of wastewater or derive from the wastewater. From second to fourth sampling, bacterial communities appear to be enriched with different bacterial genera, such as *Rheinheimera*, *Thalassospira*, *Acinetobacter*, *Pseudoxanthomonas*, *Microbacterium*, *Agrobacterium*, *Paracoccus*, *Pannonibacter*, *Achromobacter*, *Stenotrophomonas*, *Rhizobium*, *Shinella*, *Aeromonas* and others, absent in the first sampling. The most represented bacterial genera in all the analyzed bacterial communities, showing isolates in three out of the four samplings, were *Thalassospira*, *Stenotrophomonas*, *Rheinheimera*, *Pseudomonas*, *Pannonibacter*, *Microbacterium*, *Bacillus*, *Agrobacterium* and *Achromobacter*. Many of the evidenced genera, as *Pseudomonas*, *Bacillus*, *Pantoea* are tipically present as endophytes also in other plant species (Ikeda et al., 2013) (Gagne-Bourgue et al., 2013).

Concerning other studies on *P. australis* roots microbiome, Proteobacteria phylum emerged as the dominant bacterial taxa. For example, Gammaproteobacteria was observed as the most abundant taxa among roots of *P. australis* plants from a two-stage CW in Portugal (Calheiros et al., 2009). Moreover, Li and colleagues (Li et al., 2011) identified 131 clones (78.9%) affiliated to Proteobacteria among root bacterial communities of *P. australis* plants in Beijing Cuihu CW. Within the Proteobacteria group, there were sequences affiliated to *Agrobacterium*, *Rhizobium*, *Devosia*, *Janthinobacterium*, *Aeromonas*, *Pseudomonas* and *Enterobacter,* which were also highlighted among our root bacterial communities.

Bacterial taxa evidenced in *P. australis* roots communities in Calice CW could exhert important functions for the plant physiology and for the phytodepuration process. For example, *Pseudomonads* are usually found in contaminated aquifers, since they can tolerate toxic compounds (Moore et al., 2006). A good evidence of *Pseudomonas* potential in degrading toxic compounds and aiding phytodepuration was provided by the use of strain *P. putida* VM1450 for the degradation of herbicide 2,4-dichlorophenoxyacetic acid (Germaine et al., 2010). Also, *Aeromonas* isolates could be related to nitrate reduction activity and production of indole, as described for strain *Aeromonas bivalvium* 868E (Minana-Galbis, Farfan, Fuste, & Loren, 2007). *Enterobacter* strains are reported to have ability to fix nitrogen (Hallmann, Mahaffee, & Kloepper, 2011) (Tsuda, Kosaka, Tsuge, Yasuyuki, & Horino, 2001), to use phytate, to play an important role in phosphorus cycling, as well as antagonistic activity (Fuentes, Jorquera, & Mora, 2009).

Root isolates extracted from *P. australis* plants in the different samplings were tested for their growth in presence of elements (Boron, Iron, Selenium, Sodium Chloride) which constitute an issue for the wastewater treatment. The aim of such analyses was to select isolates able to grow in presence of such elements, which could suggest their utility for a reduction of such molecules in wastewater. Their ability to grow in such conditions do not directly imply that they are able to degrade or transform such compounds but represent a start-point for the further analysis of such strains and for the construction of a bacterial consortium with hypothetical potential to enhance wastewater phytodepuration. By analyzing the results for growth of bacterial isolates in presence of the so-called *Synthetic Wastewater*, similar percentages of isolates in the different samplings show levels of optical densities which indicate growth at the different concentrated SWWs. For SWW 1X, the percentages of the growing isolates are 94.11%, 87.6%, 87.93% and 88.23%, respectively from the first to the fourth sampling. For SWW 2X, the same sequences of percentages would be 92.64%, 81.6%, 77.58% and 67.64%, while for SWW 3X

percentages would be 89.7%, 61.22%, 53.44% and 50%. Such data show that there are higher percentages of growing strains in the first sampling, as if isolates with ability to grow in presence of B, Fe, Se and NaCl reduce in number along time. However, if we analyze the numbers of isolates which are able to grow with very high optical densities (around 1) in all the tested conditions (SWW 1X, SWW 2X, SWW 3X), we observe that in the first sampling, the percentage of such strains is 11.76%, in the second sampling the percentage is 12.24%, in the third one 17.24%, and finally in the fourth is 17.64%. So, on one hand, proceeding with the samplings, the number of bacterial isolates able to grow in SWW seem to diminuish, but on the other hand, strongly-growing isolates are more present in the last samplings, suggesting that time could select those strains most capable of coping with such conditions and maybe that the changing of the community requires a longer time. Considering that the concentrations of Boron, Iron, Selenium and Sodium Chloride in SWW 1X are the maximum allowed concentrations for the wastewater emission into sewers, and that, for the different samplings, at least 50% of the root isolates are able to grow in concentrations of such elements which are three-fold higher (SWWW 3X) than the maximum concentrations, bacterial communities associated to *P. australis* represent a valuable source for more detailed studies aimed at the utilization of endophytes to enhance phytodepuration.

6.6 Conclusions

In this work we analyzed bacterial communities associated to *Phragmites australis* plants and soil from the Calice pilot plant, with the aim of evaluating the influence of such communities on the phytodepuration process. Four samplings were performed in Calice, comprising one sampling before the activation of the plant. For each of the samplings, bacterial communities were extracted from the internal tissues of the plants (aerial part, stem, roots), from rhizospheric soil, and from bulk soil. Bacterial counts were determined in CFU/g for each sample and the obtained bacterial isolates were then characterized for their taxonomy and ability to grow in presence of elements that represent an issue for wastewater treatment. Also, the microbiome associated to each plant compartment and soil sample was sequenced for each of the samplings. The performed analyses allowed to highlight the predominance of *Pseudomonas* genus, characteristic from soils and water, in the analyzed cultivable bacterial communities both before and after the plant's activation. Bacterial communities were also characterized by many other genera, mainly *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Flavobacterium* and *Pantoea*. Such bacterial taxa may suggest the presence of endophytes with biotechnological relevance, as evidenced for isolates of the same genera (Germaine et al., 2006), (Minana-Galbis, Farfan, Fuste, & Loren, 2007), (Hallmann, Mahaffee, & Kloepper, 2011), (Tsuda, Kosaka, Tsuge, Kubo, & Horino, 2001), (Fuentes, Jorquera, & Mora, 2009). The presence of important endophytic isolates from a biotechnological point of view was also indicated by results obtained for the growth in presence of Boron, Iron, Selenium and Sodium Chloride. Root bacterial isolates from the four performed samplings showed noteworthy capabilities of growing in such conditions, with percentages of growing isolates that were at least of 50% in the highest tested concentrations. Taken all together, the obtained data represent a strong initial step towards the improvement of phytodepuration process by means of endophytes.

6.7 Bibliography

Calheiros C. S. C., Duque, A. F., Moura, A., Henriques, I. S., Correia, A., Rangel, A. O. S. S., & Castro, P. M. L. (2009). Bioresource Technology Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment. Bioresource Technology, 100(13), 3228–3235.

Calheiros C. S. C., Teixeira, A., Pires, C., Franco, A. R., Duque, A. F., Crispim, L. F. C., Castro, P. M. L. (2010). Bacterial community dynamics in horizontal flow constructed wetlands with different plants for high salinity industrial wastewater polishing. Water Research, 44(17), 5032–5038.

Camargo F. A. O., Bento, F. M., Okeke, B. C., & Frankenberger, W. T. (2004). Hexavalent chromium reduction by an actinomycete, Arthrobacter crystallopoietes ES 32. Biological Trace Element Research, 97(2), 183–194.

Carvalho P. N., Basto, M. C. P., & Almeida, C. M. R. (2012). Potential of Phragmites australis for the removal of veterinary pharmaceuticals from aquatic media. Bioresource Technology, 116, 497–501.

Fuentes B., Jorquera, M., & Mora, M. de la L. (2009). Dynamics of phosphorus and phytate-utilizing bacteria during aerobic degradation of dairy cattle dung. Chemosphere, 74(2), 325–331.

GagneBourgue, F., Aliferis, K. A., Seguin, P., Rani, M., Samson, R., & Jabaji, S. (2013). Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (Panicum virgatum L.) cultivars. Journal of Applied Microbiology, 114(3), 836–853.

Germaine K. J., Keogh, E., Ryan, D., & Dowling, D. N. (2009). Bacterial endophyte-mediated naphthalene phytoprotection and phytodepuration. FEMS Microbiology Letters, 296(2), 226–234.

Germaine K. J., Liu, X., Cabellos, G. G., Hogan, J. P., Ryan, D., & Dowling, D. N. (2010). Bacterial endophyte-enhanced phytodepuration of the organochlorine herbicide 2 , 4-dichlorophenoxyacetic acid, 57(2006), 302–310.

Glick B. R. (2003). Phytodepuration : synergistic use of plants and bacteria to clean up the environment, 21, 383–393.

Hallmann J., Mahaffee, W. F., & Kloepper, J. W. (2011). Endophytic Bacteria in Agricultural Crops Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology, 43(10), 895-914

He Y., Langenhoff, A. A. M., Sutton, N. B., Rijnaarts, H. H. M., Blokland, M. H., Chen, F., … Schröder, P. (2017). Metabolism of Ibuprofen by *Phragmites australis*: Uptake and Phytodegradation. Environmental Science and Technology, 51(8), 4576–4584.

Ho Y.-N., Mathew, D. C., Hsiao, S.-C., Shih, C.-H., Chien, M.-F., Chiang, H.-M., & Huang, C.-C. (2012). Selection and application of endophytic bacterium *Achromobacter xylosoxidans* strain F3B for improving phytodepuration of phenolic pollutants. Journal of Hazardous Materials, 219, 43–49.

Ikeda A. C., Bassani, L. L., Adamoski, D., Stringari, D., Cordeiro, V. K., Glienke, C., Galli-Terasawa, L. V. (2013). Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes. Microbial Ecology, 65(1), 154–160.

Jasim B., Joseph, A. A., John, C. J., Mathew, J., & Radhakrishnan, E. K. (2014). Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of Zingiber officinale. 3 Biotech, 4(2), 197–204.

Jeong H., Jeong, D. E., Kim, S. H., Song, G. C., Park, S. Y., Ryu, C. M., … Choi, S. K. (2012). Draft genome sequence of the plant growth-promoting bacterium Bacillus siamensis KCTC 13613T. Journal of Bacteriology, 194(15), 4148–4149.

Kang J. W., Khan, Z., & Doty, S. L. (2012). Biodegradation of trichloroethylene by an endophyte of hybrid poplar. Applied and Environmental Microbiology, 78(9), 3504–3507.

Lamb T. G., Tonkyn, D. W., & Kluepfel, D. A. (1996). Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Canadian Journal of Microbiology, 42(11), 1112–1120.

Li Y. H., Liu, Q. F., Liu, Y., Zhu, J. N., & Zhang, Q. (2011). Endophytic bacterial diversity in roots of *Typha angustifolia* L. in the constructed Beijing Cuihu Wetland (China). Research in Microbiology, 162(2), 124–131.

Minana-Galbis D., Farfan, M., Fuste, M. C., & Loren, J. G. (2007). *Aeromonas bivalvium* sp. nov., isolated from bivalve molluscs. International Journal of Systematic and Evolutionary Microbiology, 57(2007), 582–587.

Moore F. P., Barac, T., Borremans, B., Oeyen, L., Vangronsveld, J., Van Der Lelie, D., Moore, E. R. B. (2006). Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: the characterisation of isolates with potential to enhance phytodepuration. Systematic and Applied Microbiology, 29(7), 539–556.

Nam I.-H., Chang, Y.-S., Hong, H.-B., & Lee, Y.-E. (2003). A novel catabolic activity of *Pseudomonas veronii* in biotransformation of pentachlorophenol. Applied Microbiology and Biotechnology, 62(2–3), 284–290.

Nojiri H., Maeda, K., Sekigushi, H., Urata, M., Shintani, M., Yoshida, T., Omori, T. (2002). Organization and Transcriptional Characterization of Catechol Degradation Genes Involved in Carbazole Degradation by *Pseudomonas resinovorans* Strain CA10. Bioscience, Biotechnology, and Biochemistry, 66(4), 897–901.

O'loughlin E. J., Sims, G. K., & Traina, S. J. (1999). Biodegradation of 2-methyl, 2-ethyl, and 2-hydroxypyridine by an Arthrobacter sp. isolated from subsurface sediment. Biodegradation, 10(2), 93–104.

O'Mahony M. M., Dobson, A. D. W., Barnes, J. D., & Singleton, I. (2006). The use of ozone in the remediation of polycyclic aromatic hydrocarbon contaminated soil. Chemosphere, 63(2), 307–314.

Onaca C., Kieninger, M., Engesser, K.-H., & Altenbuchner, J. (2007). Degradation of alkyl methyl ketones by *Pseudomonas veronii* MEK700. Journal of Bacteriology, 189(10), 3759–3767.

Pérez-Montaño F., Alías-Villegas, C., Bellogín, R. A., Del Cerro, P., Espuny, M. R., Jiménez-Guerrero, I., … Cubo, T. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiological Research, 169(5–6), 325–336.

Philippot L., Raaijmakers, J. M., Lemanceau, P., & Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 11(11), 789.

Sauvêtre A., May, R., Harpaintner, R., Poschenrieder, C., & Schröder, P. (2018). Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from *Phragmites australis*. Journal of Hazardous Materials, 342, 85–95.

Sepúlveda-Torres L. D. C., Rajendran, N., Dybas, M. J., & Criddle, C. S. (1999). Generation and initial characterization of *Pseudomonas stutzeri* KC mutants with impaired ability to degrade carbon tetrachloride. Archives of Microbiology, 171(6), 424–429.

Siciliano S. D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., … Banks, M. K. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and Environmental Microbiology, 67(6), 2469–2475.

Soares M. A., Li, H. Y., Kowalski, K. P., Bergen, M., Torres, M. S., & White, J. F. (2016). Functional role of bacteria from invasive *Phragmites australis* in promotion of host growth. Microbial Ecology, 72(2), 407–417.

Stottmeister U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., … Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnology Advances, 22(1–2), 93–117.

Tsuda K., Kosaka, Y., Tsuge, S., Yasuyuki, K., & Horino, O. (2001). Evaluation of the endophyte Enterobacter cloacae SM10 isolated from spinach roots for biological control against Fusarium wilt of spinach. Journal of General Plant Pathology, 67(1), 78–84.

Vymazal J. (2011). Constructed Wetlands for Wastewater Treatment Reference Module in Earth Systems and Environmental Sciences, 45(April), 1–9.

Westerberg K., Elväng, A. M., Stackebrandt, E., & Jansson, J. K. (2000). *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol. International Journal of Systematic and Evolutionary Microbiology, 50(6), 2083–2092.

Weyens N., Van Der Lelie, D., Artois, T., Smeets, K., Taghavi, S., Newman, L., … Vangronsveld, J. (2009). Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytodepuration. Environmental Science & Technology, 43(24), 9413–9418.

Yen K. M., Karl, M. R., Blatt, L. M., Simon, M. J., Winter, R. B., Fausset, P. R., … Chen, K. K. (1991). Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. Journal of Bacteriology, 173(17), 5315–5327. https://doi.org/10.1128/jb.173.17.5315-5327.1991

Yousaf, S., Afzal, M., Reichenauer, T. G., Brady, C. L., & Sessitsch, A. (2011). Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. Environmental Pollution, 159(10), 2675–2683.

Zhang, C., Wang, B., Dai, X., Li, S., Lu, G., & Zhou, Y. (2017). Structure and function of the bacterial communities during rhizoremediation of hexachlorobenzene in constructed wetlands. Environmental Science and Pollution Research, 24(12), 11483–11492.

Zhang, C., Wang, B., Dai, X., Li, S., Lu, G., & Zhou, Y. (2017). Structure and function of the bacterial communities during rhizoremediation of hexachlorobenzene in constructed wetlands. Environmental Science and Pollution Research, 24(12), 11483–11492.

7. Conclusions

The association between plants and microorganims is known to be very ancient, and in the last decades this close interaction has been more investigated since important evidences have been highlighted: endophytes are able to influence the plant's metabolism, promote its growth, offer resistance under stress conditions, and enhance the degradation of toxic compounds. Thus, endophytes represent a relevant field of research and an important source of bioactive molecules.

In this context, the aim of this work was to analyze bacterial endophytic communities from different sources (medicinal plants tissues/seeds and hyperaccumulator plants), in order to deepen the knowledge on the composition and phenotypic traits of their communities, and to evaluate a possible use of endophytic isolates for biotechnological purposes.

The possible factors involved in structuring endophytic bacterial communities were evaluated for two congeneric medicinal plants, *E. purpurea* and *E. angustifolia*. Bacterial communities associated to these plants were evaluated for phenotypic traits, such as extracellular enzymatic activity (EEA), siderophore (SPH) and indole-3-acetic acid (IAA) production. Also, antagonistic interactions were evaluated among strains from *E. angustifolia*, and between the strains of the two plant species. The performed analyses allowed to evidence that bacterial endophytes themselves could participate in the structuring of their own communities, by means of antimicrobials production which determine the colonization of bacteria with adaptive resistant phenotypes.

The genome sequences of bacterial endophytes extracted from *Echinacea* spp. plants, exhibiting important phenotypes, such as inhibition of human opportunistic pathogens, were analyzed. Data showed that such strains present gene clusters putatively involved in secondary metabolites production, suggesting that they could be potential sources of new antibiotic molecules.

The presence and localization of endophytes within plant seeds were evidenced for *Echinacea* spp. seeds, and endophytic bacterial strains were obtained and characterized from *E. purpurea* seeds. The interesting evidences obtained from this part of the work suggest that plant seeds should be furtherly analyzed, in order to deepen the knowledge on this particular plant microbiota and to investigate on its possible biological role.

Bacterial communities associated to the hyperaccumulator plant *P. australis*, collected in a constructed wetland, were taxonomically and phenotypically characterized. Results showed that different tissues of such plants and their rhizosphere are inhabited by different cultivable bacterial communities, dominated by the genus *Pseudomonas*, and that the

composition of root cultivable bacterial communities varies in different periods of time. Phenotypic analyses performed on the root communities, interestingly highlighted that many endophytic isolates are able to thrive in presence of high concentrations of critical compounds for wastewater treatment, suggesting a possible application of such endophytes to enhance the phytodepuration process. The analyses performed on bacterial communities associated to *P. australis* plants represent an important starting point to better understand the phytodepuration process and to improve its efficiency, through a reasonable utilization of bacterial endophytes.

Together, the evidences obtained from this work underline that endophytes are indeed a valuable field of research, which allows to deepen our knowledge on the plant-microbiota interaction, and to possibly evidence important phenotypes in endophytic communities, which could help answering to different clinical and environmental issues.

8. Appendix

8.1 Spatial structuring of bacterial communities in epilithic biofilms in the Acquarossa river (Italy)

The Acquarossa site is characterized by a river with red-colored water and epilithic biofilms on the rocks around it. The biofilms are red or black colored, and co-exist very closely, without blending together. This environment captured our curiosity, in particular with regards to the red and black epilithons. Thus we decided to characterize bacterial communities present in the both epilithons, concentrating on biotic and abiotic factors that may drive the structuring of black and red epilithic biofilms. Bacterial communities from both epilithions were taxonomically and phenotipically characterized, analyzing antibiotic resistance patterns, heavy metal resistance and antagonistic interactions. The red and black epilithons turned out to be characterized by different bacteria taxa, with a predominance of iron-oxidizing strains in red epilithon and *Acinetobacter* genus in black epilithon. Among the phenotypic analyses performed, antibiotic resistance and antagonistic interactions seem to affect the structuring of both epilithic biofilms. The obtained results show that biotic factors, especially selection of population at a small scale, could be implied in the structuring of bacterial communities.

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RESEARCH ARTICLE

Spatial structuring of bacterial communities in epilithic biofilms in the Acquarossa river (Italy)

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One sentence summary: Structure of bacterial communities in black and red epilithic biofilm in the Acquarossa river (Viterbo, Italy) has been investigated with both culture dependent and independent approaches.

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ABSTRACT

Epilithic river biofilms characterize the rock surfaces along the Acquarossa river (Viterbo, Italy); they are in part red and in part black colored, maintaining a well-defined borderline. This peculiarity has raised questions about the biotic and abiotic phenomena that might avoid the mixing of the two biofilms. In this study, the structuring of bacterial communities in black and red epilithic biofilm in the Acquarossa river has been investigated with both culture dependent and independent approaches. Data obtained highlighted a (very) different taxonomic composition of black and red epilithons bacterial communities, dominated by *Acinetobacter* sp. and iron-oxidizing bacteria, respectively. The chemical characterization of both river water and biofilms revealed a substantial heavy metals pollution of the environment; heavy metals were also differentially accumulated in red and black epilithons. Overall, our data revealed that the structuring of red and black epilithons might be affected mainly by the antagonistic interactions exhibited by bacterial genera dominating the two biofilms. These findings suggest that biotic factors might be responsible for the structuring of natural bacterial communities, suggesting that there is a selection of populations at very small scale, and that different populations might compete for different niches.

Keywords: biofilm; epilithon; antagonistic interactions; *Pseudomonas*; *Acinetobacter*

INTRODUCTION

Understanding the ecological processes that lead to the structure and the function of microbial communities in the environment is a field that raised great interest in recent years, due to the crucial roles that they play in natural ecosystems, human health and industrial biotechnology (Kastman *et al.* 2016; Widder *et al.* 2016). Interestingly, biotic interactions among microbes have been widely described as a force driving the structuring of environmental communities (Maida *et al.* 2016 and references therein). The assemblage of strains in a microbial community is the result of many factors such as random drift, selection by abiotic conditions, and biotic interactions (Stoodley *et al.* 2002). Abiotic conditions have largely been studied in the past years (e.g. Mathur *et al.* 2007; Rubin and Leff 2007); they vary depending on many factors (e.g. environmental conditions) and can be

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Table 1. Basic physico-chemical variables and concentration of nitrogen and sulfur species as well as heavy metals in the Aquarossa river water.

Parameter	Acquarossa (river water)
pH	6.50
Conductivity (μS/cm 20°C)	843
Ammonium (NH_4^+) (mg/l)	0.1
Nitrites (NO_2) (mg/l)	<0.05
Nitrates (NO_3) (mg/l)	<1
Sulfates (mg/l)	<40
Sulfides (mg/l)	<0.1
COD (mg/l O_2)	<10
Cd (mg/l)	<0.01
Ni (mg/l)	<0.05
Cu (mg/l)	<0.05
As (mg/l)	<0.02
Zn (mg/l)	<0.01
Fe tot (mg/l)	1.788
Fe^{2+} (mg/l)	0.733
Fe^{3+} (mg/l)	1.05

manipulated at lab scale to understand their influence on bacterial community composition (Stubbendieck and Straight 2016). On the other hand, the complexity of biotic interactions, which play a major role by altering the structure and the degree of organization of complex communities, are challenging to investigate at lab scale and difficult to understand (Moënne-Loccoz *et al.* 2015; Battin *et al.* 2016). Epilithic biofilms are microbial communities (Battin *et al.* 2007) whose structure and composition have been investigated (e.g. Kobayashi *et al.* 2009; Ledger and Hildrew 1998) leading to the suggestion that structure and composition of river epilithic biofilms can vary in response to many factors, such as anthropogenic nutrient and organic matter (Kobayashi *et al.* 2009). Geographical factors, related for example to the altitudinal gradient, play also an important role in the composition and diversity of epilithic communities (Bartrons, Catalan and Casamayor 2012; Besemer *et al.* 2013; Wilhelm *et al.* 2015). Moreover, the interaction between prokaryotic and eukaryotic microbial communities in epilithons has been recently investigated (Zancarini *et al.* 2017).

Epilithic biofilm of the Acquarossa river (Viterbo) are particularly intriguing in terms of spatial structuring. Interestingly, they form two physically separated colored biofilms, that are in part red and in part black colored. Black and red epilithons can be found on the rocks very close to each other, but they do not blend together, maintaining a well-defined borderline. This peculiarity has raised questions about the biotic and abiotic phenomena that might avoid the mixing of the two biofilms. The Acquarossa site is characterized by the presence of an ancient Etruscan village (625–550 B.C.), historically known for its metallurgic activity (Harrison, Cattani and Turfa 2010), which caused a contamination due to the spread of significant amounts of undesirable heavy metals, especially arsenic, into the environment (Hook 2007). The river is also characterized by a high iron concentration, conferring a red color to the water and giving to the river its name Acquarossa, that in Italian stands for 'red water'. From a scientific viewpoint, there is an almost complete lack of information on this site, especially concerning the biological and environmental features; indeed, most of the available literature focuses on its archaeological and historical characteristics (e.g. Staccioli 1976; Meyers, 2003, 2013).

The few available information on the Acquarossa site are mainly focused on the biological aspects and characteristics of the whole environment; the lack of information on the microbiological aspects of the river arose great interest in this unexplored site.

For these reasons, the goal of this study is to assess the characterization of the bacterial communities inhabiting this environment, focusing on the abiotic and biotic factors that may drive the structuring of black and red epilithic biofilms. To this purpose, both a culture dependent and a culture independent approach have been applied to determine the structure and composition of bacterial communities of red and black epilithon. Physico-chemical parameters were also measured in the river water and in the two biofilms. Moreover, phenotypic characterization of the main bacterial genera detected through cultivation has been assessed, focusing on resistance patterns towards heavy metals and antibiotics, and on the antagonistic interactions between the two communities.

MATERIALS & METHODS

Site and sample collection

Samples were collected on July 2016 near the Etruscan city of 'Acquarossa', 42°28′47.0′N 12°07′17.3′E. The site of naturalistic and archaeological interest is located halfway between Viterbo and the town of Grotte Santo Stefano (Viterbo, Italy). Fig. 1a shows a detail of the Acquarossa river course. Fig. 1b and c shows details of the red and black epilithic biofilms in the sampling site. Several physico-chemical variables were measured, namely: pH, conductivity, concentrations of ammonium (NH_4^+), nitrites (NO_2), nitrates (NO_3), Chemical Oxygen Demand (COD), sulfates, sulfides, total iron content, Fe^{2+}, Fe^{3+} and heavy metals (Cu, Zn, As, Cd, Ni).

The concentrations of the following heavy metals were measured in black and red biofilms: Cu, Zn, As, Cd, Ni and total iron content.

All the analyzes mentioned above were performed by an external service. Determination of the concentration of heavy metals was performed according to UNI EN 13 657:2004 and UNI EN ISO 11 885:2009 methods (Table 1). Fe^{2+} and Fe^{3+} were measured following the Standard Methods '3500-Fe B. Phenanthroline Method' (Copyright 1999 by American Public Health Association, American Water Works Association, Water Environment Federation).

Six samples, three replicates from the red epilithon and three replicates from the black one, were collected for both culture dependent analysis, and for High-Throughput Sequencing (HTS) analysis.

Extraction of genomic DNA, HTS, sequence analysis and statistical testing

DNA extraction was performed from each sample by using PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc., Carlsbad, California, USA) following the manufacturer's instruction. Concentration and purity of extracted DNA were checked by 0.8% agarose gel electrophoresis. Bacterial 16S rRNA gene contains conserved sequences and nine hypervariable regions (V1–V9), whose lengths range from approximately 50–100 bases (e.g. Chakravorty *et al.* 2007; Petrosino *et al.* 2009). Hypervariable regions are used as molecular markers for bacterial identification in HTS analysis (e.g. Huse *et al.*

Figure 1. The Acquarossa river (A) and details of the red (B) and black (C) epilithic biofilms.

2008). For this reason, on the extracted DNAs the bacterial V4 region of 16S rRNA genes was amplified with specific primers (515F, TGYCAGCMGCCGCGGTAA; 806R GGACTACNVGGGTWTCTAAT, Caporaso *et al.* 2011) using the protocol reported in the 16S Metagenomic Sequencing Library Preparation protocol from Illumina (Illumina 2013). Library preparation and demultiplexing have been performed following Illumina's standard pipeline (Caporaso *et al.* 2012). Libraries were sequenced in a single run using Illumina MiSeq technology with pair-end sequencing strategy with MiSeq Reagent Kit v3. PCR amplification, library construction and sequencing were performed by an external company (IGA Technology Services, Udine, Italy). Sequences were merged and clustered using the UPARSE pipeline (version 10.0.240) (Edgar 2013). Reads were merged with the 'fastq_mergepairs' command setting the identity threshold to 80% to account for long overlaps (2 × 300bp, V3 – V4). Low complexity reads that may have been generated during sequencing were detected and removed using the 'filter_lowc' command before quality assessment. Merged reads were then quality checked using StreamingTrim 1.0 with a quality cut-off of 18 Phred (Bacci *et al.* 2014). An additional quality filtering step was performed using the 'fastq_filter' command of the UPARSE pipeline with a maximum expected error threshold of 1. Obtained sequences were truncated to a fixed length of 420 in order to remove PCR primers and retain only amplicons with the expected length. Finally, sequences were clustered into Operational Taxonomic Units (OTUs) by using the 'cluster_otus' command and representative sequences were taxonomically classified using the 'sintax' command along with the RDP training set (version 16). Representative sequences that were not assigned to Domain Bacteria were removed from the dataset. The OTU table was produced with the 'otutab' command.

The following analyzes were performed by using the R software version 3.4.4 (R Core Team 2014; http://www.R-project.org). Assigned OTUs were normalized by cumulative sum scaling normalization implemented in the metagenomeSeq package (version 1.16) (Paulson *et al.* 2013). Differences in OTU abundances between red and black epilithon were assessed using a zero-inflated log-normal model through the 'fitFeatureModel' function whereas different community structures were tested using permutational multivariate analysis of variance ('adonis2' function of the vegan R package, version 2.4, Dixon 2003) with 1000 permutations. Taxonomic units reporting adjusted *P* values lower than 0.05 were considered as characteristic of a biofilm type and were analyzed separately. Normalized counts were used for principal component analysis (PCA) after counts standardization ('decostand' function of the vegan package version 2.4). Environmental factors (black and red epilithon) were fitted onto the ordination analysis using the 'envfit' function of the vegan package (version 2.4). Bacterial diversity was estimated using R (vegan package version 2.4, Dixon 2003). The function was used to compute the Shannon index (H) (Hill 1973) whereas species evenness (J) (Hill 1973) was estimated as a function of the Shannon diversity and the number of OTUs detected (S), according to the Pielou's formula $J = H / \log(S)$. Differences in bacterial diversity between black and red epilithon were tested using Student's t-test ('t.test' R function). Correlation coefficients between culture dependent and culture independent methods were computed correcting the number of reads assigned to each genus by the average number of 16S rRNA gene copies as reported in the rrnDB (Stoddard *et al.* 2014). Raw data have been submitted to NCBI SRA archive under the BioProject accession PRJNA412007.

Isolation, taxonomical characterization of culturable bacterial strains and statistical analysis

Bacterial plate counts were assessed as described in Chiellini et al (2014) in Tryptic Soy Agar (TSA) medium using 1 g of biofilm of each sample. Bacterial plate counts were carried out after 48 h incubation at 21°C, and the t-test was applied using PAST3 software (Hammer, Harper and Ryan 2001) to check for any difference in the enumeration of culturable bacterial community between red and black epilithon. For each sample, 12–59 colonies were isolated on solid TSA medium and stored at −80°C in glycerol (20% final concentration) for further analysis. The variable number of isolates was determined by the number of isolated colonies that could be recovered during plate count enumeration.

Cell lysates of bacterial isolates were prepared by processing an isolated colony dissolved in 30 μl of distilled sterile water with thermal lysis (95°C for 10 min, followed by cooling on ice for 5 min). For each isolated strain, the 16S rRNA gene was amplified following the protocol described in Chiellini *et al.* (2014). Briefly, PCR amplification of 16S rRNA genes was carried out in 20-μl reactions using DreamTaq DNA Polymerase reagents (Thermofisher Scientific, Waltham, MA, USA) at the concentrations suggested by the company, and 0.5 μM of primers P0 (5′-GAGAGTTTGATCCTGGCTCAG) and P6 (5′-CTACGGCTACCTTGTTACGA) (Di Cello and Fani 1996); 1 μl of cell lysate was used as template. Amplification conditions were the following: 90-s denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 50°C and 1 min at 72°C, followed by a final extension of 10 min at 72°C. Direct sequencing of the amplified 16S rRNA genes was performed with primer P0 by an external company (IGA Technology Services, Udine, Italy). Each 16S rRNA gene sequence was submitted to GenBank and assigned an accession number from MF964679 to MF964869. Taxonomic affiliation of the 16S rRNA gene sequences was attributed using the 'classifier' tool of the Ribosomal Database Project—RDP (Cole *et al.* 2014). Alpha diversity indices (Shannon, Evenness and Chao1) were calculated using PAST3 software (Hammer, Harper and Ryan 2001) on the taxonomic composition of the cultivable fraction of bacteria

from black and red epilithon (genus level). BioEdit Software (Hall 1999) was used to align the obtained sequences together with high-quality sequences of closely related Type Strains that were previously downloaded from the RDP database. MEGA5 Software (Tamura *et al.* 2011) was used for phylogenetic tree construction, by using the Neighbor Joining algorithm; the robustness of the inferred trees was evaluated by 1000 bootstrap resampling.

Random amplified polymorphic DNA analysis.

Random amplification of DNA fragments (Williams *et al.* 1990) was independently performed on *Pseudomonas* sp. and *Acinetobacter* sp. bacterial strains. Reactions were performed in a 25-μl total volume, as described in Chiellini et al (2014) using primer 1253 (5′-GTT TCCGCCC-3′) (Mori *et al.* 1999). For each genus, strains showing the same Random amplified polymorphic DNA (RAPD) profile were grouped together into the same haplotype. Alpha diversity indices (Shannon, Evenness and Chao1) were calculated using PAST3 software (Hammer, Harper and Ryan 2001) on the results obtained from haplotype attribution, considering the number of strains attributed to each detected haplotype.

Phenotypic characterization of *Acinetobacter* and *Pseudomonas* spp. bacterial strains: resistance to antibiotics and heavy metals, and antagonistic interactions

The resistance patterns to six antibiotics and to six different heavy metals were obtained through the broth microdilution methods in Muller Hinton Broth (MHB), according to the protocols of Clinical and Laboratory Standards Institute (Jorgensen 1993). Briefly, 96-well plates were used for the experiments. Each well contained 10 µl of bacterial inoculum and 90 µl of MHB medium enriched with each tested substance at different concentrations, in order to obtain the requested final concentration in each well. The bacterial inoculum was prepared by dissolving in liquid MHB the isolated bacterial colonies after 48 h growth at 21°C in solid medium; when the suspension was adjusted to 0.5 McFarland (corresponding to 1×10^8 CFU/mL), a 1:20 dilution in MHB medium was then performed, in order to yield 5×10^6 CFU/mL in a volume of 10 µl. The final test concentration of bacteria was approximately 5×10^5 CFU/mL.

Six antibiotics belonging to different classes and having different cellular targets were chosen and tested at different concentrations: Tetracycline (0.5–1.25–2.5–5–12.5–25 μg/ml), Chloramphenicol (1–2.5–5–10–25–50 μg/ml), Rifampicin (5–10–25–50–100 μg/ml), Ciprofloxacin (0.5–1–2.5–5–10 μg/ml), Streptomycin and Kanamycin (0.5–1–2.5–5–10–50 μg/ml).

Six different heavy metals were chosen and tested at the following concentrations: Cu ($CuCl_2$ - 1–2.5–5-10 mM), Ni ($NiCl_2$ 5–10-15–25 mM), Zn ($ZnSO_4$ 5–10-15–25 mM), Cd ($Cd(NO_3)_2$ 1–2.5–5-10 mM), As (V) (KH_2AsO_4 0.5–1-2.5–5-7.5–10-12.5–25 mM) and As (III) ($NaAsO_2$ 0.5–1-2.5–5-10–15) mM. Results from Broth microdilution methods applied for antibiotics and heavy metals were validated by using TECAN microplate reader (Tecan, Durham, USA) at 600 nm wavelength, after 48 h incubation at 21°C.

Antagonistic interactions among 36 bacterial strains were tested by the cross-streak method (Arasu, Veeramuthu and Savarimuthu 2013; Thirumurugan and Vijayakumar 2015) using a 36 × 36 (total 1296) array of tests, as described in Maida et al (2016). All tests were performed in TSA medium. Antagonism assays were performed both with petri dishes with or without a septum separating the two hemi-cycles. In petri dishes without septum, the inhibitory activity might be due to both the presence of volatile and of molecules diffusing in the agar medium; in petri dishes containing a central septum, the inhibition is due to the production of volatile compounds only, since the septum physically separates the two chambers hosting the tester and the target strains, respectively.

Table 2. Concentration (mg/kg) of heavy metals in red and black epilithic biofilms collected from the Acquarossa river.

Heavy metal (mg/kg)	Red epilithon	Black epilithon
Cd	<1	<1
Ni	<5	54
Cu	<5	17
As	7	17
Zn	14	108
Fe tot	54 314	11 668

Statistical analysis of phenotypic results

Phenotypic characterization results were organized in a table with different colors corresponding to different levels of growth/inhibition. For both heavy metals and antibiotic resistance pattern experiments, a positive control consisting on the bacterial inoculum in MHB medium was assessed to verify the growth of each strain in the absence of heavy metal or antibiotic. A negative control, consisting of MHB medium, was also set up in triplicate in each microplate, to verify the absence of any external contamination. The OD_{600} value measured after 48 h incubation at 21°C for the positive control, was considered as the 100% value. All the other measured values corresponding to the different concentrations of each tested substance, were reported as percentage of growth in proportion to the positive control. The data matrix containing the data of growth expressed as % on the positive control has been used for statistical analysis (UPGMA with Bray Curtis distance measure, and PCA) with PAST3 software (Hammer, Harper and Ryan 2001), as described in Mengoni et al (2014).

The results from the cross-streak inhibition assay were organized in two distinct inhibition matrices representing the test performed with both petri dishes with or without septum; in each matrix, rows stand for a target strains and each column stands for a tester strain, as described in Maida et al (2016). The inhibition values corresponding to three different inhibition levels observed during the cross-streak experiment are complete (red), strong (orange), weak (yellow) and absence (white) of inhibition.

RESULTS

Physico-chemical characterization of river water and epilithic biofilms

The values of the measured water variables are resumed in Table 1; the concentration of heavy metals tested in the biofilms is shown in Table 2. The pH of the river water was 6.5, and the conductivity was 1133 µS/cm. Data obtained revealed that water samples collected in the Acquarossa river were enriched in iron (total content 1.788 mg/l). The Fe^{2+} and Fe^{3+} content in the Acquarossa river water was 0.733 and 1.05 mg/l, respectively. The total iron content within epilithic biofilm matrix was about four orders of magnitude higher in respect to river water content. Moreover, total iron content of red epilithic biofilm was

Table 3. Mean values ±SEM of richness and diversity indices calculated for both type of biofilms (n = 3). *P* values resulting from Student t-test are also shown.

Index	Mean ± SEM		*P*
	Red epilithon	Black epilithon	
Shannon	3.86 ± 0.121	3.73 ± 0.666	0.86
Simpson	0.95 ± 0.001	0.87 ± 0.070	0.34
Richness	784.67 ± 183.550	870.33 ± 217.428	0.78
Evenness	0.59 ± 0.007	0.56 ± 0.094	0.79

five times higher than black epilithic biofilm. Quantification of Cd, Ni, Cu, As and Zn revealed that these heavy metals accumulated in the epilithic biofilms. Particularly, the heavy metals content within the biofilm matrix was almost 100 times higher for Cd, 100–1000 times higher for Ni, Cu and As, and up to 10^4 times higher for Zn respect to the river water.

HTS analysis

Sequencing yielded 6 804 040 paired sequences (2 × 300 bp) with a mean of 567 003 sequences per pair. More than 50% of the initial pairs were correctly merged (1 822 737 sequences) with a mean of 151 894 sequence *per* sample. Quality filtering step produced 1 238 851 high-quality sequences that were clustered into 1850 OTUs. Representative sequences for each OTU were correctly classified into 276 genera, 212 families, 114 orders, 84 classes and 35 phyla according to the RDP database (see Materials and Methods). Sequences were correctly mapped to OTUs with 80 262 sequences per sample on average.

Samples from red and black epilithon showed similar degree of bacterial diversity according to the main indices computed (Table 3). Indeed, no significant differences were found between the two biofilms studied (Student's t-test). The taxonomic distribution of bacterial phyla in each biofilm, obtained through HTS of the 16S rRNA gene, is reported in Fig. 2. Both epilithons were characterized by a dominance of *Proteobacteria* (ranging from 41% to 89%), followed by *Bacteroidetes* and *Acidobacteria* (ranging from 0.5% to 20% and from 2% to 37%, respectively). Replicates from red epilithon appeared to be more homogeneous in respect to replicates from black epilithon. This finding was also confirmed by a principal component analysis (PCA, Fig. 3) revealing that black epilithon samples were more different than those coming from the red one, spanning a higher range of the plot. However, both multivariate analysis of variance and environmental fitting on PCA did not support a statistically significant difference in the whole community assemblage ($P = 0.1$; environmental fitting, $R^2 = 0.8$; $P = 0.1$).

At a finer scale, 14 OTUs were more represented in red epilithon samples whereas only one was more abundant in black epilithon (zero-inflated log-normal model, $p < 0.05$, adjusted using false discovery rate correction) (Fig. 4). Interestingly, while *Gammaproteobacteria* appeared the most abundant class in red epilithon (Fig. 2), at OTU level, there was a dominance by members of *Betaproteobacteria*, as members of *Gallionellaceae* family (15%), *Sideroxydans* species (11%) and *Gallionella* species (11%). On the contrary *Acinetobacter* was the dominant genus in black epilithon.

Culturable bacterial community analysis and taxonomic diversity

Analysis of the concentration of heterotrophic cultivable bacteria in red and black epilithic biofilm samples revealed that they contained from 5.9×10^4 to 7.5×10^7 CFU/g (Table 4). No significant differences between the two epilithons were detected ($p > 0.5$).

To check the structure and the composition of the cultivable bacterial communities isolated from the two epilithons, we used a two-step strategy. First, we amplified the 16S rRNA gene from bacterial isolates randomly chosen coming from the two epilithons. Once we obtained a phylogenetic affiliation, a RAPD analysis was carried out to check if the different bacterial isolates corresponded to the same or different strains.

Phylogenetic analysis of 16S rRNA gene sequences from the bacterial isolates obtained (Fig. 5) revealed that the black epilithon was dominated by bacteria affiliated to *Acinetobacter* sp. (56.6% *vs* 6.5% of red epilithon), whereas *Pseudomonas* sp. was prevalent in red epilithon (53.2% *vs* 8.5% of black epilithon). *Curtobacterium* sp. and *Sphingobacterium* sp. were detected in black epilithon samples, but with low percentages (7.8% and 2.3% respectively). *Bacillus* sp. and *Aeromonas* sp. were two other bacterial genera contributing to the differentiation of the two kinds of biofilm; *Bacillus* sp. was mostly present in black epilithon (12.4%) with respect to red epilithon (3.2%) while *Aeromonas* sp. showed a greater abundance in red epilithon (16.1%) with respect to black epilithon (1.6%). Even though the Chao1 index was higher in black epilithon than in the red one (11.33 *vs* 9), and the Evenness value was higher in red epilithon (0.5313) than in the black one (0.4212), the differences were not statistically significant ($p = 0.07$ in both cases). On the contrary, Shannon index was significantly different in the two epilithons ($p = 0.006$), showing a value higher in red than in black epilithon (1.565 *vs* 1.533, respectively).

A good correlation between the taxonomic composition (at the genus level) detected with both culture dependent and culture independent methods was found (Fig. S1, Supporting Information). *Pseudomonas* and *Acinetobacter* were among the most abundant genera detected in both black and red epilithons, with the former exhibiting a higher presence in the cultured isolates than in HTS analysis.

Due to the dominance of cultivable *Acinetobacter* or *Pseudomonas* in the two epilithons, we further inspected the genetic structure and the degree of strain sharing of the *Acinetobacter* and *Pseudomonas* populations associated to the two epilithons using the RAPD technique. The analysis was performed on a total of 77 and 44 *Acinetobacter* sp. and *Pseudomonas* sp. isolates, respectively. Each RAPD fingerprinting obtained was compared with the other ones to split the bacterial isolates into groups (or haplotypes), very likely corresponding to different strains. Data revealed that the 77 *Acinetobacter* and the 44 *Pseudomonas* strains were split into 12 and 19 RAPD haplotypes, respectively, and the two epilithons harbored distinct populations of *Pseudomonas* and *Acinetobacter* (Tables S1a and b, Supporting Information). Moreover, the higher values of richness and diversity indices calculated for the cultivable fraction of bacterial communities in red and black biofilms suggested that the genetic diversity of *Pseudomonas* community was higher than that of the *Acinetobacter* one (Table S2, Supporting Information). Very interestingly, the two epilithons did not share any *Pseudomonas* and/or *Acinetobacter* strain, suggesting the existence of a hidden/unknown factor driving the structuring of these communities. The phylogenetic analysis on *Acinetobacter* sp. (Fig. S2, Supporting Information)

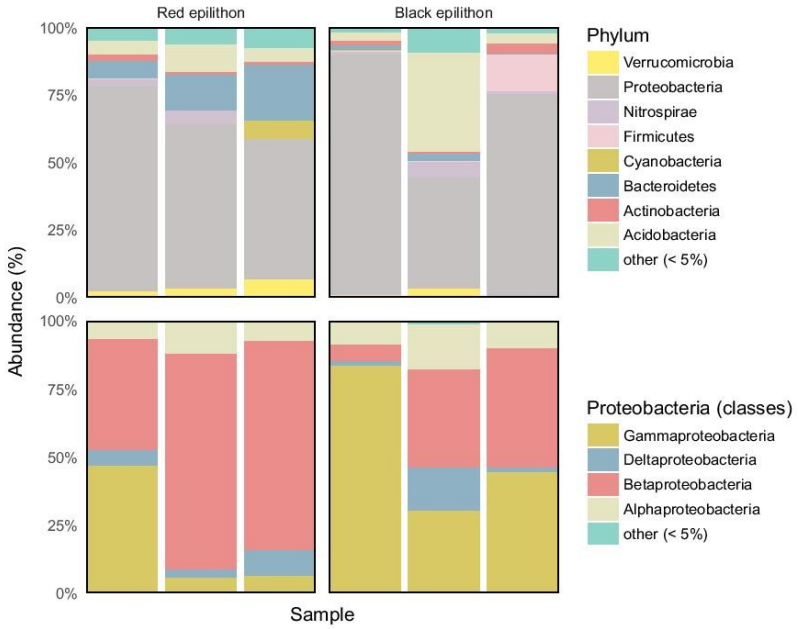


Figure 2. Affiliation of 16S rRNA sequences obtained from red (left panels) and black (right panels) epilithic biofilms. Phyla with relative abundance lower than 0.5% were grouped together and labelled as 'Other taxa'.

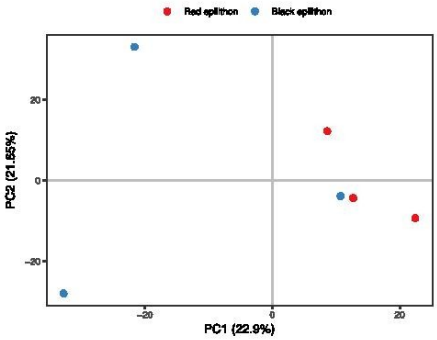


Figure 3. PCA analysis of HTS data on samples collected in red and black epilithons.

and *Pseudomonas* sp. strains (Fig. S3, Supporting Information), confirmed the separation of the strains in the different haplotypes detected by RAPD analysis. Indeed, data revealed that isolates exhibiting the same RAPD profile, clustered together in the phylogenetic tree. Moreover, the phylogenetic analysis confirmed the higher genetic diversity of *Pseudomonas* in respect to *Acinetobacter* strains.

Phenotypic characterization of *Pseudomonas* and *Acinetobacter* community

In order to obtain insights into the (different) phenotypic features of *Acinetobacter* and *Pseudomonas* strains, some phenotypic tests (resistance to heavy metals and antibiotics, antagonistic interactions) were carried out on a panel of 13 *Acinetobacter* sp. strains (3 from red epilithon and 10 from black epilithon) and 23 *Pseudomonas* sp. strains (8 from black epilithon and 15 from red epilithon), representative of all the identified RAPD haplotypes.

The *Pseudomonas* and *Acinetobacter* strains exhibited different resistance patterns to antibiotics (Table S3, Supporting Information); this was particularly evident for Streptomycin and Rifampicin. Indeed, most *Pseudomonas* strains grew at Streptomycin concentrations up to 10–50 μg/ml, while most *Acinetobacter* strains grew up to 5–10 μg/ml. Similarly, *Pseudomonas* strains were able to grow at Rifampicin concentrations up to 5–10 μg/ml, while *Acinetobacter* strains were sensitive to all the tested concentrations of this antibiotic.

Concerning heavy metals, *Pseudomonas* strains were more sensitive to Arsenate than *Acinetobacter* ones; on the contrary, *Pseudomonas* could grow on Cadmium (up to 5 mM), while *Acinetobacter* were sensitive to all the Cadmium tested concentrations. When analyzed separately, heavy metals resistance patterns did not reveal any strong separation between *Acinetobacter* and *Pseudomonas* communities (Fig. 6a); conversely, antibiotic resistance patterns allowed a strong and complete separation between the two distinct communities (Fig. 6b).

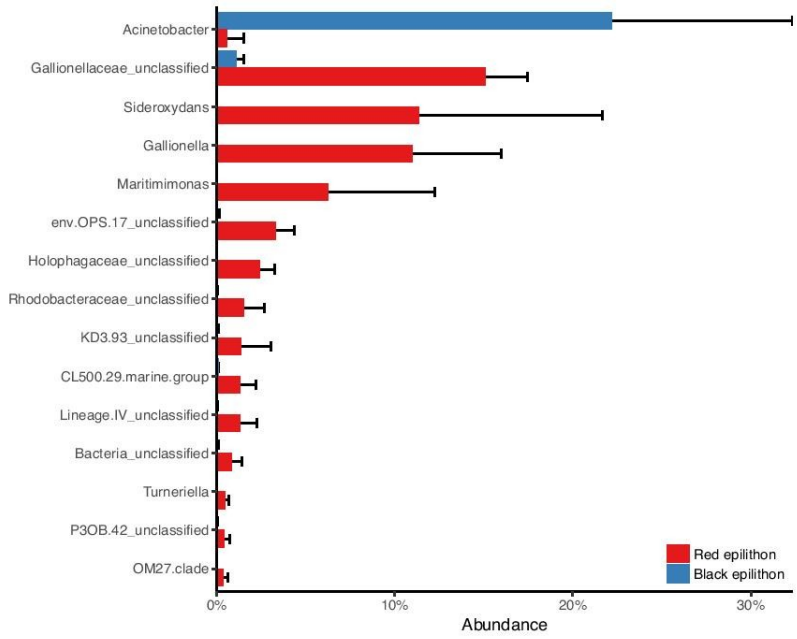


Figure 4. Top—HTS analysis results of bacterial phyla distribution within samples collected in red and black epilithon. Phyla with a relative abundance lower than 0.5% were pooled together and labelled as 'Other'. Bottom—Distribution of classes belonging to Proteobacteria phylum.

Table 4. Viable counts of heterotrophic bacteria in red and black epilithic biofilms.

Sample ID	Type of biofilm	Bacterial plate count (CFU/g)	Number of isolated strains
1	Black	$2.08 \pm 0.09 \times 10^7$	63
5	Black	1.65×10^5	35
6	Black	$2.63 \pm 0.02 \times 10^6$	106
2	Red	$7.53 \pm 3.5 \times 10^7$	38
3	Red	$1.73 \pm 0.4 \times 10^6$	39
4	Red	$5.88 \pm 4.4 \times 10^4$	12

Antagonistic interactions experiments carried out between *Acinetobacter* sp. and *Pseudomonas* sp. and between strains belonging to the same genus (*Acinetobacter vs Acinetobacter* and *Pseudomonas vs Pseudomonas*) using single-chambered petri dishes highlighted a strong inhibitory activity of *Pseudomonas* sp. strains against the *Acinetobacter* strains and a moderate inhibitory activity between *Pseudomonas* sp. strains (Table 5, upper part). Moreover, since it is known that bacterial strains can produce volatile organic compounds that may inhibit the growth of other bacteria (Papaleo *et al.* 2013), the same experiments were carried out using two-chambered petri dishes with a central septum physically separating the tester strain from the target ones. Data obtained are shown in Table 5 (lower part) whose analysis revealed that the *Pseudomonas* sp. strains were less active against *Acinetobacter* strains; moreover, the inhibition among *Pseudomonas* strains disappeared completely. On the other side, *Acinetobacter* sp. strains exhibited an almost complete absence of inhibition activity against *Pseudomonas*, either with single-chambered or two-chambered petri dishes, with the only exception represented by *Acinetobacter* strain 1.7, which showed a slightly moderate inhibitory activity *vs Pseudomonas* strains when grown on single-chambered petri dishes.

DISCUSSION

The Acquarossa river (Viterbo, Italy) represents a still unexplored site of naturalistic interest. To the best of authors' knowledge this is the first study exploring the structure and complexity of bacterial communities of rock biofilms along the river course, focusing on both chemical and microbiological aspects. Data obtained in this work shed some light on the role that biotic factors might play in driving the structuring of the bacterial communities of rock biofilms.

On the basis of HTS data, iron-oxidizing bacteria, mainly related to *Sideroxydans* sp. and *Gallionellaceae* represent an important fraction of the entire microbiota of the red-colored biofilms. It is known that freshwater iron-oxidizing bacteria are a group of bacteria associated to aqueous environments containing appreciable concentrations of Fe(II) (Emerson, Fleming and McBeth 2010; Emerson *et al.* 2013). The analysis of Fe^{2+} and Fe^{3+} content in the Acquarossa river water revealed that almost 60% of the total iron content (1.05/1.788 mg/l) is in the oxidized Fe^{3+}

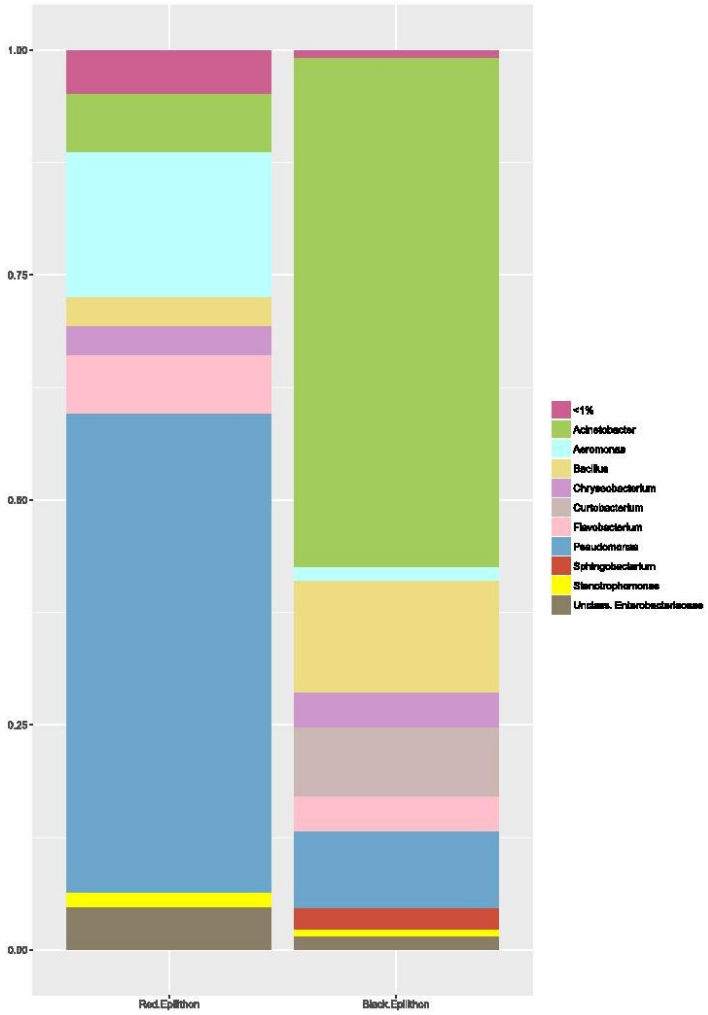


Figure 5. Bacterial genera distribution in black and red epilithons obtained by analysing culturable bacterial communities.

form, while the 40% is in the form of Fe^{2+}. The presence of high amounts of Fe^{2+} in the river is favoured by pH (about 6.5 in the flowing water) (Hem and Cropper 1960) thus sustaining the activity of iron-oxidizing bacteria (Emerson, Fleming and McBeth 2010) and deposition of iron hydroxides in biofilms dominated by these microorganisms. Therefore, the red color shown by this biofilm might be related to the presence of iron hydroxides that accumulates within the biofilm matrix (presumably due to the formation of Fe_2O_3) (Fig. 1c).

The color of the black epilithon might be due to the presence of other compounds in the river water. One possible explanation might be related to the presence of iron sulfides, which are known to form a black colored precipitate (Berner 1964) and that can be the result of the combination of Fe^{2+} with S (Table 1) by sulfate-reducing bacteria in the biofilm matrix. HTS data revealed the presence of sulfate-reducing bacteria affiliated to *Nitrospirae* and *Deltaproteobacteria* both in red and black epilithic biofilms (Fig. 2). Among them, the analysis of OTUs at genus level revealed the presence of sulfate reducing *Desulfobulbus* and *Desulfuromonas*. However, the multivariate analysis of variance did not show any significant difference in the whole community assemblage between the two epilithons ($p = 0.1$), and as a consequence, there are not significant differences in sulfate-reducing bacteria distribution within the two biofilms. Hence, it might be possible that other bacterial and/or archaeal groups not analyzed in this work could carry out the chemical reaction leading to the production of black precipitates characterizing this biofilm.

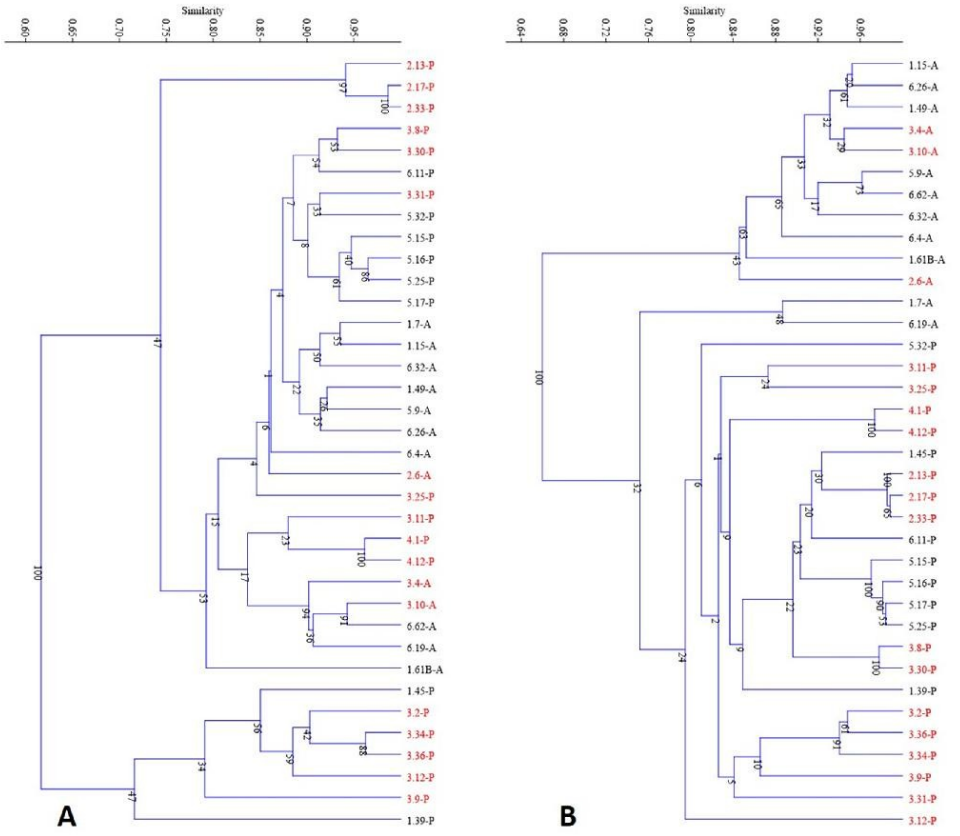


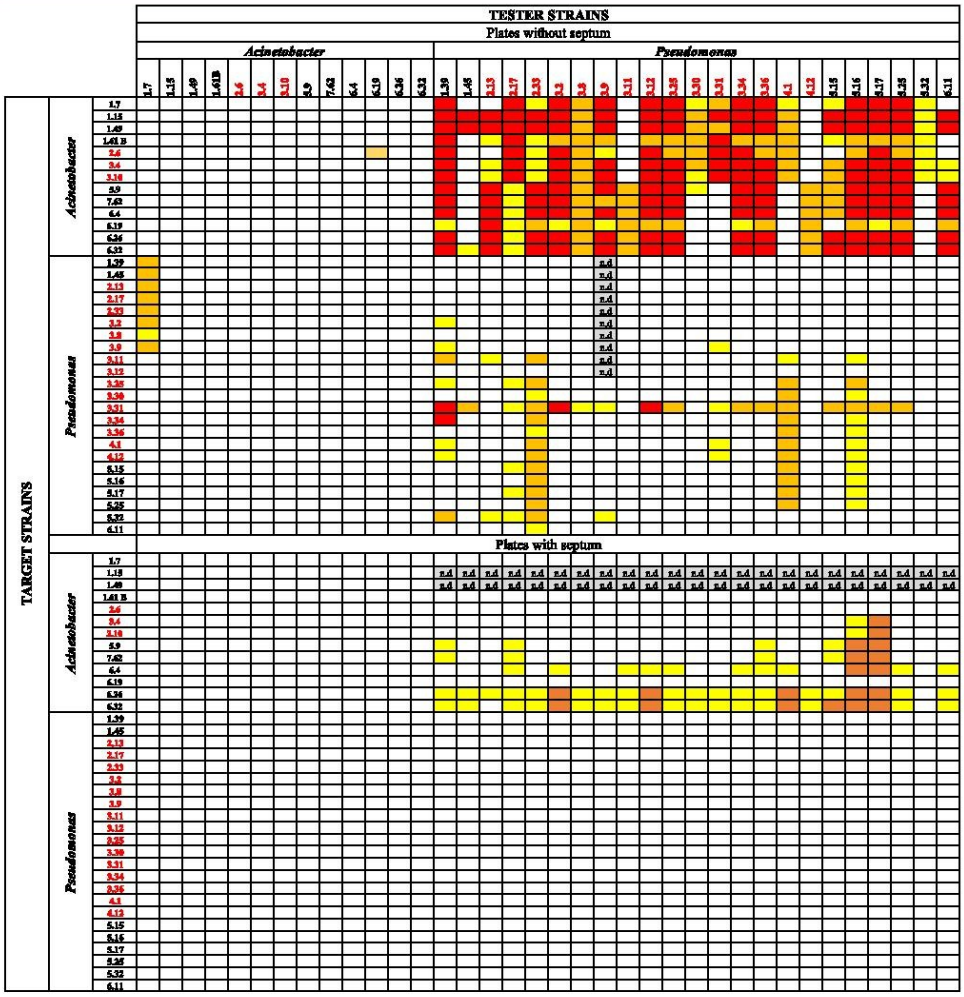
Figure 6. UPGMA analysis (Bray Curtis distance) performed on the heavy metal data matrix (A) and on the antibiotic data matrix (B) obtained by means of the broth microdilution methods.

The prevalence of *Proteobacteria* in red and black epilithons might be expected since *Proteobacteria* dominance in epilithic biofilm has been already reported from different environments (e.g. Bartrons, Catalan and Casamayor 2012; Ragon *et al.* 2012). Moreover, it has been reported that a limited number of dominant bacterial phyla (<10) prevails in rock biofilm bacterial communities (McNamara and Mitchell 2005). The bacterial communities in black and red epilithons were dominated by *Acinetobacter* and unclassified *Gallionellaceae*, *Sideroxydans* sp. and *Gallionella* sp., respectively. The HTS data partially agree with those obtained using culture-dependent approach: indeed, strains affiliated to *Acinetobacter* spp. were more abundant in black epilithon samples, whereas *Pseudomonas* sp. prevailed in red epilithon ones (Fig. 5). This discrepancy in the red epilithon might be explained as bacteria belonging to the genera *Gallionella* and *Sideroxydans* don't grow on the TSA medium, hence, they might have not been isolated by the culture conditions used in this work. Indeed, they are considered 'iron bacteria' because they are able to oxidize iron (Emerson, Fleming and McBeth 2010) and live in environments with high iron concentration, preferentially at the oxic–anoxic interfaces.

Bacterial plate counts gave comparable results in both red and black epilithon samples revealing that there was not a significant difference between the two biofilms. This result suggest that the fractions of cultivable and non-cultivable bacterial communities are comparable in the two environments and do not contribute to the differences between the red and black epilithon.

The complete absence of RAPD haplotype sharing between red and black epilithon both for *Acinetobacter* and *Pseudomonas*, suggests that the two communities are genetically differentiated at the strain level. These findings are in agreement with previous data suggesting that when two communities co-exist in the same environment and are spatially and physically in contact with each other, there could be some selective pressure that create and/or maintain a barrier to the free intermix of bacterial strains (Chiellini *et al.* 2014 and references therein). This selective pressure might likely be due to biotic conditions such as the

Table 5. Results from the cross-streak inhibition assay performed with petri dishes without (upper) or with (lower) septum. Rows stand for target strains and each column stands for a tester strain. The inhibition values corresponding to three different inhibition levels are as follows: complete (red), strong (orange), weak (yellow) and absence (white) of inhibition; n.d. stands for 'not determined' tests. Red and black numbers indicate strains isolated from red and black epilithon, respectively.



production of antibacterial compounds, as previously shown for other bacterial communities (Mengoni *et al.* 2014; Maida *et al.* 2016).

Concerning the resistance to antibiotics and heavy metals, UPGMA performed on data from heavy metals (Fig. 6a) and antibiotics (Fig. 6b) resistance patterns, revealed quite different scenarios. Concerning heavy metals, *Acinetobacter* and *Pseudomonas* populations share similar resistance patterns (Table S3, B, Supporting Information). Overall, these findings might suggest that the presence of heavy metals in such environment (abiotic factor) might not be a driving force for the evolution of the two separated communities inhabiting the red and the black epilithons. On the contrary, the resistance patterns towards antibiotics (Table S3, A, Supporting Information) were completely different between the two populations; indeed, *Acinetobacter* and *Pseudomonas* cluster separately (Fig. 6a). Hence, the resistance pattern towards antibiotics (biotic factor) might represent (at least) one of the factors responsible for the physical separation of the different bacterial communities in the

two biofilm types. This is in agreement with data obtained with other reports concerning the structuring on bacterial communities isolated from different compartments (Mengoni *et al*. 2014). *Pseudomonas* spp. population showed higher resistance levels towards the tested antibiotics in respect to the *Acinetobacter* spp. one. The comparison of our results with other published data is not easy to perform due to the scarcity of similar studies at environmental scale. One example can be given by *Acinetobacter* sp. and *Pseudomonas* sp. strains associated with fish and water from Congonhas river, that revealed no significant differences in resistance patterns towards Streptomycin at concentration of 10 μg/ml (Sousa and Silva-Souza 2001).

The RAPD and phylogenetic analysis performed for the two bacterial groups suggested that *Pseudomonas* constitutes a panmictic population and dominates the red epilithon, while *Acinetobacter* is a clonal population that dominates the black epilithon. Bacteria are naturally organized in clonal populations due to binary fission; however, the occurrence of horizontal gene transfer might shift the genetic structure of populations to panmictic (Vogel *et al*. 2003).

Moreover, it is known that in natural communities the release of toxic/antagonistic compounds synthesized by some bacteria can prevent the invasion from other (micro)organisms by creating an inhospitable zone for competitors (Stubbendieck and Straight 2016; Stubbendieck, Vargas-Bautista and Straight 2016). The competition for space and resources and the production of molecules with inhibiting potential against other organisms represent an advantage for the colonization of a niche by bacteria, as previously suggested by the few studies on this issue (Pérez-Gutiérrez *et al*. 2013).

It has also been reported that antagonistic interactions between bacterial strains belonging to the same or to different taxa might play a role in driving the structuring of microbial communities (Maida *et al*. 2016). Cross-streaking experiments (Table 5) performed in this work demonstrated that *Pseudomonas* sp. strains are able to inhibit the growth of *Acinetobacter* sp. strains by synthesizing diffusible (and very likely not volatile) antibiotic compounds. The ability of *Pseudomonas* sp. strains in synthetizing antibiotic compounds has been previously documented (e.g. Raaijmakers, Weller and Thomashow 1997; Haas and Keel 2003). This finding strongly supports the importance of the antagonistic interaction (biotic factor) as a driving force for the assembly of microbial communities in those environments that are spatially structured, such as biofilms (Pérez-Gutiérrez *et al*. 2013). This might suggest that the molecule(s) synthesized by *Pseudomonas* strains and able to inhibit the growth of *Acinetobacter* can diffuse within the biofilm and might be responsible for the structuring of the two distinct biofilms characterizing the black and the red epilithons. Indeed, diffusion has been previously demonstrated as being the prevalent transport process for molecules in biofilms (Stewart 2003). Considering that the black and red epilithons never mix but they are spatially located close to each other, 'diffusion' might be the main mechanism, if not the only one, acting in these environments, since molecules can easily diffuse/flow between cells; indeed, in aquatic environment—such as the Acquarossa river—molecules produced by bacteria are carried off the cell and there is a low probability that they might reach neighbor bacteria (Watnick and Kolter 2000).

CONCLUSION

The structuring of bacterial communities in black and red epilithic rock biofilms in Acquarossa river (Viterbo, Italy) has been investigated by means of cultivation and unculturable dependent approaches. In both cases, differences in taxonomic composition of black and red epilithon were highlighted. While black epilithon is dominated by *Acinetobacter* sp., bacteria inhabiting red epilithon are most iron-oxidizing strains. The proportion of culturable and non-culturable fractions of the community, as well as the resistance patterns towards heavy metals, do not seem to affect the differential structuring of the communities. On the other side, antibiotic resistance patterns and, in a larger proportion, the antagonistic interactions between the dominant bacterial genera seem to affect the whole structuring of red and black epilithic biofilms. These findings enforce the role of biotic factors as responsible for the structuring of natural bacterial communities. Overall, the study suggests that there is a selection of population at very small scale, and that different population might compete for different niches.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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REFERENCES

Arasu MV, Veeramuthu D, Savarimuthu I. Antibacterial and antifungal activities of polyketide metabolite from marine *Streptomyces* sp. AP-123 and its cytotoxic effect. *Chemosphere* 2013;**90**:479–87.

Bacci G, Bazzicalupo M, Benedetti A *et al*. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. *Mol Ecol Resour* 2014;**14**:426–34.

Bartrons M, Catalan J, Casamayor EO. High bacterial diversity in epilithic biofilms of oligotrophic mountain lakes. *Microb Ecol* 2012;**64**:860–9.

Battin TJ, Sloan WT, Kjelleberg S *et al*. Microbial landscapes: new paths to biofilm research. *Nat Rev Microbiol* 2007;**5**:76.

Battin TJ, Besemer K, Bengtsson MM *et al*. The ecology and biogeochemistry of stream biofilms. *Nat Rev Microbiol* 2016;**14**:251–63.

Berner RA. Iron sulfides formed from aqueous solution at low temperatures and atmospheric pressure. *J Geol* 1964;**72**:293–306.

Besemer K, Singer G, Quince C *et al*. Headwaters are critical reservoirs of microbial diversity for fluvial networks. *P Biol Sci* 2013;**280**:20131760.

Caporaso JG, Lauber CL, Walters WA *et al*. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *P Natl Acad Sci USA* 2011;**108**:4516–22.

Caporaso JG, Lauber CL, Walters WA *et al*. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;**6**:1621–4.

Chakravorty S, Helb D, Burday M *et al*. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Meth* 2007;**69**:330–9.

Chiellini C, Maida I, Emiliani G *et al.* Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. *Int Microbiol* 2014;**17**:165–74.

Cole JR, Q Wang JA, Fish B *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 2014;**42**:D633–42.

Di Cello F, Fani R. A molecular strategy for the study of natural bacterial communities by PCR-based techniques. *Minerva Biotecnol* 1996;**8**:126–34.

Dixon P. VEGAN, a package of R functions for community ecology. *J Veg Sci* 2003;**14**:927–30.

Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat methods* 2013;**10**:996–8.

Emerson D, Fleming EJ, McBeth JM. Iron-oxidizing bacteria: an environmental and genomic perspective. *Annu Rev Microbiol* 2010;**64**:561–83.

Emerson D, Field EK, Chertkov O *et al.* Comparative genomics of freshwater Fe-oxidizing bacteria: implications for physiology, ecology, and systematics. *Front Microbiol* 2013;**4**:254.

Haas D, Keel C. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Ann Rev Phytopathol* 2003;**41**:117–53.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95 /98/NT. *Nucl Acid S* 1999;**41**:95–98.

Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 2001;**4**:1–9.

Harrison AP, Cattani I, Turfa JM. Metallurgy, environmental pollution and the decline of Etruscan civilisation. *Environ Sci Poll Res* 2010;**17**:165–80.

Hem JD, Cropper WH. Survey of ferrous-ferric chemical equilibria and redox potentials. In: *Chemistry of Iron in Natural Water, Geological Survey Water-supply Paper 1459*. Washington: United States Government Printing Office, 1960, 1459.

Hill MO. Diversity and evenness: a unifying notation and its consequences. *Ecology* 1973;**54**:427–32.

Hook D. The composition and technology of selected Bronze Age and Early Iron Age copper alloy artefacts from Italy. In: Bietti Sestieri AM, Macnamara E (eds). *Prehistoric Metal Artefacts From Italy (3500–720 BC) in the British Museum (British Museum Research Publication 159)*. London: British Museum, 2007;308–23.

Huse SM, Dethlefsen L, Huber JA *et al.* Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLOS GENET* 2008;**4**:e1000255.

Illumina. 16S Metagenomic sequencing library preparation. In: *Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*. Illumina, 2013, 1–28. https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Jorgensen HJ, NCCLS Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, NCCLS-M7. *Infect Dis Clin N Am* 1993; **7**:393–-409.

Kastman EK, Kamelamela N, Norville JW *et al.* Biotic interactions shape the ecological distributions of *Staphylococcus* species. *MBio* 2016;**7**:e01157–16.

Kobayashi Y, Kim C, Yoshimizu C *et al.* Longitudinal changes in bacterial community composition in river epilithic biofilms: influence of nutrients and organic matter. *Aquat Microb Ecol* 2009;**54**:135–52.

Ledger ME, Hildrew AG. Temporal and spatial variation in the epilithic biofilm of an acid stream. *Freshwater Biol* 1998;**40**:655–70.

Maida I, Chiellini C, Mengoni A *et al.* Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant *Echinacea purpurea*. *Environ Microbiol* 2016;**18**:2357–65.

Mathur J, Bizzoco RW, Ellis DG *et al.* Effects of abiotic factors on the phylogenetic diversity of bacterial communities in acidic thermal springs. *Appl Environ Microb* 2007;**73**:2612–23.

McNamara CJ, Mitchell R. Microbial deterioration of historic stone. *Front Ecol Environ* 2005;**3**:445–51.

Mengoni A, Maida I, Chiellini C *et al.* Antibiotic resistance differentiates *Echinacea purpurea* endophytic bacterial communities with respect to plant organs. *Res Microbiol* 2014;**165**:686–94.

Meyers GE. Etrusco-Italic monumental architectural space from the Iron Age to the Archaic period: an examination of approach and access. *Doctoral dissertation*. https://repositories.lib.utexas.edu/handle/2152/784, 2003.

Meyers GE. Approaching monumental architecture: mechanics and movement in Archaic Etruscan palaces. *Pap Brit Sch Rome* 2013;**81**:39–66.

Moënne-Loccoz Y, Mavingui P, Combes C *et al.* Microorganisms and biotic interactions. In: *Environmental Microbiology: Fundamentals and Applications*. Netherlands: Springer, 2015, 395–444.

Mori E, Liò P, Daly S *et al.* Molecular nature of RAPD markers amplified from Haemophilus influenzae Rd genome. *Res Microbiol* 1999;**150**:83–93.

Papaleo MC, Romoli R, Bartolucci G *et al.* Bioactive volatile organic compounds from Antarctic (sponges) bacteria. *New Biotechnol* 2013;**30**:824–38.

Paulson JN, Stine OC, Bravo HC *et al.* Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* 2013;**10**:1200–2.

Pérez-Gutiérrez RA, López-Ramírez V, Islas A *et al.* Antagonism influences assembly of a Bacillus guild in a local community and is depicted as a food-chain network. *ISME J* 2013;**7**:487.

Petrosino JF, Highlander S, Luna RA *et al.* Metagenomic pyrosequencing and microbial identification. *Clin Chem*; 2009;**55**:856–66.

R Core Team. R: A language and environment for statistical computing. In: *R Foundation for Statistical Computing*. Vienna, Austria: R Core Team, 2014.

Raaijmakers JM, Weller DM, Thomashow LS. Frequency of antibiotic-producing Pseudomonas spp. in natural environments. *Appl Environ Microb* 1997;**63**:881–7.

Ragon M, Fontaine MC, Moreira D *et al.* Different biogeographic patterns of prokaryotes and microbial eukaryotes in epilithic biofilms. *Mol Ecol* 2012;**21**:3852–68.

Rubin MA, Leff LG. Nutrients and other abiotic factors affecting bacterial communities in an Ohio River (USA). *Microb Ecol* 2007;**54**:374–83.

Sousa JAD, Silva-Souza ÂT. Bacterial community associated with fish and water from Congonhas River, Sertaneja, Paraná, Brazil. *Braz Arch Biol Techn* 2001;**44**:373–81.

Staccioli RA. Considerazioni sui complessi monumentali di Murlo e di Acquarossa. *Publications de l'École française de Rome* 1976;**27**:961–72.

Stewart PS. Diffusion in biofilms. *J Bacteriol* 2003;**185**:1485–91.

Stoddard SF, Smith BJ, Hein R *et al.* rrn DB: improved tools for interpreting rRNA gene abundance in bacteria and archaea

and a new foundation for future development. *Nucleic Acids Res* 2014;**43**:D593–8.
Stoodley P, Sauer K, Davies DG *et al*. Biofilms as complex differentiated communities. *Ann Rev Microbiol* 2002;**56**:187–209.
Stubbendieck RM, Vargas-Bautista C, Straight PD. Bacterial communities: interactions to scale. *Front Microbiol* 2016;**7**:1234.
Stubbendieck RM, Straight PD. Multifaceted interfaces of bacterial competition. *J Bacteriol* 2016;**198**:2145–55.
Tamura K, Peterson D, Peterson N *et al*. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731–9.
Thirumurugan D, Vijayakumar R. Characterization and structure elucidation of antibacterial compound of Streptomyces sp. ECR77 isolated from East Coast of India. *Curr Microbiol* 2015;**70**:1–11.
Vogel J, Normand P, Thioulouse J *et al*. Relationship between spatial and genetic distance in *Agrobacterium* spp. in 1 cubic centimeter of soil. *Appl Environ Microb* 2003;**69**:1482–7.
Watnick P, Kolter R. Biofilm, city of microbes. *J Bacteriol* 2000;**182**:2675–9.
Widder S, Allen RJ, Pfeiffer T *et al*. Challenges in microbial ecology: building predictive understanding of community function and dynamics. *ISME J* 2016;**10**:2557.
Wilhelm L, Besemer K, Fragner L *et al*. Altitudinal patterns of diversity and functional traits of metabolically active microorganisms in stream biofilms. *ISME J* 2015;**9**:2454–64.
Williams JG, Kubelik AR, Livak KJ *et al*. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990;**18**:6531–5
Zancarini A, Echenique-Subiabre I, Debroas D *et al*. Deciphering biodiversity and interactions between bacteria and microeukaryotes within epilithic biofilms from the Loue River, France. *Sci Rep* 2017;**7**:4344.

8.2 Plant-endophytes interaction influences the secondary metabolism in *Echinacea purpurea* (L.) Moench: an in vitro model

The plant microbiota has gained more and more relevance in the last decades, since it has been highlighted that it may strongly influence the plants' physiology in many aspects. In particular, endophytes associated to medicinal plants could play an important role in the plant's production of secondary metabolites with therapeutic properties.

The analysis of the endophytic influence of medicinal plants' production of therapeutic molecules is of great importance in order to better understand the plant-microbiota interaction and to possibly obtain medicinal plants with improved capacity of producing such molecules.

Echinacea medicinal plants are largely used in many countries for respiractory infections. Alkamides characterize the plants' bioactive metabolites and are responsible for many of the immunomodulant effects of the plant. For example, alkamides increase the TNF mRNA expression in macrophages and monocytes binding the cannabinoid CB2 receptor20 and decrease mitogens-induced interleukin-2 secretion in Jurkat-T cells.

The aim of this work was to explore the involvement of the endophytic communities of *E. purpurea* plants in the regulation of bioactive compound (alkamide) accumulation. For this purpose, axenic *E. purpurea* plants were inoculated with bacterial endophytes extracted from *E. purpurea* stem and leaves. Very interestingly, the bacterial inoculation determined a higher expression of the branched-chain amino acids (BCCA) decarboxylase gene, involved in alkamides production, showing that the production of the plant's secondary metabolites and its therapeutical properties could indeed be influenced by the bacterial endophytes.

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Plant-endophytes interaction influences the secondary metabolism in *Echinacea purpurea* (L.) Moench: an *in vitro* model

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The influence of the interaction(s) between the medicinal plant *Echinacea purpurea* (L.) Moench and its endophytic communities on the production of alkamides is investigated. To mimic the *in vivo* conditions, we have set up an infection model of axenic *in vitro E. purpurea* plants inoculated with a pool of bacterial strains isolated from the *E. purpurea* stems and leaves. Here we show different alkamide levels between control (not-inoculated) and inoculated plants, suggesting that the alkamide biosynthesis may be modulated by the bacterial infection. Then, we have analysed the branched-chain amino acids (BCCA) decarboxylase gene (GenBank Accession #LT593930; the enzymatic source for the amine moiety formation of the alkamides) expression patterns. The expression profile shows a higher expression level in the inoculated *E. purpurea* tissues than in the control ones. These results suggest that the plant-endophyte interaction can influence plant secondary metabolism affecting the therapeutic properties of *E. purpurea*.

Echinacea purpurea (L.) Moench (Asteraceae) is a medicinal plant with immune-modulatory and anti-inflammatory properties, whose roots and aerial parts are frequently used in Europe and North America for the preparation of therapeutic extracts for common cold[1]. It is rich in various phytochemicals including caffeic acid derivatives, alkamides and polysaccharides[2]. The concentrations of these bioactive compounds are species-specific and they may vary due to several factors such as plant material, cultivation techniques, plant tissue treatment, extraction methods and phytosanitary status[3]. Recently, the attention has been focused on the plant microbiota and its role in the production of secondary metabolites[4,5]. Many studies aim to investigate the influence of endophytic fungi on the production of plant bioactive molecules[6,7] but the interest for the bacterial endophytes is considerably increasing[8]. Genomics and proteomics approaches have been applied to deepen the understanding of the plant-endophyte interaction[9]. Differential protein accumulations have been revealed in the proteome of *in vitro*-grown *Zea mays*[10] and Chinese hybrid poplar clone 741[11] inoculated or not-inoculated with *Herbaspirillum seropedicae* and *Paenibacillus* sp., respectively. In particular, the role of the endophytes is investigated related to agricultural aspects (*e.g.* plant-growth promoting and biocontrol) and very few studies have been conducted on medicinal plants[3,12]. *E. purpurea* root extracts are reported to stimulate macrophage TNF-α production but the extracts obtained from *in vitro*-grown axenic *E. purpurea* do not induce the same result, supporting the hypothesis that it is originated from the interaction with bacterial endophytes[12]. Our previous study shows that different compartments of *E. purpurea*, namely stems and leaves (SL), roots (R) and the rhizosphere (RS), share very few strains[13]. This finding has been shown to be related to the antagonism existing between strains inhabiting the different compartments[14] and to the degree of resistance to antibiotics[15]. Moreover, the presence of distinct bacterial communities in plant compartments could account for the different bioactive

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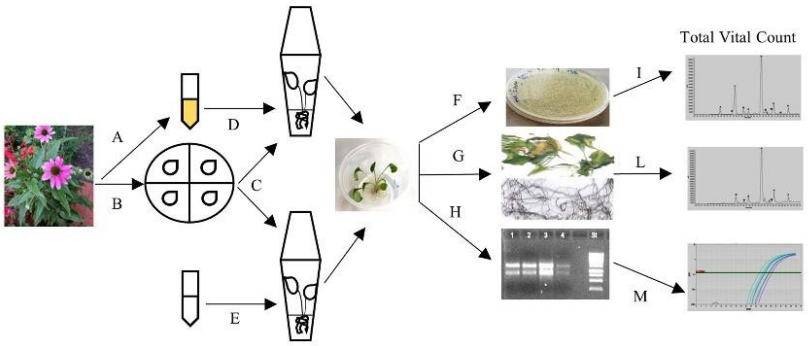

Figure 1. *In vitro* model system setting up to study the interaction between *Echinacea purpurea* plants and their stem/leaves endophytic bacteria. (**A**) Bacterial endophytes were isolated from the aerial compartment of *E. purpurea* plants. (**B**) *E. purpurea* seeds were provided by the common garden at the "Il Giardino delle Erbe", Casola Valsenio, Italy and surface-sterilized. (**C**) Seeds were germinated in De Wit Culture tubes containing 5 ml of Linsmaier & Skoog Medium (LS) including vitamins. After root formation, the seedlings were transferred in Wavin flasks containing 50 ml of LS solid medium, supplemented with 3% sucrose and maintained in a plant growth chamber for a photoperiod of 16 h light a day. (**D,E**) After about 2 months, five *E. purpurea* plants were inoculated with 8 × 106 bacterial endophytes isolated from SL compartment of *E. purpurea* plants (**D**); five plants were used as control and were inoculated with sterilized saline solution (**E**). After 45 days, SL and root (R) samples from control and infected plants, were collected separately and sterilized. (**F–H**) Samples were then, separated in different aliquots (R and SL pooled separately). (**I**) One aliquot of each tissue was immediately used for the *in planta* bacterial growth analysis. (**L**) One aliquot of each tissue was weighed and dried at 60 °C to be used for *n*-hexane extracts preparation. (**M**) One aliquot of each tissue was ground to a fine powder in liquid nitrogen and successively used for RNA extraction.

compounds found in the various plant organs. Therefore, *E. purpurea* represents an interesting and useful *in vitro* model for plant-bacterial interaction studies on the production of pharmacological relevant secondary metabolites as the alkamides.

Alkamides (alkylamides; fatty acid amides) are lipophilic compounds chemically composed of two moieties, an amine moiety acylated by a fatty acid-derived one[16]. They are important bioactive compounds dissimilarly distributed in the different compartments of *E. purpurea*[17]. Indeed, a notable difference is reported for the total alkamide content between aerial parts and roots, which is mainly due to a larger presence of non-tetraene alkamides in roots than in other plant organs[18]. The main *Echinacea* spp. alkamides are the isomeric dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutylamides[19]. These compounds increase the TNF mRNA expression in macrophages and monocytes binding the cannabinoid CB2 receptor[20]. Furthermore, the alkylamides decrease mitogens-induced interleukin-2 secretion in Jurkat-T cells[21] and show an *in vitro* inhibitory activity of the 5-lipoxygenase[22] and the cyclooxygenase-1 and 2 enzymes[23].

Recently, a pyridoxal phosphate-dependent (PLP) decarboxylating enzyme belonging to the Class II tryptophan synthase family that utilizes branched-chain amino acids (BCAA) as substrate has been suggested[24]. Isotope labelling analyses have revealed the generation of isobutylamine and 2-methylbutylamine (*i.e.* the amine moiety of the *E. purpurea* alkamides) from valine and isoleucine, respectively. PLP decarboxylase-like proteins have been identified in the proteome of *E. purpurea* through *in silico* analyses and their transcript levels correlated with alkamide accumulation patterns in *E. purpurea* tissues. Then, a valine decarboxylase (VDC), potentially involved in the generation of the amine moieties of the alkamides, has been identified.

The aim of this work is to check the involvement of the endophytic communities of *E. purpurea* plants in the regulation of bioactive compound (alkamide) accumulation. To this purpose, we set up an *in vitro* model system in which axenic *E. purpurea* plants, *in vitro* germinated from sterilized seeds, are inoculated with a pool of selected endophytes previously isolated from the aerial compartment of *E. purpurea* plants cultivated in open field (Fig. 1). We have evaluated the biochemical alkamide profiles estimating different alkamide levels in control (not-inoculated) and inoculated plants. We have also found that the level of *VDC* gene expression is higher in the inoculated *E. purpurea* tissues than in the control ones, establishing a close relationship between the endophyte presence and alkamide levels.

Results

Bacterial endophytes tend to re-colonize the native niche during plant infection. A pool of thirty-seven bacterial strains (Supplementary Table 1), isolated from the SL compartment of *E. purpurea* plants, was used to inoculate five axenic *in vitro* 2-months old *E. purpurea* plants each with 6 or 7 leaflets; five plants of the same age, used as control, were inoculated with sterilized saline solution. The infection experiment was repeated three times. Forty-five days after the infection, plants were analysed for bacterial colonization estimating

Compound	Peak[a]	Organ	Alkamides
1	**A**	SL	undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide
2	**A**	SL	undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide
3	**B**	SL	undeca-2*E*,4*Z*-diene-8,10-diynoic acid methylbutylamide
4	**B**	SL	undeca-2*Z*,4*E*-diene-8,10-diynoic acid methylbutylamide
5	**D**	R, SL	trideca-2*E*,7*Z*-diene-8,10-diynoic acid isobutylamide
6	**D**	SL	dodeca-2*E*,4*Z*,10*E*-triene-8-ynoic acid isobutylamide
7	**G**	R, SL	dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide
8	**G**	R, SL	dodeca-2*E*,4*E*,8*Z*,10*E* tetraenoic acid isobutylamide
9	**K**	R, SL	dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid methylbutylamide
10	**K**	R, SL	dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid methylbutylamide
11	**L**	R, SL	dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide
12	**N**	R, SL	dodeca-2*E*,4*E*-dienoic acid isobutylamide
13	**O**	R	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylbutylamide
14	**O**	R	dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylbutylamide
15	**P**	R	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide

Table 1. Identification of alkamides detected in roots (R) and stem/leaves (SL) of control and infected *E. purpurea* plants. Compound numbers are referred to peaks in the chromatograms of Supplementary Figs 1 and 2. Peaks **C**, **E**, **F**, **H**, **I**, **J**, and **M** remained unidentified. [a]Two alkamide isomers are present under the same peak: **A** (**1**, **2**), **B** (**3**, **4**), **G** (**7**, **8**), **K** (**9**, **10**), and **O** (**13**, **14**). The peak **D** is generated by the coelution of two not isomer alkamides (**5**, **6**). The *E*/*Z* stereochemistry is indicated in accordance with the literature[3,26–28].

the total viable count (TVC) as Colony Forming Units (CFU)/g into the host R and SL tissues. Data obtained revealed that the highest CFU/g was detected in the SL compartment (7.06 ± 6.50 log CFU/g), and the lowest one in the roots of infected plants (6.70 ± 5.82 log CFU/g; $P < 0.001$). This finding could indicate that the endophytes tended to re-colonize the native niche (SL compartment). The absence of bacteria in the control plant tissues and in the washing solutions confirmed the use of an axenic plant model and a successful sterilization procedure, respectively.

Alkamide profiling analysis in different organs of control and infected plants. The alkamide profiles of both control and infected R and SL extracts of *E. purpurea* pooled plants were investigated by means of high performance liquid chromatography (HPLC) coupled to a photo diode array (PDA)/ultraviolet (UV) detector and electrospray ionization tandem mass spectrometry (ESI-MS/MS). The LC-PDA/UV chromatograms of both *E. purpurea* R and SL extracts showed different alkamide profiles (Supplementary Figs 1 and 2). In particular, the SL extracts appeared to be richer in alkamide content, with twelve identified compounds (**1**-**12**, Table 1), respect to the R samples (compounds **5**, **7**-**12**, Table 1) even though for the peaks **A**, **B**, **G**, **K**, and **O** two isomers can occur and their exact identification was not possible based on MS fragmentation pathway. Some alkamides present in the SL extract (peaks **A**, **B**, **C**, **E**, **F**, **H**, **I**, and **M**) were not found in the R one. On the other hand, two R extracts showed the presence of the alkamides **13**/**14** (peak **O**) and **15** (peak **P**), which were absent in the SL ones. In all R and SL samples, the most representative alkamides were a mixture of the two co-eluting isomers dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**7**) and dodeca-2*E*,4*E*,8*Z*,10*E* tetraenoic acid isobutylamide (**8**). During the alkamide chromatography of both R and SL extracts, polyacetylene amides (peaks **A**, **B**, **D**, **O**, and **P**) elute early, followed by tetraenes (peaks **G** and **K**), trienes (peak **L**), and finally dienes (**N**). Interestingly, the analysis of the alkamide content in the *E. purpurea* plant organs also revealed the presence of seven alkamides not previously reported (peaks **C**, **E**, **F**, **H, I, J**, and **M**), but ascribable to the alkamide class due to the characteristic absorbance at 260 nm and typical ESI-MS and MS/MS spectra (Supplementary Table 3). A tentative level estimation of the main alkamides was performed on control and infected R and SL extracts by measuring the peaks area and calculating the mean of the replicates (Supplementary Table 4). Data were submitted to Principal Component Analysis (PCA), and the result was reported in Fig. 2: the vectors accounting for the R extracts were differentially oriented than those of the SL ones ($F = 10.42$; $P < 0.001$). In particular, the R samples presented C12 diene-diyne alkamides (peak **O** and **P**), whilst the SL samples contained C11 diene-diyne alkamides (peak **A** and **B**). Both samples showed a prevalence of C12 tetraene alkamides (peak **G** and **K**). Furthermore, PCA showed a discrimination among control and infected samples both for R and SL extracts and suggested that such differences were mainly created by the amount of the most abundant alkamide isomers **7** and **8** (peak **G**). The comparison of means (Supplementary Table 4) revealed that the amount of these alkamide isomers was significantly different for all samples (Tukey HSD $P < 0.001$). A relative estimation of the alkamide isomers **7** and **8** in the R and SL infected samples was also performed in respect to the levels of the relative control samples showing that the infection resulted in an increase of alkamide levels of about 70% in SL and 87% in R infected samples compared to the controls.

BCCA decarboxylases genetic expression in different organs of control and infected plants. The relative quantification of *VDC* gene expression in the SL and R tissues of the control and infected *E. purpurea* plants was calculated in respect to the ubiquitin E2 (*UbE2*) gene expression in the three biological replicates. The amplification efficiency was optimal as achievable by the R^2 and slope values ($slope_{VDC} = -3.113$; $R^2_{VDC} = 0.904$; $slope_{UbE2} = -3.182$;

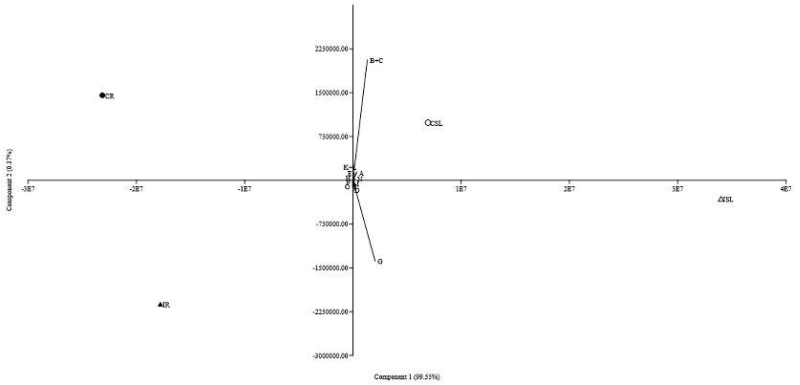

Figure 2. Principal Component Analysis of alkamide relative estimations of the four different *Echinacea purpurea* extracts. Letters on vectors indicate the HPLC peaks accounting for the differentiation of samples (see text for details). CSL, stem/leaves extract from control plants; ISL, stem/leaves extract from infected plants, CR, root extract from control plants; IR: root extract from infected plants.

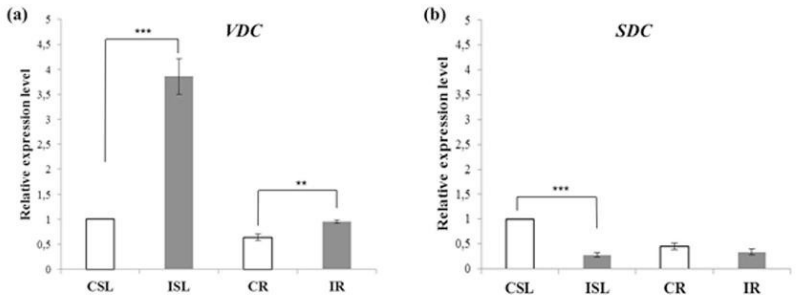

Figure 3. Gene expression of (**a**) valine decarboxylase (*VDC*) and (**b**) serine decarboxylase (*SDC*) in control and infected samples of *Echinacea purpurea* plants. Expression levels were normalized to expression in CSL. Data report average from three independent experiments (with 3 technical replicates each). Error bars: standard error of the mean (s.e.m.). Comparison between infected samples and the relative controls was determined by 2-tailed t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). CSL, stem/leaves extract from control plants; ISL, stem/leaves extract from infected plants, CR, root extract from control plants; IR: root extract from infected plants.

$R^2_{UbE2} = 0.988$). As depicted in Fig. 3a, the *VDC* gene expression levels were higher in the SL samples than the R ones ($P < 0.01$). The *VDC* transcription level in the infected SL samples was about 4 times more than in the SL control tissues ($P < 0.001$). Also, the expression level resulted increased in R infected tissues respect to the relative controls even if at a minor extent ($P < 0.01$). In parallel, the involvement of an enzyme (serine decarboxylase SDC, GenBank Accession #LT593931.1) not decarboxylating valine or isoleucine (to form isobutylamine or 2-methylbutylamine, the amine moieties of the *E. purpurea* alkamides) but utilizing the serine as substrate to generate ethanolamine[24] was evaluated. The transcription of the *SDC* gene (slope$_{SDC} = -3.135$; $R^2_{SDC} = 0.982$) appeared down-regulated in the infected SL samples respect to the SL control ones whilst the *SDC* expression levels were similar between control and infected R samples (Fig. 3b).

Discussion

In this work we set up an *in vitro* system to study the influence of the interaction between bacterial endophytes and *E. purpurea* plants on the production of plant bioactive compounds.

The infection data revealed the bacterial tendency to reach their niche of origin passing through the R apparatus since a significant number of bacteria was detected also in the R tissues of the infected plants. This was in agreement with the current literature, according to which one of the ways used by endophytes to reach the aerial parts of the host plant was their migration through the xylem vascular system[25] once that they have entered the

plant roots. Hence, we speculated that all the endophytes could reach the SL compartment. However, we were not able to consider a post-infection time longer than 45 days since it would have induced several plant damages. Therefore, our data confirmed the hypothesis that differences in bacterial strains distribution between R and SL of *E. purpurea* could be related to the physiological conditions existing in the different plant tissues and organs representing a specific ecological niche, as previously suggested[13].

On the basis of data obtained in this work, the R and SL extracts displayed different alkamide profiles, with twelve and ten compounds, respectively identified comparing their HPLC elution orders, ESI-MS/MS, and PDA/UV spectra with data reported in the literature[3,26–29]. The most abundant alkamides were the 2,4-diene type compounds and the two isomers dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**7**) and dodeca-2*E*,4*E*,8*Z*,10*E* tetraenoic acid isobutylamide (**8**) being predominant, according to previous studies performed on *in vivo* plants[30,31]. Moreover, seven alkamides (peaks **C**, **E**, **F**, **H**, **I**, **J**, and **M**) remained not identified, and among these one alkamide (peak **F**) was present only in the infected SL compartment. To our knowledge, these alkamides were not previously reported in *E. purpurea*.

The estimated total alkamide level was higher in the SL samples than in R ones, apparently in contrast with the literature data reporting that the *E. purpurea* roots contain more alkamides than the leaves[2]. However, the alkamide content in SL samples could be higher than the reported leaves amount since it was the sum of the leaves and the vegetative stems. In fact, in line with the literature[32], the R and SL extracts were differentiated by the presence of the C12 and C11 diene-diyne alkamides (mainly reported in the stem), respectively. On the other hand, Qu *et al.*[18] reported that the tetraene alkamides **7** and **8** accounted for the 75% in the aerial part and only for the 9% in the roots of *E. purpurea* plants cultivated into a field and these estimates resulted discordant with our results (about 50 and 70%, respectively). To this concern, one possible explanation could be that, to the best of our knowledge, this study is the first to estimate the *E. purpurea* alkamide level in an *in vitro* model whose experimental conditions could influence plant genetics and/or biochemical synthesis. However, the estimated alkamide level was higher in both infected R and SL, with a relative increase of alkamides **7** and **8** about 87% and 70% respectively, when compared to the controls, suggesting that the alkamide biosynthesis was modulated by the *E. purpurea* endophyte infection.

Concerning the *VDC* gene expression profiles, the highest expression level was detected in the infected *E. purpurea* tissues. By comparing results concerning both the chemical profiling analysis and the *VDC* gene expression data, it could be observed that the use of the proposed *in vitro* infection model system allowed us to demonstrate that the infection of *E. purpurea* axenic plants with their endophytes influenced the alkamide levels. In fact, both alkamide content and *VDC* transcription level resulted increased after the infection. The transcriptional up-regulation in the infected R samples was lower than the expected one: this result could be explained considering that the increase of the alkamide quantity in the infected R might be due to the transcriptional regulation of other enzymes required for the biosynthesis of the alkamide ammine and fatty acid moieties[24]. Interestingly, the SDC enzyme did not seem to contribute to the increase of alkamide levels confirming the specificity of the enzymatic source for the amine moiety formation of the alkamides (*i.e.* the *VDC* decarboxylase) as reported by Rizhsky *et al.*[24].

The main objective of this work was to develop an *in vitro* model to study the role of the interaction between *E. purpurea* and its endophytes in the modulation of the plant secondary metabolism. Bacterial communities differed substantially between *E. purpurea* organs[13] probably exerting a host selectivity able to modulate the community structure as reported for endophytic fungi[33]. In fact, the *E. purpurea* endophytes inoculated in axenic plants tended to re-colonize the native niche whose specific properties probably were influenced in turn by the natural endophytes. Chemical profiles and *VDC* genetic expression resulted quantitatively different between the control and infected plants, in particular in the SL compartment. Therefore, despite the *in vivo* status during bacterium-host interactions was difficult to mimic *in vitro*, the infection with *E. purpurea* SL endophytes modulated the characteristics resulting in axenic conditions, at least to some extent.

Consequently, the whole body of data obtained in this work strongly suggested that the secondary metabolism in *E. purpurea* was influenced by the plant-endophyte interaction thus possibly contributing to the therapeutic properties of this medicinal plant.

Materials and Methods

Bacterial cultures and plant material. Bacterial endophytes were isolated from the aerial compartment (stem and leaves) of *E. purpurea* plants grown at the "Il Giardino delle Erbe", Casola Valsenio, Italy, as previously reported[13]. Stock cultures were grown at 30 °C on tryptone soy agar (TSA; Bio-Rad, USA) solid medium or tryptone soy broth (TSB, Bio-Rad, USA) liquid medium. *E. purpurea* seeds were provided by the "Il Giardino delle Erbe".

Seed sterilization and plating. Seeds were surface sterilized in order to prevent any unwanted fungal or bacterial growth. Seeds were immersed in a 70% (v/v) ethanol for one minute and, subsequently, in a 5% sodium hypochlorite solution for eight minutes. They were then rinsed three times with sterile distilled water, kept overnight at 4 °C in the dark for growth synchronization and then germinated in De Wit Culture tubes (LAB Associates BV, The Netherlands) containing 5 ml of Linsmaier & Skoog Medium (LS) including vitamins (Duchefa Biochemie, The Netherlands) at 24 ± 1 °C in the dark. After root formation, the seedlings were transferred in Wavin flasks (LAB Associates BV, The Netherlands) containing 50 ml of LS solid medium, supplemented with 3% sucrose, for a photoperiod of 16 h light a day for a minimum of two months. In order to validate the sterility of the obtained model system, cultivable endophyte multiplication into host tissues was checked: both shoots and roots were separately collected, washed in saline solution (0,9% NaCl, washing solution), surface sterilized in 1% (v/v) hypochlorite for 8 min and rinsed three times with sterile distilled water. Both samples were homogenized in saline solution and five replications of 100 µl of the homogenates were plated on TSA medium. Bacterial growth was scored after three days of plate incubation at 30 °C.

Plant infection. *Inocula* of bacterial endophytes, isolated from SL compartment of *E. purpurea* plants, were incubated for three days at 30 °C in horizontal position and in agitation. The bacterial suspensions were then adjusted to 8×10^8 CFU/ml ($OD_{600} = 1$). The optical density (OD) was measured in a biophotometer (Eppendorf, Germany). The pool generated from 100 µl of each diluted 1:10 OD_{600} suspension cultures was then centrifuged at 4000 rpm for 20 minutes and the pellet suspended in a correspondent volume of 0.9% saline solution. Five 2-months old *E. purpurea* plants were infected with 100 µl of bacterial suspension culture. Five plants were used as control and were infected with 100 µl of sterilized saline solution. Plants were then incubated in the growth chamber at 24 ± 1 °C. After 45 days, SL and R samples from control and infected plants, were collected separately, firstly washed in saline solution and then sterilized in 1% (v/v) hypochlorite for 8 min. Then, both tissue samples were washed three times with sterile distilled water and separated in different aliquots. R and SL aliquots were weighed and dried at 60 °C to be used for *n*-hexane extract preparation. R and SL aliquots of fresh material were ground to a fine powder in liquid nitrogen and successively stored at − 80 °C for RNA extraction. Finally, 1.0 g of fresh R and SL tissues were immediately used for the *in planta* bacterial growth analysis. The experiment was performed in triplicate.

***In planta* bacterial growth analysis.** In order to evaluate endophytes multiplication into host tissues, 1.0 g of each sample was homogenized in saline solution and 100 µl of the homogenate were serially diluted up to 10^{-7}/ml cells. Five replications of each dilution were plated on TSA medium. The washing solution and the distilled water after the last wash were also diluted to check the presence of bacterial cells on the surface of the tissues and the outcome of the sterilization procedure. Bacterial growth was scored after two, three and four days of incubation of the plates at 30 °C.

Sample preparation for HPLC analysis of alkamides. Dried and powdered R and SL of control and infected *E. purpurea* plants from three independent experiments were pooled and extracted at room temperature with *n*-hexane (1.0 g of dried drug in 30 ml of solvent for three times, every 24 h) as detailed in Supplementary Table 5. Solutions of each *n*-hexane residue from R and SL samples were then prepared dissolving the respective *n*-hexane extract in an opportune volume of methanol and then centrifuging the mixture. Finally, 20 µl of each supernatant solution (2.0 mg/ml) were injected for HPLC-PDA/UV-ESI-MS/MS analysis. The experiment was performed in duplicate.

HPLC-PDA/UV-ESI-MS/MS analyses. Qualitative HPLC-PDA/UV-ESI-MS/MS analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and a LCQ Advantage ion trap mass spectrometer (ThermoFinnigan) equipped with Xcalibur 3.1 software. Analyses were performed using a 4.6 × 250 mm, 4 µm, Synergi Fusion-RP column (Phenomenex). The eluent was a mixture of methanol (solvent A) and a 0.1% v/v aqueous solution of formic acid (solvent B). A linear gradient of increasing 55% to 85% A was developed within 45 min. The column was successively washed for 15 min with methanol and equilibrated with 55% A for 10 min. Elution was performed at a flow rate of 0.8 ml/min with a splitting system of 2:8 to MS detector (160 ml/min) and PDA detector (640 ml/min), respectively. The volume of the injected methanol solutions was 20 µl. Analyses were performed with an ESI interface in the positive mode. The ionization conditions were optimized and the parameters used were as follows: capillary temperature, 270 °C; capillary voltage, 29.0 V; tube lens offset, 50.0 V; sheath gas flow rate, 60.00 arbitrary units; auxiliary gas flow rate, 3.00 arbitrary units; spray voltage, 4.50 kV; scan range of m/z 150–1200. N_2 was used as the sheath and auxiliary gas. PDA data were recorded with 200–600 nm range with preferential channel as the detection wavelength 260 nm.

Quantitative real time PCR (qRT-PCR) analysis. Total RNA from R and SL was extracted from *E. purpurea* tissues using the RNeasy Micro Kit (Qiagen, USA) and quantified by Qubit® 2.0 fluorimeter. One µg of total RNA for each sample was reverse-transcribed using the Quantitect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen) including a treatment with 1X gDNA Wipeout Buffer to remove any remaining DNA. The relative abundance of the *E. purpurea* cDNA for BCAA decarboxylases (*VDC*, GenBank Accession #LT593930; *SDC*, GenBank Accession #LT593931.1) was determined on a QUANTSTUDIO 7 FLEX (Applied Biosystems, USA) using the QuantiNova SYBR Green PCR Kit (Qiagen, USA). Ubiquitin E2 genetic expression was used as internal reference to normalize mRNA content. The quantification of the expression was measured by the comparative Ct ($2^{-\Delta\Delta Ct}$) method[34]. Target gene expression was relative to the control SL cDNA, which has been adopted as calibrator. The experiment was conducted in triplicate. Primer3 software[35] was used to design primers specific to *SDC* template (epa_locus_952_iso_8_len_1801_ver_2) as available in the Medicinal Plant Genomics Resource (http://medicinalplantgenomics.msu.edu). The primer sequences for the *VDC* gene were reported in Rizhsky *et al.*[24]. All primers listed in Supplementary Table 2 were synthesized by Eurofins Genomics (Ebersberg, Germany). Independent RT-PCR products were sequenced to control for primer specificity.

Statistical analyses. Means and standard deviations of the bacterial TVC data of the three biological replicates were estimated and compared by one-way analysis of variance between R and SL samples. To evaluate whether the level estimations of the alkamides (mean peak area values) were useful in reflecting the chemical relationships between R and SL samples (controls and infected ones), a PCA was performed[36]. One-way analysis of variance followed by Tukey test was used to compare peak area values between control and infected plants and to identify the alkamides mainly responsible of the differences between the samples. Comparison of the qRT-PCR data of the three biological replicates was determined by 2-tailed t-test. $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Error bars are shown as s.e.m. The analyses were performed by using the modules present in the PAST program, version 3.15[37].

Data availability. The Authors declare that all the data supporting the findings of this study are available within the manuscript and its Supplementary Material and from the Corresponding Author on request.

References

1. Karsch-Volk, M., Barrett, B. & Linde, K. Echinacea for preventing and treating the common cold. *Jama* **313**, 618–619 (2015).
2. Manayi, A., Vazirian, M. & Saeidnia, S. Echinacea purpurea: Pharmacology, phytochemistry and analysis methods. *Pharmacognosy reviews* **9**, 63–72 (2015).
3. Pellati, F. *et al*. Chromatographic methods for metabolite profiling of virus- and phytoplasma-infected plants of Echinacea purpurea. *Journal of agricultural and food chemistry* **59** (2011).
4. Braga, R. M., Dourado, M. N. & Araujo, W. L. Microbial interactions: ecology in a molecular perspective. *Brazilian journal of microbiology: [publication of the Brazilian Society for Microbiology]* **47**(Suppl 1), 86–98 (2016).
5. Tanvir, R., Javeed, A. & Bajwa, A. G. Endophyte bioprospecting in South Asian medicinal plants: an attractive resource for biopharmaceuticals. *Applied microbiology and biotechnology* **101** (2017).
6. Aly, A. H., Debbab, A. & Proksch, P. Fungal endophytes: unique plant inhabitants with great promises. *Applied microbiology and biotechnology* **90**, 1829–1845 (2011).
7. Zhai, X. *et al*. The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medical plants. *Critical reviews in microbiology* **43**, 238–261 (2017).
8. Brader, G., Compant, S., Mitter, B., Trognitz, F. & Sessitsch, A. Metabolic potential of endophytic bacteria. *Current opinion in biotechnology* **27**, 30–37 (2014).
9. Kaul, S., Sharma, T. & M, K. D. "Omics" Tools for Better Understanding the Plant-Endophyte Interactions. *Frontiers in plant science* **7**, 955 (2016).
10. Ferrari, C. S. *et al*. Expressed proteins of Herbaspirillum seropedicae in maize (DKB240) roots-bacteria interaction revealed using proteomics. *Applied biochemistry and biotechnology* **174**, 2267–2277 (2014).
11. Scherling, C., Ulrich, K., Ewald, D. & Weckwerth, W. A metabolic signature of the beneficial interaction of the endophyte paenibacillus sp. isolate and *in vitro*-grown poplar plants revealed by metabolomics. *Molecular plant-microbe interactions: MPMI* **22**, 1032–1037 (2009).
12. Todd, D. A. *et al*. Ethanolic Echinacea purpurea Extracts Contain a Mixture of Cytokine-Suppressive and Cytokine-Inducing Compounds, Including Some That Originate from Endophytic Bacteria. *PloS one* **10**, e0124276 (2015).
13. Chiellini, C. *et al*. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants Echinacea purpurea and Echinacea angustifolia. *International microbiology: the official journal of the Spanish Society for Microbiology* **17**, 165–174 (2014).
14. Maida, I. *et al*. Antagonistic interactions between endophytic cultivable bacterial communities isolated from the medicinal plant Echinacea purpurea. *Environmental microbiology* **18**, 2357–2365 (2016).
15. Mengoni, A. *et al*. Antibiotic resistance differentiates Echinacea purpurea endophytic bacterial communities with respect to plant organs. *Research in microbiology* **165**, 686–694 (2014).
16. Greger, H. Alkamides: a critical reconsideration of a multifunctional class of unsaturated fatty acid amides. *Phytochem Rev* **15**, 729–770 (2016).
17. Barnes, J., Anderson, L. A., Gibbons, S. & Phillipson, J. D. Echinacea species (Echinacea angustifolia (DC.) Hell., Echinacea pallida (Nutt.) Nutt., Echinacea purpurea (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *The Journal of pharmacy and pharmacology* **57**, 929–954 (2005).
18. Qu, L., Chen, Y., Wang, X., Scalzo, R. & Davis, J. M. Patterns of Variation in Alkamides and Cichoric Acid in Roots and Aboveground Parts of Echinacea purpurea (L.) Moench. *HortScience: a publication of the American Society for Horticultural Science* **40**, 1239–1242 (2005).
19. Woelkart, K. & Bauer, R. The role of alkamides as an active principle of echinacea. *Planta medica* **73**, 615–623 (2007).
20. Raduner, S. *et al*. Alkylamides from Echinacea are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. *The Journal of biological chemistry* **281**, 14192–14206 (2006).
21. Sasagawa, M., Cech, N. B., Gray, D. E., Elmer, G. W. & Wenner, C. A. Echinacea alkylamides inhibit interleukin-2 production by Jurkat T cells. *International immunopharmacology* **6**, 1214–1221 (2006).
22. Muller-Jakic, B. *et al*. *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from Echinacea and Achillea species. *Planta medica* **60**, 37–40 (1994).
23. Clifford, L. J., Nair, M. G., Rana, J. & Dewitt, D. L. Bioactivity of alkamides isolated from Echinacea purpurea (L.) Moench. *Phytomedicine: international journal of phytotherapy and phytopharmacology* **9**, 249–253 (2002).
24. Rizhsky, L. *et al*. Integrating metabolomics and transcriptomics data to discover a biocatalyst that can generate the amine precursors for alkamide biosynthesis. *The Plant journal: for cell and molecular biology* **88**, 775–793 (2016).
25. Hardoim, P. R. *et al*. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and molecular biology reviews: MMBR* **79**, 293–320 (2015).
26. Cech, N. B. *et al*. High performance liquid chromatography/electrospray ionization mass spectrometry for simultaneous analysis of alkamides and caffeic acid derivatives from Echinacea purpurea extracts. *Journal of chromatography A* **1103**, 219–228 (2006).
27. He, X. G., Lin, L. Z., Bernart, M. W. & Lian, L. Z. Analysis of alkamides in roots and achenes of Echinacea purpurea by liquid chromatography electrospray mass spectrometry. *Journal of Chromatography A* **815**, 205–211 (1998).
28. Hohmann, J. *et al*. Alkamides and a neolignan from Echinacea purpurea roots and the interaction of alkamides with G-protein-coupled cannabinoid receptors. *Phytochemistry* **72**, 1848–1853 (2011).
29. Spelman, K., Wetschler, M. H. & Cech, N. B. Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry Echinacea purpurea utilizing HPLC-ESI-MS. *Journal of pharmaceutical and biomedical analysis* **49**, 1141–1149 (2009).
30. Binns, S. E., Livesey, J. F., Arnason, J. T. & Baum, B. R. Phytochemical variation in echinacea from roots and flowerheads of wild and cultivated populations. *Journal of agricultural and food chemistry* **50**, 3673–3687 (2002).
31. Chen, C. L., Zhang, S. C. & Sung, J. M. Caffeoyl phenols and alkamides of cultivated Echinacea purpurea and Echinacea atrorubens var. paradoxa. *Pharm Biol* **47**, 835–840 (2009).
32. Perry, N. B., van Klink, J. W., Burgess, E. J. & Parmenter, G. A. Alkamide levels in Echinacea purpurea: a rapid analytical method revealing differences among roots, rhizomes, stems, leaves and flowers. *Planta medica* **63**, 58–62 (1997).
33. Heilmann-Clausen, J. *et al*. Citizen science data reveal ecological, historical and evolutionary factors shaping interactions between woody hosts and wood-inhabiting fungi. *The New phytologist* **212**, 1072–1082 (2016).
34. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).
35. Untergasser, A. *et al*. Primer3–new capabilities and interfaces. *Nucleic acids research* **40**, e115 (2012).
36. Cserhati, T. Data evaluation in chromatography by principal component analysis. *Biomedical chromatography: BMC* **24**, 20–28 (2010).
37. Hammer Ø H. D., R. P. PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9 (2001).

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Author Contributions

V.M., E.R.G., A.M., R.F., P.B. and F.F. conceived and designed the work. V.M. and P.B. conceived and planned the experiments. V.M. and M.D.L. carried out all the experiments. R.V.B.R. and E.M. were involved in data collection. V.M. and A.M. performed the data analysis. V.M., M.D.L., A.M., R.F., L.P. and P.B. contributed to the interpretation of results. V.M. and P.B. wrote the first draft of the manuscript. All authors were involved in critical revision and approval of the final version.

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8.3 Draft genome sequences of Antarctic bacterial strains able to inhibit human opportunistic pathogens

Extreme environments represent a very promising field for the research of biotechnologically relevant microorganisms. In fact, in order to survive harsh environmental conditions, bacteria may develop particular phenotypic traits, such as the production of antimicrobial molecules. Thus, extreme environments may represent a rich source of antibiotic producing bacteria. For these reasons, we decided to explore bacterial strains from Antarctic environment.

The strains *Flavobacterium* sp. TAB 87, *Pseudomonas* sp. TAA 207, and *Pseudomonas* sp. TAD 18 were phenotypically characterized, and showed ability to completely inhibit 40 strains, most of which affiliated to *Burkholderia cenocepacia* and *B. multivorans*, that determine severe infections in immunocompromised patients. Considering their important inhibiory activities, we decided to deeply characterize such strains, by determining their genome sequences. Analyses revealed the presence of gene clusters involved in inhibitory activities, such as polyketide synthase, nonnribosomal peptide synthase, and terpene synthase. Overall, the obtained data confirmed that Antarctic bacteria could indeed represent important sources of antibiotic molecules, which could help finding solutions for the antibiotic resistance issue.

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Draft Genome Sequence of *Flavobacterium* sp. Strain TAB 87, Able To Inhibit the Growth of Cystic Fibrosis Bacterial Pathogens Belonging to the *Burkholderia cepacia* Complex

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We report here the draft genome sequence of the *Flavobacterium* sp. TAB 87 strain, isolated from Antarctic seawater during a summer campaign near the French Antarctic station Dumont d'Urville (60°40′S, 40°01′E). It will allow for comparative genomics and the fulfillment of both fundamental and application-oriented investigations. It allowed the recognition of genes associated with the production of bioactive compounds and antibiotic resistance.

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Antarctic bacteria are cold-adapted microorganisms that have evolved peculiar features to overcome barriers for growth at low temperatures. They are driving scientific interest, both in the field of ecological sciences as they play a key role in maintaining proper ecosystem functions, and in the clinical setting, since they are known to produce molecules able to exert antibacterial activity in order to withstand strongly adapted competitors. Indeed, it was recently demonstrated that many Antarctic bacteria exhibited the ability to counteract the growth of other Antarctic strains (1) and, more intriguingly, of some human pathogens belonging to the *Burkholderia cepacia* complex (BCC) (2–5), which represent a serious threat among immunocompromised patients, especially those affected by cystic fibrosis (CF).

Here, we report the draft genome sequence of *Flavobacterium* sp. strain TAB 87, a Gram-negative bacterium belonging to the family *Flavobacteriaceae* (6). The strain was isolated from seawater during a summer campaign near the French Antarctic station Dumont d'Urville (60°40′ S, 40°01′ E). The genome analysis of this Antarctic strain enables both fundamental and application-oriented investigations. Indeed, this strain completely inhibited the growth of 40 BCC strains belonging to 18 different bacterial species, most of which belonged to the species *Burkholderia cenocepacia* and *Burkholderia multivorans*, two of the most important CF pathogens. Moreover, some of the antimicrobial compounds produced were volatile organic compounds (VOCs), according to previous observations (3, 4). The draft genome sequence of *Flavobacterium* sp. TAB 87 was determined by the Institute of Applied Genomics and IGA Technology Services Srl (University of Udine, Italy) through a paired-end approach using an Illumina (Solexa) Genome Analyzer II platform. A total of 19,040,534 paired-end reads (average coverage, 1,004×) were initially obtained, those with low quality were trimmed with Streaming Trim (7), and those remaining were assembled with SPAdes genome assembler version 3.6.1 (8), which generated a total of 5,056 contigs. Those contigs <1,000 bp were discarded, while the others were embedded in the final version of the draft genome, which is 3,827,405 bp long and harbors 38 contigs (the longest of which is 1,014,695 bp long). The G+C content is 65.5%, similar to that of other *Flavobacterium* genomes sequenced so far. Annotation was performed by using Prokka (9), which, among all the predicted genes (3,365), identified a total of 3,323 protein-coding genes, 3 rRNA-coding genes, and 39 tRNA-coding genes.

Moreover, we screened the genome sequence for the presence of genetic traits involved in secondary metabolite biosynthesis. The analysis was performed within antiSMASH shell (10), revealing that the *Flavobacterium* sp. TAB 87 genome harbors four interesting gene clusters: a type I and a type III polyketide synthase (PKS) and two terpene biosynthetic gene clusters. Additionally, the genome sequence was analyzed through CARD (11), leading to the identification of *cfrA* and *Staphylococcus aureus* parE, two genes conferring resistance to florfenicol and fluoroquinolones, respectively.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at GenBank under the accession no. LLWK00000000. The version described in this paper is version LLWK01000000.

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REFERENCES

1. **Mangano S, Michaud L, Caruso C, Brilli M, Bruni V, Fani R, Lo Giudice AL.** 2009. Antagonistic interactions between psychrotrophic cultivable bacteria isolated from Antarctic sponges: a preliminary analysis. Res Microbiol **160:**27–37. http://dx.doi.org/10.1016/j.resmic.2008.09.013.
2. **Papaleo MC, Perrin E, Maida I, Fondi M, Fani R, Vandamme P.** 2010. Identification of species of the *Burkholderia cepacia* complex by sequence analysis of the *hisA* gene. J Med Microbiol **59:**1163–1170. http://dx.doi.org/10.1099/jmm.0.019844-0.
3. **Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice AL, Michaud L, Mangano S, Bartolucci G, Romoli R, Fani R.** 2012. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv **30:**272–293. http://dx.doi.org/10.1016/j.biotechadv.2011.06.011.
4. **Maida I, Fondi M, Papaleo MC, Perrin E, Orlandini V, Emiliani G, de Pascale D, Parrilli E, Tutino ML, Michaud L, Lo Giudice A, Romoli R, Bartolucci G, Fani R.** 2014. Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds. Extremophiles **18:**35–49. http://dx.doi.org/10.1007/s00792-013-0590-0.
5. **Lo Giudice A, Fani R.** 2015. Cold-adapted bacteria from a coastal area of the Ross Sea (Terra Nova Bay, Antarctica): linking microbial ecology to biotechnology. Hydrobiologia **761:**417–441. http://dx.doi.org/10.1007/s10750-015-2497-5.
6. **Holmes B, Owen RJ, McMeekin TA.** 1984. Genus *Flavobacterium* Bergey, Harrison, Breed, Hammer and Huntoon. 1923:97^{AL}, p 353–361. *In* Krieg NR, Holt JG (ed), Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, MD.
7. **Bacci G, Bazzicalupo M, Benedetti A, Mengoni A.** 2014. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. Mol Ecol Resour **14:**426–434. http://dx.doi.org/10.1111/1755-0998.12187.
8. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA.** 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol **19:**455–477. http://dx.doi.org/10.1089/cmb.2012.0021.
9. **Seemann T.** 2012. Prokka: prokaryotic genome annotation system. Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia.
10. **Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R.** 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res **39**(Suppl 2):W339–W346. http://dx.doi.org/10.1093/nar/gkr466.
11. **McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJV, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T, Wright GD.** 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother **57:**3348–3357. http://dx.doi.org/10.1128/AAC.00419-13.

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Draft Genome Sequences of the Antimicrobial Producers *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18 Isolated from Antarctic Sediments

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We report here the draft genome sequence of the *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18 strains, isolated from Antarctic sediments during a summer campaign near coastal areas of Terra Nova Bay (Antarctica). Genome sequence knowledge allowed the identification of genes associated with the production of bioactive compounds and antibiotic resistance. Furthermore, it will be instrumental for comparative genomics and the fulfillment of both basic and application-oriented investigations.

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Antarctica provides one of the largest unexplored sources of biodiversity. Here, the continuous environmental challenges led to extremely adapted living forms that may be sources of potentially novel, untapped gene functions. Particularly, it has been shown how microorganisms are claimed to be a reservoir of biotechnologically relevant molecules, such as antibiotics (1–6).

Here, we report the genome sequences of two *Pseudomonas* sp. strains, TAA207 and TAD18, isolated from Antarctic sediments during a summer campaign near the coastal areas of Terra Nova Bay (Antarctica). These bacteria have been screened for antimicrobial activity against human pathogens. The results obtained show how they totally inhibited 40 strains belonging to the *Burkholderia cepacia* complex (BCC), most of which are affiliated to the species *Burkholderia cenocepacia* and *Burkholderia multivorans*, two of the most important pathogens in immunocompromised patients affected by cystic fibrosis disease. Also, they produce antibiofilm molecules acting against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (7).

Both genome sequences of *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18 were determined through a paired-end approach using the Illumina/Solexa genome analyzer IIx platform at the Institute of Applied Genomics and IGA Technology Services Srl (University of Udine, Italy). A total of 11,007,120 and of 12,698,315 reads were obtained for *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18, respectively. Low-quality sequences were trimmed with StreamingTrim (8), and the remaining were assembled with SPAdes genome assembler version 3.6.1 (9). Only contigs longer than 1,000 bp were embedded in the final version of the draft genomes, which are 4,900,197-bp long for *Pseudomonas* sp. TAA207 (72 contigs, 453× average coverage, 57% GC content) and 4,917,586-bp long for *Pseudomonas* sp. TAD18 (82 contigs, average coverage 521×, 57.24% GC content).

Annotation was then performed using Prokka (10), which identified 4,379 and 4,403 genes for *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18, respectively. Among these, 4,028 are protein-encoding genes in the former organism, and 4,220 are in the latter one.

The genome sequences allowed comparative genomics analysis to check for the presence of genetic traits involved in secondary metabolite biosynthesis. The analysis was performed within the antiSMASH shell (11), revealing that both genomes harbor gene clusters encoding molecules involved in inhibitory activities. Particularly, the two strains embed gene clusters similar to those coding for aryl-polyene, terpene, bacteriocin, and nonribosomal peptide synthase. Additionally, *Pseudomonas* sp. TAA207 contains a cluster involved in microcin production.

Further, we investigated the possibility that both strains possess antibiotic resistance genes in their pool by probing their sequences in the Comprehensive Antibiotic Resistance Database (CARD). The outcome yields strong indications that both genomes have genes coding for general efflux pumps, alongside several genes conferring resistance to specific classes of antibiotics, including chloramphenicol, fluoroquinilone, beta-lactam, trimethoprim, tetracycline, polymyxin, aminoglycoside, and rifampin.

Nucleotide sequence accession numbers. The whole-genome shotgun projects of *Pseudomonas* sp. TAA207 and *Pseudomonas* sp. TAD18 have been deposited at GenBank under the accession numbers LLWJ00000000 and LLWI00000000, respectively. The

versions described in this paper are the first versions, LLWJ01000000 and LLWI01000000.

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REFERENCES

1. **Mangano S, Michaud L, Caruso C, Brilli M, Bruni V, Fani R, Lo Giudice AL.** 2009. Antagonistic interactions between psychrotrophic cultivable bacteria isolated from Antarctic sponges: a preliminary analysis. Res Microbiol **160:**27–37. http://dx.doi.org/10.1016/j.resmic.2008.09.013.
2. **Papaleo MC, Perrin E, Maida I, Fondi M, Fani R, Vandamme P.** 2010. Identification of species of the *Burkholderia cepacia* complex by sequence analysis of the *hisA* gene. J Med Microbiol **59:**1163–1170. http://dx.doi.org/10.1099/jmm.0.019844-0.
3. **Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice AL, Michaud L, Mangano S, Bartolucci G, Romoli R, Fani R.** 2012. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv **30:**272–293. http://dx.doi.org/10.1016/j.biotechadv.2011.06.011.
4. **Maida I, Fondi M, Papaleo MC, Perrin E, Orlandini V, Emiliani G, de Pascale D, Parrilli E, Tutino ML, Michaud L, Lo Giudice A, Romoli R, Bartolucci G, Fani R.** 2014. Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds. Extremophiles **18:**35–49. http://dx.doi.org/10.1007/s00792-013-0590-0.
5. **Lo Giudice A, Fani R.** 2015. Cold-adapted bacteria from a coastal area of the Ross Sea (Terra Nova Bay, Antarctica): linking microbial ecology to biotechnology. Hydrobiologia **761:**417–441. http://dx.doi.org/10.1007/s10750-015-2497-5.
6. **Presta L, Inzucchi I, Bosi E, Fondi M, Perrin E, Miceli E, Tutino ML, Lo Giudice A, de Pascale D, Fani R.** 2016. Draft genome sequence of *Flavobacterium* sp. strain TAB 87, able to inhibit the growth of cystic fibrosis bacterial pathogens belonging to the *Burkholderia cepacia* complex. Genome Announc **4**(3): http://dx.doi.org/10.1128/genomeA.00410-16.
7. **Papa R, Selan L, Parrilli E, Tilotta M, Sannino F, Feller G, Tutino ML, Artini M.** 2015. Anti-biofilm activities from marine cold adapted Bacteria against staphylococci and *Pseudomonas aeruginosa*. Front Microbiol **6:**1333. http://dx.doi.org/10.3389/fmicb.2015.01333.
8. **Bacci G, Bazzicalupo M, Benedetti A, Mengoni A.** 2014. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. Mol Ecol Resour **14:**426–434. http://dx.doi.org/10.1111/1755-0998.12187.
9. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA.** 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol **19:**455–477. http://dx.doi.org/10.1089/cmb.2012.0021.
10. **Seemann T.** 2012. Prokka: prokaryotic genome annotation system. Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia.
11. **Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R.** 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res **39**(suppl 2):W339–W346. http://dx.doi.org/10.1093/nar/gkr466.