UNIVERSITY OF MILAN

PHYLOSOPHY DOCTORATE SCHOOL IN

FOOD SYSTEMS

XXXII CYCLE

Department of Food, Environment and Nutritional Science

PLANT ASSOCIATED BACTERIA: A SUSTAINABLE RESOURCE TO MINIMIZE WATER FOOTPRINT IN AGRICULTURE

AGR16

Valentina Riva

Supervisor: Prof.ssa Sara Borin

Coordinator: Prof.ssa Ella Pagliarini

Academic year 2018-2019

"Cuando el agua se mueve el cosmos interviene. El agua recibe la fuerza de los planetas, la transmite al suelo y a todas las criaturas. El agua es un órgano mediador entre las estrellas y nosotros"

– El botón de nácar –

ABSTRACT

Water scarcity is one of the major worldwide problems affecting in particular agriculture, an intensive water consumer activity. In the frame of the EU H2020 MADFORWATER project, aimed to develop integrated technological and management solutions to reduce water footprint in agriculture, this PhD thesis focused on plant-associated bacteria to improve the productivity of crops growing under drought conditions and wastewater phytodepuration efficiency to allow water reuse for irrigation purposes. A large collection of 681 bacterial strains was isolated from the root apparatus of six plants species adapted to dry soils or other unconventional environmental conditions. The isolates have been taxonomically identified and functionally characterized in order to select the most promising strains exploitable in *in vivo* experimentations. The isolate collection was compared with a literature-derived dataset of bacterial families identified in the plant microbiome by molecular methods. The comparison demonstrated that we were able to bring into culture members of 20% of the bacterial families detected by culture-independent approaches, partially confirming on the plant biome recent concepts emerged on others environmental microbiota. Moreover, the collection included ubiquitous and potentially beneficial core members of the plant microbiota and satellite taxa, which could have a role in sustaining plant growth under peculiar environmental conditions. The bacterial community associated to *Argania spinosa*, a drought tolerant tree relevant for the ecology and economy of Morocco, was described for the first time, by culture dependent and independent approaches. The Plant Growth Promoting (PGP) potential of isolates obtained from the root and soil environment of Argan revealed a particular abundance of PGP related traits in strains isolated from the residuesphere fraction, providing a putative explanation to the tradition of local farmers of using argan litter as soil amendment.

A selection of strains was tested in *in vivo* experiments aimed to evaluate their potential to improve plant growth, water use efficiency and fruit productivity. Five PGP bacteria were applied on tomato plants, cultivated in greenhouse under optimal and deficit irrigation and plant physiology, biomass and fruit yield were compared with control, not bacterized plants. All the bacterial inocula showed at different extent to have the potential capacity to alleviate plant water stress, improving different plant and soil parameters, but no statistically significant effect was reported on plant productivity. Further experiments are needed to confirm the results, nevertheless it was demonstrated the importance of performing long-term experiments to obtain reliable and applicable data about the real efficacy of PGP crop application.
A second selection of PGP strains significantly showed in microcosm constructed wetlands the ability to tolerate metal contaminated wastewaters and to enhance the azo-dye phytodepuration capacity of *Juncus acutus* plants. These bacteria have thus the potential to improve the quality of water treated in low cost systems, and will be selected in the project for experimentation at higher pilot scale.
This work demonstrated the relevance of the plant microbiota in sustaining plant growth, and provided a collection of strains which need to be further evaluated but could potentially be exploited to mitigate water shortage effects in agriculture.

RIASSUNTO

La scarsa disponibilità di acqua rappresenta uno dei problemi più rilevanti a colpire principalmente il settore agricolo, posto oggigiorno sotto pressione dai cambiamenti climatici e dal continuo aumento della popolazione mondiale. Nel contesto del progetto EU H2020 MADFORWATER, il cui scopo è quello di sviluppare soluzioni tecnologiche e manageriali al fine di ridurre l'impronta idrica in agricoltura, la presente tesi di dottorato si è focalizzata sull'utilizzo di batteri associati alle piante per migliorare la produttività di colture sottoposte a condizioni di stress idrico e l'efficienza di fitodepurazione delle acque reflue per permettere il riuso di acqua per scopi irrigui. Un'ampia collezione composta da 681 ceppi batterici è stata isolata dall'apparato radicale di sei specie vegetali adattate a vivere in suoli aridi o altre condizioni ambientali non convenzionali. Gli isolati batterici sono stati tassonomicamente identificati e caratterizzati a livello funzionale al fine di selezionare i ceppi più promettenti da poter testare in esperimenti *in vivo*. La collezione di isolati è stata confrontata con un dataset, ottenuto dalla letteratura, di famiglie batteriche identificate tra il microbioma delle piante tramite tecniche molecolari. Il confronto ha dimostrato che siamo stati in grado di ottenere in coltura membri del 20% delle famiglie batteriche ritrovate con approcci coltura-indipendenti, parzialmente confermando, sul bioma delle piante, un recente concetto emerso con altri microbiomi ambientali. La collezione inoltre è risultata composta da membri ubiquitari e potenzialmente benefici del cosiddetto “core” del microbioma della pianta ma anche da taxa satelliti, i quali possono avere un ruolo di supporto alla crescita di piante sottoposte a particolari condizioni ambientali. La comunità batterica di *Argania spinosa*, un albero tollerante a condizioni di siccità di rilevante importanza per l'ecologia e l'economia del Marocco, è stata descritta per la prima volta utilizzando approcci coltura-dipendenti e indipendenti. Lo studio del potenziale di promozione della crescita vegetale

(PGP) degli isolati batterici ottenuti dai campioni di radice e di suolo influenzato dalla pianta di argan, ha rivelato una particolare abbondanza di abilità PGP nei ceppi isolati dalla frazione residuosfera, il che offre una possibile spiegazione alla tradizione dei contadini locali che utilizzano la lettiera degli alberi di argan come ammendante per i suoli agricoli. Una selezione di isolati batterici è stata testata in esperimenti *in vivo* per valutare il loro potenziale nel migliorare la crescita vegetale, l'efficienza di utilizzo dell'acqua e la produttività delle piante. Cinque batteri PGP sono stati applicati a piante di pomodoro, coltivate in serra e sottoposte a condizioni irrigue ottimali e di deficit irriguo: la fisiologia, la biomassa e la resa in frutti di queste piante sono stati confrontati con piante non batterizzate. Tutti gli inoculi batterici hanno mostrato, a diversi livelli, di avere una potenziale capacità di alleviare lo stress idrico, promuovendo diversi parametri della pianta e del suolo, ma nessun effetto statisticamente significativo è stato riportato sulla produttività. Ulteriori esperimenti sono necessari per confermare i risultati, tuttavia è stata dimostrata l'importanza dell'effettuare esperimenti a lungo termine per ottenere risultati affidabili riguardanti la reale efficienza di applicazione di batteri PGP sulle colture agrarie. Una seconda selezione di ceppi PGP, testati in microcosmi riproducenti impianti di fitodepurazione, ha mostrato una significativa capacità di tollerare la presenza di metalli in acque reflue e di aumentare la capacità di piante della specie *Juncus acutus* di fitodepurare coloranti azoici. Questi batteri hanno quindi un potenziale nel migliorare la qualità delle acque trattate in impianti a basso costo, e si prevede la loro selezione, all'interno del progetto, per effettuare esperimenti in impianti pilota.

Questo lavoro ha dimostrato l'importanza del microbioma delle piante nel sostenere la crescita vegetale e ha fornito una collezione di ceppi batterici che, in seguito ad ulteriori caratterizzazioni, può potenzialmente essere sfruttato per mitigare l'effetto della carenza idrica in agricoltura.

TABLE OF CONTENTS

- ***Abstract*** *I*
- ***Riassunto*** *III*
- ***Introduction*** *1*

  Chapter I: Exploitation of rhizosphere microbiome services: improvement of plant drought tolerance *1*

  Chapter II: Microbial assisted phytodepuration for water reclamation: environmental benefits and threats *15*

- ***Aims and objectives*** *51*
- ***Results*** *55*

  Chapter III: Investigation of plant microbiome through culture-dependent and independent techniques *55*

  Chapter IV: Unveiling the microbiota diversity of the xerophytic tree *Argania spinosa* L. Skeels *81*

  Chapter V: Plant growth promoting effect of bacteria inocula on tomato plants cultivated under different water conditions during long-term greenhouse experiment *127*

  Chapter VI: Root bacteria recruited by *Phragmites australis* in constructed wetlands have the potential to enhance azo-dye phytodepuration *161*

- ***General conclusion*** *209*
- ***Implications and future directions*** *215*
- ***Appendices*** *217*

INTRODUCTION

Chapter I

Exploitation of rhizosphere microbiome services: improvement of plant drought tolerance

[Published as book chapter in *Methods in Rhizosphere Biology Research* (pp. 105-132). Springer, Singapore. Riva, V., Terzaghi, E., Vergani, L., Mapelli, F., Zanardini, E., Morosini, C., Raspa, G., Di Guardo, A. and Borin, S., 2019. Exploitation of Rhizosphere Microbiome Services]

Exploitation of rhizosphere microbiome services: improvement of plant drought tolerance

Drought is one of the major environmental stresses that limit plant growth and agricultural productivity around the world. Because of the ongoing global warming and climate changes, it is projected that the land area affected by drought will increase by twofold and water resources will decline by 30% by 2050 (Falkenmark 2013). Finding efficient strategies for the mitigation of drought stress is therefore increasingly gaining importance.

Microbial Mechanisms Responsible of the Improvement of Plant Drought Tolerance

Plants may cope with water scarcity through different physiological strategies;

nevertheless, drought is among the most destructive abiotic stresses, since its multidimensional action affects the whole plant levels. Furthermore, most of the approaches suggested for controlling the negative effects of drought stress in plants, such as breeding for tolerant varieties and genetic engineering, are time-consuming and cost-intensive. In this scenario, plant-associated microbes with the capacity to improve drought tolerance in crop plants have received considerable attention in the last years. It has been reported that mycorrhizal fungi and PGPR recruited by the plant in the rhizosphere can contribute to alleviate abiotic stresses of the host improving plant physiological processes associated with drought resistance. Many research works characterized the activity of isolated microbial strains, identifying several mechanisms putatively responsible of the promotion activity, briefly overviewed in the following paragraph (see recent reviews by Ngumbi and Kloepper 2016; Soussi et al. 2016; Kumar and Verma 2018).

Several bacterial strains have been isolated from plant rhizosphere and characterized *in vivo* for their capacity to improve drought tolerance to a variety of plants. Some PGPR strains were reported to be able to promote root growth and to alter root architecture, with the consequences to increase

the root surface area, in turn leading to improve water and nutrient uptake. PGPR demonstrated, moreover, the capacity to promote also shoot growth, hence potentially decreasing the yield losses due to the plant response to drought stress, which inhibits shoot growth aiming to decrease water loss by evapotranspiration. Other PGPR drought protection mechanisms involve a strict plant-bacteria relationship, inducing plant cells to increase the content of osmoprotectant compounds (e.g., proline, free amino acids, sugars) or ROS (reactive oxygen species)-scavenging enzymes, both conferring higher tolerance toward drought-induced damages. The most studied and widespread mechanisms hypothesized to explain PGPR activity in increasing drought tolerance in plants are, nevertheless, the control of plant growth regulators. Lowering the concentration of ethylene, which is overproduced in response to stressful conditions, by 1-aminocyclopropane-1-carboxylate (ACC) deaminase, is considered to be one of the major mechanisms employed by PGP bacteria to favor plant growth under stress conditions. Other metabolites produced by PGP bacteria and potentially involved in supporting plant growth under stress conditions include phytohormones that modulate root development (i.e., indole-3-acetic acid), osmolytes that contribute to reduce cell dehydration, siderophores, and volatile compounds (Vurukonda et al. 2016). Additional mechanisms of PGPR growth promotion are directed to alleviate other environmental challenges that the plant is experiencing in dry lands. The production of exopolysaccharides (EPS), which protect roots from mechanical stress determined by dry soil compactness, can indeed play an important role under water deficit. Salt stress is, moreover, strictly related to drought, since high salt concentrations in the soil are very frequent in arid and semiarid regions. Salinity stress is one of the most common abiotic stress factors in modern agriculture, and PGP microbes can have several direct and indirect mechanisms of growth promotion (reviewed by Kumar and Verma 2018).

Approaches, Techniques, and Results

PGPR Isolation Sources

Several recent ecological studies have found that microbial symbionts can confer habitat-specific stress tolerance to host plants, suggesting that the basis for the stress tolerance-enhancing effects of microbial symbionts is the coevolution of plant and microbes under harsh environmental conditions. Thus, it is a good strategy to look for plant-beneficial microorganisms that confer resistance to a specific environmental stress from the environments where that stress is a regular phenomenon. Water availability and drought gradients in soil were described to shape the structure of plant-associated bacterial communities. It was observed that the microbial diversity decreases along with water shortage and the relative abundance of the different bacteria change: under arid conditions, a higher proportion of Gram-positive bacteria, especially represented by the phylum Actinobacteria and the genus *Bacillus*, was observed by metataxonomic analyses (PCR fingerprinting and 16S rRNA sequencing) (Kavamura et al. 2013; Köberl et al. 2011). Recent advances in sequencing technologies and metagenomics have enabled us to explore and compare the microbial diversity that is associated with extreme environments and gain insight into how microorganisms survive in these harsh ecological niches (Lebre et al. 2017). Bacteria that colonize the rhizosphere of plants in dry environments may have undergone a selective pressure in order to survive. In addition, these bacteria may confer a certain level of tolerance to plants, as well as other functions such as plant growth promotion and soil maintenance due to their function and strategy in the ecosystem. Marasco and co-workers (2012) showed a variation in the distribution of bacteria into the endosphere and rhizosphere compared to uncultivated soil associated with *Capsicum annuum* plants under desert farming conditions, indicating that the enrichment of specific bacterial taxa is given by the plant itself. Moreover, most of the bacteria (88%) isolated from the root system of pepper plants exhibited multiple PGP activities and stress

resistance capabilities, indicating that they can be active and hence express their PGP features in vivo under water stress conditions. Likewise, bacterial communities isolated from *Salicornia* plants grown under hypersaline ecosystems in Tunisia showed resistance to high-temperature, osmotic, and saline stresses and were able to perform different plant growth-promoting activities (Mapelli et al. 2013). Plants adapted to extreme environmental features, thus, represent ideal sites for discovering novel biotechnological agents which could be exploited to sustain other plants, including agricultural crops, to counteract water shortage in arid land agriculture.

Autochthonous or Allochthonous PGPR?

One main question is if a PGPR strain isolated from a plant species would be equally efficient in promoting the growth when inoculated to a different species. This issue has important practical and economic consequences related to the formulation of microbial products to be applied on specific or broad range of crop plants. Different research works showed the cross-compatibility of PGPR strains, allowing their exploitation as biofertilizers tailored for arid lands. *Rhodococcus* sp., a Grampositive bacterium isolated from the rhizosphere of olive tree growing in south Tunisia, was able to promote root fresh biomass of tomato plants (Marasco et al. 2013); rhizospheric and endophytic *Pseudomonas* sp. isolated from the native halotolerant coastal plant *Suaeda salsa* were observed to be responsible for strengthening growth and salt stress responses of rice plants (Yuan et al. 2016); *Pseudomonas putida* MTCC5279 isolated from the desert regions of Rajasthan was able to increase drought resistance in chickpea (Tiwari et al. 2016). These evidences lead to consider that the nature of the plant-bacteria interaction in dry environments can have a limited level of specificity and that PGPR isolates may determine resistance to water stress in plants other than the one of the original isolation, increasing their application potential. It is, therefore, apparent that in relation to plant resistance to water stress, a feature of primary evolutionary importance for all plants, a cross compatibility

between PGPR and different plant models exists at least on a short term (Marasco et al. 2013). On the contrary, when PGPR isolates were applied on the same plant species but growing in different environmental contexts, the results were contradictory, and in some cases the strains showed different levels of promotion efficacy. Some strains were reported to promote plant growth under both irrigated and drought conditions (Gagné-Bourque et al. 2016), while others were specifically able to enhance plant growth only under stress and resulted ineffective in plant growth promotion under optimal irrigation conditions (Rolli et al. 2014; Chen et al. 2017). These findings suggest that the promotion activity of bacteria may be either stress-dependent or stress-independent.

In Vivo and In Vitro Testing of Drought Tolerance Promotion

Isolated bacterial strains can be tested in vitro for traits that could be associated to plant growth promotion (PGP) (Marasco et al. 2012). Plate and tube assays are available and generally adopted to select the strains having PGP potential (Table 1): the production of hormones (the auxin indole acetic acid), ACC-deaminase enzyme, bioavailable nutrients (ammonia, soluble phosphate, iron chelating siderophores), and other compounds (EPS) is routinely tested by most of the authors. Besides potential PGP traits, the strains could also be characterized *in vitro* for the capacity to tolerate abiotic conditions typical of arid soils, i.e., water and osmotic stress, high salt concentrations, and temperature extremes (Table 1). *In vitro* tests are easy to be performed and are useful to select the most promising strains among large collections of isolates. The PGP effect is nevertheless the result of a strict relation between bacteria and plant; therefore, other complex factors drive the efficacy of a strain. *In vivo* experiments under drought conditions are, for this reason, required.

According to *in vitro* PGP tests and the ability of the isolates to cope with several abiotic stresses (Table 1), the most promising strains should be further tested in pot experiments for their potential in growth promotion and

plant drought tolerance improvement. Using surface-sterilized seeds, an established concentration of bacteria is supplied to plants by seed biopriming or by liquid inocula provided to seedlings. During the period of growth under controlled conditions, water-deficit stress is imposed to plants withholding water for a certain time (from 7 to 18 days depending on plant variety) or maintaining soil moisture at constant low levels (Zolla et al. 2013). When plants are harvested, different parameters are measured to evaluate the potential of PGP bacteria to improve drought tolerance in plants. In particular, the parameters to take into account are (i) vegetative parameters including shoot and root lengths, shoot and root dry weight, and number of leaves and nodes and (ii) productive parameters like fruits yield per plant and fruits quality.

Table 1: *In vitro* screening of plant growth-promoting activities and stress resistance capabilities

***In vitro* screening of plant growth promoting activities**	**Methods**
Production of indole-3-acetic acid	Bric et al., 1991
Solubilise insoluble phosphate compounds	Ahmad et al., 2008
Ammonia synthesis	Cappuccino and Sherman, 1992
Protease activity	Nielsen and Sørensen, 1997
ACC-deaminase activity	Belimov et al., 2005
Atmospheric nitrogen fixation ability	Penrose and Glick, 2003
Production of exopolysaccharides	Santarella et al., 2008
Siderophore production	Schwyn and Neilands, 1987
***In vitro* screening of stress resistance capabilities**	**Methods**
Resistance to salt	Adding 5, 8, and 10% sodium chloride to culture media and incubating the plate at 30∘C for 7 days
Resistance to high temperature	Verifying grow at 4, 42, and 50∘C in solid media placed in incubators for 7 days
Tolerance to osmotic stress	Adding 10–20% polyethylene glycol (PEG) to the original liquid media

In order to evaluate how the inoculated strain promoted plant growth and alleviate water stress, it is also possible to measure leaf physiological parameters, possibly using not destructive methods, including net

photosynthesis, evapotranspiration, and stomatal conductance. Chen et al. (2017) for instance, measured free proline, soluble sugars, malondialdehyde (MDA), and total chlorophyll from leaves of wheat plants inoculated with the PGP strain LTYR-11ZT. The proline concentration in leaves, which usually increases drastically under drought stress, was reduced with the inoculation of the PGP strain: this decrease could be indicative of a less damage in wheat plants in the presence of the bacterium. Similarly, wheat plants colonized with LTYR-11ZT strain had significantly lower MDA contents compared with non-inoculated control under drought stress: MDA level can reflect the degree of cell membrane damage, so the bacteria inoculation can help in overcoming this kind of damage. About soluble sugars, which drastically increase under water-deficit conditions, it is known that they play a role in drought tolerance maintaining cell osmotic turgor: the presence of the PGP bacterium further increased the concentration of sugars allowing better osmotic adjustment in the host plants. Finally, chlorophyll concentration is considered as an important indicator of drought tolerance in plants: this study revealed that chlorophyll level was increased in treated plants under stress conditions, suggesting that the PGP bacterium helped plants adapt to drought stress. Another approach was used by Gagné-Bourque and co-workers (2016) with *Brachypodium* plants inoculated with *Bacillus subtilis* strain: they set up a DNA methylation assay, and they showed an increase in the abundance of methyl transferases involved in the maintenance and regulation of DNA methylation, suggesting that this bacterium could potentially act at the epigenetic level to increase drought stress tolerance in plants.

Study of Plant-Bacteria Relationship

To understand and compare the “holistic” responses of the bacteria and plant system under water-deficit conditions, a powerful tool is the study of their metatranscriptome, consisting of the entire set of transcripts that are expressed with in a meta-organism in a particular developmental stage or

under different environmental conditions. Using quantitative real-time PCR, upregulation of drought-response genes DREB2B-like, DHN3-like, and LEA-14-A-like in the aerial parts of *Brachypodium* was identified when plants were inoculated with a PGPR *Bacillus subtilis* strain (Gagné-Bourque et al. 2016). Using microarray analysis, a set of drought signaling response genes were discovered to be downregulated under drought stress in *Arabidopsis thaliana* plants colonized by a PGPR *Pseudomonas chlororaphis* strains compared to non-inoculated plants (Cho et al. 2013). Yuan et al. (2016), using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) tool for predicting *S. salsa*-associated microbiome functions under salinity stress, discovered an enrichment of diverse two-component systems and ATP-binding cassette transporters, which may provide a selective advantage for bacterial adaptation to adverse conditions. More recent in situ applications of "omics" technologies provided key insights into the responses of xerotolerant microorganisms in their natural xeric environments. The microarray-based GeoChip technology, which encompasses an array of functional genes that are involved in metabolism and stress responses, has been efficaciously applied to directly measure xeric stress responses (Chan et al. 2013).

A critical step in the interaction between beneficial bacteria and the host plants is the efficient root colonization by inoculated bacteria. To play an effective role in plant growth promotion, a bacterial strain must be able to respond to the plant rhizodepositions and colonize its root apparatus entering in association/competition with the resident rhizosphere microbial community. The potential ability of PGP isolates to efficiently colonize plant root system can be tested *in vivo* by performing an adhesion assay. To evaluate the colonization pattern of the tested PGP strain, one of the most exploited strategies is to manipulate it in order to obtain a gfp-tagged strain. To have the possibility to perform long-term adhesion test, the tagging should be chromosomal rather than plasmidic, in order to minimize the appearance of not fluorescent revertants. Plant roots are exposed for a

period of time (i.e., 16 h) to the bacterial suspension of the fluorescent mutant and then analyzed by confocal microscopy (Mapelli et al. 2013; Chen et al. 2017). For strains reluctant to genetic manipulation, a culture-dependent monitoring of colonization could be performed, but the colonization in this case can result overestimated due to the presence of a cultivable endogenous plant microbiota difficult to be distinguished from the inoculated PGP strain. Inoculants could be, in alternative, quantified in the rhizosphere metagenome by quantitative real-time PCR (qPCR) assay, basing on sequence signatures previously identified in the strain genome (Gagné-Bourque et al. 2016).

The abovementioned methods could also be useful to detect the vertical transmission of the PGP strains initially recruited from the rhizosphere, evaluating their presence into the seeds and in the offspring tissues. Vertical transmission of PGPR to the plant progeny is very interesting as it enables a plant with an established endophytic community to pass bacteria with beneficial characteristic to the next generation and ensures the presence of a beneficial microbiota at an early stage of seedling growth. Bacteria having the potential to establish as endophytes have other qualities that make them of particular interest, like their advantage of being relatively protected from the competitive high-stress environment of the soil, since they live in the interior parts of the plants. As compared to rhizospheric bacteria, endophytic inocula showed positive results in the promotion of plant growth and stress tolerance induction in plant under harsh environmental condition (Naveed et al. 2014); however, it has not been resolved whether plants benefit more from an endophyte than from a rhizospheric bacterium. It was proved that endophytes might exhibit phytopathogenic effects under certain conditions, and rhizosphere bacteria might also be able to colonize the internal parts of the plant (Hardoim et al. 2015). This is because microbial population dynamics are affected by a variety of factors, such as plant species, soil type, and biotic/abiotic factors.

Discussion

Scaling Up from Laboratory to the Field: The Importance of the Residing Soil Microbiota

Many factors, known and unknown, influence the interaction between plants, soil, and microbiota. The relationship is complex and dynamic, and this is the reason why bacterial inoculants often perform well in controlled laboratory experiments and then fail to give a beneficial effect in natural agricultural settings. One possible explanation is that laboratory experiments are often conducted using sterilized soil substrates, while the microbial inoculants face competition with the native soil microbiota when inoculated to natural soil. Plant inoculation with selected bacterial strains in natural soil conditions could fail to achieve expected outcomes because of the competition with native microbial communities and the limited colonization efficiency. Moreover, it is well known that the behavior of microorganisms as pure cultures could be different from their behavior in a microbial community. It has been hypothesized that it is better to leverage on the natural community rather than trying to transplant microorganisms (East 2013). Zolla et al. (2013) unraveled indeed the importance of the soil microbiome as a whole in alleviating drought stress, against the convention of a single bacterial application, demonstrating the ability of a sympatric soil microbiome to increase Arabidopsis growth under water-deficit conditions.

Synthetic PGPR Communities

Many different PGPR strains have been characterized up to now, discovering very different mechanisms of plant growth promotion and protection toward drought. A possible approach to exploit this high functional diversity is to inoculate plants with a mixture of strains having different putative beneficial traits. The combination on inoculants may not necessarily produce additive or synergistic effects but could, instead, generate a competitive situation in the rhizosphere without benefitting the plant. To

better understand this complexity, a novel strategy was recently proposed to reconstruct and transfer into the rhizosphere the functional microbial groups instead of individual isolates. Synthetic communities are indeed a powerful tool to investigate fundamental principles in natural systems as they are more similar to natural conditions compared to pure cultures, but at the same time they have a reduced complexity and higher controllability. Niu and co-workers (2017) inoculated maize axenic plants with seven strains representing three of the four most dominant phyla found in maize roots and by tracked by a selective culturing method the abundance of each strain during root colonization in maize seedlings. This method allowed to study the dynamics and function of root bacterial assemblages, leading to identify key species necessary for plant protection.

References

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.). Soil Biol Biochem 37:241–250

Bric JM, Bostock RM, Silverstone SE (1991) Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57:535–538

Cappuccino JC, Sherman N (1992) Negative staining. In: Cappuccino JC, Sherman N (eds) Microbiology: a laboratory manual, 3rd edn. Benjamin/Cummings, Redwood City, pp 125–179

Chan Y, Van Nostrand JD, Zhou J, Pointing SB, Farrell RL (2013) Functional ecology of an Antarctic dry valley. Proc Natl Acad Sci 110:8990–8995

Chen C, Xin K, Liu H, Cheng J, Shen X, Wang Y, Zhang L (2017) Pantoea alhagi, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat. Sci Rep 7:41564

Cho SM, Kang BR, Kim YC (2013) Transcriptome analysis of induced systemic drought tolerance elicited by Pseudomonas chlororaphis O6 in Arabidopsis thaliana. Plant Pathol J 29:209

East R (2013) Soil science comes to life. Nature 501:S18

Falkenmark M (2013) Growing water scarcity in agriculture: future challenge to global water security. Phil Trans R Soc A 371:20120410

Gagné-Bourque F, Bertrand A, Claessens A, Aliferis KA, Jabaji S (2016) Alleviation of drought stress and metabolic changes in timothy (Phleum pratense L.) colonized with Bacillus subtilis B26. Front Plant Sci 7:584

Hardoim PR, Van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Sessitsch A (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79:293–320

Köberl M, Müller H, Ramadan EM, Berg G (2011) Desert farming benefits from microbial potential in arid soils and promotes diversity and plant health. PLoS One 6:e24452

Kumar A, Verma JP (2018) Does plant—microbe interaction confer stress tolerance in plants? A review. Microbiol Res 207:41–52

Lebre PH, De Maayer P, Cowan DA (2017) Xerotolerant bacteria: surviving through a dry spell. Nat Rev Microbiol 15:285

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D., Borin, S. (2013). Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *BioMed research international*, *2013*.

Marasco R, Rolli E, Vigani G, Borin S, Sorlini C, Ouzari H, Daffonchio D (2013) Are drought resistance promoting bacteria cross-compatible with different plant models? Plant Signal Behav 8:e26741

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., Abou-Hadid, A., El-Behairy, U., Sorlini, C., Cherif, A., Zocchi, G., Daffonchio, D. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PloS one*, *7*(10), e48479.

Naveed M, Hussain MB, Zahir ZA, Mitter B, Sessitsch A (2014) Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regul 73:121–131

Ngumbi E, Kloepper J (2016) Bacterial-mediated drought tolerance: current and future prospects. Appl Soil Ecol 105:109–225

Nielsen P, Sørensen J (1997) Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in Paenibacillus polymyxa and Bacillus pumilus strains from barley rhizosphere. FEMS Microbiol Ecol 22:183–192

Niu B, Paulson JN, Zheng X, Kolter R (2017) Simplified and representative bacterial community of maize roots. PNAS 114:2450–2459

Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminasecontaining plant growth-promoting rhizobacteria. Physiol Plant 118:10–15

Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Pierotti Cei F (2014) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stressdependent trait. Environ Microbiol 17:316–331

Santaella C, Schue M, Berge O, Heulin T, AchouakW(2008) The exopolysaccharide of Rhizobium sp. YAS34 is not necessary for biofilm formation on Arabidopsis thaliana and Brassica napus roots but contributes to root colonization. Environ Microbiol 10:2150–2163

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Soussi A, Ferjani R, Marasco R, Guesmi A, Cherif H, Rolli E, Mapelli F, Ouzari H, Daffonchio D, Cherif A (2016) Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. Plant Soil:357–370

Tiwari S, Lata C, Chauhan PS, Nautiyal CS (2016) Pseudomonas putida attunes morphophysiological, biochemical and molecular responses in Cicer arietinum L. during drought stress and recovery. Plant Physiol Biochem 99:108–117

Yuan Z, Druzhinina IS, Labbé J, Redman R, Qin Y, Rodriguez R, Lin F (2016) Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. Sci Rep 6:32467

Zolla G, Badri DV, Bakker MG, Manter DK, Vivanco JM (2013) Soil microbiomes vary in their ability to confer drought tolerance to Arabidopsis. Appl Soil Ecol 68:1–9

Chapter II

Microbial assisted phytodepuration for water reclamation: environmental benefits and threats

[Published in *Chemosphere*, *241*, p.124843. Riva, V., Riva, F., Vergani, L., Crotti, E., Borin, S. and Mapelli, F., 2020. Microbial assisted phytodepuration for water reclamation: Environmental benefits and threats.]

Abstract

Climate changes push for water reuse as a priority to counteract water scarcity and minimize water footprint especially in agriculture, one of the highest water consuming human activities. Phytodepuration is indicated as a promising technology for water reclamation, also in the light of its economic and ecological sustainability, and the use of specific bacterial inocula for microbial assisted phytodepuration has been proposed as a further advance for its implementation. Here we provided an overview on the selection and use of plant growth promoting bacteria in Constructed Wetland (CW) systems, showing their advantages in terms of plant growth support and pollutant degradation abilities. Moreover, CWs are also proposed for the removal of emerging organic pollutants like antibiotics from urban wastewaters. We focused on this issue, still debated in the literature, revealing the necessity to deepen the knowledge on the antibiotic resistance spread into the environment in relation to treated wastewater release and reuse. In addition, given the presence in the plant system of microhabitats (e.g. rhizosphere) that are hot spot for Horizontal Gene Transfer, we highlighted the importance of gene exchange to understand if these events can promote the diffusion of antibiotic resistance genes and antibiotic resistant bacteria, possibly entering in the food production chain when treated wastewater is used for irrigation. Ideally, this new knowledge will lead to improve the design of phytodepuration systems to maximize the quality and safety of the treated effluents in compliance with the 'One Health' concept.

1. Introduction

Water scarcity is a highly relevant and still unresolved global issue. In 2018, the World Economic Forum ranked the water crisis among the top 3 global risks for the third consecutive year (WEF, 2018) and, given the continuous world population growth and the impact of climate change, global water

demand is predicted to increase of 55% by 2050 (UN, 2015; Gain et al., 2016; Ercin et al., 2014). Among anthropogenic activities, agriculture is the most water-consuming one, using for irrigation purposes about 75% of the world's freshwater (FAO, 2015; Sato et al., 2013; Falkenmark, 2013). The consequences of water scarcity on agriculture are particularly relevant in countries currently facing an intense growth of population and economy and characterized by harsh environmental conditions, such as those located in the Middle East and North Africa (MENA) region that is among the most arid areas of the world. Here, the economic growth is forecast to determine an increase in water demand of 47% by 2035 (Frascari et al., 2018), intensifying the competition among different water-consuming sectors. This may negatively affect the allocation of freshwater to agriculture and, eventually, threaten food security. Furthermore, climate changes have already intensified phenomena like inconstant rainfall distribution and drought in Europe (Lehner et al., 2006; NOAA, 2018), exacerbating their consequences on agriculture and promoting the development of alternative solutions also on those territories once considered safe in terms of water availability (Rolli et al., 2015).

In this context, pursuing an improved water reuse is a priority to fight water crisis and minimize water footprint in agriculture (Van der Hoek et al., 2002) and, accordingly, the reuse of treated wastewaters is a priority for the European water management policy (SCHEER, 2017). In fact, non-conventional water resources (e.g. wastewaters) can offer complementary supplies to alleviate water shortage in areas where renewable water sources are particularly limited (Qadir et al., 2007) and it is estimated that at least twenty million hectares of cultivated lands worldwide are irrigated with wastewaters (Bouaroudj et al., 2019). Unfortunately, this approach frequently implies the release of untreated wastewaters in agriculture fields, and eventually in the food chain (Garner et al., 2016), representing an issue particularly in the least developed countries (LDCs) and in the MENA region.

Among wastewater treatment technologies, constructed wetlands (CWs) are engineering systems based on the purifying processes that occur in natural wetlands, i.e. the ability to remove pollutants and nutrients present in the water that flows through, thus enhancing water quality (Carvalho et al., 2017). Compared to the conventional wastewater treatment processes, CW systems need low building and maintenance costs, require less external energy and can be implemented at both small and large scales: these features make them highly suitable for wastewater treatment in the LDCs (Mahmood et al. 2013). CWs can be used as unique treatment to cleanup wastewaters produced by small communities, being effective in decreasing chemical and biochemical oxygen demand (COD and BOD) and removing total suspended solids (TSS) and nutrients (i.e. ammonia and phosphorus) (Vymazal, 2010; Wang et al., 2018). Moreover, CWs can be used as tertiary treatment for the final cleaning step in conventional wastewater treatment plants, which are not specifically designed for the removal of micropollutants (e.g. emerging organic contaminants – EOCs- like pharmaceuticals and personal care products) (Verlicchi and Zambello, 2014; Li et al., 2014). The CW system is indeed suitable for the improvement of water quality of different types of wastewaters including domestic (Wang et al., 2018) and industrial (Wu et al., 2015) ones.

As in natural wetlands, in CW systems several physicochemical and biological processes take place spontaneously at the same time, allowing the removal of organic and inorganic compounds by soil/sediment sorption and sedimentation, phytodegradation and plant uptake. In addition, the soil/sediment matrices and the plant root apparatus provide a niche for the development of microbial communities that are pivotal for the nutrient and pollutant removal from wastewaters and can at the same time sustain plant growth (Faulwetter et al., 2009) (Fig. 1). Recently, different studies have demonstrated that the addition of specific bacteria to the plant species used in CWs can be a promising strategy to boost the phytodepuration performances (Prum et al., 2018; Rehman et al., 2018; Syranidou et al.,

2016), given their capacity to promote plant growth and remove different pollutants (see chapter 2 and 3 of this review).

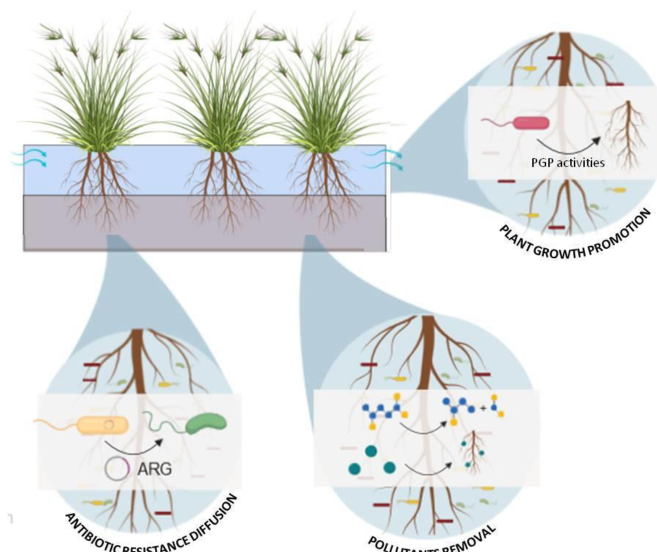


Figure 1. Bacteria mediated processes in phytodepuration system. Bacterial populations can play a beneficial effect in phytodepuration systems acting as Plant Growth Promoting strains and contributing to the degradation of micropollutants of emerging concerns (e.g. pharmaceuticals). On the other side, bacteria are involved in the phenomenon of antibiotic resistance diffusion into the environment through Horizontal Gene Transfer events, which allow the spread of antibiotic resistance genes.

CWs have been proposed as a solution to reduce contaminants of emerging concern like antibiotics, antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), typically present in wastewaters containing human and animal stools (Chen and Zhang, 2013). Nevertheless, contrasting results are reported in the literature on this topic, as shown in the chapter 4 of this review. The possible occurrence of horizontal gene transfer (HGT), the mechanism supporting the spread of ARGs (Jechalke et al., 2014) is an issue in phytodepuration technology (Fig. 1), considering that it can be enhanced in the bacterial community inhabiting plant rhizosphere (Chen et

al., 2019). This critical aspect urgently needs to be addressed, since the spread of antibiotic resistance through treated wastewater used in agriculture could increase the presence of environmental ARB that can potentially be recruited by the plant and eventually enter the food production chain.

In this review, we propose some considerations on the advantages and the possible risks correlated with the use of selected bacterial inocula in CWs for microbial assisted phytodepuration to improve nutrient and micropollutant removal, focusing specifically on the threat of antibiotic resistance diffusion into the environment amplified by HGT events.

2. PGP bacteria support to plant growth under adverse conditions

When growing in adverse environments plants face a number of growth-limiting factors, such as water scarcity, high salinity, reduced nutrients bioavailability and toxicity of polluted water and soil (Soussi et al., 2016). These conditions determine oxidative stress, reduced photosynthetic rate and plant growth, thereby affecting negatively plant productivity and related ecosystem services, including soil and water depuration (Khan et al., 2015). Plant growth-promoting (PGP) bacteria are known to establish an intimate association with the plant rhizosphere and endosphere, where they can sustain plant growth and development by counteracting the effects of stress conditions through a variety of mechanisms that have been extensively reviewed and can be categorized as direct and indirect (Abilash et al., 2016; de Bashan et al., 2012; Riva et al., 2019). PGP bacteria can directly promote plant growth acting as biofertilizers by increasing the bioavailability of key nutrients including nitrogen, phosphorus and iron. Moreover, PGP bacteria sustain the growth of associated plants by mechanisms that interfere with the production and regulation of phytohormones, such as auxins and ethylene, orchestrating root development and plant biotic and abiotic stress response (Backer et al., 2018).

2.1 Mechanisms relevant for plant growth promotion in CWs

The adverse conditions encountered by plants growing in constructed wetland (CW) ecosystems seem to be mostly related to the toxicity of the pollutants contaminating the wastewaters, including pharmaceuticals, azo-dyes and metals in high concentrations. These compounds are often slightly hydrophobic and can easily enter the roots and be translocated through the xylem to the plant tissues, where they determine an increase of the reactive oxygen species (ROS) and the activation of the plant stress response supported by an increase of the respiration rate (Ferreira et al., 2014; Christou et al., 2016; Mesa-Marín et al., 2018). Such physiological changes alter the carbon balance against plant biomass production, eventually inducing a reduction of the depuration rate in the CW system (Ferreira et al., 2016). PGP bacteria ability to counteract abiotic stress and to enhance the development of the root system represents thereby a valid biotechnological resource to sustain plant development and enhance the detoxification process (Vergani et al., 2017). The ability of indole-3-acetic acid (IAA) producing bacteria to promote the development of the root apparatus represents a significant advantage for the plant that can explore an increased volume of soil for water and nutrient uptake (Backer et al., 2018), but can be also exploited to increase the phytodepuration rate of wastewaters. Plant associated bacteria also interfere with the production of stress and senescence related hormone, i.e. ethylene, through the expression of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Nascimento et al., 2018). This enzyme hydrolyses the immediate precursor of ethylene in plants, thereby lowering the concentration of endogenous ethylene related to stress response in polluted waters and indirectly promoting plant growth. However, the mechanisms underlying microbial-assisted phytoremediation remain widely unclear and variable, depending on the bacteria and plant species and on the type of

contamination. Different studies report that plant stress tolerance and phytoremediation performances benefit from the enhancement of antioxidant enzymes activity (e.g. catalase, superoxide dismutase, glutathione reductase) triggered by the inoculated bacteria (Rajkumar et al., 2012; Backer et al., 2018). This type of mechanism assumes particular interest when the target pollutants in the wastewaters are hydrophilic organic contaminants and metals that can be taken up by the roots, translocated and eventually metabolized within the plant tissues. Alternatively, PGP bacteria can exert a protective effect towards the plant, decreasing roots respiration rate and reducing the energy consumption for antioxidant enzymes related to the stress response, hence leading to higher root biomass production and increased phytoremediation potential (Mesa Marin et al., 2018).

3. PGP bacteria application in phytodepuration

As previously mentioned, in phytodepuration systems like CWs the pollutants present in wastewaters can impair plant growth and performance. PGP bacteria have the potential to improve the efficiency of CW plants by i) decreasing stress response and in turn increasing plant growth and services and ii) degrading phytotoxic compounds (Faulwetter et al., 2009). The chemical pollutants occurring in wastewaters are indeed hardly degradable compounds and the supplementation in CWs of microbes owing specific metabolic pathways able to degrade chemical compounds and to promote plant growth has been indicated as a strategy to accelerate and enhance wastewaters depuration (Shehzadi et al., 2014; Wu et al., 2015).

3.1 Methods for PGP bacteria selection, application in CWs and testing

The successful exploitation of PGP bacteria in CW systems foresees different research steps, including the isolation of the proper bacterial strains, their taxonomic identification in order to omit potential pathogens, the functional characterization to select the most promising ones. The whole

procedure must also include the assessment of bacterial effects on the mesocosm-scale CW system and the quality of the treated wastewater effluent. A survey of the peer-reviewed articles published in the last 10 years on this topic showed that different approaches can be adopted in the pipeline that leads from bacteria selection (Table 1) to bacteria inoculum (Table 2) and performance validation (Table 3).

Different authors performed the PGP bacteria selection establishing a collection of strains from the endosphere or rhizosphere of wetland plant species commonly used in phytodepuration systems (Kabra et al., 2013; Saleem et al., 2018; Salgado et al., 2018. Table 1). PGP bacteria were isolated from the same plant species used in the CW to be bioaugmented (Ijaz et al., 2015) or from a different plant species grown in CWs treating the wastewater type of interest (Syranidou et al., 2016). Alternatively, PGP bacteria used in CWs were isolated from a site polluted with the target contaminant that had to be removed from the wastewater (Lingua et al., 2015; Prum et al., 2018; Rehman et al., 2018). The selection of the most promising strains suitable for assisted phytodepuration was then performed by analyzing the isolates for the potential capacity to tolerate/degrade the target contaminants and for the plant growth promotion ability both *in vitro* and *in vivo* (Table 1).

Once the best candidates were selected, pilot scale experiments were implemented to validate their possible application in CWs. As summarized in Table 2, most of the studies applied a consortium of two or more bacteria rather than single strain inocula to CW system, and the bioaugmentation was usually performed once, at the beginning of the experiment. The bacteria inoculation was performed by adding a cell suspension of determined concentration directly into the wastewater (Salgado et al., 2018; Shehzadi et al., 2014) or supplied around the plant root apparatus (Lingua et al., 2015; et al., Syranidou et al., 2016). As an alternative, before placing the plant in the CWs, it was possible to dip the root apparatus in the bacterial cells suspension as proposed by Prum et al. (2018).

Different chemical parameters were measured to verify the efficacy of the PGP bacteria inoculation in enhancing the CW depuration performance (Table 3). Obviously, the pollutant removal in the effluent must be evaluated, comparing wastewaters treated with inoculated and non-inoculated plants in CWs. Chemical and biological oxygen demands (COD and BOD) were routinely measured as general parameters that provide an indication of effluent quality. Moreover, the target pollutants in CWs, varying according to the wastewater origin, were quantified. For instance, heavy metals and emerging organic contaminants (EOCs) such as pharmaceuticals and personal care products were monitored in industrial and domestic wastewater effluents (Carvalho et al., 2014; Syranidou et al., 2016; Zhang et al., 2018). In addition, the quality of the treated wastewater was assessed through ecotoxicological tests using model microbes, plants or fishes (Table 3).

To complete the analysis of the bacterial inocula effect on a CW system, plant growth promotion was also evaluated, generally measuring plant dry biomass, root and shoot lengths and ROS concentration (Table 3). Furthermore, a key aspect for the validation of the PGP bacteria to be used in assisted phytodepuration was the persistence of the inoculum into the system and its capacity to establish a tight bacteria-plant interaction. Such analyses can be performed using different molecular methods as reviewed by Rilling and coworkers (2018) and showed in Table 3.

Table 1 (next page): Overview of the scientific literature reporting *in vivo* bioaugmentation experiments in Constructed Wetlands (CWs). The table includes CW type (FTW = floating treatment wetland; VF = Vertical flow; SF = subsurface flow), wastewater type, plant species and the criteria applied for the selection bacteria in each study. ACC = 1-Aminocyclopropane-1-Carboxylate deaminase activity, P sol. = phosphate solubilization, sid. = siderophore production, IAA = Indole-3-acetic acid production

References	CW type	Wastewater type	Plant species	Bacteria applied for bioaugmentation in CW		
				Bacterial isolation site	**Characterization of pollutant tolerance/degradation**	**Characterization of PGP potential**
Ijaz et al. (2015)	microcosm (FTW)	sewage effluent	*Brachiaria mutica*	*Brachiaria mutica* shoot	COD/BOD removal	ACC, P sol., sid.
Kabra et al. (2013)	mesocosm	textile effluent	*Glandularia pulchella*	*Glandularia pulchella* root	dye decolorization	-
Lingua et al. (2015)	mesocosm	nitrate polluted water	*Phragmites australis*	forest soil	-	IAA, sid., P sol., *in vivo P. australis* promotion
Prum et al. (2018)	mesocosm (VF)	arsenic polluted water	*Echinodorus cordifolius*	arsenic contaminated soil	arsenic tolerance	-
Rehman et al. (2018)	microcosm (FTW)	oil field wastewater	*Brachiara mutica/Phragmites australis*	crude oil-contaminated site	hydrocarbon degradation	IAA, ACC, sid., P sol.
Saleem et al. (2018)	microcosm (FTW)	phenol polluted water	*Typha domingensis*	Plant rhizosphere and shoot	phenol degradation	ACC
Salgado et al. (2018)	mesocosm	domestic wastewater	*Typha latifolia*	*Typha domingensis* plant	COD/nitrogen/phosphorus removal	-
Shezhadi et al. (2014)	mesocosm (VF)	textile effluent	*Typha domingensis*	wetland plants roots	textile effluent degradation	ACC
Syranidou et al. (2016)	microcosm (static)	EOCs and metals polluted water	*Juncus acutus*	*Juncus acutus* endosphere	BPA/antibiotic/metals tolerance	IAA, ACC, P sol., sid., organic acid production
Watharkar et al. (2015)	mesocosm (static)	textile effluent	*Pogonatherum crinitum*	*Petunia grandiflora* rhizosphere	dye decolorization	-
Zhao et al. (2016)	mesocosm (SF)	sewage effluent	*Acorus calamus*	CW/commercial bacteria	nitrogen removal	-

Table 2. List of the different methods applied in the literature for the inoculation of bacteria in a CW system. The table reports for each reference the type of inoculum used, the way and the frequency of the microbe addition to CW.

References	Bacteria inoculation methods in CW		
	Inoculum type	**Inoculation method**	**Frequency of inoculation**
Ijaz et al. (2015)	consortium	wastewater inoculation	once
Kabra et al. (2013)	single strain	soil inoculation	daily (for 15 days)
Lingua et al. (2015)	single strain	bacterial suspension around root apparatus	once
Prum et al. (2018)	consortium	root dipping	once
Rehman et al. (2018)	consortium	wastewater inoculation	once
Saleem et al. (2018)	consortium	wastewater inoculation	once
Salgado et al. (2018)	consortium	wastewater inoculation	once
Shehzadi et al. (2014)	consortium	wastewater inoculation	once
Syranidou et al. (2016)	consortium	bacterial suspension around plant base	once
Watharkar et al. (2015)	single strain	cell immobilization	once
Zhao et al. (2016)	consortium	bacterial suspension around plant base	thrice

Table 3. List of the parameters measured in the literature to assess the effect of bacteria inoculation in CW systems. The table refers to different bacteria-mediated effects possibly enhancing CW performance (i.e. pollutant removal, effluent toxicity, plant growth promotion) besides inoculum persistence.

References	Bacterial effect assessment			
	Pollutant removal	**Effluent toxicity**	**Plant growth promotion**	**Inoculum persistence**
Ijaz et al. (2015)	BOC, COD, TS, TDS, TSS, DO, oil and grass, SO4, Cl, total N, PO4, Na, K, metals	Fish toxicity test	-	Plating + RFLP analyses
Kabra et al. (2013)	dyes, COD, BOD, TOC	Plant toxicity test	-	-
Lingua et al. (2015)	nitrogen, ammonium	-	N° of stems/sprouts/leaves; stem length; shoot and root fresh/dry biomass and weights	Plating
Prum et al. (2018)	arsenic	-	ROS and IAA levels	-
Rehman et al. (2018)	COD, BOD, Na, K, metals, residual oil	Fish toxicity test	fresh and dry biomass, root and shoot length	Plating + RFLP analyses
Saleem et al. (2018)	phenol, COD, BOD, TOC	-	fresh and dry biomass	Plating + RFLP analyses
Salgado et al. (2018)	COD, NH4, PO4	-	-	-
Shehzadi et al. (2014)	dyes, COD, BOD, TOC, TDS, TSS	Ames test	fresh and dry biomass	Plating + RFLP analyses
Syranidou et al. (2016)	metals, BPA, antibiotics	-	dry biomass	ARISA
Watharkar et al. (2015)	dyes, BOD, COD	Plant and fish toxicity test	-	-
Zhao et al. (2016)	nitrogen, COD, BOD	-	-	high-throughput sequencing

3.2 PGP bacteria contribution to increase plant performances

PGP bacteria can improve the fitness of plants used in CW systems. As reported by Shehzadi and coworkers (2014), the inoculation of the wetland plant *Typha domingensis* with the endophytic strains *Microbacterium arborescens* TYSI04 and *Bacillus pumilus* PIRI30 in a vertical flow CW reactor promoted plant growth besides improving the depuration of the effluent. In the system, textile effluent negatively influenced the development of *T. domingensis,* however the endophytes inoculation reduced the toxic effects of textiles, restoring the plant biomass production up to the values of *T. domingensis* plants irrigated with clean tap water and used as control in the study. Likewise, the addition of a bacterial consortium to a floating treatment wetland enhanced *Brachiara mutica* and *Phragmites australis* growth that was normally inhibited by the presence of oil-contaminated water (Rehman et al., 2018). In the study, bacterial inoculation restored plant health and increased root (36-46%) and shoot (35-36%) biomass and root (15-29%) and shoot (4-21%) length, enabling these two plant species to cope with the oil-induced stress.

The influence of PGP bacteria on wetland plants can be also indirectly evaluated by measuring the decrease in plant content of antioxidative enzymatic activity, which is linked to the oxidative stress induced in plants (Prum et al., 2018; Syranidou et al., 2016). It is known that PGP bacteria stimulate enzymatic and non-enzymatic antioxidant responses that scavenge ROS compounds related to stress condition (Marasco et al., 2013; Wang et al., 2012). The monitoring of antioxidative enzymatic activity has been used by Syranidou et al. (2016) in a microcosm scale experiment designed for the depuration of water artificially contaminated with metals, bisphenol-A and antibiotics to simulate urban wastewaters. The authors showed a significant reduction of activity of several enzymes involved in anti-oxidant defense in *Juncus acutus* plants inoculated with a consortium of endophytic PGP bacteria.

3.3 PGP bacteria contribution to nutrient removal

CWs are often used as secondary treatment systems for municipal wastewater cleanup in rural communities where wastewater treatment facilities are not present due to the high cost of treatment processes (Ghrabi et al., 2011; Fountoulakis et al., 2017). Phytodepuration is also used as tertiary treatment when the common treatment plant is not efficient enough in the achievement of legal standards for organic matter and nutrient removal to allow the safe discharging of treated water in the environment (Ijaz et al., 2015). Wetland plants, such as P*hragmites* and *Typha* spp., are able to uptake nitrogen and phosphorus from wastewaters (Vymazal, 2010; Fountoulakis et al., 2017). However, the addition of bacteria in CWs can help plants in the removal of these contaminants and contribute to organic matter decrease by the indigenous microbial communities, improving the quality of treated wastewaters (Ijaz et al., 2015; Salgado et al., 2018).

Salgado and coworkers (2018) investigated the effects of the application of four indigenous rhizobacteria to *Typha latifolia* on the performance of a vertical flow CW system treating domestic wastewaters. The study showed that the system removed over 75% of the organic matter, ammonium and phosphate and it had better performance than i) *T. latifolia* CW mesocosms without bacteria inoculation and ii) CW mesocosms established using plant with sterilized root surface supplemented by the four selected rhizobacteria. This interesting experiment suggests that the inoculation of the selected indigenous rhizobacterial strains activated the overall microbiome associated to *T. latifolia* in the bioaugmented plants, resulting in a synergic effect that significantly improved the effluent quality. Another study analyzed the removal of nitrogen from nitrate polluted and urban wastewaters using pilot scale-CWs enriched with non-indigenous bacteria aiming to achieve the legal standards of nitrogen emission in surface waters and to prevent the risk of eutrophication in lakes and rivers (Zhao et al., 2016). The addition of the selected PGP bacteria improved the performance of the system and

ameliorated the efficiency of denitrification up to 10% during the wastewater phytodepuration process.

3.4 PGP bacteria contribution to micropollutant removal

Wastewaters contain several categories of micropollutants, including metals and organic contaminants of emerging concern (e.g. pharmaceuticals, personal care products, pesticides), which pose serious risks for human health if released into the environment (Li et al., 2017; Pruden et al., 2012; Tezel and Pavlostathis, 2015; Zuccato et al., 2000). In this review, we focused on EOCs that are the prevalent class of micropollutants occurring in urban wastewater and show a relationship with the antibiotic resistance diffusion in the environment, as illustrated in chapter 4. EOCs that are frequently present in wastewater at trace concentrations and are hardly removed by common wastewater treatment plants, which are not tailored for their elimination (Gorito et al., 2017). CWs are proposed as tertiary treatment for the removal of micropollutants from industrial and municipal effluents (Verlicchi and Zambello, 2014; Garcia-Rodríguez et al., 2014; Wu et al., 2015), basing on the capacity of certain plant species to accumulate organic and inorganic compounds from water and considering their potential association in the rhizosphere and endosphere with pollutant degrading bacteria (Borruso et al., 2017). Bacterial degradation has been demonstrated to be the main process involved in micropollutant removal in CWs treating urban wastewaters (Carvalho et al., 2014; Li et al., 2016; Zhang et al., 2018), hence through the addition of microbial inocula having a specific degrading pathway it is in principle possible to achieve treated effluents at reuse grade. In a recent study, the endophytic bacteria *Rhizobium radiobacter* and *Diaphonobacter nitroreducens*, isolated from reed plants and characterized for plant growth promotion and carbamazepine degradation capacities, were inoculated to hairy root cultures of *Armoracia rusticana*. The inoculated hairy root cultures showed higher carbamazepine removal rate (21% and 10% for the cultures

inoculated with *Rhizobium radiobacter* and *Diaphonobacter nitroreducens* respectively) compared to the non-inoculated one (Sauvêtre et al., 2018) and, even though the plant-endophyte synergy needs to be further investigated, these data clearly indicated that plant associated microbes could be successfully exploited for municipal wastewater treatment in phytodepuration system. A CW mesocosm experiment for the depuration of urban wastewaters was recently described by Saleem and coworkers (2018): in a floating treatment wetland, *T. domingensis* was able to remove a small amount of phenol from the polluted water, however the addition of three phenol-degrading bacterial strains to the system significantly improved the phenol removal, which increased from 0,146 g/m^2/day to 0,166 g/m^2/day. Other studies have demonstrated that the addition of bacteria inocula to plants can be also effective for the treatment of textile wastewaters, contaminated by toxic dyes. In two different pilot scale experiments, Kabra et al. (2013) and Watharkar et al. (2015) inoculated respectively plants of *Glandularia pulchella* and *Pogonatherum crinitum* with two isolates belonging to the species *Pseudomonas monteilii* and *Bacillus pumilus*. The results highlighted a synergic effect of the plant and bacteria components, showing that inoculated plants were more efficient in dye removal and BOD and COD decrease compared to i) non-inoculated plants and ii) reactor systems with the presence of the sole bacterium without the plant.

An additional advantage of assisted phytodepuration is that the combined use of plants and bacteria is useful to deal with mixed pollution. Syranidou et al. (2016) investigated the potential of endophytic bacteria to sustain the removal of metals and emerging organic pollutants, such as bisphenol-A and antibiotics, by *Juncus acutus* plants in CWs. The results showed that beneficial effect of the bacteria in phytodepuration was especially evident when high concentration of contaminants where used, significantly improving the removal of organics and metals in shorter time by inoculated plants compared to the non-inoculated ones.

4. Antibiotic resistance dimension in phytodepuration

The majority of antibiotics are not completely metabolized and absorbed by animal and human's bodies, thus they are discharged in municipal and hospital wastes in their original or transformed chemical species (Huang et al., 2017; Berglund et al., 2014). As other EOCs, antibiotics escape wastewater treatment systems, which are not specifically designed to remove these compounds, thus representing micropollutants in treated effluents (Luo et al., 2014). Antibiotics are considered "pseudo-persistent" contaminants because of their constant addition through municipal wastewaters at low concentrations into the environment (Gorito et al., 2017) and can reach biologically active amounts due to accumulation as reported for the waters and sediments of Italian rivers where they have been measured at ppm and ppb concentrations respectively (Zuccato et al., 2000). The principal concern for their release is related to the selection and spread of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) (Rizzo et al., 2013; Liang et al., 2018; Yan et al., 2019), as already hypothesized by Sir Alexander Fleming, the discoverer of the first antibiotic, during his Nobel Lecture in 1945. Indeed, antibiotic resistance is nowadays one major concern threatening human and animal health due to the emergence of multi-drug resistant pathogens, which cannot be eliminated by pharmacological therapy with serious consequences for the overall society. The genetic determinants of antibiotic resistance (i.e. ARGs) are typically present in mobile genetic elements (MGE) and are considered micropollutants themselves since, if acquired by a receiving sensitive cell, can produce a new antibiotic resistant mutant.

The study of the antibiotic resistance dimension in the environment has to include the processes occurring in wastewater treatment plants (WWTPs), identified as one of the main sources of ARB and ARGs (Amos et al. 2015; Czekalski et al. 2014). The inadequacy of WWTPs in antibiotic removal from wastewaters has been identified as a major cause for the ubiquitous

occurrence of ARB and ARGs in different environmental compartments (Amos et al., 2015; Tang et al.; 2016; LaPara et al., 2011; Ju et al., 2019). For instance, recent findings suggest that the use of struvite as crop fertilizer can increase the diversity and the concentration of ARGs in the microbiome inhabiting soil and plant microhabitats, i.e. roots and leaves (Chen et al., 2017). Moreover, WWTPs are a suitable environment for antibiotic resistance selection and spread due to the continuous contact between the bacteria involved in the biological treatment processes and the antibiotics present in the wastewaters at sub-inhibitory concentrations (Rizzo et al., 2013). The presence of sub-lethal concentrations of antibiotics in WWTPs and in their effluents is a peculiar ecological driver favoring the ARB selection and the outbreak of antibiotic resistance (Andersson and Hughes, 2014). Other EOCs (e.g. biocides) and metals are typical stressors in WWTPs that might exert selective pressure contributing to the enrichment of ARGs and ARB through selection or co-selection events (Berendonk et al., 2015; Di Cesare et al. 2016). Indeed, the numerous bacteria thriving in wastewater, WWTPs and related habitats (e.g. biofilm, sludge) can host a myriad of ARGs and a better wastewater treatment technology is required to limit the risk of antibiotic resistance spread.

4.1 ARB & ARG threat in CWs

Phytodepuration of wastewater has gained increasing attention as low-cost and eco-friendly system for water reclamation and reuse, producing effluent of high quality for irrigation purposes (Petroselli et al., 2015; Petroselli et al. 2017). Several works have highlighted the efficacy of CWs in limiting the diffusion of antibiotics, ARGs and ARB (Hijosa-Valsero et al., 2011; Chen et al., 2015) by removing antibiotics from wastewaters with different mechanisms such as biodegradation, plant uptake, substrate absorption, photolysis and volatilization (Liu et al., 2013; Chen et al., 2016a). Chen and Zhang (2013) demonstrated that CWs used as tertiary treatment of WWTP were more effective than biological filter and UV disinfection for ARG

removal. Chen et al. (2016a) proved that different mesocosm-scale CWs treating raw domestic wastewaters reached a removal efficiencies of total antibiotics from the aqueous phase comprised between 75.8 and 98.6%, and those of total ARGs varied between 63.9 and 84%. Berglund and coworkers (2014) assessed the effluent quality of surface-flow experimental wetlands exposed to antibiotics at concentrations commonly found in wastewaters, showing that the CW system did not promote the dispersal of ARGs and leading to hypothesize that the low ARG concentration detected was probably due to a background genetic resistance present in the wetlands.

The fate of ARGs in CWs and their effluents is extremely influenced by the operating conditions of the CW (e.g. plant species, substrate type, flow type and velocity, system configuration) and environmental factors (e.g. temperature) (Liu et al., 2013; Chen and Zhang 2013; Chen et al., 2016a; Fang et al., 2017; Huang et al., 2017). For instance, the relative abundance of tetracycline resistance genes (*tet*) seemed to be higher in the CW effluents of an up-flow system compared to down-flow treatment (Huang et al., 2017). Likewise, the use of different substrates can influence the removal of *tet* genes, as shown by Liu and coworkers (2013) in CWs established using volcanic (CW1) or zeolite (CW2) substrates. The results of the study indicate that the absolute abundance of *tet* and the universal bacterial 16S rRNA genes decreased in both CW1 and CW2 effluents, while the relative abundance of *tet* genes increased in CW1, pointing out an increase of the resistant populations over the total bacterial community.

During the summer, an increase of ARGs concentration was registered in wastewater effluent compared to the winter period (Fang et al., 2017). Indeed, temperature has a key role for the regulation of several microbial processes and metabolisms in CWs, eventually influencing the microbial community structure (Truu et al., 2009). High temperature and solar irradiation positively influenced the degradation of different pharmaceuticals such as diclofenac, ibuprofen, ketoprofen, naproxen, salicylic acid, triclosan, and carbamazepine (Li et al., 2014) and antibiotics possibly share the same

fate. On the other side, higher concentrations of ARGs were reported in natural and constructed wetlands during the summer period compared to winter (Li et al., 2019). Nevertheless, the influence of temperature on ARG fate is under debate and a different study revealed that low temperatures promote antibiotic resistance and horizontal gene transfer (HGT) of integron-associated ARGs (Miller et al., 2014).

A further issue on the efficacy of CWs in restricting ARGs and ARB diffusion in the environment has been overlooked until recently. The natural microbial community associated to CW plants could become itself a reservoir for specific ARGs due to the continuous exposition to antibiotics and ARB present in the raw or partially treated wastewater. In a ten years-experiment, Fang et al. (2017) showed that CW system could promote the increase of ARG level in the effluent mostly because of the accumulation of these genes into CW sediment. Similar results were obtained by Song and coworkers (2018) using synthetic wastewater, with negligible ARG abundance, as CW inflow. The effluent presented a lower abundance of ARGs compared to the CW sediments, however the ARG abundance was higher in the effluent compared to the inflow, unveiling the role of the natural bacterial community associated to *Oenanthe javanica* plants in the antibiotic resistance diffusion.

4.2 Role of horizontal gene transfer (HGT) in the ARG diffusion into the environment and within the plant microbiome

HGT is a crucial mechanism involved in evolution that consists in the movement of genetic material across different organisms. In bacteria it is generally divided in three main mechanisms, i.e. transformation, transduction and conjugation (Aminov 2001; Van Elsas et al., 2003; Baltrus 2013). In the last few years HGT has been increasingly investigated in relation to the spread of ARGs that are frequently linked to MGE (Petrovich et al., 2018) and could be finally internalized by pathogenic bacteria representing a risk for human health (Song et al., 2018; Headd and Bradford 2018).

HGT events have been examined in many different studies mimicking environmental conditions (Qiu et al., 2018; Baur et al., 1996; Hasegawa et al., 2018). For instance, Klümper and colleagues (2015) showed the possible spread of different plasmids in a soil bacterial community highlighting the importance of HGT in this environment. In a recent study, MGEs have been discovered in metal contaminated soil amended with pig manure (Garbisu et al., 2018), in agreement with the previous knowledge that manure's utilization as fertilizer in crops may enhance the abundance of ARGs in the soil microbiota by HGT (Chen et al., 2016b; Gotz and Smalla 1997). The frequency of HGT events depends on many different factors, beside the specific molecular nature of the mobile element and donor/receiving strains. As a general factor, HGT could be enhanced by selective pressure due to anthropogenic activities that release into the environment different classes of pollutants, e.g. metal or antibiotics under the minimal inhibitory concentration (MIC) (Santos et al., 2018). Furthermore, HGT events are enhanced in environments with high cell density and metabolic activity (Ulrich et al., 2015). Conjugation, for example, may depend on the ratio of donor to recipient strains and on the dimension and the conformation of the plasmid (Zeaiter et al., 2018). The HGT promoting conditions can occur in specific hot spots in natural and engineered ecosystems, mostly characterized by the presence of dense aggregates of colonies where cell-to-cell contact is boosted. Notably, even low concentrations of antibiotics were proved to determine the aggregation of bacterial cells in aquatic ecosystems, thus creating conditions prone to cell-cell DNA transfer (Corno et al., 2014; Eckert et al., 2019).

Biofilm and mycosphere are two examples of environmental hot spots for HGT events. Qui and colleagues (2018) visualized ARGs transfer through conjugation in an activated sludge bacterial biofilm, and several studies underlined that conjugation and transformation events happen at a higher frequency when bacterial cultures are in biofilm rather than in the planktonic state (Madsen et al., 2012). Another HGT hot spot is the mycelia produced

by Fungi that provide a high amount of nutrients and a wide surface to which bacterial cells can adhere, enhancing the frequency of the conjugation events (Berthold et al., 2016). High concentrations of MGEs have been detected In water ecosystems like WWTPs (Petrovich et al., 2018), where the frequency of HGT events can be increased due to the presence of a dense microbial population and sublethal concentrations of antibiotics acting as selective pressure toward the resistance acquisition. WWTP effluents can influence the spread of ARGs through MGEs in aquatic sediments (Chu et al., 2018) and it has been demonstrated that the release of tertiary treated urban wastewater can increase ARG concentration in the surface layers of the receiving water bodies (LaPara et al., 2011). Moreover, Di Cesare and co-authors (2016) demonstrated that chemical disinfection of urban wastewaters, treated in different WWTPs of Northern Italy, could favour the survival of bacterial populations resistant to antibiotics, able to form cell aggregates and harbouring different ARGs.

Major HGT hot spots related to the plant niche are rhizosphere, phyllosphere and spermosphere (Aminov 2011; Chen et al., 2019; Van Elsas et al., 2003) and, accordingly, HGT events in the plant microbiome have been extensively reported. Conjugation between exogenous and indigenous bacteria have been detected in bacterial communities of poplar plants (Ulrich et al., 2015) while plasmid transfer from *Pseudomonas putida* to indigenous bacteria have been studied on the surface of alfalfa sprout (Mølbak et al., 2003). Likewise, Björklöf and colleagues (1995) demonstrated that conjugative plasmid transfer occur in the phyllosphere of bean plants. Microcosm scale experiments showed that conjugation events can occur in barley seedling sphermosphere, where the transfer of plasmid RP4 from a *Pseudomonas* strain donor to the indigenous bacteria has been detected (Sorensen and Jensen 1998).

In the frame of phytodepuration, the rhizosphere is the most crucial hot spot of HGT events. In phytodepuration systems such as CWs, the root apparatus and its associated microbial community are continuously exposed

to substances (i.e. pharmaceutical, biocides, metals) promoting selection and co-selection events. Moreover, in this habitat bacterial populations are metabolically active due to the high nutrient content, live in high concentration and tight contact and harbour abundant MGEs (Chen et al., 2019). Plasmid transfer has been studied in barley and pea rhizosphere (Mølbak et al., 2003; Sørensen and Jensen 1998) and a higher relative abundance of IncP-1 plasmid has been detected in lettuce rhizosphere in comparison with bulk soil (Jechalke et al., 2014). Accordingly, a recent study suggests that plasmid transfer in rhizosphere soil of maize seedlings occurs with higher abundance than in bulk soil (Zhu et al., 2018). The chemical composition of root exudates, including sugars, organic acids and amino acids, enhances HGT and plasmid transfer events in the rhizosphere (Zhu et al., 2018), as specifically shown for nodulation-inducing flavonoid (Ling et al., 2016). The investigation of HGT events in the rhizosphere of plants in phytodepuration systems is still neglected, revealing a gap of knowledge that should be taken into account for the overall evaluation of this wastewater treatment technology, especially in the frame of ARG spread into the environment.

Even if there is massive evidence on HGT occurrence and ARGs presence in the environment, limited information is still available on their relationship in both natural and human impacted ecosystems, although previous studies suggest that HGT events, such as conjugation, happen in nature at several orders of magnitude higher than *in vitro* studies (Davies and Davies 2010). According to the available literature, HGT events in plant microbiome are influenced by different abiotic factors such as pH, temperature, micro- and macro-nutrients, oxygen and moisture content, and by biotic agents such as the presence of antagonistic, syntrophic or competing organisms (Van Elsas et al., 2003). Unfortunately, the high variability of such parameters over time and space makes extremely difficult the comprehension of the spread and dynamics of antibiotic resistance in the environmental niches where HGT

can take place, hampering the modelling of antibiotic resistance fate that could be useful for successful water reuse management strategies.

5. Concluding remarks and research perspectives

In this review, we presented the advantages related to the implementation of microbial assisted phytodepuration. Bacterial inocula have indeed the potential to contribute to the biological wastewater cleanup in phytodepuration systems that nowadays are proposed as a low maintenance solution for water reclamation at suitable quality for water reuse in agriculture. Thanks to different PGP mechanisms, bacteria are able to support the growth of wetland plants improving their service in term of nutrient and pollutant removal, through the action of a more developed root apparatus and higher biomass. Microorganisms, enriched in plant rhizosphere, can also degrade recalcitrant micropollutants, such as pharmaceuticals, that are not targeted by common wastewater treatment plants, directly improving the effluent quality.

On the other side, we evidenced the possible peculiar risk of this technology in terms of antibiotic resistance diffusion in the environment. Designed wetlands seem to have a high efficiency in the removal of pollutants of emerging concerns, such as antibiotics, ARGs and ARB, although this is still debated given some contrasting results present in the literature. We highlighted that the pollutant removal processes in phytodepuration are performed in the rhizosphere, which is reported as a hot spot for HGT that could drive the diffusion of ARGs and the ARB. Although the root apparatus of plants used in phytodepuration of urban wastewaters is constantly exposed to ARB and low concentration of biocides and antibiotics, we point out that HGT of ARGs is still not sufficiently investigated in this type of wastewater treatment facilities. In particular, we recommend the need of a deep characterization of the antibiotic resistance profile and the related genetic determinants for the bacteria selected as promising inocula for assisted CW phytodepuration, characterization that is lacking in all the

literature works discussed in this review. Some authors demonstrated a role of the natural community associated to plants in CWs in increasing the ARG concentration in the effluent, making important to clarify the dynamics of antibiotic resistance in response to the addition of bacterial inocula in such systems. The investigation of such aspects is pivotal in the frame of the 'One-Health' approach, especially when the treated wastewater is reused for irrigation purposes, entering the food production chain and eventually contributing to the diffusion of ARGs that could be acquired by pathogenic bacteria.

Even though further studies are necessary to deepen our knowledge on the above-mentioned phenomena, assisted phytodepuration can be considered a valuable approach able to enhance the quality of treated wastewater and it can be part of a wider strategy to boost water reuse worldwide.

References

Aminov RI, 2011. Horizontal gene exchange in environmental microbiota. Frontiers in microbiology, 2, 158.

Amos GC, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, Zhang L, Singer AC, Gaze WH, Wellington EM, 2015. Validated predictive modelling of the environmental resistome. The ISME journal, 9(6), 1467.

Andersson DI, Hughes D, 2014. Microbiological effects of sublethal levels of antibiotics. Nature Reviews Microbiology, 12(7), 465.

Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, Subramanian S, Smith D L, 2018. Plant Growth-Promoting Rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Frontiers in Plant Science, 23; 9: 1473.

Baltrus DA, 2013. Exploring the costs of horizontal gene transfer. Trends in ecology & evolution, 28(8), 489-495.

Baur B, Hanselmann K, Schlimme W, Jenni B, 1996. Genetic transformation in freshwater: Escherichia coli is able to develop natural competence. Applied and Environmental Microbiology, 62(10), 3673-3678.

Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons MN, Kreuzinger N, Huovinen P, Stefani S, Schwartz T,

Kisand V, Baquero F, Martinez JL, 2015. Tackling antibiotic resistance: the environmental framework. Nature Reviews Microbiology, 13(5), 310.

Berglund B, Khan GA, Weisner SE, Ehde PM, Fick J, Lindgren PE, 2014. Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. Science of the total environment, 476, 29-37.

Berthold T, Centler F, Hübschmann T, Remer R, Thullner M, Harms H, Wick LY, 2016. Mycelia as a focal point for horizontal gene transfer among soil bacteria. Scientific reports, 6, 36390.

Björklöf K, Suoniemi A, Haahtela K, Romantschuk M, 1995. High frequency of conjugation versus plasmid segregation of RP1 in epiphytic Pseudomonas syringae populations. Microbiology, 141(10), 2719-2727.

Borruso L, Esposito A, Bani A, Ciccazzo S, Papa M, Zerbe S, Brusetti L, 2017. Ecological diversity of sediment rhizobacteria associated with Phragmites australis along a drainage canal in the Yellow River watershed. Journal of soils and sediments, 17(1), 253-265.

Bouaroudj S, Menad A, Bounamous A, Ali-Khodja H, Gherib A, Weigel DE, Chenchouni H, 2019. Assessment of water quality at the largest dam in Algeria (Beni Haroun Dam) and effects of irrigation on soil characteristics of agricultural lands. Chemosphere, 219, 76-88. https://doi.org/10.1016/j.chemosphere.2018.11.193

Carvalho PN, Arias C, Brix H, 2017. Constructed wetlands for water treatment: New developments. Water 9*(*6), 397.

Carvalho PN, Basto MCP, Almeida CMR, Brix H, 2014. A review of plant–pharmaceutical interactions: from uptake and effects in crop plants to phytoremediation in constructed wetlands. Environmental Science and Pollution Research, 21(20), 11729-11763.

Czekalski N, Gascón Díez E, Bürgmann H. 2014. Wastewater as a point source of antibioticresistance genes in the sediment of a freshwater lake. The ISME Journal 8: 1381–1390.

Chen H, Zhang M, 2013. Effects of advanced treatment systems on the removal of antibiotic resistance genes in wastewater treatment plants from Hangzhou, China. Environmental science & technology, 47(15), 8157-8163.

Chen J, Liu YS, Su HC, Ying GG, Liu F, Liu SS, He LY, Chen ZF, Yang YQ, Chen FR, 2015. Removal of antibiotics and antibiotic resistance genes in rural wastewater by an integrated constructed wetland. Environmental Science and Pollution Research, 22(3), 1794-1803.

Chen J, Ying GG, Wei XD, Liu YS, Liu SS, Hu LX, He LY, Chen ZF, Chen FR, Yang, YQ, 2016a. Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: effect of flow configuration and plant species. Science of the Total Environment, 571, 974-982.

Chen Q, An X, Li H, Su J, Ma Y, Zhu YG, 2016b. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environment international, 92, 1-10.

Chen QL, An XL, Zhu YG, Su JQ, Gillings MR, Ye ZL, Cui L, 2017. Application of struvite alters the antibiotic resistome in soil, rhizosphere, and phyllosphere. Environmental science & technology, 51(14), 8149-8157.

Chen QL, Cui HL, Su JQ, Penuelas J, Zhu YG, 2019. Antibiotic resistomes in plant microbiomes. Trends in plant science, 24 (6), 530-541.

Christou A, Antoniou C, Christodoulou C, Hapeshi E, Stavrou I, Michael C, Fatta-Kassinos D, Fotopoulos V, 2016. Stress-related phenomena and detoxification mechanisms induced by common pharmaceuticals in alfalfa (Medicago sativa L.) plants. Science of the Total Environment 557–558 652–664.

Chu BT, Petrovich ML, Chaudhary A, Wright D, Murphy B, Wells G, Poretsky R, 2018. Metagenomics reveals the impact of wastewater treatment plants on the dispersal of microorganisms and genes in aquatic sediments. Applied and Environmental Microbiology, 84(5), e02168-17.

Corno G, Coci M, Giardina M, Plechuk S, Campanile F, Stefani S, 2014. Antibiotics promote aggregation within aquatic bacterial communities. Frontiers in microbiology, 5, 297.

Davies J, Davies D, 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Review, 74(3), 417-433.

de-Bashan LE, Hernandez J-P, Bashana Y, 2012. The potential contribution of plant growth-promoting bacteria to reduce environmental degradation – A comprehensive evaluation. Applied Soil Ecology 61 171– 189.

Di Cesare A, Fontaneto D, Doppelbauer J, Corno G, 2016. Fitness and recovery of bacterial communities and antibiotic resistance genes in urban wastewaters exposed to classical disinfection treatments. Environmental science & technology, 50(18), 10153-10161.

Eckert EM, Quero GM, Di Cesare A, Manfredini G, Mapelli F, Borin S, Fontaneto D, Luna GM, Corno G, 2019. Antibiotic disturbance affects aquatic microbial community composition and foodweb interactions but not community resilience. Molecular ecology.

Ercin AE, Hoekstra AY, 2014. Water footprint scenarios for 2050: A global analysis. Environment international, 64, 71-82.

Falkenmark M, 2013. Growing water scarcity in agriculture: future challenge to global water security. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 371(2002), 20120410.

Fang H, Zhang Q, Nie X, Chen B, Xiao Y, Zhou Q, Liao W, Liang X, 2017. Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. Chemosphere, 173, 99-106.

FAO (Food and Agriculture Organization of the United Nations). 2015. Aquastat Information System on Water and Agriculture. FAO, Rome, Italy.

Faulwetter JL, Gagnon V, Sundberg C, Chazarenc F, Burr MD, Brisson J, Camper AK, Stein OR, 2009. Microbial processes influencing performance of treatment wetlands: a review. Ecological engineering, 35(6), 987-1004.

Ferreira RA, Duarte JG, Vergine P, Antunes CD, Freire F, Martins-Dias S, 2014. Phragmites sp. physiological changes in a constructed wetland treating an effluent contaminated with a diazo dye (DR81). Environmental Science Pollution Research 21 9626–9643.

Ferreira RA, Roma-Rodrigues C, Davies LC, Sá-Correia I, Martins-Dias S, 2016. A quantitative proteomic approach to highlight Phragmites sp. adaptation mechanisms to chemical stress induced by a textile dyeing pollutant. Science of the Total Environment 573 788–798.

Fleming, A. in Nobel Lectures: Physiology or Medicine 1942–1962 83–93 (Elsevier, 1964).

Fountoulakis MS, Daskalakis G, Papadaki A, Kalogerakis N, Manios T, 2017. Use of halophytes in pilot-scale horizontal flow constructed wetland treating domestic wastewater. Environmental Science and Pollution Research, 24(20), 16682-16689.

Frascari D, Zanaroli G, Motaleb MA, Annen G, Belguith K, Borin S, Choukr-Allah R, Gibert C, Jaouani A, Kalogerakis N, Karajeh F, Ker Rault PA, Khadra R, Kyriacou S, Li WT, Molle B, Mulder M, Oertlé E, Ortega CV, 2018. Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt, Morocco, and Tunisia. Integrated Environmental Assessment Management 14(4), 447-462.

Gain AK, Giupponi C, Wada Y, 2016. Measuring global water security towards sustainable development goals. Environmental Research Letters 11(12), 2-13.

Garbisu C, Garaiyurrebaso O, Lanzén A, Álvarez-Rodríguez I, Arana L, Blanco F, Smalla K, Grohmann E, Alkorta I, 2018. Mobile genetic elements and antibiotic resistance in mine soil amended with organic wastes. Science of the total environment, 621, 725-733.

Garcia-Rodríguez A, Matamoros V, Fontàs C, Salvadó V, 2014. The ability of biologically based wastewater treatment systems to remove emerging organic contaminants—a review. Environmental Science and Pollution Research, 21(20), 11708-11728.

Garner E, Zhu N, Strom L, Edwards M, Pruden A, 2016. A human exposome framework for guiding risk management and holistic assessment of recycled water quality. Environmental Science: Water Research & Technology, 2(4), 580-598.

Ghrabi A, Bousselmi L, Masi F, Regelsberger M, 2011. Constructed wetland as a low cost and sustainable solution for wastewater treatment adapted to rural settlements: the Chorfech wastewater treatment pilot plant. Water Science and Technology, 63(12), 3006-3012.

Gorito AM, Ribeiro AR, Almeida CMR, Silva AM, 2017. A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation. Environmental pollution, 227, 428-443.

Gotz A, Smalla K, 1997. Manure enhances plasmid mobilization and survival of Pseudomonas putida introduced into field soil. Applied and Environmental Microbiology, 63(5), 1980-1986.

Hasegawa H, Suzuki E, Maeda S, 2018. Horizontal Plasmid Transfer by Transformation in Escherichia coli: Environmental Factors and Possible Mechanisms. Frontiers in microbiology, 9.

Headd B, Bradford SA, 2018. Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. Frontiers in microbiology, 9, 2122.

Hijosa-Valsero M, Fink G, Schlüsener MP, Sidrach-Cardona R, Martín-Villacorta J, Ternes T, Bécares E, 2011. Removal of antibiotics from urban wastewater by constructed wetland optimization. Chemosphere, 83(5), 713-719.

Huang X, Zheng J, Liu C, Liu L, Liu Y, Fan H, 2017. Removal of antibiotics and resistance genes from swine wastewater using vertical flow constructed wetlands: effect of hydraulic flow direction and substrate type. Chemical Engineering Journal, 308, 692-699.

Ijaz A, Shabir G, Khan QM, Afzal M, 2015. Enhanced remediation of sewage effluent by endophyte-assisted floating treatment wetlands. Ecological Engineering, 84, 58-66.

Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K, 2014. Fate and effects of veterinary antibiotics in soil. Trends in microbiology, 22(9), 536-545.

Ju F, Beck K, Yin X, Maccagnan A, McArdell CS, Singer HP, Johnson DR, Zhang T, Bürgmann H, 2019. Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. The ISME journal, 13(2), 346.

Kabra AN, Khandare RV, Govindwar SP, 2013. Development of a bioreactor for remediation of textile effluent and dye mixture: a plant–bacterial synergistic strategy. Water research, 47(3), 1035-1048.

Khan MIR, Fatma M, Per TS, Anjum NA, Khan NA, 2015. Salicyli cacid-induced abiotic stress tolerance and underlying mechanisms in plants. Frontiers in Plant Science, 6:462.

Klümper U, Riber L, Dechesne A, Sannazzarro A, Hansen LH, Sørensen SJ, Smets BF, 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. The ISME journal, 9(4), 934–945.

LaPara TM, Burch TR, McNamara PJ, Tan DT, Yan M, Eichmiller JJ, 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environmental science & technology, 45(22), 9543-9549.

Lehner B, Döll P, Alcamo J, Henrichs T, Kaspar F, 2006. Estimating the impact of global change on flood and drought risks in Europe: a continental, integrated analysis. Climate Change 75, (3), 273-299.

Li LG, Xia Y, Zhang T, 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. The ISME Journal 11: 651–662.

Li S, Zhang R, Hu J, Shi W, Kuang Y, Guo X, Sun W, 2019. Occurrence and removal of antibiotics and antibiotic resistance genes in natural and constructed riverine wetlands in Beijing, China. Science of The Total Environment, 664, 546-553.

Li Y, Wu B, Zhu G, Liu Y, Ng WJ, Appan A, Tan SK, 2016. High-throughput pyrosequencing analysis of bacteria relevant to cometabolic and metabolic degradation of ibuprofen in horizontal subsurface flow constructed wetlands. Science of the Total Environment, 562, 604-613.

Li Y, Zhu G, Ng WJ, Tan SK, 2014. A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: design, performance and mechanism. Science of the Total Environment, 468, 908-932.

Liang Y, Zhu H, Bañuelos G, Shutes B, Yan B, Cheng X, 2018. Removal of sulfamethoxazole from salt-laden wastewater in constructed wetlands affected by plant species, salinity levels and co-existing contaminants. Chemical Engineering Journal, 341, 462-470.

Ling J, Wang H, Wu P, Li T, Tang Y, Naseer N, Zheng H, Masson-Boivin C, Zhong Z, Zhu J, 2016. Plant nodulation inducers enhance horizontal gene transfer of Azorhizobium caulinodans symbiosis island. Proceedings of the National Academy of Sciences, 113(48), 13875-13880.

Lingua G, Copetta A, Musso D, Aimo S, Ranzenigo A, Buico A, Gianotti V, Osella D, Berta G, 2015. Effect of arbuscular mycorrhizal and bacterial inocula on nitrate concentration in mesocosms simulating a wastewater treatment system relying on phytodepuration. Environmental Science and Pollution Research, 22(23), 18616-18625.

Liu L, Liu C, Zheng J, Huang X, Wang Z, Liu Y, Zhu G, 2013. Elimination of veterinary antibiotics and antibiotic resistance genes from swine wastewater in the vertical flow constructed wetlands. Chemosphere, 91(8), 1088-1093.

Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S, Wang XC, 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of the total environment, 473, 619-641.

Madsen JS, Burmølle M, Hansen LH, Sørensen SJ, 2012. The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunology & Medical Microbiology, 65(2), 183-195.

Mahmood Q, Pervez A, Zeb BS, Zaffar H, Yaqoob H, Waseem M, Zahidullah, Afsheen S.2013. Natural Treatment Systems as Sustainable Ecotechnologies for the Developing Countries. BioMed Research International, Article ID 796373.

Marasco R, Rolli E, Vigani G, Borin S, Sorlini C, Ouzari H, Zocchi G, Daffonchio D, 2013. Are drought-resistance promoting bacteria cross-compatible with different plant models?. Plant signaling & behavior, 8(10), e26741.

Mesa-Marín J, Fernández Del-Saz N, Rodríguez-Llorente ID, Redondo-Gómez S, Pajuelo E, Ribas-Carbó M, Mateos-Naranjo E, 2018. PGPR reduce root respiration and oxidative stress enhancing Spartina maritima root growth and heavy metal rhizoaccumulation. Frontiers in Plant Science, 9:1500.

Miller JH, Novak JT, Knocke WR, Pruden A, 2014. Elevation of antibiotic resistance genes at cold temperatures: implications for winter storage of sludge and biosolids. Letters in applied microbiology, 59(6), 587-593.

Mølbak L, Licht TR, Kvist T, Kroer N, Andersen SR, 2003. Plasmid transfer from Pseudomonas putida to the indigenous bacteria on alfalfa sprouts: characterization, direct quantification, and in situ location of transconjugant cells. Applied and Environmental Microbiology, 69(9), 5536-5542.

Nascimento FX, Rossi MJ, Glick BR, 2018. Ethylene and 1-Aminocyclopropane-1-carboxylate (ACC) in plant–bacterial interactions. Frontiers in Plant Science, 9:114.

NOAA (National Centers for Environmental Information Report). 2017. State of the Climate: Global Climate. (April 14, 2018)

Petroselli A, Giannotti M, Arcangeletti E, Palomba F, Marras T, 2015. The integrated system of phytodepuration of Sile River Natural Park. International journal of phytoremediation, 17(11), 1038-1045.

Petroselli A, Giannotti M, Marras T, Allegrini E, 2017. Integrated system of phytodepuration and water reclamation: A comparative evaluation of four municipal wastewater treatment plants. International journal of phytoremediation, 19(6), 563-571.

Petrovich M, Chu B, Wright D, Griffin J, Elfeki M, Murphy BT, Poretsky R, Wells G, 2018. Antibiotic resistance genes show enhanced mobilization through suspended growth and biofilm-based wastewater treatment processes. FEMS microbiology ecology, 94(5), fiy041.

Pruden A, Arabi M, Storteboom HN, 2012. Correlation Between Upstream Human Activities and Riverine Antibiotic Resistance Genes. Environ. Sci. Technol. 46, 11541-11549.

Prum C, Dolphen R, Thiravetyan P, 2018. Enhancing arsenic removal from arsenic-contaminated water by Echinodorus cordifolius− endophytic Arthrobacter creatinolyticus interactions. Journal of environmental management, 213, 11-19.

Qadir M, Sharma BR, Bruggeman A, Choukr-Allah R, Karajeh F, 2007. Non-conventional water resources and opportunities for water augmentation to achieve food security in water scarce countries. Agricultural water management, 87(1), 2-22.

Qiu Y, Zhang J, Li B, Wen X, Liang P, Huang X, 2018. A novel microfluidic system enables visualization and analysis of antibiotic resistance gene transfer to activated sludge bacteria in biofilm. Science of the total environment, 642, 582-590.

Rajkumar M, Sandhya S, Prasad MNV, Freitas H, 2012. Perspectives of plant-associated microbes in heavy metal phytoremediation. Biotechnology advances, 30(6), 1562-1574.

Rehman K, Imran A, Amin I, Afzal M, 2018. Inoculation with bacteria in floating treatment wetlands positively modulates the phytoremediation of oil field wastewater. Journal of hazardous materials, 349, 242-251.

Rilling JI, Acuña JA, Nannipieri P, Cassan F, Maruyama F, Jorquera MA, 2018. Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria. Soil Biology and Biochemistry, 130, 205-219.

Riva V, Terzaghi E, Vergani L, Mapelli F, Zanardini E, Morosini C, Raspa G, Di Guardo A, Borin S, 2019. Exploitation of Rhizosphere Microbiome Services. In Methods in Rhizosphere Biology Research (pp. 105-132). Springer, Singapore.

Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, Michael I, Fatta-Kassinos D, 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Science of the total environment, 447, 345-360.

Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Gandolfi C, Casati E, Previtali F, Gerbino R, Pierotti Cei F, Borin S, Sorlin C, Zocchi G, Daffonchio D, 2014. Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environmental Microbiology, 17(2), 316–331.

Saleem H, Rehman K, Arslan M, Afzal M, 2018. Enhanced degradation of phenol in floating treatment wetlands by plant-bacterial synergism. International journal of phytoremediation, 20(7), 692-698.

Salgado I, Cárcamo H, Carballo ME, Cruz M, del Carmen Durán M, 2018. Domestic wastewater treatment by constructed wetlands enhanced with bioremediating rhizobacteria. Environmental Science and Pollution Research, 25(21), 1-8.

Santos L, Ramos F, 2018. Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. International journal of antimicrobial agents, 52(2), 135-143.

Sato T, Qadir M, Yamamoto S, Endo T, Zahoor A, 2013. Global, regional, and country level need for data on wastewater generation, treatment, and use. Agricultural Water Management, 130, 1-13.

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.

SCHEER (Scientific Committee on Health Environment and Emerging Risks) Scientific advice on Proposed EU minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge; European Union: Brussels, 2017.

Shehzadi M, Afzal M, Khan MU, Islam E, Mobin A, Anwar S, Khan QM, 2014. Enhanced degradation of textile effluent in constructed wetland system using Typha domingensis and textile effluent-degrading endophytic bacteria. Water research, 58, 152-159.

Song HL, Zhang S, Guo J, Yang YL, Zhang LM, Li H, Yang XL, Liu X, 2018. Vertical up-flow constructed wetlands exhibited efficient antibiotic removal but induced antibiotic resistance genes in effluent. Chemosphere, 203, 434-441.

Sørensen SJ, Jensen LE, 1998. Transfer of plasmid RP4 in the spermosphere and rhizosphere of barley seedling. Antonie van Leeuwenhoek, 73(1), 69-77.

Soussi A, Ferjani R, Marasco R, Guesmi A, Cherif H, Rolli E, Mapelli F, Ouzari HI, Daffonchio D, Cherif A, 2016. Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. Plant and Soil, 405(1-2), 357-370.

Syranidou E, Christofilopoulos S, Gkavrou G, Thijs S, Weyens N, Vangronsveld J, Kalogerakis N, 2016. Exploitation of endophytic bacteria to enhance the phytoremediation potential of the wetland helophyte Juncus acutus. Frontiers in microbiology, 7, 1016.

Tang J, Bu Y, Zhang XX, Huang K, He X, Ye L, Shan Z, Ren H, 2016. Metagenomic analysis of bacterial community composition and antibiotic resistance genes in a wastewater treatment plant and its receiving surface water. Ecotoxicology and environmental safety, 132, 260-269.

Tezel U, Pavlostathis SG, 2015. Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. Current Opinion in Biotechnology, 33:296–304.

Truu M, Juhanson J, Truu J, 2009. Microbial biomass, activity and community composition in constructed wetlands. Science of the total environment, 407(13), 3958-3971.

Ulrich A, Becker R, Ulrich K, Ewald D, 2015. Conjugative transfer of a derivative of the IncP-1α plasmid RP4 and establishment of transconjugants in the indigenous bacterial community of poplar plants. FEMS microbiology letters, 362(23), fnv201.

UN (United Nations). 2015. Washington, DC. Transforming Our World: the 2030 Agenda for Sustainable Development.

Van der Hoek W, Hassan MU, Ensink JH, Feenstra S, Raschid-Sally L, Munir S, Aslam R, Ali N, Hussain R, Matsuno Y, 2002. Urban Wastewater: A valuable Resource for agriculture: A case study from Haroonabad, Pakistan. IWMI. (Vol. 63).

Van Elsas JD, Turner S, Bailey MJ, 2003. Horizontal gene transfer in the phytosphere. New phytologist, 157(3), 525-537.

Vergani L, Mapelli F, Zanardini E, Terzaghi E, Di Guardo A, Morosini C, Raspa G, Borin S. Phyto-rhizoremediation of polychlorinated biphenyl contaminated soils: an outlook on plant-microbe beneficial interactions. Science of the Total Environment 2017; 575: 1395-1406.

Verlicchi P, Zambello E, 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Science of the Total Environment, 470, 1281-1306. https://doi.org/10.1016/j.scitotenv.2013.10.085

Vymazal J, 2010. Constructed wetlands for wastewater treatment: five decades of experience. Environmental science & technology, 45(1), 61-69.

Wang CJ, Yang W, Wang C, Gu C, Niu DD, Liu HX, Wang YP, Guo, JH, 2012. Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting rhizobacterium strains. PLoS One, 7(12), e52565.

Wang J, Tai Y, Man Y, Wang R, Feng X, Yang Y, Chen Z, Vymazal J, Cai N, 2018. Capacity of various single-stage constructed wetlands to treat domestic sewage under optimal temperature in Guangzhou City, South China. Ecological Engineering, 115, 35-44.

Watharkar AD, Khandare RV, Waghmare PR, Jagadale AD, Govindwar SP, Jadhav JP, 2015. Treatment of textile effluent in a developed phytoreactor with immobilized bacterial augmentation and subsequent toxicity studies on Etheostoma olmstedi fish. Journal of hazardous materials, 283, 698-704.

WEF (World Economic Forum). 2018. Global risks 2018. Davos-Klosters (CH): Creating a Shared Future in a Fractured World.

Wu S, Wallace S, Brix H, Kuschk P, Kirui WK, Masi F, Dong R, 2015. Treatment of industrial effluents in constructed wetlands: challenges, operational strategies and overall performance. Environmental Pollution, 201, 107-120.

Yan W, Xiao Y, Yan W, Ding R, Wang S, Zhao F, 2019. The effect of bioelectrochemical systems on antibiotics removal and antibiotic resistance genes: a review. Chemical Engineering Journal.

Zeaiter Z, Mapelli F, Crotti E, Borin S, 2018. Methods for the genetic manipulation of marine bacteria. Electronic Journal of Biotechnology, 33, 17-28.

Zhang L, Lyu T, Zhang Y, Button M, Arias CA, Weber KP, Brix H, Carvalho PN, 2018. Impacts of design configuration and plants on the functionality of the microbial community of mesocosm-scale constructed wetlands treating ibuprofen. Water research, 131, 228-238.

Zhao X, Yang J, Bai S, Ma F, Wang L, 2016. Microbial population dynamics in response to bioaugmentation in a constructed wetland system under 10 C. Bioresource technology, 205, 166-173.

Zhu H, Zhang L, Li S, Wang Y, Sun S, Chen J, Kou S, Jin Q, Xiao M, 2018. The rhizosphere and root exudates of maize seedlings drive plasmid mobilization in soil. Applied Soil Ecology, 124, 194-202.

Zuccato E, Calamari D, Natangelo M, Fanelli R, 2000. Presence of therapeutic drugs in the environment. The lancet, 355(9217), 1789-1790.

AIMS AND OBJECTIVES

Water scarcity is one of the most relevant problems worldwide and agriculture is the activity most vulnerable toward a potential decrease in water availability, since it is an intensive water consumer (Rolli et al., 2014). Drought is indeed one of the major limitations to food production and is expected to cause serious plant growth problems for crops on more than 50% of the Earth's arable lands by 2050 (Raiten & Combs; 2019). Water resource management is therefore one of the most pressing environmental issues, especially in countries already characterized by very high levels of water stress, such as the Mediterranean African Countries (MACs) and worldwide relevant in the frame of the global warming threat. Agriculture, that has an important role in the economy of MA countries, accounts for 80-85% of freshwater consumption (Frascari et al., 2018). Moreover, MACs face different challenges in their strategy to improve food security: rapid population growth, urbanization and harsh environmental conditions inducing increasing pressure on water resources quantity and quality that will be likely exacerbated by political instability and climate change (Frascari et al., 2018). In this framework, MADFORWATER project (https://www.madforwater.eu/), which this PhD thesis is a part, has the objective to develop an integrated set of technological and management instruments to reduce water vulnerability in Egypt, Morocco and Tunisia. To reach the general aim of MADFORWATER project, this PhD thesis is focusing on plant-associated microorganisms and in particular Plant Growth Promoting (PGP) bacteria aiming to exploit them to improve i) crop productivity in drought affected soils, ii) wastewater phytodepuration efficacy. PGP bacteria are components of the plant-associated communities and establish a positive interactive dialogue with the plant, leading to a better performance of the plant holobiont, composed by the plant and its microbiome, in particular under environmental stress conditions (Compant et al., 2019; also reviewed in the

introduction section of this PhD thesis). The specific aim of this work was to assess the usefulness of PGP bacteria in reducing water footprint in agriculture through two envisaged innovation directions. The first direction is to increase water use efficiency in crops and mitigate yield losses induced by drought, the second is to improve wastewater depuration technologies in order to use recycled water for irrigation. The successful exploitation of PGP bacteria i) in agriculture for increasing crop resistance to drought stress and ii) in wastewater depuration to improve the efficiency of Constructed Wetland (CW) systems as tertiary treatment to allow the municipal reuse in agriculture, foresees different research steps. This process includes the isolation of proper bacterial strains, their taxonomic identification in order to exclude potential pathogens, the functional characterization and the *in vivo* experimentation with the most promising strains: all these steps were included in the present PhD thesis.

A large collection of bacterial strains have been established from the rhizosphere and endosphere of several plants sampled in stressed environment of MACs and selected between i) plants experiencing drought condition i.e. *Argania spinosa*, ii) plants irrigated with treated wastewaters i.e. *Sorghum bicolor* and *Medicago sativa,* iii) plants used in phytodepuration system for tertiary treatment of domestic wastewaters i.e. *Phragmites australis* and *Typha domingensis*. It is indeed known that plants growing under drought stress and in general under adverse environmental conditions, are strongly supported by plant microbiota, potentially enriched in bacteria able to counteract the stress by developing several adaptations strategies and to boost plant growth and productivity (Soussi et al., 2016).

In **Chapter III** the large bacterial collection was taxonomically compared to a composed dataset referred to plant microbiome described through culture-independent DNA sequence-based techniques. Aim of the work was the evaluation of the bacterial taxa diversity that could be brought into culture from the overall plant microbiome evaluated by culture-independent methods. According to a recent study (Martiny, 2019) it appears that the

overlap between cultured and uncultured microbial diversity is greater than previously thought (Amann et al., 1995; Brock et al., 1987), but no comparison was, up to now, performed on plant-associated bacterial communities. The possibility to isolate a vast part of the plant microbiome would indeed allow to deeper understand the functions and roles of bacteria in their relation with the plant and laying the groundwork for microbial exploitation in plant growth promotion.

Chapter IV focused on *A. spinosa* microbiome that is still uncharacterized, despite the economic value and the crucial ecological role of this tree species. Moreover, as xerophilic plant, *A. spinosa* harbors a microbial community that could represent a source of microbial resources potentially useful for environmental and agriculture biotechnologies applications. The phylogenetic composition of the bacterial communities associated to the argan root system was analyzed by 16S rRNA gene high throughput sequencing and, in parallel, the functional diversity of the cultivable microbiome was characterized in terms of PGP potential.

In order to evaluate the potential of plant-associated bacteria to improve plant growth under drought stress condition, a long-term greenhouse experiment was conducted with tomato plants inoculated with different selected PGP bacteria isolated from the root apparatus of extremophilic plants. The experiment, described in **Chapter V**, had the specific aim to test the response of bacterized tomato plants to water shortage not only in terms of biomass improvement but also as crop productivity, which is rarely analyzed in literature studies, but is of primary importance in light of the potential applicability of the PGP strains to improve agriculture in dry regions.

In **Chapter VI**, in the frame of wastewater reuse improvement, the bacterial collection isolated from *P. australis* plants grown in a CW system was analyzed and characterized for the ability to promote plant growth, aiming to select bacterial strains that could improve plant growth and, as a consequence the phytodepuration service. Besides PGP activity, the strains

were characterized also for the capacity to tolerate and/or modify micropollutants, which can be present in municipal and industrial wastewaters and are known to escape the biological wastewater treatment. As model micropollutants, textile azo-dyes, metals and bisphenol A had been chosen. The most promising strains were selected for a microcosm scale experiment, with the aim to test their suitability as candidate inoculants for the removal of the azo-dyes from textile wastewaters.
General conclusions and future directions of this work were finally summarized in the final chapters of the thesis.

Amann, R.I., Ludwig, W. and Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.*, *59*(1), pp.143-169

Brock, T.D., 1987. The study of microorganisms in situ: progress and problems. In *Symp. Soc. Gen. Microbiol.* (Vol. 41, pp. 1-17).

Compant, S., Samad, A., Faist, H. and Sessitsch, A., 2019. A review on the plant microbiome: Ecology, functions and emerging trends in microbial application. *Journal of advanced research*, *19, pp. 29-37.*

Frascari, D., Zanaroli, G., Motaleb, M.A., Annen, G., Belguith, K., Borin, S., Choukr-Allah, R., Gibert, C., Jaouani, A., Kalogerakis, N. and Karajeh, F., 2018. Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt, Morocco, and Tunisia. *Integrated environmental assessment and management*, *14*(4), pp.447-462.

Martiny, A.C., 2019. High proportions of bacteria are culturable across major biomes. *The ISME Journal*, p.1.

Raiten, D.J. and Combs, G.F., 2019. Nutritional ecology: Understanding the intersection of climate/environmental change, food systems and health. *Agriculture for Improved Nutrition: Seizing the Momentum*, p.68.

Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Pierotti Cei F (2014) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress dependent trait. Environ Microbiol 17:316–331

Soussi A, Ferjani R, Marasco R, Guesmi A, Cherif H, Rolli E, Mapelli F, Ouzari H, Daffonchio D, Cherif A (2016) Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. Plant Soil:357–370.

RESULTS

Chapter III

Investigation of plant microbiome through culture-dependent and independent techniques

Abstract

A recent study demonstrated the culturability of the majority of the bacterial taxa across environments, suggesting that the established assumption that only 1% of bacterial diversity is culturable is no longer correct. In the present study, this new hypothesis was tested on plant biome, comparing a large bacterial collection isolated from different plant species with a composed dataset of plant-associated bacterial taxa identified in the literature with culture-independent techniques. The overlap between cultured and uncultured bacterial families associated to plant roots corresponded to 20% of the whole data set, meaning that the 1% of the paradigm was an underestimation, but, on the other hand, it was not possible asserted that the majority of plant microbiome was culturable. However, despite the too limited set of culturing conditions applied in this work, it was possible obtain in culture both common and uncommon plant-associated bacterial families, which allows to study and understand i) how plant can benefit from the common bacterial partners and ii) the role of satellite taxa found associated to specific plant species under certain environmental conditions.

1. Introduction

Plants live in association with a plethora of microorganisms, also referred to a second genome of the plant, having important roles for plant health and productivity (Berendsen et al., 2012; Turner et al., 2013a; Compant et al., 2019). The bacterial community of plants is really complex and dynamic and its assembly it can be considered as resulting from a hierarchy of events (Philippot et al., 2013). Starting from the soil, which is considered as a microbial seed bank (Lennon and Jones, 2011), the structure of plant-microbiome is firstly shaped by physico-chemical properties of the soil, together with biogeographical processes. Then, the location where plants grown and, finally, the plant genotype determine which members of this reservoir of bacteria can grow and thrive in association with plant roots.

Furthermore, this bacterial community can also vary in time, during the plant life cycle and according to the environmental conditions (Perez-Jaramillo et al., 2016; Reinhold-Hurek et al., 2015; Xu et al., 2018). Plant microbiome is thus a complex community and understanding recruiting mechanisms and the functionality of plant-microbe interactions can lead to a better understand the plant as a meta-organism and how plants can benefit from their microbial partners (Geisen et al., 2019). However, it is generally asserted that most of the bacteria are currently considered "unculturable", thus limiting our ability to functionally understand these bacterial communities: isolation-based methods, according to Amann et al. (1995) and Brock et al. (1987) only select for easily cultivable and fast-growing taxa, which can represent as little as 1% of the entire diversity. However, a recent study conducted by Martiny (2019) questioned this 1% paradigm, demonstrating that almost all bacteria diversity has been brought into culture when the commonly used standard similarity threshold of 97% in the 16S rRNA gene sequence is adopted. This suggests that using the right culturing conditions and approaches, all taxa are theoretically cultivable. For instance, a Japanese research group working with intestinal microbiota was recently able to obtain novel gut bacteria using a combination of comprehensive genomics and conventional culturing methods, indicating that a number of bacteria hitherto considered unculturable are potentially culturable using commercially available media (Ito et al., 2019). Moreover, a study on forest soil compared the amplicon-based community profiles with the cultivable fraction of bacteria using eight different media (VanInsbergen et al., 2013), revealing that approximately 22% of the complex forest soil microbiota was cultivable *in vitro*.

In his work, Martiny proved his hypothesis by considering 40 bacterial communities across six major biomes: human associated, marine water, marine sediment, freshwater, soil and air biome. However, no information about plant biome was provided in this study. The overlap between cultured and uncultured plant microbiota could be greater in the plant niche than

previously thought. In fact, the evaluated bacterial community associated to *Arabidopsis thaliana* showed that the 60% of the taxa identified with culture-independent technique had corresponding sequence matches in the culture collection (Schlaeppi and Bulgarelli, 2015). However, this hypothesis should be verified looking at a broader range of information and not limiting the investigation to a single laboratory cultivated plant species. Aiming to obtain indications in order to proof this hypothesis, we evaluated the wide bacterial collection isolated within this PhD thesis from plants of different species (*Phragmites australis*, *Typha domingensis*, *Persicaria* sp., *Sorghum bicolor*, *Medicago sativa*, *Argania spinosa*), grown under pristine (forest and field), and anthropized (phytodepuration system and polluted river) conditions, in different countries (Morocco, Tunisia, Egypt and Italy) hence subjected to different soil and environmental conditions. Based on the 16S rRNA gene sequences of the isolates, taxa isolated from endosphere and rhizosphere of plants were compared, at family level, to the composition of plant microbiome described in different studies through culture-independent methods.

2. Material and methods

2.1. Plant sample collection

Plant roots and root surrounding soils were collected in six sampling campaigns using sterile tools and were transferred to the laboratories of the University of Milan within 48 hours. a) In two different constructed wetland plants treating municipal wastewaters and located in Morocco (Drarga, Souss-Massa region) and Egypt, the root apparatus of *Phragmites australis* and *Typha domingensis* were sampled; b) *Persicaria* sp. was sampled in highly anthropized and polluted waters of the Lambro River in Northern Italy; c) *Argania spinosa* was collected in a protected historical forest close to

Agadir (Morocco); d) *Sorghum bicolor* and *Medicago sativa* were collected in Northern Tunisia in a field irrigated with treated wastewaters.
Plant species and sampling countries were synthesized in Table 1.

2.2. Bacteria collection establishment

All the plant samples were collected in triplicate and were pooled and homogenized prior to bacteria isolation with the exception of the three samples of *S. bicolor* that were separately treated. Collection of endophytic and rhizosphere bacteria were obtained from surface sterilized roots. In particular, endophytic bacteria collections were isolated from *P. australis* plants collected in Morocco and Egypt, *T. domingensis*, *M. sativa* and *A. spinosa*, while rhizobacteria collections were obtained with *P. australis* collected in Morocco, *Persicaria* sp., *S. bicolor* and *A. spinosa* samples (Table 1). To obtain endophytic bacteria collections, plant root surface (1 gram/specimen) were sterilized with the following protocol: roots were firstly vigorous washed in physiological solution (0.9% NaCl) for 10 minutes and then threated with 70% ethanol for 3 min, 1% sodium hypochlorite for 5 min, 70% ethanol for 30 sec and finally rinsed four times for 2 min in sterile distilled water before a final washing step in sterile distilled water for 30 min. To confirm root surface sterility, 100 µl of the last rinsing water was plated on the same solid medium subsequently used for bacteria isolation. Roots were then smashed with sterile mortar and pestle and 1 gram of the resulted root tissue homogenate was suspended in 9 ml of physiological solution, serially diluted and plated in triplicate.
Rhizospheric bacteria were isolated from the few millimeters of soil strictly attached to the root apparatus of four plant specimens: one gram of the pooled soil (except for *S. bicolor*) was subjected to the same protocol described for the root tissue homogenate.
After 48 hours of incubation at 30°C, bacteria colonies were picked and spread three times on the same solid medium in order to obtain pure

bacterial cultures. Isolates were selected on the basis of colony morphology to maximize diversity and minimize redundancy in the culture collection. The established collections were cryopreserved in 25% glycerol stocks at -80°C. As showed in Table 1, the majority of bacteria were isolated on 869 medium diluted 1:10, suited for the isolation of plant-associated bacteria (Barac et al., 2004). The bacteria collection of *Persicaria* sp. was isolated on R2A medium (0.5 g/l peptone casein, 0.5 g/l yeast extract, 0.5 g/l proteose peptone, 0.5 g/l dextrose, 0.5 g/l starch, 4.8 g/l diphosphate potassium, 0.032 g/l magnesium sulphate, 4.8 g/l sodium pyruvate, 15 g/l agar) supplemented with a mixture of metals (nickel 40ug/l, arsenic 20ug/l and lead 20ug/l) to simulate the contaminated environment of origin. Finally, from the rhizosphere samples of *S. bicolor*, a 1-aminocyclopropane-1-carboxylate (ACC)-deaminase enrichment was performed to isolate bacteria with ACC deaminase activity, generally considered one of the main bacterial Plant Growth Promoting traits (Penrose and Glick 2003).

2.3. Bacteria genotyping and identification

The genomic DNA of each isolate was extracted through boiling cell lysis (Ferjani et al., 2015) The bacteria collections were de-replicated by fingerprinting analyses of the 16S-23S rRNA Intergenic Transcribed Spacer (ITS) region (Mapelli et al., 2013): isolates which showed the same ITS band pattern, which is species-subspecies specific, were grouped in ITS clusters and at least one representative strain per each ITS cluster has been further taxonomically identified through 16S rRNA gene amplification (Mapelli et al., 2013). Partial 16S rRNA sequences were obtained from Macrogen, Rep. of South Korea. Nucleotide sequences were edited in Chromas Lite 2.01 and compared with those deposited in the GeneBank database, using BLAST suite.

Table 1. Summary of the different isolate bacteria collections considered in the present study. The code of the collection includes information about the plant species (PA= *Phragmites australis*; TD = *Typha domingensis*; MS = *Medicago sativa*; SB = *Sorghum bicolor*; AS = *Argania spinosa*; P = *Persicaria* sp.), the fraction (R = rhizosphere; E = endosphere) and in some cases also the country of origin (CWM = constructed wetland Morocco; CWE = constructed wetland Egypt; L= Lambro River). The medium used for bacteria isolation was specified in the fourth column: 869 1:10 = 869 medium diluted 1:10; ACC-d = ACC-deaminase enrichment; R2A+Me = R2A + metals mixture (nickel 40ug/l, arsenic 20ug/l and lead 20ug/l). The sixth and seventh columns indicate if the plants grown in pristine or anthropized place and if they are aquatic or terrestrial plants. Water type used for irrigation was specified in the eighth column: WW = wastewater; TWW = treated wastewater; n.i. = non irrigated; river = river water. Finally, the number of bacteria isolated for each collection and the total number of isolates were indicated.

Code	Plant species	Fraction	Medium	Country	Environment	Plant type	Irrigation	N° of isolates
CWM-PA-R	*P. australis*	R	869 1:10	Morocco	Constructed wetland	Aquatic	TWW	72
CWM-PA-E	*P. australis*	E	869 1:10	Morocco	Constructed wetland	Aquatic	TWW	80
CWE-PA-E	*P. australis*	E	869 1:10	Egypt	Constructed wetland	Aquatic	WW	78
CWE-TD-E	*T. domingensis*	E	869 1:10	Egypt	Constructed wetland	Aquatic	WW	80
MS-E	*M. sativa*	E	869 1:10	Tunisia	Field	Terrestrial	TWW	78
SB-R	*S. bicolor*	R	ACC-d	Tunisia	Field	Terrestrial	TWW	98
AS-R	*A. spinosa*	R	869 1:10	Morocco	Forest	Terrestrial	n.i.	70
AS-E	*A. spinosa*	E	869 1:10	Morocco	Forest	Terrestrial	n.i.	80
L-P-R	*Persicaria sp.*	R	R2A+Me	Italy	Polluted River	Aquatic	river	45
								Tot=681

2.4. Plant microbiome dataset identified with culture-independent techniques

Twelve works found in the literature were selected to compose a dataset of plant microbiome identified through culture-independent techniques. The

selection of the literature works took into account the plant species, the root fractions that was analyzed and the environment where plant grown, in order to obtain a bacterial collection comparable to our isolate collections. Six studies about aquatic plants and six studies about terrestrial plants were indeed selected.

Table 2. Summary of the different bacteria collections of the literature identified with culture-independent methods considered in the present study. The analyzed fractions are indicated in the third column: R = rhizosphere, E = endosphere. Water type used for irrigation was specified in the seventh column: WW = wastewater; TWW = treated wastewater; n.i. = non irrigated; river = river water, n.s.= not specified.

Ref.	Plant species	Fraction	Country	Environment	Plant type	Irrigation
Ansola et al., 2014	*T. latifolia, S. atrocinerea*	R	Spain	Constructed and natural wetland	Aquatic	TWW
Chow et al., 2002	*P. contorta*	R	British Columbia	Forest	Terrestrial	n.i.
Dai et al., 2014	*Caragana microphylla*	E	China	Desert grassland	Terrestrial	Freshwater
el Zahar Haichar et al., 2008	*T. aestivum, Z. mays, B. napus, M. truncatula*	R	n.s.	Pots placed in growth chamber	Terrestrial	n.s.
Filion et al., 2004	*P. mariana*	R	Québec	Tree nursery	Terrestrial	n.s.
Guo et al., 2015	*T. angustifolia*	R	Miyun County	Constructed wetland	Aquatic	TWW
Ibekwe et al., 2007	*Typha* sp., *S. californicus*	R	California	Constructed wetland	Aquatic	river
Li et al., 2010	*P. australis*	E	China	Constructed wetland	Aquatic	WW
Li et al., 2013	*P. australis, T. angustifolia*	E	China	Constructed wetland	Aquatic	WW
Pisa et al., 2011	*Saccharum* spp	R	Brazil	Field	Terrestrial	Freshwater
Ulrich et al., 2008	*Populus* sp	E	Germany	Field	Terrestrial	Freshwater
Zhang et al., 2017	*P. australis, T. angustifolia*	R	China	Constructed wetland	Aquatic	WW

As described in Table 2, aquatic plants analyzed by culture-independent techniques (Ansola et al., 2014; Guo et al., 2015; Ibekwe et al., 2007; Li et al., 2010; Li et al., 2013; Zhang et al., 2017) comprised *Phragmites australis*, *Thypa* spp, *Salix atrocinerea* and *Schoenoplectus californicus* sampled from constructed and natural wetlands. The group of terrestrial plants was composed by trees grown in a forest environment, i.e. *Pinus contorta* and *Picea mariana, Populus* sp (Chow et al., 2002; Filion et al., 2004; Ulrich et al., 2008), species of the family Leguminosae highly tolerant to drought and salt stress, i.e. *Caragana microphylla* (Dai et al., 2014) and crop of agricultural interest, i.e. *Triticum aestivum, Zea mays, Brassica napus, Medicago truncatula* and *Saccharum* spp (el Zahar Haichar et al., 2008; Pisa et al., 2011). The literature studies were also selected according to the culture-independent methods used for the identification of the plant microbiome. High throughput sequencing techniques were not considered suitable for our aim because they usually describe the bacterial community at phylum/class level, not allowing the bacterial phylogenetic identification at deeper levels. To verify the overlap between cultured and uncultured plant microbiota we considered appropriate the comparison of bacterial communities at family level. The literature data were indeed chosen among studies that performed 16S rRNA pyrosequencing, denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone library analysis.

3. Results and discussion

The whole bacterial collection was composed by 681 bacterial isolates. Table 1 showed the number of bacteria isolated from each collection: almost all the collections were represented by around 80 isolates, while *S. bicolor* collection was composed by 98 strains and *Persicaria* sp. collection by 45 isolates.

In order to combine a data set of plant microbiome described at family level, a literature research was conducted on different plant species embracing

aquatic (Ansola et al., 2014; Guo et al., 2015; Ibekwe et al., 2007; Li et al., 2010; Li et al., 2013; Zhang et al., 2017) and terrestrial plants (Dai et al., 2014; Chow et al., 2002; el Zahar Haichar et al., 2008; Filion et al., 2004; Pisa et al., 2011; Ulrich et al., 2008) growing in pristine or anthropized environments (Table 2). The data discussed in these selected works were obtained by culture-independent techniques such as 16S rRNA pyrosequencing, denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone library analysis.

Overall, a minor part of the bacterial phyla detected in the plant microbiomes by DNA-based methods were recovered in this work by conventional culturing techniques from a variety of plants and environmental conditions. The isolate collection obtained in the present work collectively contained 20% of the families present in the combined literature data sets (Table 3): only 22 over 109 families detected in the literature by cultivation-independent analyses were indeed present in our isolate collections. Notably, the isolate collection comprised Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Table 3, Figure 1 and Figure 2), which represented the dominant phyla associated to the plant root apparatus (Philippot et al., 2013; Sànchez et al., 2017; Turner et al., 2013a; Turner et al., 2013b). Phyla missing from the isolate collection, such as Acidobacteria, Chlamydiae, Chloroflexy, Fusobacteria, Gemmatimonadetes, Nitrospirae and Verrucromicrobia, were nevertheless minor components of the plant microbiota, the so-called rare biosphere, as detected by cultivation independent methods (Table 3).

Table 3: List of bacteria families associated to plant root apparatus according to literature data. In the first column are indicated the phylogenetic group: Acido = Acidobacteria; Alphaproteo = Alphaproteobacteria; Bacteroidetes; Betaproteo = Betaproteobacteria; C = Chlamydiae; Chl = Chloroflexi; Deltaproteo = Deltaproteobacteria; E = Epsilonproteobacteria; Firmicutes; F = Fusobacteria; Gamma = Gammaproteobacteria; G = Gemmatimonadales; N = Nitrospirae; V = Verrucomicrobia. Studies indicated with a number are terrestrial plants works: 1 = Dai et al. (2014); 2 =

Chow et al. (2002); 3 = el Zahar Haichar et al. (2008); 4 = Filion et al. (2004); 5 = Pisa et al. (2011); 6 = Ulrich et al. (2008). Studies indicated with a letter are aquatic plants works: a = Ansola et al. (2014); b = Guo et al. (2015); c = Ibekwe et al. (2007); d = Li et al. (2010); e = Li et al. (2013); f = Zhang et al. (2017). Isolate collections are indicated with the code explained in Table 1. In bold character are indicated the families found in isolate collections in the present work. The blue square indicates the collection where the family was found.

		Terrestrial plants										**Aquatic plants**										
GROUP	**FAMILY**	**1**	**2**	**3**	**4**	**5**	**6**	SB-R	AS-R	AS-E	MS-E	a	b	c	d	e	f	CWM-PA-R	CWM-PA-E	CWE-PA-E	CWE-TD-E	L-P-R
Acido	Acidimicrobiaceae												■									
	Acidobacteriaceae				■																	
	Acidomicrobiaceae		■		■																	
	Holophagaceae				■							■										
Actino	Beutenbergiaceae						■															
	Godoniaceae				■																	
	Kineosporiaceae						■															
	Konexibacteriaceae				■																	
	Microbacteriaceae			■	■		■		■													
	Micrococcaceae			■		■			■	■												
	Micromonosporaceae					■																
	Mycobacteriaceae				■	■																
	Nitriliruptoraceae											■										
	Nocardioidaceae				■	■	■															■
	Pseudonocardiaceae				■	■																
	Rubrobacteriaceae					■																
	Sporichthyaceae				■																	
	Streptomycetaceae			■	■	■			■													
Alphaproteo	Acetobacteriaceae		■			■	■									■						
	Aurantimonadaceae						■															
	Bradyrhizobiaceae	■		■		■						■	■		■							
	Brucellaceae			■														■				
	Caulobacteraceae	■	■	■			■					■			■				■			
	Chromobacteriaceae													■								
	Erythrobacteraceae	■		■																		
	Hyphomicrobiaceae					■									■	■						
	Methylobacteriaceae					■	■															
	Methylocystaceae														■							
	Phyllobacteriaceae	■		■		■				■												
	Rhizobiaceae	■	■	■		■	■	■	■										■			
	Rhodobacteraceae											■	■			■						
	Rhodospirillaceae			■		■									■	■						
	Sphingomonadaceae	■	■	■		■	■					■					■				■	
	Xanthobacteraceae					■																
Bacteroidetes	Bacteroidaceae														■	■						
	Chitinophagaceae					■									■	■						
	Clostridiaceae													■	■							
	Crocinitomicaceae														■							
	Cryomorphaceae											■						■				
	Cytophagaceae												■									
	Eubacteriaceae													■	■							
	Flavobacteriaceae		■			■	■		■	■		■	■			■	■	■	■		■	■
	Flexibacteraceae						■															
	Ignavibacteriaceae												■									
	Paludibacteraceae														■							

	Porphyromonadaceae													■								
	Prevotellaceae														■							
	Prolixibacteraceae														■							
	Saprospiraceae												■									
	Sphingobacteriaceae					■	■															
Betaproteo	Alcaligenaceae						■						■									
	Burkholderiaceae	■	■	■		■	■					■					■					
	Comamonadaceae		■	■		■	■		■	■		■	■		■	■						
	Gallionellaceae		■										■									
	Hydogenophylaceae				■							■										
	Methylophylaceae											■										
	Neisseriaceae				■		■					■				■						
	Nitrosomonadaceae				■								■			■						
	Oxalobacteraceae			■	■	■	■					■			■							
	Rhodocyclaceae		■	■								■	■		■	■						
C	Parachlamydiaceae				■																	
Chl	Anaerolineaceae											■										
	Caldilineaceae												■									
	Ktedonobacteraceae											■										
Deltaproteo	Bacteriovoracaceae		■																			
	Bdellovibrionaceae					■																
	Desulfobacteraceae											■			■							
	Desulfobulbaceae											■				■						
	Desulfomicrobiaceae														■							
	Desulfovibrionaceae												■		■	■						
	Desulfuromonadaceae		■																			
	Geobacteraceae											■		■								
	Mixococcaceae		■			■																
	Peleobacteriaceae														■							
	Polyangiaceae				■							■										
	Syntrophaceae											■	■		■							
	Syntrophorhabdaceae											■										
E	Campylobacteriaceae														■	■						
	Helicobacteraceae											■										
Firmicutes	Alicyclobacillaceae					■																
	Bacillaceae	■			■	■		■	■	■				■			■	■	■		■	■
	Enterococcaceae	■						■			■											
	Gemmatimonadaceae													■								
	Lachnospiraceae													■								
	Natranaerobiaceae													■								
	Paenibacillaceae	■		■	■	■									■							
	Peptococcaceae													■		■						
	Peptostreptococcaceae						■					■										
	Planococcaceae																■	■				
	Ruminococcaceae													■								
	Tissierellaceae																■					
F	Fusobacteriaceae													■	■							
Gammaproteo	**Aeromonadaceae**											■		■	■	■						■
	Chromatiaceae															■		■				
	Competibacteraceae																■					
	Enterobacteriaceae		■	■				■				■	■		■		■	■	■	■	■	
	Legionaellaceae		■																			
	Methylococcaceae					■						■		■								
	Moraxellaceae			■					■			■										■
	Pseudomonadaceae				■		■	■	■	■	■	■	■		■	■	■	■	■			■
	Shewanellaceae													■								
	Sinobacteraceae					■						■	■	■	■	■						
	Succinivibrionaceae											■										
	Thiotricaceae														■							
	Xanthomonadaceae	■	■	■	■		■	■	■	■		■	■			■		■	■		■	
G	Gemmatimonadaceae					■																
N	Nitrospiraceae					■										■						
V	Opitutaceae											■										
	Verrucomicrobiaceae			■	■							■										

Results were confirmed even at lower taxonomic rank since bacterial families found in most of the isolate collections, such as Xanthomonadaceae, Flavobacteriaceae, Pseudomonadaceae and Enterobacteriaceae, were also found in several (more than 50%) culture-independent studies (Table 3) and thus it is possible to consider them as the core plant microbiota, comprising keystone microbial taxa tightly associated to plants regardless plant genotype soil or environmental conditions and containing essential functions genes for the fitness of the plant holobiont (Compant et al., 2019). In particular, the Pseudomonadaceae family, comprising 26% of the isolate collection, is largely described in culture-independent studies and is a well-recognized dominant member of the root microbiota, able to use a large number of substances as energy or carbon sources, tolerant to toxic compounds and able to promote plant growth directly and indirectly (Chow et al., 2002; Li et al., 2010; Philippot et al., 2013). An isolate collection that includes the core plant microbiome creates the opportunity to study key community members through genomic and physiological investigations: isolates are indeed source for laboratory models in physiological studies and are relevant to determine the role of genes and functions discovered with molecular methods.

Some representative families of plant microbiome such as Burkholderiaceae, Bradyrhizobiaceae and Rhizobiaceae, known to be nutritionally versatile species with plant growth promoting and biological disease control attributes (Chow et al., 2002), were not found in our isolates collection, while, conversely, rare families like Chromatiaceae, Cryomorphaceae and Brucellaceae, were obtained in culture (Table 3). Microbial taxa that occur in a reduced number of plants, called satellite taxa, can be defined on the basis of geographical range and habitat specificity and their importance is increasingly recognized as drivers of key functions for the ecosystem (Compant et al., 2019). In our isolate collection 50% of the families were found in single plant microbiomes. They could be therefore considered satellite taxa, demonstrating a high specificity of the plant microbiome.

Studies of the last decade have indeed revealed highly complex microbial assemblages associated with different plants (Reinhold-Hurek et al., 2015; Hardoim et al., 2015), influenced by several factors including climate, local mineralogy, soil pH, plant species and agricultural measures (Hirsch and Mauchlin, 2012).
The specificity of microbiome associated to aquatic plants growing in phytodepuration systems, i.e. constructed wetlands, is particularly remarked. In phytodepuration plants, the environmental conditions that affect the root-microbial community are indeed so various that it is difficult to identify a core microbiome associated to these plant species (Pietrangelo et al., 2018; Sànchez et al., 2017). Hydraulic design, availability of organic matter, plant species, temperature, dissolved oxygen, wastewater and substrate type are among others some of the environmental factors that play a role in the microbial composition of wetland plants (Pietrangelo et al., 2018; Sànchez et al., 2017). Considering separately the microbial community of wetland and terrestrial plants of our study the percentage of isolates recovered in pant microbiome described with culture-independent techniques, is slightly lower for wetland plants (18%) than for terrestrials (22%). The high specificity of bacterial community associated to wetland plants, is evident also among our bacterial collections isolated from *P. australis* and *T. domingensis* in CW of Morocco and Egypt. Comparing endophytic bacteria, isolated from surface-sterilized roots of these plants, we observed a specific bacterial community composition (Fig.1). The strains isolated from *P. australis* collected from CW plant located in Morocco belonged to 6 families: Bacillaceae (48%), Pseudomonadaceae (24%), Xanthomonadaceae (8%), Rhizobiaceae (6%), Flavobacteriaceae (4%) and Caulobacteriaceae (1%) (Fig.1). Other reports showed abundance of isolates belonging to Bacillaceae and Pseudomonadaceae families in the root-microbiota of this plant species (Chen et al., 2012; Soares et al., 2016), however, the composition of the endophytic community of *P. australis* growing in CW plant located in Egypt was completely different and mainly represented by Leuconostocaceae

(80%) and Streptococcaceae (17%) (Fig.1). Lactic acid-producing bacteria belonging to Leuconostocaceae and Streptococcaceae families was described in different plant niches as neutral association (Makarova et al., 2006; Siezen et al., 2008; van Baarlen and Siezen, 2009), however, it is uncommon that the nearly whole bacteria collection isolated from the endosphere of wetland plants is represented by lactic acid bacteria. These bacterial families were indeed not detected in culture-independent reports analyzed for the composition of the plant-microbiome data set (Table 1). Lactic acid-producing bacteria are primary constituent of many starter cultures used for the manufacturing of fermented foods and beverages (Makarova et al., 2006; Siezen et al., 2008) and even if they are not usually described as intestinal inhabitants, they can survive passage through the gastrointestinal tract (Dal Bello et al., 2003; Walter et al., 2001; Maruo et al., 2006) reaching urban wastewater depuration systems.

Endophytic bacterial community isolated from *T. domingensis* in CW in Egypt was characterized by different bacterial families in comparison to the collection isolated from *P. australis*, even if in both cases the plants were grown in a CW system that received municipal wastewaters (Fig.1). *T. domingensis* collection was indeed manly represented by members of the families Enterobacteriaceae (71%) and Flavobacteriaceae (25%). The results showed that the endosphere is a peculiar habitat having reduced phylogenetic diversity due to a strict plant-bacteria relationship (Compant et al., 2019; Hirsch and Mauchline, 2012), but in the case of plants used in wastewater depuration a stronger influence could be due to the environmental factors that characterized the overall CW system, like operational parameters and wastewater biotic and abiotic composition.

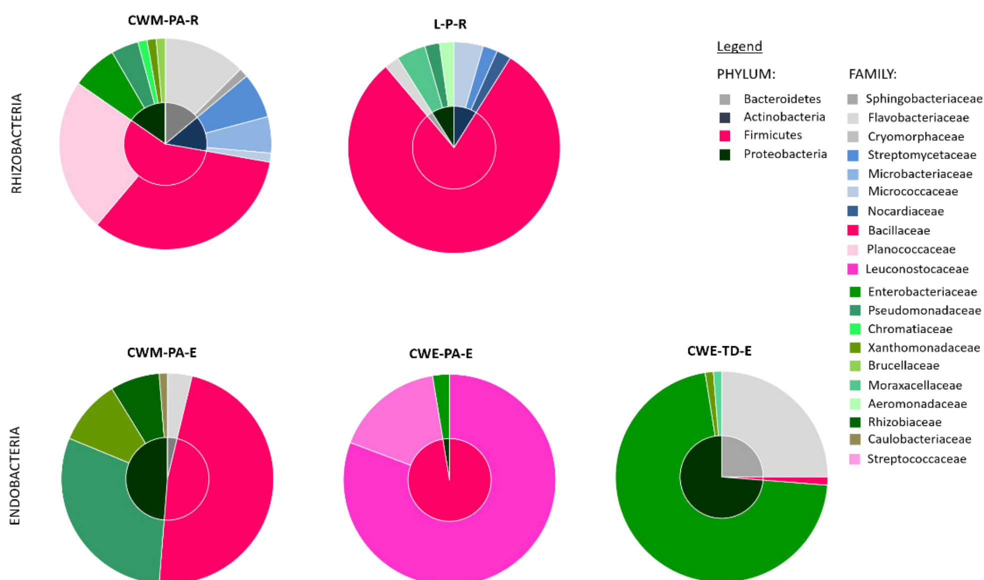


Figure 1: Taxonomic distribution of 16S rRNA sequences of culturable rhizospheric and endophytic bacteria isolated from aquatic plants, *P. australis*, *T. domingensis* and *Persicaria* sp. The inner pie graph shows the strain taxonomic identification according to phylum and the outer ring represent their identity at family level.

Culture-independent studies analyzed for the data set composition asserted the predominance of Proteobacteria in plant microbiome of both aquatic and terrestrial plants. This observation was confirmed in our isolate collections obtained from terrestrial plants since rhizobacteria isolated from *S. bicolor* and endobacteria isolated from *M. sativa* and *A. spinosa* were dominated by Gammaproteobacteria.

The bacterial collection obtained from the endosphere of *M. sativa* cultivated in Tunisia and irrigated with TWW was composed by only two families: 76% of the isolates were assigned to the Pseudomonadaceae family and 24% to Enterococcaceae (Fig.2). The presence of Enterococcaceae in the collection could be due to the fact that *M. sativa* plants were irrigated with TWW and

Enterococci survived to wastewater treatment (Da Costa et al., 2006) could have found a suitable niche in the root apparatus of this plant. *Enterococci* are commonly isolated from clinical sources or municipal wastewaters, however they have been identified on different plant species (Ott et al.,2001)

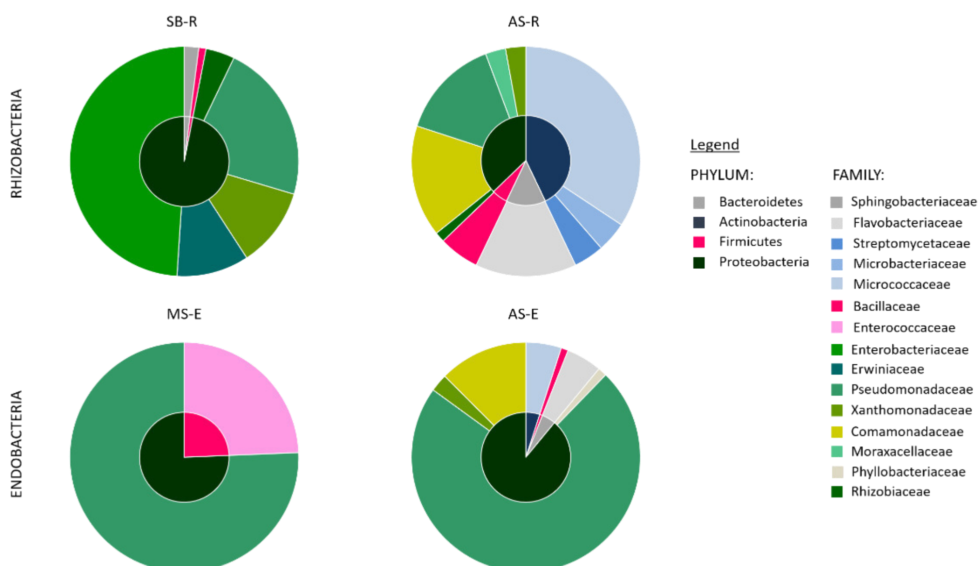


Figure 2: Taxonomic distribution of 16S rRNA sequences of culturable rhizospheric and endophytic bacteria isolated from terrestrial plants, *S. bicolor*, *A. spinosa* and *M. sativa*. The inner pie graph shows the strain taxonomic identification according to phylum and the outer ring represent their identity at family level.

The collection of rhizobacteria with ACC-deaminase activity isolated from *S. bicolor* was mainly represented by Proteobacteria (93%, Fig.2), which were also dominant in the endophyte isolate collection of *A. spinosa* (89%), obtained in a different medium without the selection given by the preliminary enrichment for ACC-deaminase harbouring bacteria. The *A. spinosa* rhizosphere, compared with the endosphere, contained less Proteobacteria (37%) with a higher percentage of Actinobacteria (43%) (Fig.2). These relative abundances were confirmed by Illumina sequencing analyses of 16S rRNA gene performed on the same samples used for the cultivation approach (Chapter IV). As showed in Figure 3, the endophytic community of *A. spinosa* was mainly composed by Proteobacteria (50%) and

Bacteroidetes (26%) while the bacterial community associated to the rhizopshere fraction was affiliated to Actinobacteria (32%) and Proteobacteria (28%). However, a disparity in the representation of different bacterial phyla, classes and genera between isolate collection and clone library can be observed (Ulrich et al., 2008; VanInsberghe et al., 2013).

In the bacterial collections isolated from aquatic plants, a different aboundance was observed in comparison to cultivation independent literature data. The Firmicutes phylum was indeed the dominant group in the isolate collections while molecular methods resulted in identifying the predominance of Proteobacteria (Ansola et al., 2014; Li et al., 2010; Sànchez et al., 2017). Limits related to both culture-dependent and independent analyses techniques could contribute to explain the difference in the relative abundance of the different taxa identified within bacteria communities. Nutrient media represent a determiant factor for the isolation of certain bacterial phyla: for instance, VanInsberghe and collegues (2013) observed that rare community members of forest soil samples were easily cultivable under nutrient-rich conditions, while abundant taxa were typically slow-growing members adapted to competition under oligotrophic conditions. Some of the collections obtained in this work, moreover, were obtained after an initial enrichment step for bacteria with PGP traits (i.e. ACC deaminase activity), which strongly selected for taxa adapted to grow in the specific condition. Conversely, i) cell lysis recalcitrance of certain phyla, that might results underrepresented in DNA-based methods and ii) the preferential amplification of certain bacterial groups with universal primers can represent limits in culture-independent methods that can affect the results about the microbiome composition. Moreover, except for *A. spinosa* plants, the comparison of bacterial diversity estimated by isolate collection vs sequence catalogues was not applied on the same plant species collected from the same environment, hence the specific influence of plant genotype and environmental parameters on the community structure could not be evaluated.

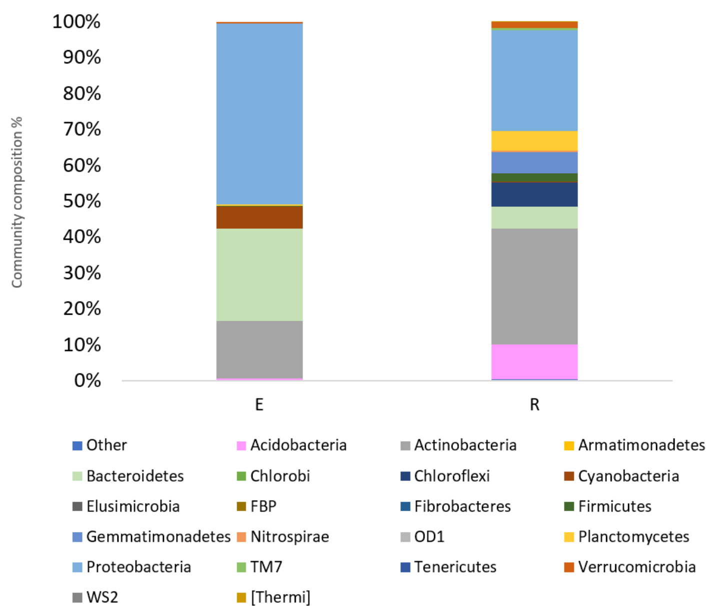


Figure 3: composition of the bacterial community associated to A. spinosa roots. The distribution of bacterial phyla is based on data obtained by Illumina Sequence analyses of 16S rRNA gene. “E” indicates endosphere fraction; “R” indicates rhizosphere fraction.

4. Conclusion

In a recent study (Martiny, 2019), it was demonstrated that members of almost all the bacterial species identified by cultivation-independent methods in major biomes (human, marine water and sediments, freshwater, soil and air) could be obtained in culture, thus questioning the consolidated assumption that 99% of the bacterial diversity is unculturable. In the present study, a large bacteria collection isolated from root endosphere and rhizosphere of plants of different species and habitats, from aquatic to terrestrial, was identified and compared to a composed dataset of plant-associated bacterial taxa identified in the literature with culture-independent techniques. Aim of the comparison was to prove the hypothesis proposed by Martiny (2019) applying it to the plant biome. Bacterial community associated to plants is very complex and dynamic, and presents high

specificity according to the peculiar environmental conditions: understanding the role of plant microbiome by studying cultivable bacterial strains could thus lead to gain deeper knowledge of the plant biome, provided that the isolated strains can describe the overall bacterial diversity of the microbiome.

The culture collection collectively represented 20% of the total bacterial families identified with culture-independent techniques. This percentage indicates that the old established paradigm of only 1% of cultivable bacteria in environmental samples underestimated the bacterial diversity that can be isolated using common culture-dependent methods; on the other hand, the obtained data do not support the conclusion that the majority of the bacterial diversity associated to the plant biome can be cultivated, as Martiny (2019) proved on other environmental biomes. The results could be nevertheless affected by the fact that the phylogenetic diversity of the plant-associated bacterial community is strongly dependent on many factors like the plant genotype and the specific environmental factors, making intrinsically difficult the identification of a plant microbiome common to the whole plant biome. However, in this study we observed that among the combined data set, it was possible to obtain in culture members of the core plant microbiome composed by bacterial families found in most of the analyzed plant species, together with members of satellite taxa found associated to single or few plant species grown in peculiar environmental conditions. This is an important result because, thanks to this kind of isolate collections, it is possible to better understand the function of i) the core plant microbiome that may have an important role for plant health since it is recurrent in different plant genotypes and environments but also ii) the satellite taxa that can specifically help the growth of particular plant species under certain environmental conditions.

Furthermore, in the present study it was observed that the relative abundance of phyla within the bacterial community can be different between isolate collection and clone library because of intrinsic limit of the two

methods. In particular, in the present study a too limited set of culturing conditions was applied, which could have led the selection of the taxa adapted to the specific medium or incubation conditions besides the most relevant taxa of each bacterial community. However, the study on the microbiome associated to the plant species *A. spinosa*, analyzed by applying both culture-dependent and independent techniques on the same rhizosphere and endosphere samples, demonstrated that the relative abundance of the bacterial phyla identified by cultivation vs molecular analyses was maintained between the two approaches. These results, when confirmed at lower taxonomic depth, would confirm the results obtained by Martiny et al. (2019).

References

Amann, R.I., Ludwig, W. and Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.*, *59*(1), pp.143-169.

Ansola, G., Arroyo, P., de Miera, L. E. S. 2014. Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands. *Science of the Total Environment*, *473*, 63-71.

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

Berendsen, R.L., Pieterse, C.M. and Bakker, P.A., 2012. The rhizosphere microbiome and plant health. *Trends in plant science*, *17*(8), pp.478-486.

Brock, T.D., 1987. The study of microorganisms in situ: progress and problems. In *Symp. Soc. Gen. Microbiol.* (Vol. 41, pp. 1-17).

Chen, W.M., Tang, Y.Q., Mori, K. and Wu, X.L., 2012. Distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted waters. Aquatic biology, 15(2), pp.99-110.

Chow, M.L., Radomski, C.C., McDermott, J.M., Davies, J. and Axelrood, P.E., 2002. Molecular characterization of bacterial diversity in Lodgepole pine (Pinus contorta) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. *FEMS microbiology ecology*, *42*(3), pp.347-357.

Compant, S., Samad, A., Faist, H. and Sessitsch, A., 2019. A review on the plant microbiome: Ecology, functions and emerging trends in microbial application. *Journal of advanced research*, *19, pp. 29-37.*

Da Costa, P. M., Vaz-Pires, P., Bernardo, F. 2006. Antimicrobial resistance in Enterococcus spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water research*, *40*(8), 1735-1740.

Dai, J.X., Liu, X.M. and Wang, Y.J., 2014. Diversity of endophytic bacteria in Caragana microphylla grown in the desert grassland of the Ningxia Hui autonomous region of China. *Genet. Mol. Res*, *13*(2), pp.2349-2358.

Dal Bello, F., Walter, J., Hammes, W.P. and Hertel, C., 2003. Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. Microbial ecology, 45(4), pp.455-463.

el Zahar Haichar, F., Marol, C., Berge, O., Rangel-Castro, J.I., Prosser, J.I., Balesdent, J., Heulin, T. and Achouak, W., 2008. Plant host habitat and root exudates shape soil bacterial community structure. *The ISME journal*, *2*(12), p.1221.

Ferjani, R., Marasco, R., Rolli, E., Cherif, H., Cherif, A., Gtari, M., Boudabous, A., Daffonchio, D. and Ouzari, H.I., 2015. The date palm tree rhizosphere is a niche for plant growth promoting bacteria in the oasis ecosystem. *BioMed research international*, *2015*.

Filion, M., Hamelin, R. C., Bernier, L., St-Arnaud, M. 2004. Molecular profiling of rhizosphere microbial communities associated with healthy and diseased black spruce (Picea mariana) seedlings grown in a nursery. *Appl. Environ. Microbiol.*, *70*(6), 3541-3551.

Geisen, S., Briones, M.J., Gan, H., Behan-Pelletier, V.M., Friman, V.P., de Groot, G.A., Hannula, S.E., Lindo, Z., Philippot, L., Tiunov, A.V. and Wall, D.H., 2019. A methodological framework to embrace soil biodiversity. *Soil Biology and Biochemistry*, *136*, p.107536.

Guo, Y., Gong, H., Guo, X. 2015. Rhizosphere bacterial community of Typha angustifolia L. and water quality in a river wetland supplied with reclaimed water. *Applied microbiology and biotechnology*, *99*(6), 2883-2893.

Hardoim, P.R., Van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M. and Sessitsch, A., 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.*, *79*(3), pp.293-320.

Hirsch, P.R. and Mauchline, T.H., 2012. Who's who in the plant root microbiome?. *Nature biotechnology*, *30*(10), p.961.

Ibekwe, A. M., Lyon, S. R., Leddy, M., Jacobson-Meyers, M. 2007. Impact of plant density and microbial composition on water quality from a free water surface constructed wetland. *Journal of applied microbiology*, *102*(4), 921-936.

Ito, T., Sekizuka, T., Kishi, N., Yamashita, A. and Kuroda, M., 2019. Conventional culture methods with commercially available media unveil the presence of novel culturable bacteria. *Gut microbes*, *10*(1), pp.77-91.

Lennon, J.T. and Jones, S.E., 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature reviews microbiology*, *9*(2), p.119.

Li, Y. H., Zhu, J. N., Zhai, Z. H., Zhang, Q. 2010. Endophytic bacterial diversity in roots of Phragmites australis in constructed Beijing Cuihu Wetland (China). *FEMS microbiology letters*, *309*(1), 84-93.

Li, Y. H., Zhu, J. N., Liu, Q. F., Liu, Y., Liu, M., Liu, L., Zhang, Q. 2013. Comparison of the diversity of root-associated bacteria in Phragmites australis and Typha angustifolia L. in artificial wetlands. World Journal of Microbiology and Biotechnology, 29(8), 1499-1508

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, Pavlov, A., Pavlova, N.,Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y.,Rohksar, D.,Lucas, S.Huang, K., Goodstein, D.M.,Hawkins,T., Plengvidhya, V. , Welker, D.,Hughes, J., Goh, Y.,Benson, A., Baldwin, K., Lee, J.-H., Díaz-Muñiz I.,Dosti,B.,Smeianov, V., Wechter, W.,Barabote,R., Lorca, G., Altermann, E., Barrangou, R.,Ganesan, B., Xie, Y.,Rawsthorne, H.,Tamir,D. , Parker, C., Breidt, F., Broadbent, J.,Hutkins, R.,O'Sullivan,D.,Steele, J., Unlu, G.,Saier, M., Klaenhammer, T., Richardson, P.,Kozyavkin, S., Weimer, B., Mills, D. 2006. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences*, *103*(42), 15611-15616.

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D. and Borin, S., 2013. Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *BioMed research international*, *2013*.

Martiny, A.C., 2019. High proportions of bacteria are culturable across major biomes. *The ISME Journal*, p.1.

Maruo, T., Sakamoto, M., Toda, T. and Benno, Y., 2006. Monitoring the cell number of Lactococcus lactis subsp. cremoris FC in human feces by real-time PCR with strain-specific primers designed using the RAPD technique. International journal of food microbiology, 110(1), pp.69-76.

Ott, E. M., Müller, T., Müller, M., Franz, C. M. A. P., Ulrich, A., Gabel, M., Seyfarth, W. 2001. Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *Journal of Applied Microbiology*, *91*(1), 54-66.

Penrose, D. M., Glick, B. R. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiologia plantarum*, *118*(1), 10-15.

Pérez-Jaramillo, J.E., Mendes, R. and Raaijmakers, J.M., 2016. Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant molecular biology*, *90*(6), pp.635-644.

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

Pietrangelo, L., Bucci, A., Maiuro, L., Bulgarelli, D. and Naclerio, G., 2018. Unravelling the composition of the root-associated bacterial microbiota of Phragmites australis and Typha latifolia. *Frontiers in microbiology*, *9*, p.1650.

Pisa, G., Magnani, G. S., Weber, H., Souza, E. M., Faoro, H., Monteiro, R. A., Daros, E., Baura, V., Bespalhok, J.P., Pedrosa, F.O., Cruz, L. M. 2011. Diversity of 16S rRNA genes from bacteria of sugarcane rhizosphere soil. *Brazilian Journal of Medical and Biological Research*, *44*(12), 1215-1221.

Reinhold-Hurek, B., Bünger, W., Burbano, C.S., Sabale, M. and Hurek, T., 2015. Roots shaping their microbiome: global hotspots for microbial activity. *Annual review of phytopathology*, *53*, pp.403-424.

Sánchez, O., 2017. Constructed wetlands revisited: microbial diversity in the–omics era. *Microbial ecology*, *73*(3), pp.722-733.

Schlaeppi, K. and Bulgarelli, D., 2015. The plant microbiome at work. *Molecular Plant-Microbe Interactions*, *28*(3), pp.212-217.

Siezen, R.J., Starrenburg, M.J., Boekhorst, J., Renckens, B., Molenaar, D., van Hylckama Vlieg, J.E. 2008 Genomescale genotype-phenotype matching of two Lactococcus lactis isolates from plants identifies mechanisms of adaptation to the plant niche. Appl Environ Microbiol 74: 424–436

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

Turner, T.R., James, E.K. and Poole, P.S., 2013 (a). The plant microbiome. *Genome biology*, *14*(6), p.209.

Turner, T.R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., Osbourn, A., Grant, A. and Poole, P.S., 2013 (b). Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. *The ISME journal*, *7*(12), p.2248.

Ulrich, K., Ulrich, A. and Ewald, D., 2008. Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS microbiology ecology*, *63*(2), pp.169-180.

Van Baarlen, P. and Siezen, R.J., 2009. Genomics of plant-associated microbes. *Microbial biotechnology*, *2*(4), p.406.

VanInsberghe, D., Hartmann, M., Stewart, G.R. and Mohn, W.W., 2013. Isolation of a substantial proportion of forest soil bacterial communities detected via pyrotag sequencing. *Appl. Environ. Microbiol.*, *79*(6), pp.2096-2098.

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K. and Hammes, W.P., 2001. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol., 67(6), pp.2578-2585.

Xu, L., Naylor, D., Dong, Z., Simmons, T., Pierroz, G., Hixson, K.K., Kim, Y.M., Zink, E.M., Engbrecht, K.M., Wang, Y. and Gao, C., 2018. Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. *Proceedings of the National Academy of Sciences*, *115*(18), pp.E4284-E4293.

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.

Chapter IV

Unveiling the microbiota diversity of the xerophyte *Argania spinosa* L. Skeels

Abstract

The microbial communities inhabiting the root system of the xerophytic tree *Argania spinosa* is still uncharacterized despite the ecological rule and the economic value in Northern Africa of this plant species. In this work, the bacterial community of *A. spinosa* was analyzed with both culture-dependent and independent methods, revealing a different distribution of phylogenetic groups according to their degree of association with the plant: endosphere, rhizosphere, soil surrounding roots, bulk soil and residuesphere (i.e. litter). To investigate the microbiome associated to this drought tolerant tree as source of novel microbial resources of interest for a tailored management of arid lands, the plant growth promotion potential (PGP) of the isolate collection was *in vitro* characterized. Most of the multivalent isolates showing the higher PGP score were identified in the residuesphere. We hypothesized that these bacteria can contribute to the litter effect played by the argan tree potentially providing a scientific explanation of the argan litter use as agricultural soil amendment. Moreover, these bacterial strains could be suggested as candidates for future studies aimed at setup inoculants for sustainable agriculture in arid lands.

Introduction

Argania spinosa L. Skeels is a xerophilic plant species, endemic from Northern Africa and especially present in the Agadir area in South Western Morocco. Archaeobotanists demonstrated the high importance of this tree for the economy of Morocco since the past centuries (Ruas et al. 2016). Nowadays, the plant has a pivotal economic value for this country due to the production of argan oil that is worldwide requested by the cosmetic industry. Moreover, argan oil is traditionally used for food consumption and it has been proposed as a nutraceutical since the characterization of the fruit flesh content led to the identification of several phenolic compounds with antioxidant activity (Charrouf et al. 2005; Khallouki et al. 2015). The leaves

and fruits of argan tree are exploited as forage, and the plant is considered the base of a peculiar agrosystem, which includes argan tree, goat and barley, having a great socio-economic value for South Western Morocco (Nouaim and Chaussod, 1994a).

Noteworthy, argan trees are able to grow on low fertile soils, mining ions and nutrients from the deep soil layers and increasing their concentration in the top soil as a consequence of litter deposition, *i.e.* litter effect (Nouaim and Chaussod, 1994b). Indeed, the use of litter composed by argan leaves as soil amendments is widespread among farmers in South Western Morocco according to tradition and the local availability of this organic fertilizer (Dr. Choukrallah personal communication). The portion of soil that is more influenced by the decomposition of the litter, or other organic supplement, is defined as 'residuesphere' (Magid et al. 2006) and it is highly relevant in terms of soil fertilization because it represents the soil niche with highest mineralization rates (Christel et al. 2016). Litter decomposition depends upon several factors including their origin (e.g. leafy substrate, woody debris, root materials) and a recent study showed that leaf litter plays a strongest plant growth promotion effect compared to the addition of root litter into the soil, likely due to the different carbon composition of these litter types (Fu et al. 2017). An aspect that has not yet been explored is the possible role played by the litter associated microbes as plant growth promoters, in relation to the litter effect.

In fact, plant growth and adaptation to the occurring environmental conditions are strongly supported by the plant microbiome (Theis et al. 2016). In the past years, extremophilic plants and their associated microbiota have been largely studied (Jha et al., 2012; Pampurova and Van Dijck, 2014; Soussi et al. 2016) aiming at the exploitation of beneficial microbe-plant interactions to boost plant growth and productivity under harsh conditions such as soil salinity (Marasco et al. 2016; Sgroy et al. 2009; Soldan et al. 2019; Tiwary et al. 2011) and water shortage (Cherni et al. 2019; Egamberdieva et al., 2011; Marasco et al., 2012; Frascari et al.

2018, Rolli et al. 2015; Rolli et al. 2017). In this framework, experimental protocols have been established in the last years to effectively combine plant seeds and extremophilic microbes able to cope with desiccation and to promote the plant growth under drought conditions (Vilchez and Manzanera, 2011). Among extremophilic plants, xerophytes (e.g. cacti, argan, resurrection plants) are adapted to long-term survival under severe water scarcity, by means of several mechanisms like the decrease of transpiration surface and stomatal closure. Previous studies suggested a key role of endophytic bacteria in terms of plant adaptation to drought (Pampurova et al. 2014, Puente et al. 2009), however studies on the diversity and composition of the microbiota associated to xerophytes are still scarce. Surprisingly, we realized that no data are available for the xerophilic species *Argania spinosa* L. Skeels despite its economic value and the crucial ecological role it plays in the native region, where it cannot be replaced by other tree species and it represents a unique tool to counteract desertification (Louati et al. 2019).

In this work we studied the microbiota inhabiting plant and soil fractions collected along a gradient that includes micro-niches i) intimately associated to *A. spinosa* L. Skeels plant (*i.e.* endosphere, rhizosphere, root-surrounding soil), ii) not associated to the plant (*i.e.* bulk soil) and iii) indirectly influenced by the plant being partially composed by its leafy residue and the associated microbes (*i.e.* litter, from here on defined as residuesphere). The phylogenetic composition of the overall argan bacterial communities was disentangled by 16S rRNA gene high-throughput sequencing. Furthermore, we focused on the cultivable microbiota establishing a large bacteria collection which functional diversity was characterized *in vitro* in terms of Plant Growth Promotion (PGP) potential. These bacteria might be expoited in the future for research on plant adaptation under lack of water and the development of biofertilizers adapted to conditions of drought and soil salinity.

Materials and methods

Root system sampling and processing

Argania spinosa L. Skeels root system was collected in a field located in the protected Argan forest within the farm of IAV Hassan II, the Horticulture campus of Agadir in the southern part of Morocco. The permission to conduct the sampling was granted by Prof. Redouane Choukr-Allah. The local climate is arid Mediterranean, with an average annual rainfall of 200 mm restricted to the winter months (December-January). The texture of the soil in the sampling site is loamy sand, rich in organic matter and a high pH 8.5 with a saturated past conductivity of 1.8 (Prof. Redouane Choukr-Allah, personal communication). Argan roots were collected at 30 cm depth. Bulk soil, *i.e.* the portion of soil not influenced by any plant root exudates, was collected at 2 meters from argan trees, where no visible plants were present. Residuesphere, *i.e.* the portion of soil influenced by the decomposition of residue (Magid et al. 2006; in our study it was composed by a mixture of leaf litter and soil), was collected below the tree crown at 3-5 cm depth after the removal of the surface material. All samples were collected from triplicate argan tree using sterile tools and were processed within 48 h from the time of collection. Rhizosphere soil was separated from the sampled roots in sterile conditions and the clean roots were then surface sterilized, as previously described (Cherif et al. 2015). Five washes with sterile water were performed to remove any trace of reagents used. The wash solution from the last rinse was cultured in plates containing 1:10 869 medium (Barac et al. 2004) to determine the efficiency of sterilization.

Bacteria isolation, cultivation and identification

Bacteria were isolated from each plant/soil/residuesphere fraction, after pooling and homogenization of the samples collected from the replicate

plants (n=3). For endophytic bacteria isolation, the root tissues were smashed with sterile mortar and pestle after the above-described sterilization procedure. One gram of the resulting soil/root tissue was suspended in 9 ml of physiological solution (0.9% NaCl), diluted in 10-fold series and plated on 1:10 869 medium supplemented with cycloheximide 0.1 g/L to prevent fungal growth. Colonies were randomly picked after two days of incubation at 30 °C and were spread three times on the same medium to obtain pure cultures. The purified strains were stored at -80°C in 1:10 869 medium supplemented with 25% glycerol for later use. A collection of 371 bacterial isolates was obtained. Strain code includes information on the plant species ('A' for *Argania spinosa*), the medium used for the isolation ('8' for 1:10 869) and the fraction ('E/R/SSR/B/Re' for endosphere/rhizosphere/root surrounding soil/bulk soil and residuesphere) followed by progressive numbers. The genomic DNA of each isolate was extracted through boiling cell lysis and the bacteria collection has been de-replicated by ITS-PCR fingerprinting (16S-23S rRNA Internal Transcribed Spacer-PCR, Daffonchio et al. 2000) using ITS-F (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-R (5'- GCCAAGGCATCCACC -3') primers as previously described (Mapelli et al. 2013). Bacterial isolates were grouped according to their ITS-PCR fingerprint profile and one representative strain per each 'ITS group' has been selected for subsequent physiological characterization. Bacterial strains were identified through 16S rRNA gene amplification and partial sequencing (Macrogen, Rep. of South Korea) as previously described (Mapelli et al. 2013). The 16S rRNA gene sequences of the bacterial isolates were subjected to BLAST search (using blastn program) and were deposited in the European Nucleotide Archive under the accession numbers LS991221-LS991231 (endosphere), LS991172-LS991220 (rhizosphere), LS991066-LS991120 (root surrounding soil), LS991015-LS991065 (bulk soil) and LS991121-LS991171 (residuesphere).

In vitro characterization of the PGP potential of cultivable bacteria

In vitro screening for the presence of activities related to plant growth promotion (PGP) was performed for one representative strain per each 'ITS group' identified in the bacteria collection (n=219). The solubilization of inorganic phosphate and the production of siderophores, ammonia, protease and exopolysaccharides (EPS) were assessed as described in detail by Cherif et al. (2015). The production of esterase was conducted using tributyrin agar plates and scoring the strains as positive in presence of a solubilization halo, as previously described (Kumar et al. 2012). The results of the PGP in vitro tests for each taxonomic group (i.e. Family) present in the collection were visualized as a heat-map (Babicki et al. 2016).

Metagenomic DNA extraction

For the rhizosphere, root-surrounding soil, bulk soil and residuesphere fractions, the metagenomic DNA was extracted from a 0.5 g of sample using the PowerSoil DNA Isolation Kit (MoBio Inc., CA, USA). To obtain metagenomic DNA of endophytes, one gram of root was surface-sterilized (as described above) and crushed using liquid nitrogen as previously reported by Cherif et al. (2015). The DNA was extracted using a DNeasy Plant Max Kit (Qiagen). The DNA concentration of each sample was assessed using a Qubit™ flurometer with dsDNA HS kit (ThermoFisher).

Quantification of bacteria by quantitative PCR (qPCR)

qPCR reactions were performed on metagenomic DNA in polypropylene 96-well plates using a BIORAD CFX Connect™ Real-Time PCR Detection System by the amplification of 16SrRNA universal bacterial gene (Terzaghi et al. 2019) using primers 357F (5'-CCCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') with the following conditions: 0.3 µM of each primer, 7.5 µl SsoAdvanced™Universal SYBR®Green Supermix (BIORAD), 1 µl DNA template, 15 µl final volume. PCR thermal conditions were 3 min at 98°C, followed by 35 cycles of 98°C for 1 min, 30 s at 58°C,

and 72°C for 1 min. Standards were prepared through tenfold serial dilutions of the plasmid pCR®II-TOPO® carrying the 16S rRNA gene of the strain *Asaia stephensi* (Favia et al. 2007) and cloned into TOP10 *Escherichia coli* competent cells (TOPO® TA Cloning® Kit, ThermoFischer Scientific). The plasmids were isolated from LB over-night cultures of the transformant *E. coli* and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) to determine the number of 16S rRNA copies contained. Standard curves were constructed with a series of dilutions ranging from $2x10^8$ to $2x10^4$ 16S rRNA copies per microliter. All the standards and the samples were run in triplicate. R^2 and amplification efficiency of the qPCR assay were 1,000 and 90% respectively. Statistical analysis of qPCR results was performed by one-way ANOVA test using the aov function of the R software (R Core Team 2012).

Illumina high-throughput analysis of 16S rRNA gene

Illumina tag analysis of the V3-V4 hypervariable regions of the 16S rRNA gene was performed on the metagenomics DNA by BioFab (Italy), using primers IlluminaF (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG) and IlluminaR (GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). The obtained sequences were analysed using a combination of the VSEARCH (Rognes et al. 2016) and the QIIME v1.9 (Caporaso et al. 2010) software. Raw forward and reverse reads for each sample were assembled into paired-end reads considering a minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using PEAR - Paired-End reAd mergeR (https://sco.h-its.org/exelixis/web/software/pear/doc.html). The paired reads were then quality filtered, the primer sequences were removed and the individual sample files were merged in a single fasta file. Chimeras were removed using both de-novo and reference-based detection. For reference chimera

detection, the "Gold" database containing the chimera-checked reference database in the Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/) was used. QIIME was used to generate the operational taxonomic units (OTUs) of 97% sequence identity. Taxonomy was assigned to the representative sequences of the OTUs in QIIME using UClust (Edgar, 2010) and searching against the latest version of the SILVA database 128 (Quast et al. 2013). Finally, an OTU table (*i.e.*, a sample x OTU count matrix with a tab containing the taxonomic affiliation of each OTU) was created. The OTU table and the phylogenetic tree were calculated with FastTree2 (Price et al. 2010) using default parameters and the PyNast-aligned representative sequences as an input. The OTU table and the phylogenetic tree were used as inputs for the subsequent analyses of alpha- and beta-diversity. The sequence reads were deposited in the NCBI SRA database under the BioProject ID: PRJNA484110.

Diversity and phylogenetic composition of the microbiota and statistical analyses

The phylogenetic compositional differences of the bacterial communities inhabiting the different types of samples analyzed in this study were investigated both on the cultivable fraction (based on the 16S rRNA sequences of the isolated strains, n=371) and on the entire bacterial microbiota (based on the Illumina 16S rRNA gene dataset). For the cultivable bacteria, the 16S rRNA gene sequences of each 'ITS group' representative strain were aligned using the Clustal X software (Thompson et al. 1997) and the output file was used to define operational taxonomic units at 97% of identity (OTU_{97}) using DOTUR (Schloss et al. 2009). On the other hand, to test the differences of the overall bacterial compositional among the fractions, we performed a Permutational multivariate analysis of the variance (PERMANOVA) on the Bray-Curtis distance matrix generated from the Illumina 16S rRNA gene dataset, considering the 'Fraction' (five

levels: 'Endosphere', 'Rhizosphere', 'Soil Surrounding Root', 'Bulk soil' and 'Residuesphere') as categorical variable. Bray-Curtis distance matrix was used also to perform a Principal Coordinates Analysis (PCoA) and a Canonical Analysis of Principal coordinates (CAP). Statistical analyses were conducted in PRIMER v. 6.1, PERMANOVA++ for PRIMER routines (Anderson et al. 2008). Richness, i.e. number of OTU_{97}, Shannon and dominance indices were calculated using the PAST software (Hammer et al. 2001) and their statistical difference was evaluated with the analysis of variance (ANOVA) and Tukey's mean grouping considering the index as response variable and 'Fraction' as explanatory categorical variable.

Results and discussion

α- and β-diversity and phylogenetic classification of the total bacterial communities associated to Argania spinosa

The structure of the overall bacterial communities associated to the *A. spinosa* root system (E; R, SSR fractions), bulk soil (B) and residuesphere (Re) was disentangled by Illumina sequencing of 16S rRNA gene. A total of 2236 OTU_{97} were identified, the rarefaction curves of the libraries were assessed and all samples had a coverage of more than 99% (Supplementary Figure 1). The number of OTU_{97} (richness) was significantly lower in the endophytic bacterial community (236±51) compared to the other fractions (R: 1825±66, SSR: 1759±119, B: 1468±12, Re: 1864±80) as shown in Figure 1A. Likewise, the endophytic bacterial community was characterized by lower diversity (according to the Shannon index values, Figure 1B) and a higher dominance (Figure 1C) of few bacterial populations. The lower richness and diversity of the endosphere bacterial community is in agreement with previous reports on the model plant *A. thaliana* (Lundberg et al. 2012) and different crops growing under both conventional agriculture and desert farming conditions (Edwards et al. 2015; Marasco et al. 2012; Marasco et al. 2018a). Bulk soil, *i.e.* the portion of soil not influenced by root

exudates, hosted a less rich bacterial community compared to the other soil (R, SSR) and residuesphere (Re) fractions (Figure 1A) while the higher diversity was detected in the bacterial community that inhabits the residuesphere (Figure 1B).

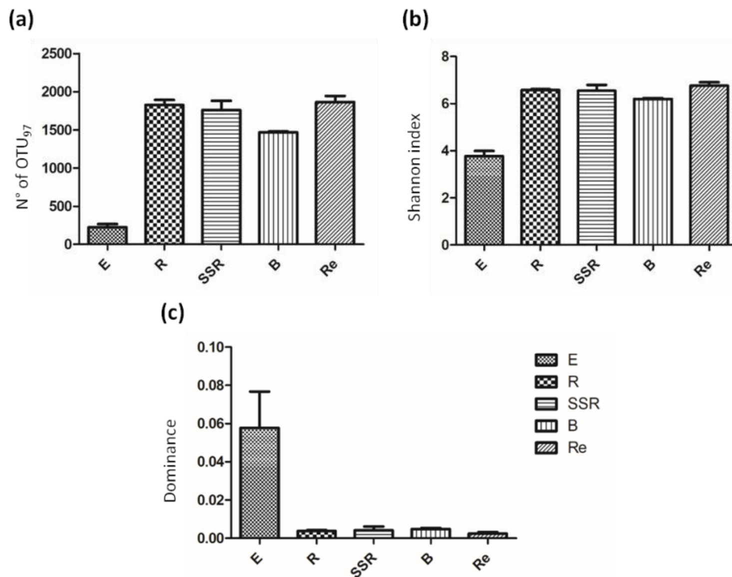


Figure 1. α-diversity indices of the total bacterial communities associated to *Argania spinosa*. (**a**) Richness, expressed as number of OTU_{97}; **(b)** Shannon index and **(c)** Dominance.

The lower richness of the bacterial community inhabiting the bulk soil compared to those of the rhizospheric (R) and root-surrounding soils (SSR), which are influenced by the release of root exudates, is common in desertic areas (Miniaci et al., 2007; Kumar et al., 2016; Kumar et al., 2017), in contrast to what is generally reported for conventional agriculture systems (Berg and Smalla 2009). Indeed, it has been proposed that the nurturing effect played by plants on soil bacterial communities becomes more evident under harsh conditions, favoring the establishment of a more diverse and rich assembly of bacterial populations around plant roots due to higher

nutrient availability (*i.e.* root exudates) compared to the nutrient-poor desertic soil (Mapelli et al. 2018).

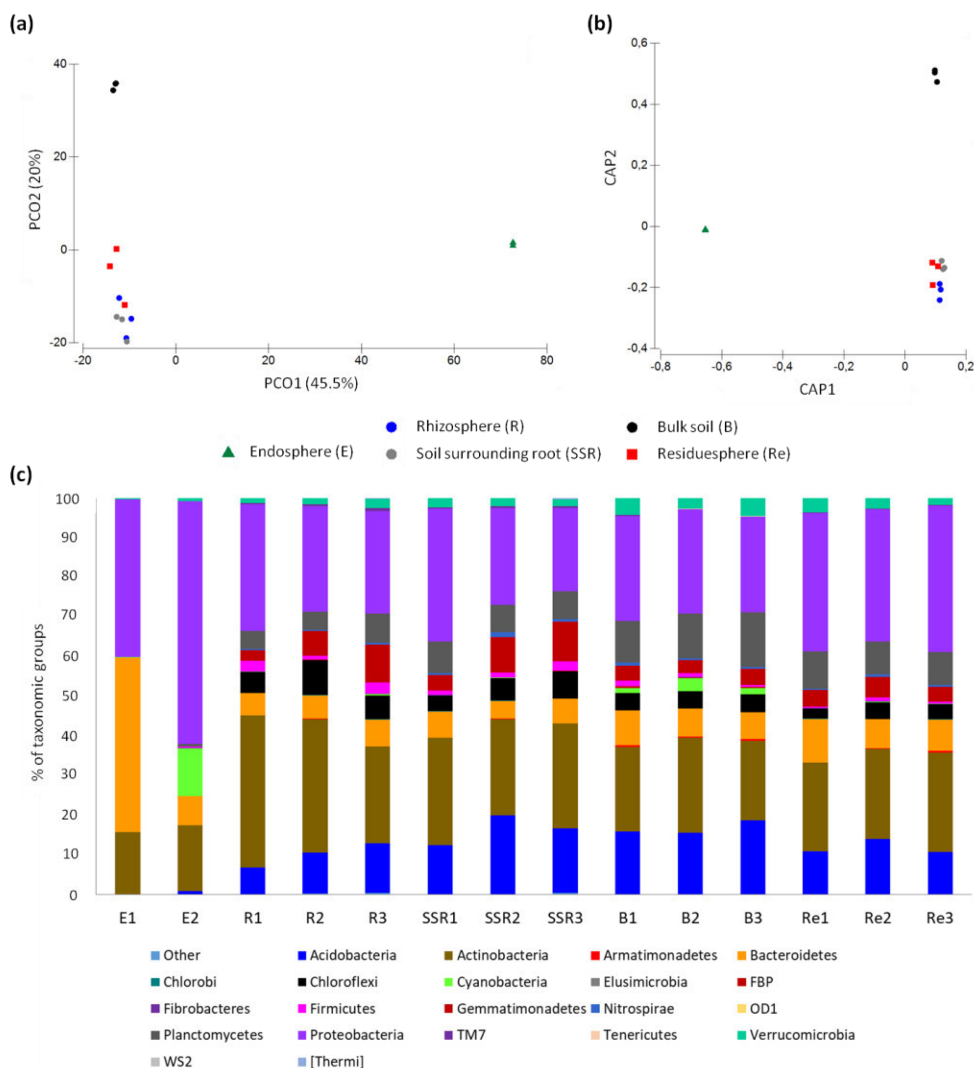


Figure 2. Phylogenetic classification and β-diversity of the total bacterial communities associated to *Argania spinosa*. (a) Principal coordinates analysis (PCoA) and **(b)** Constrained analysis of principal coordinates (CAP) of the bacterial communities inhabiting the *A. spinosa* endosphere (E), rhizosphere (R), root-surrounding soil (SSR), bulk soil (B) and residuesphere (Re) fractions. PCoA and CAP were calculated from the OTU_{97} table generated by 16S rRNA gene Illumina sequencing. **(c)** Relative abundance of different taxonomic groups (at the Phylum level) in the bacterial communities.

The Principal Coordinate Analysis (PCoA) and the Constrained analysis of principal coordinates (CAP) showed that the bacterial communities are clustered according to the fraction type, and unveiled a clear separation of the endosphere (E) and bulk soil (B) bacterial communities compared to those inhabiting the R, SSR and Re niches (Figure 2A-B).

The categorical variable 'Fraction' significantly influenced the composition of the bacterial communities in the analyzed samples (PERMANOVA, $F_{4, 9} = 6.27$; $p = 0.001$). In particular, as it could be inferred by the PCoA, the pairwise PERMANOVA results confirmed that the endosphere and the bulk soil hosted significantly different assembly of bacterial populations while the bacterial microbiota of the rhizosphere, root-surrounding and residuesphere could not be significantly distinguished (Table 1). The pattern of beta-diversity observed reflects the selective effect played by the plant through the release of root exudates and the complex signaling mechanism it establishes with the soil dwelling microflora. In fact, plants actively select rhizosphere competent bacterial populations (R and SSR fractions) starting from the initial pool of bacteria present in the bulk soil (B) enriching beneficial microorganisms in proximity of its root system. Moreover, among the rhizosphere and rhizoplane colonizers only a subset of bacterial populations is able to enter the root tissues showing an endophytic lifestyle (Hardoim et al. 2008), and the sharp separation of the endosphere bacterial communities highlights a high specialization of the dominant taxa in root tissues of *A. spinosa*. In our study, the bacterial communities inhabiting residuesphere samples could not be significantly discriminated from those of the rhizosphere and root-surrounding soil ones by 16S rRNA Illumina sequencing. The residuesphere object of this study was a mixture of argan leafy substrate and soil, collected below the tree crown, and according to the results of the PCoA we hypothesize a selective effect played by the plant on the residuesphere-colonizing bacterial community (Figure 2A-B). Although it is less studied compared to the plant microbiome, it is known that litter types influence the structure of microbial communities (Fu et al.

2017) and a recent study showed that also the diameter of twig shapes the fungi and bacteria assemblages associated to litter (Angst et al. 2018). In the argan root system the phylogenetic groups were differentially distributed, as particularly evident in the case of the endosphere microbiota that is resulted dominated by Proteobacteria, Bacteroidetes and Actinobacteria (Figure 2C), in accordance to previous high-throughput taxonomic characterization of the endophytic bacterial communities (Bulgarelli et al. 2012; Lundberg et al. 2012; Edwards et al. 2015).

Table 1. Pair-wise PERMANOVA for the 16S rRNA gene-based Illumina dataset. Post-hoc test performed on the distance matrix generated according to OTU_{97} distribution in the different fractions ('E', 'R', 'SSR', 'B', 'Re') bacterial communities (16S rRNA gene-based Illumina dataset). Asterisk (*) indicates significant differences between samples. E= endosphere; R= rhizosphere; SSR= soil surrounding root; B= bulk soil; Re= residuesphere.

Groups	**T**	***P***
E, R	2.6707	0.019*
E, SSR	2.6055	0.026*
E, B	3.5484	0.009*
E, Re	3.0397	0.018*
R, SSR	0.92395	0.492
R, B	2.7382	0.009*
R, Re	1.4644	0.119
SSR, B	2.6329	0.011*
SSR, Re	1.4192	0.156
B, Re	3.0824	0.01*

The prevalence of Proteobacteria and Actinobacteria was observed also in the soil and residuesphere fractions, while the abundance of Bacteroidetes phylum decreased. Other phyla, such as Chloroflexi, Gemmatimonadetes, Verrucomicrobia and Acidobacteria, which were not detected or were present below 1% of the total community of the endosphere, were important components of the bacterial communities in the different soil fractions (R, SSR, B) and the residuesphere (Re) as shown in Figure 2C. These Phyla

are known to colonize soil much more efficiently than root surface and interior tissues (Edwards et al. 2015; Lundberg et al. 2012). For example, Chloroflexi and Acidobacteria were among the most abundant taxa in soils associated to cork oak, a tree able to tolerate drought, sampled in different climate regions (Reis et al. 2019). Furthermore, the relative abundance of Chloroflexi and Acidobacteria over the total bacterial community was significantly higher in rhizospheric soil compared to the endosphere in a study that extensively analyzed the microbiome of grapevine plants (Marasco et al. 2018). The different Classes of the Proteobacteria phylum were unevenly distributed in the endosphere and the plant associated soils (R, SSR), bulk soil and residuesphere (Table 2). Gammaproteobacteria and Betaproteobacteria were mostly retrieved from the endosphere metagenome and decreased in the other fractions (Table 2). Gammaproteobacteria was reported as the dominant taxon within the endosphere described in both leaves and branches for different arborous crops such as the *Olea* and *Citrus* genera (Mina et al. 2019). The phylogenetic affiliation of OTU_{97} in the residuesphere, with a prevalence of Proteobacteria and Actinobacteria, resembles that recently reported for the *Salix caprea* litter (Angst et al. 2018). The authors also observed a shift of the Proteobacteria classes in the residuesphere niche during the decomposition process, revealing a higher abundance of Alphaproteobacteria, the same class retrieved in the argan Re samples, in the later phases of decomposition according to the preference of several Alphaproteobacteria taxa for oligotrophic conditions (Angst et al. 2018). Besides the significant differences observed comparing alpha- and beta-diversity values and the phylogenetic structure of the bacterial communities colonizing the different plant, soil and residuesphere niches, the abundance of the 16S rRNA gene amplified from the extracted metagenome (Figure 3) was significantly different according to the ‘Fraction’ factor (Table 3). However, the pairwise test performed on these data revealed that the bacterial abundance was significantly different only comparing the

endosphere fraction to the bulk soil and residuesphere (Table 3B). The fact that we could not detect significant differences of the bacterial community abundance among the soil fraction contrasts the general knowledge on the rhizosphere enrichment effect on the microbial community that leads to a higher density of bacteria in plant-associated soil fractions fueled by carbon-substrates released by roots (Vacheron et al. 2013). Our relatively small set of data did not allow us to elaborate a convincing explanation to justify the observed deviation from an effect that has been described independently from the plant species and also in ecosystems subjected to different stress types like pollution (Terzaghi et al. 2019) and xeric conditions (Marasco et al. 2018b). Given the scarcity of data on xerophytes, we can hypothesize that arborous species such as A. spinosa present a rhizodeposition profile able to select a peculiar microbiota from the bulk soil but not sufficient to sustain the typical flourishing observed in the rhizosphere niche.

Table 2. Distribution of the taxonomic groups (at the Class level) according to the phylogenetic affiliation of the OTU$_{97}$ detected by 16S rRNA Illumina sequencing. The relative abundance of each group is expressed as percentage over the total bacterial community, E= endosphere; R= rhizosphere; SSR= root-surrounding soil; B= bulk soil; Re= residuesphere.

Phylum	Class	E1	E2	R1	R2	R3	SSR1	SSR2	SSR3	B1	B2	B3	Re1	Re2	Re3
Other	Other	0,0000	0,0000	0,1388	0,2883	0,4698	0,0854	0,1708	0,3702	0,1317	0,1317	0,1424	0,0890	0,1424	0,1459
Acidobacteria	Acidobacteria-5	0,0000	0,0000	0,0107	0,0783	0,0925	0,0498	0,1424	0,1673	0,0214	0,0214	0,0142	0,0249	0,0463	0,0356
Acidobacteria	Acidobacteria-6	0,0036	0,8898	3,6269	4,4241	7,8623	7,3071	11,3895	9,6882	4,9900	4,5273	5,3815	6,0507	7,0829	5,0114
Acidobacteria	Acidobacteriia	0,0000	0,0000	0,0142	0,0071	0,0178	0,0392	0,0178	0,0320	0,0036	0,0071	0,0036	0,0285	0,0285	0,0285
Acidobacteria	Solibacteres	0,0000	0,0000	1,0962	0,6264	1,4878	1,3454	0,7866	1,8152	2,6303	2,1355	2,0216	1,2208	1,2564	1,5447
Acidobacteria	Sva0725	0,0000	0,0000	0,2598	0,3951	0,2669	0,2385	0,2100	1,5483	0,8791	0,9396	0,6691	0,2919	1,1318	0,3737
Acidobacteria	[Chloracidobacteria]	0,0000	0,0000	1,5874	4,7373	1,7262	3,1748	6,7091	1,6764	6,8871	7,4459	10,0157	2,9114	4,0504	3,3279
Acidobacteria	iii1-8	0,0000	0,0000	0,0783	0,1068	0,9468	0,2598	0,5659	1,3312	0,4378	0,3274	0,4663	0,2705	0,3595	0,3132
Actinobacteria	Acidimicrobiia	0,0320	0,6869	1,4415	1,4557	1,5874	1,1639	1,3383	1,9896	1,0962	1,1639	1,2884	1,2315	1,0251	0,9966
Actinobacteria	Actinobacteria	15,7496	15,9240	25,2954	15,5182	10,6243	17,4829	7,1861	7,6950	9,3181	12,1405	8,3749	15,8350	13,3079	18,9813
Actinobacteria	MB-A2-108	0,0000	0,0000	0,1566	0,8862	1,1283	0,2242	1,1496	1,7511	0,6585	0,5019	0,5979	0,0961	0,4449	0,2349
Actinobacteria	Nitriliruptoria	0,0000	0,0000	0,0498	0,1175	0,4022	0,0819	0,0819	0,8649	0,0854	0,0925	0,0819	0,0605	0,0783	0,0747
Actinobacteria	Rubrobacteria	0,0000	0,0000	4,0575	9,3572	7,2822	2,9328	8,3144	10,0085	7,0010	6,4280	5,8763	1,5767	4,6519	2,2494
Actinobacteria	Thermoleophilia	0,0071	0,0000	7,3534	6,3283	3,4809	5,1680	6,2785	4,1643	3,0681	3,6589	3,8297	3,5877	3,1819	2,5520
Armatimonadetes	Armatimonadia	0,0000	0,0000	0,0392	0,1175	0,0427	0,0747	0,0498	0,0498	0,3986	0,4129	0,3915	0,0819	0,1673	0,4271
Armatimonadetes	[Fimbriimonadia]	0,0000	0,0000	0,0000	0,0178	0,0178	0,0036	0,0107	0,0036	0,0142	0,0107	0,0285	0,0000	0,0036	0,0498
Bacteroidetes	At12OctB3	0,0000	0,0000	0,0071	0,0036	0,0071	0,0071	0,0142	0,0071	0,0320	0,0071	0,0712	0,0142	0,0036	0,0036
Bacteroidetes	Cytophagia	0,8115	0,9752	2,1498	2,2138	1,9860	2,7370	1,9967	1,3739	6,2856	4,9722	4,3601	3,5023	3,0218	3,3528
Bacteroidetes	Flavobacteriia	11,8522	1,3525	1,2635	1,6088	0,6442	0,6442	0,3880	0,6086	0,0214	0,0071	0,0463	1,9825	0,7724	0,5268

Phylum	Class	E1	E2	R1	R2	R3	SSR1	SSR2	SSR3	B1	B2	B3	Re1	Re2	Re3
Bacteroidetes	Sphingobacteriia	30,8478	0,1602	0,5196	0,5196	0,3915	0,7403	0,6122	0,6727	0,1602	0,1068	0,1851	1,6515	0,6905	0,8293
Bacteroidetes	[Rhodothermi]	0,0000	0,0000	0,0036	0,0000	0,7652	0,0000	0,0036	1,3667	0,0000	0,0036	0,0071	0,0000	0,0036	0,0249
Bacteroidetes	[Saprospirae]	0,4698	4,8405	1,6372	1,4379	2,9043	2,4310	1,3917	2,2565	2,2850	1,8223	2,1071	3,7265	2,7442	3,0787
Chlorobi	Unc. Chlorobi	0,0000	0,0000	0,0285	0,0178	0,0107	0,0036	0,0569	0,0071	0,0142	0,0214	0,0285	0,0569	0,0285	0,0356
Chlorobi	OPB56	0,0000	0,0000	0,0214	0,0392	0,1495	0,0534	0,0676	0,0178	0,0534	0,0392	0,0676	0,0712	0,0819	0,0498
Chloroflexi	Unc. Chloroflexi	0,0000	0,0000	0,0641	0,1424	0,1103	0,0819	0,0605	0,0997	0,0783	0,0747	0,0712	0,0142	0,0819	0,0854
Chloroflexi	Anaerolineae	0,0036	0,0000	0,1388	0,2064	0,2136	0,1068	0,1246	0,2563	0,1068	0,1459	0,1032	0,0890	0,1815	0,2242
Chloroflexi	C0119	0,0000	0,0000	0,0534	0,0676	0,0320	0,0142	0,0107	0,0071	0,0427	0,0392	0,0534	0,0107	0,0320	0,0214
Chloroflexi	Chloroflexi	0,0000	0,0000	0,5552	1,5483	0,4841	0,6229	0,6727	0,4912	1,6088	1,4166	1,3240	0,2349	0,7581	0,7190
Chloroflexi	Ellin6529	0,0000	0,0000	0,5481	0,8507	0,3310	0,4734	0,5979	0,3203	0,1673	0,2064	0,1958	0,2527	0,3559	0,3061
Chloroflexi	Gitt-GS-136	0,0000	0,0000	0,1388	0,1637	0,7937	0,1068	0,1495	0,6763	0,0142	0,0249	0,0249	0,0997	0,0641	0,0676
Chloroflexi	S085	0,0000	0,0000	0,3097	0,5196	0,3595	0,2171	0,3630	0,7261	0,1388	0,1210	0,1068	0,1566	0,1673	0,1317
Chloroflexi	SAR202	0,0000	0,0000	0,0000	0,0392	0,0569	0,0000	0,0641	0,0534	0,0036	0,0107	0,0000	0,0000	0,0071	0,0000
Chloroflexi	TK10	0,0000	0,0000	0,8008	1,5020	0,6371	0,5766	1,4949	0,8756	0,6051	0,6798	0,7546	0,4378	0,7332	0,4556
Chloroflexi	TK17	0,0000	0,0000	0,1602	0,1851	0,3524	0,1495	0,1530	0,5125	0,0285	0,0427	0,0356	0,1780	0,0605	0,0676
Chloroflexi	Thermomicrobia	0,0036	0,0036	2,5484	3,5841	2,6125	1,5554	1,9576	2,7691	1,5910	1,5874	1,7725	1,0678	1,8188	1,7511
Cyanobacteria	Unc. Cyanobacteria	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,3061	0,7154	0,5161	0,0000	0,0000	0,0000
Cyanobacteria	4C0d-2	0,0000	0,0000	0,0000	0,0107	0,0463	0,0214	0,1210	0,0605	0,0356	0,0107	0,0107	0,0036	0,0142	0,0000
Cyanobacteria	Chloroplast	0,1602	12,0907	0,0819	0,0427	0,2420	0,0819	0,0036	0,0071	0,3132	0,8257	0,2990	0,0107	0,0854	0,0107
Cyanobacteria	Nostocophycideae	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0498	0,4235	0,0392	0,0000	0,0000	0,0000
Cyanobacteria	Oscillatoriophycideae	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,4413	0,9823	0,5944	0,0000	0,0071	0,0000
Cyanobacteria	Synechococcophycideae	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,1139	0,3666	0,0997	0,0000	0,0000	0,0036

Phylum	Class	E1	E2	R1	R2	R3	SSR1	SSR2	SSR3	B1	B2	B3	Re1	Re2	Re3
Elusimicrobia	Elusimicrobia	0,0000	0,0000	0,0142	0,0036	0,0427	0,0178	0,0000	0,0356	0,0107	0,0071	0,0214	0,0107	0,0142	0,0249
FBP	Unc. FBP	0,0000	0,0000	0,0178	0,0747	0,0427	0,0071	0,0285	0,0605	0,3951	0,2278	0,2705	0,0320	0,0392	0,1281
Fibrobacteres	Fibrobacteria	0,0000	0,1353	0,0249	0,0214	0,0569	0,0320	0,0498	0,0747	0,0356	0,0569	0,0249	0,0463	0,0249	0,0000
Firmicutes	Bacilli	0,0000	0,2634	2,7157	1,0073	2,8723	1,1817	1,2315	2,3064	1,3703	0,9254	0,5303	0,3559	1,0322	0,4164
Gemmatimonadetes	Gemm-1	0,0000	0,0000	0,4058	0,7937	0,2705	0,5873	1,1425	0,1459	1,0856	0,8044	0,8079	0,3880	0,6371	0,6549
Gemmatimonadetes	Gemm-2	0,0000	0,0000	0,0605	0,1495	0,1317	0,0783	0,3274	0,3524	0,0071	0,0071	0,0107	0,0712	0,1281	0,0392
Gemmatimonadetes	Gemm-3	0,0000	0,0000	0,8222	1,8508	4,8690	1,0037	0,9859	4,5736	1,0784	0,8613	1,0464	1,6622	1,2671	1,0144
Gemmatimonadetes	Gemm-5	0,0000	0,0000	0,6122	1,2030	1,9967	0,9040	2,7264	2,7620	0,3310	0,2563	0,3559	0,9859	1,1140	0,6371
Gemmatimonadetes	Gemmatimonadetes	0,0000	0,0000	0,7617	2,1640	2,1640	1,3810	3,7799	2,1854	1,3561	1,3383	1,8401	1,2208	2,0501	1,3739
Nitrospirae	Nitrospira	0,0000	0,0000	0,2171	0,3808	0,4591	0,4164	1,2244	0,5873	0,7581	0,5766	0,5232	0,2883	0,5552	0,4912
OD1	Unc. OD1	0,0000	0,0000	0,0000	0,0000	0,0285	0,0000	0,0000	0,0498	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Planctomycetes	Phycisphaerae	0,0036	0,1210	1,9149	2,4559	3,9223	3,8546	4,0504	3,0253	7,5491	7,6025	10,5638	5,4278	4,5985	4,5629
Planctomycetes	Planctomycetia	0,0071	0,6335	2,7335	2,1249	3,3919	4,1714	2,8260	4,1038	2,9470	3,6553	3,2247	3,9329	3,7407	3,6945
Proteobacteria	Alphaproteobacteria	5,5488	13,9415	21,7896	16,5860	15,4221	19,7110	11,0585	12,6993	21,3304	21,8003	19,6078	20,1666	20,0420	24,5302
Proteobacteria	Betaproteobacteria	2,5982	26,4664	3,0609	4,0824	4,4206	4,5487	6,9334	4,3672	2,1818	1,8472	1,9505	4,4312	4,9188	4,1429
Proteobacteria	Deltaproteobacteria	0,0427	2,8011	3,0431	3,7550	3,0681	4,4348	4,1856	2,2957	1,8935	1,7298	1,5589	4,9046	4,3565	4,3423
Proteobacteria	Gammaproteobacteria	31,4885	17,9207	4,0006	2,1320	2,9470	4,8156	2,2921	1,6301	1,0642	0,9290	0,8756	5,3780	3,9614	3,9970
TM7	Unc. TM7	0,0000	0,0000	0,0107	0,0000	0,4235	0,0107	0,0000	0,0178	0,0000	0,0071	0,0000	0,0000	0,0000	0,0000
TM7	SC3	0,0000	0,0000	0,0000	0,0000	0,0997	0,0000	0,0000	0,1495	0,0036	0,0036	0,0036	0,0000	0,0000	0,0036
TM7	TM7-1	0,0000	0,0000	0,1246	0,0641	0,1851	0,1246	0,1530	0,1103	0,2598	0,0178	0,0819	0,0925	0,1246	0,0356
TM7	TM7-3	0,0036	0,0000	0,1851	0,4841	0,1103	0,1459	0,2100	0,0569	0,0000	0,0071	0,0178	0,1424	0,1175	0,0890
Tenericutes	Mollicutes	0,0000	0,0000	0,0036	0,0071	0,0000	0,0071	0,0036	0,0000	0,0676	0,0285	0,0498	0,0000	0,0285	0,0000

Phylum	Class	E1	E2	R1	R2	R3	SSR1	SSR2	SSR3	B1	B2	B3	Re1	Re2	Re3
Verrucomicrobia	Opitutae	0,0214	0,2527	0,3203	0,3524	0,5517	0,4734	0,4520	0,4556	0,2883	0,2776	0,4129	0,7368	0,5730	0,3381
Verrucomicrobia	Verrucomicrobiae	0,3346	0,2954	0,3097	0,2207	0,7047	0,2883	0,1708	0,2100	0,0427	0,0285	0,0285	0,6549	0,3168	0,2207
Verrucomicrobia	[Pedosphaerae]	0,0000	0,0000	0,2812	0,6229	0,6763	0,6407	0,8827	0,8471	0,5446	0,3630	0,5552	0,6656	0,6620	0,4129
Verrucomicrobia	[Spartobacteria]	0,0107	0,2456	0,3132	0,2812	0,3844	0,8862	0,5374	0,3097	3,2816	2,0430	3,5806	1,4771	1,0037	0,7332
WS2	SHA-109	0,0000	0,0000	0,0214	0,0178	0,0142	0,0214	0,0427	0,0249	0,0000	0,0000	0,0000	0,0107	0,0214	0,0214
[Thermi]	Deinococci	0,0000	0,0000	0,0000	0,0000	0,1780	0,0000	0,0000	0,2420	0,0000	0,0000	0,0000	0,0000	0,0000	0,0036
		100	100	100	100	100	100	100	100	100	100	100	100	100	100

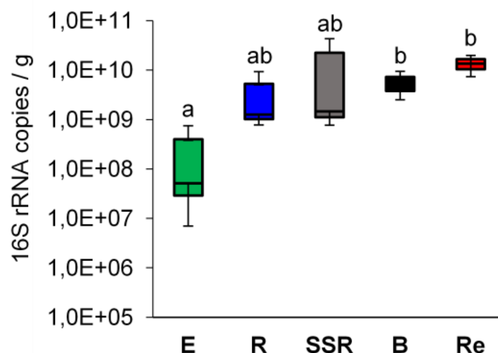


Figure 3. Evaluation of bacterial population abundance in the different plant, soil and residuesphere fractions through qPCR. The graph indicates the number of amplified 16S rRNA gene copies per gram of root tissue (E), soil (R, SSR, B) or residuesphere (Re).

Table 3. Comparison of the bacterial population abundance in the different plant and soil fractions evaluated as 16S rRNA copies number by qPCR. (A) Main-test and **(B)** pairwise comparisons were performed according to the fraction types (E= endosphere; R= rhizosphere; SSR= root-surrounding soil; B= bulk soil; Re= residuesphere). Asterisk (*) indicates significant differences between samples.

(A)

	Df	Sum_Sq	Mean_Sq	F_value	Pr(>F)
fraction	4	92.354	230.885	48.466	0.01962*
Residuals	10	47.639	0.47639		

(B)

fraction	diff	lwr	upr	**p**
E-B	-18831730	-37378665	-0.02847953	**0.0462268***
R-B	-0.3737773	-22284708	148091617	0.9600296
Re-B	0.4039798	-14507137	225867334	0.9478002
SSR-B	-0.1323617	-19870552	172233184	0.9992018
R-E	15093957	-0.3452978	336408920	0.1278523
Re-E	22871529	0.4324594	414184637	**0.0152834***
SSR-E	17508114	-0.1038821	360550487	0.0665269
Re-R	0.7777572	-10769363	263245067	0.6523767
SSR-R	0.2414157	-16132778	209610917	0.9918603
SSR-Re	-0.5363415	-23910350	131835199	0.8701704

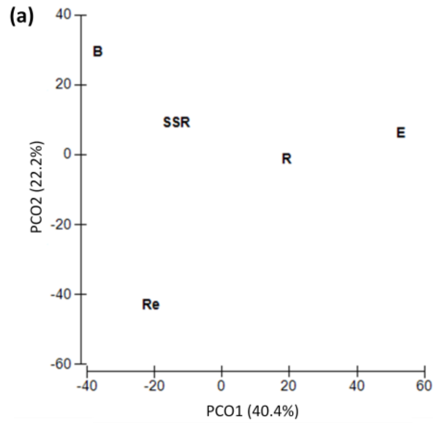
Distribution of phylogenetic groups and plant growth promotion potential within the bacterial collection established from the Argania spinosa root system, bulk soil and residuesphere.

There are many evidences that plant associated bacteria are able to sustain plant growth under adverse environmental conditions (Riva et al. 2019; Soussi et al. 2016), however no report exists in the literature on the identity and the PGP potential of the argan tree associated bacteria. We established a collection of 371 bacterial isolates from the root system (E-R-SSR fractions) of *A. spinosa*, the bulk soil and the residuesphere composed by soil and *A. spinosa* leafy substrate. The sequencing of the 16S rRNA gene of the isolates allowed to i) phylogenetically identify the bacteria (Supplementary Table 1) and ii) cluster the cultivable bacteria in OTU_{97} (according to the % of identity of the 16S rRNA gene sequence) and describe their shift along a gradient of relationship with the plant. The PCoA performed on the cultivable microbiota OTU_{97} showed that the samples are distributed according to the level of association to the *A. spinosa* root system, with the bacteria isolated from the residuesphere that are more diverse from those isolated from the other niches, including both the soil fractions and endosphere (Figure 4A). A similar trend was observed by investigating the cultivable bacterial community of plants grown under drought conditions (Marasco et al. 2012) although residuesphere was not included in the analysis. The 371 bacterial isolates were uniformly distributed among the five microhabitats of origin (number of isolates per fraction, Figure 4B). We observed for the cultivable microbiota a trend of the alpha-diversity indices similar to that calculated on the 16S rRNA Illumina dataset. For example, the richness (number of OTU_{97}) and the Shannon index were lower in the endophytic bacteria sub-collection compared to the soil and residuesphere ones (Figure 4B), in agreement to what detected for the overall bacterial community composition. These data are corroborated by the diversity indices of a bacterial collection established from pepper

plants growing under desert farming conditions (Marasco et al. 2012). Similarly, the residuesphere sub-collection had a higher number of OTU_{97} and Shannon index (Figure 4B), in coherence with the data obtained on the 16S rRNA Illumina dataset in particular for the latter ecological index.

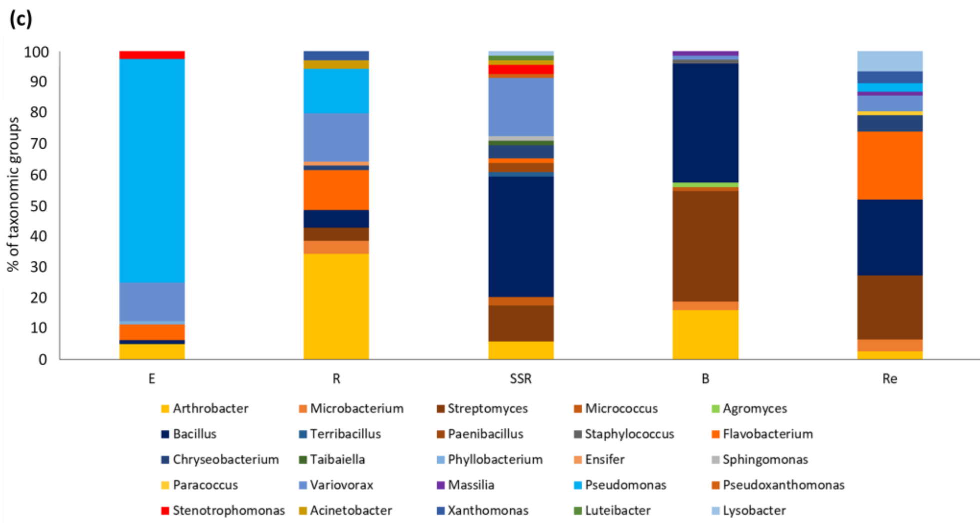
The presence and the distribution of the bacterial genera in the collection established from the root (E), soil (R, SSR, B) and residuesphere (Re) fractions reflected the same pattern obtained by the alpha-diversity indices calculated by the OTU_{97} clustering. The taxonomic composition of the cultivable communities showed that R, SSR and B soil fractions were overall more similar compared to the E and Re sub-collections and that the endosphere hosted a clearly different composition of cultivable bacteria compared to the other environmental niches (Table 4, Figure 4C). Moreover, the residuesphere hosted the more complex bacterial community in terms of bacterial genera (Figure 4C). The richness of OTU_{97} detected both in the total and cultivable fraction of the bacterial microbiota associated to the argan residuesphere, besides the higher taxonomic diversity of the sub-collection, suggests that this niche could potentially harbor novel microbial resources of interest for a tailored management of arid lands in the frame of sustainable agriculture.

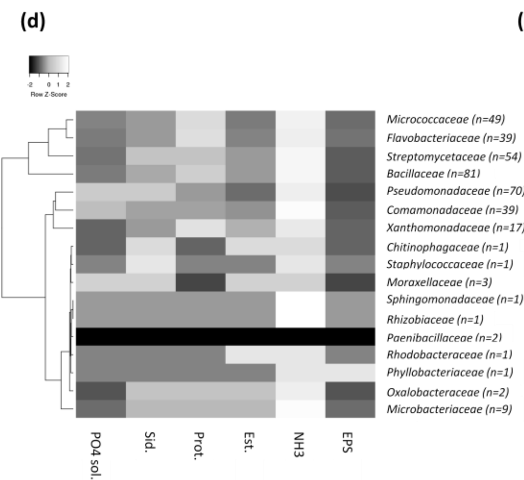
As shown in Figure 4C and in Table 4, the bacteria isolated from the endosphere mainly belonged to the genus *Pseudomonas*, making the Class Gammaproteobacteria the most abundant within the cultivable endophytes of *A. spinosa*, in accordance to the taxonomic classification of endophytes selected by other tree plants, i.e. date palms, in desert-farming agro-ecosystems (Cherif et al. 2015). Moreover, this is in agreement with the high percentage of OTU_{97} identified through 16S rRNA Illumina sequencing in the *A. spinosa* microbiota and belonging to this class. On the contrary, the Alphaproteobacteria class was abundantly retrieved in the 16S rRNA Illumina libraries generated from soils fraction and residuesphere, its representatives were scarcely recorded in the bacteria collection.



(b)

Fraction	OTU_{97}	Isolates (n)	Dominance	Shannon
E	10	80	0,5084	1,1630
R	24	70	0,0752	2,8430
SSR	27	69	0,0720	2,9390
B	20	75	0,1122	2,4980
Re	35	77	0,0467	3,2980





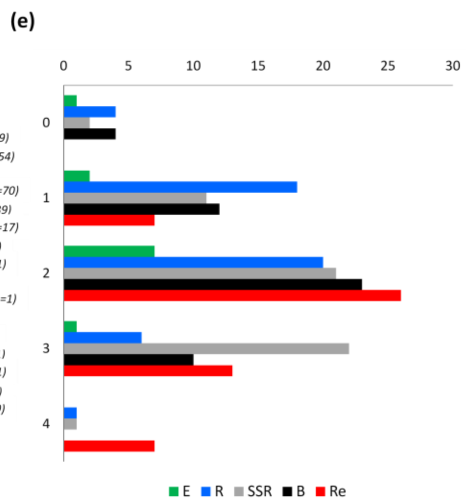


Figure 4. Cultivable bacteria isolated from *Argania spinosa* root system, bulk soil and residuesphere. (a) Principal coordinates analysis (PCoA) of the cultivable bacterial communities inhabiting the *A. spinosa* endosphere (E), rhizosphere (R), soil surrounding root (SSR), bulk soil (B) and residuesphere (Re) fractions. PCoA was calculated from the OTU_{97} table obtained from the 16S rRNA gene sequences of the isolates. The analysis has been performed on the entire bacterial collection (n= 371). **(b)** α-diversity indices calculated on the cultivable bacterial communities. **(c)** Relative abundance of bacterial genera in the cultivable communities isolated from the different fractions. **(d-e)** Number of plant growth promotion (PGP) traits expressed by the strains isolated from different fractions.

Indeed only 4 isolates were classified as Alphaproteobacteria, and they were divided in 4 genera (i.e. *Phyllobacterium*, *Ensifer*, *Sphingomonas*, *Paracoccus*) each peculiar of a different niche (Table 4). Likewise, the Bacteroidetes phylum, abundantly detected in the total bacterial community of the endosphere, were scarcely represented (n=4) in the correspondent sub-collection of isolates while it became more relevant in the residuesphere collection that included isolates of the *Flavobacterium* genus (n=17) (Table 4). Actinobacteria were mainly isolated from the *A. spinosa* rhizosphere (n=30) and bulk soil (n=43), where the most abundant genera were *Arthrobacter* and *Streptomyces* respectively (Table 4). Some of the genera detected in the established bacteria collection (e.g. *Arthrobacter, Microbacterium*) include species that are well adapted to the extreme conditions of dry soil where argan trees thrive and can act as plant growth promoters (Manzanera et al. 2015; SantaCruz-Calvo et al. 2013). The established bacteria collection (n=371) was dereplicated by means of ITS-PCR fingerprinting to reduce genotype redundancy, classifying the isolates in 219 ITS groups. One strain from each ITS groups (n=219) was tested *in vitro* for a set of direct and indirect activities related to Plant Growth Promotion (PGP). The results of these assays (reported in a detail in Supplementary Table 2) showed that the higher numbers of positive isolates were observed in the *Bacillaceae*, *Flavobacteriaceae*, *Micrococcaceae*, *Streptomycetaceae* and *Pseudomonadaceae* families (Figure 4D, Supplementary Table 2).

The bacteria affiliated to these taxa were widely reported as plant growth promoting bacteria selected in the endosphere and/or rhizosphere by a range of different plant species under extreme conditions such as soil salinity and water scarcity (Marasco et al. 2016; Soldan et al. 2019). In the present study we investigated the PGP potential of the isolates limiting the

Table 4. Phylogenetic affiliation of the cultivable bacteria associated to the *Argania spinosa* root system (E, R, SSR fractions), bulk soil (B) and residuesphere (Re). The table indicates the number of isolates classified at the Phylum/Class and at the Genus levels.

Phylum/Class	E	R	SSR	B	Re	Genus	E	R	SSR	B	Re
Actinobacteria	4	30	14	43	21	*Arthrobacter*	4	24	4	12	2
						Microbacterium	0	3	0	2	3
						Streptomyces	0	3	8	27	16
						Micrococcus	0	0	2	1	0
						Agromyces	0	0	0	1	0
Bacilli	1	4	30	30	19	*Bacillus*	1	4	27	29	19
						Terribacillus	0	0	1	0	0
						Paenibacillus	0	0	2	0	0
						Staphylococcus	0	0	0	1	0
Bacteroidetes	4	10	5	0	21	*Flavobacterium*	4	9	1	0	17
						Chryseobacterium	0	1	3	0	4
						Taibaiella	0	0	1	0	0
Alphaproteobacteria	1	1	1	0	1	*Phyllobacterium*	1	0	0	0	0
						Ensifer	0	1	0	0	0
						Sphingomonas	0	0	1	0	0
						Paracoccus	0	0	0	0	1
Betaproteobacteria	10	11	13	2	5	*Variovorax*	10	11	13	1	4
						Massilia	0	0	0	1	1
Gammaproteobacteria	60	14	6	0	10	*Pseudomonas*	58	10	0	0	2
						Pseudoxanthomonas	0	0	1	0	0
						Stenotrophomonas	2	0	2	0	0
						Acinetobacter	0	2	1	0	0
						Xanthomonas	0	2	0	0	3
						Luteibacter	0	0	1	0	0
						Lysobacter	0	0	1	0	5
Total n° of isolates	80	70	69	75	77		80	70	69	75	77

screening to the biofertilization capacity (i.e. phosphate solubilization activity and the production of siderophores and ammonia), the occurrence of traits related to biocontrol activities and also important for organic matter degradation in the residuesphere (i.e. protease and esterase activities) and the production of exopolysaccharides that can ameliorate the water holding capacity of soil and promote the stability of soil aggregates (Mapelli et al. 2012). Extremophilic EPS-producing bacteria isolated from desert and saline systems were able to produce biosurfactants/bioemulsifiers that under controlled laboratory conditions proved to increase water retention of a sandy soil (Raddadi et al. 2018), an aspect of great interest to reverse desertification. However, in this study EPS production was detected only in one strain, *Phyllobacterium ifriqiyense* A-8E16 (Supplementary Table 2), an endophyte previously isolated from the root nodule of two leguminous plant species in Southern Tunisia (Mantelin et al. 2016). Ammonia production was a common trait in the bacteria collection, with 199 positive isolates over the tested 219, in accordance with previous reports of bacteria associated to the halophyte *Salicornia* (Mapelli et al. 2013). A high number of positive isolates were detected also for siderophore production (n=61) while those showing phosphate solubilization capacity (n=27) were less abundant. Protease and esterase activities were traits widely spread in the bacteria collection (n=109 and n=39, respectively; Supplementary Table 2). These are cell wall degrading enzymes with a potential role in biocontrol (a PGP indirect mechanism) but they are also involved in the degradation of the organic matter and are in fact abundant among the strains isolated from the residuesphere, the niche where plant cell material is primarily degraded. Likewise, Egamberdieva and coauthors (2016) found a higher abundance of cell wall degrading enzymes among bacteria isolated from hydrochar-supplemented soil compared to the control one. Interestingly, in their work the authors could detect a significant increase in soybean growth and higher diversity of soybean rhizospheric bacteria exclusively in the soil subjected to the hydrochar addition, suggesting a combined plant growth promotion

effect due to nutrient supply and the stimulation of a more effective rhizospheric community (Egamberdieva et al. 2016).
The results of the PGP assays were used to calculate for each isolate a 'PGP score', reporting the total number of positive activities. Isolates that did not harbor PGP traits were detected in all the fractions except the residuesphere sub-collection (Figure 4E), while none of the tested bacteria displayed the complete set of PGP activities (Supplementary Table 2). Most of the isolates showed a 'PGP score' comprised between 1 and 3 (Figure 4E) and they were present in all the sub-collections obtained by the root, soils and residuesphere. Noteworthy, most of the bacterial strains showing a 'PGP score' of 4 were isolated from the residuesphere fraction (n=7) and were not present within endosphere and bulk soil sub-collections. We did not detect a clear fractioning of the PGP potential associated to the cultivable bacteria isolated from *A. spinosa* in a specific ecological niche, however the fact that the multivalent isolates with the higher PGP score were mostly identified in the residuesphere led us to hypothesize that these bacteria play a central role in the argan litter effect (Nouaim and Chaussod, 1994b), as recently suggested also for the addition of biochar (Egamberdieva et al. 2016). Previous evidences are in agreement with our hypothesis and showed that the residuesphere is enriched in ammonia oxidizing bacteria (AOB) able to increase the bioavailability of nitrogen for the plant, a critical aspect in arid environments (Marcos et al. 2016). We propose that the high PGP potential observed among the residuesphere bacterial isolates act synergically with the nutrient addition exerted when litter is supplemented to the soil, although specific studies are required to validate our hypothesis and possibly provide a novel scientific interpretation behind the traditional use of argan litter by Moroccan farmers as soil amendment for sustainable agriculture.

Conclusions

The xerophite plant microbiome is a still overlooked source of microbial resources potentially useful for environmental and agriculture biotechnologies application. Currently, no reports are available concerning the bacterial communities associated to the tree *Argania spinosa* although UNESCO has defined their forest in South Morocco as a biosphere reserve (Louati et al. 2019). The 16S rRNA Illumina sequencing dataset showed that the root system portions of *A. spinosa* hosted different bacterial communities according to their degree of association with the plants. Trends of beta-diversity could be observed both considering the entire bacterial communities described by 16S rRNA Illumina sequencing and the cultivable fraction obtained from the endosphere (E), the root-associated soils (R-SSR), the bulk soil (B) and the residuesphere (Re). The latter environmental niche is indirectly subjected to the plant influence, being partially composed by its leafy litter, and it was colonized by cultivable bacteria community clearly distinguished from the other analyzed samples. Our data showed that the root system of *A. spinosa* and the residuesphere developed from its litter are associated to a high number of bacteria endowed with direct and indirect plant growth promotion activities. In particular, we report here that according to our *in vitro* screening, the residuesphere of *A. spinosa* is colonized by several bacterial strains with a high PGP potential that, in concert with its root system, could contribute to the litter effect previously described for this tree species (Nouaim and Chaussod, 1994b), possibly providing a novel scientific interpretation behind the traditional use of argan litter by Moroccan farmers as soil amendment. Residuesphere bacterial isolates could become, moreover, candidates for future studies aimed at setup inoculants for sustainable agriculture in arid lands.

References

Anderson, M., Gorley, R.N. and Clarke, R.K., 2008. *Permanova+ for primer: Guide to software and statisticl methods*. Primer-E Limited.

Angst, Š., Baldrian, P., Harantová, L., Cajthaml, T. and Frouz, J., 2018. Different twig litter (Salix caprea) diameter does affect microbial community activity and composition but not decay rate. *FEMS microbiology ecology*, *94*(9), p.fiy126.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J.R., Maciejewski, A. and Wishart, D.S., 2016. Heatmapper: web-enabled heat mapping for all. *Nucleic acids research*, *44*(W1), pp.W147-W153.

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

Berg, G. and 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), pp.1-13.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E.V.L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E. and Peplies, J., 2012. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature*, *488*(7409), p.91.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, *7*(5), p.335.

Charrouf, Z., Hilali, M., Jauregui, O., Soufiaoui, M. and Guillaume, D., 2007. Separation and characterization of phenolic compounds in argan fruit pulp using liquid chromatography–negative electrospray ionization tandem mass spectroscopy. *Food Chemistry*, *100*(4), pp.1398-1401.

Cherif, H., Marasco, R., Rolli, E., Ferjani, R., Fusi, M., Soussi, A., Mapelli, F., Blilou, I., Borin, S., Boudabous, A. and Cherif, A., 2015. Oasis desert farming selects environment-specific date palm root endophytic communities and cultivable bacteria that promote resistance to drought. *Environmental microbiology reports*, *7*(4), pp.668-678.

Cherni, M., Ferjani, R., Mapelli, F., Boudabous, A., Borin, S. and Ouzari, H.I., 2019. Soil parameters drive the diversity of Citrus sinensis rhizosphere microbiota which exhibits a potential in plant drought stress alleviation. *Applied soil ecology*, *135*, pp.182-193.

Christel, W., Zhu, K., Hoefer, C., Kreuzeder, A., Santner, J., Bruun, S., Magid, J. and Jensen, L.S., 2016. Spatiotemporal dynamics of phosphorus release, oxygen consumption and greenhouse gas emissions after localised soil amendment with organic fertilisers. *Science of the Total Environment*, *554*, pp.119-129

Daffonchio, D., Cherif, A. and Borin, S., 2000. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the “Bacillus cereus group”. *Appl. Environ. Microbiol.*, *66*(12), pp.5460-5468.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), pp.2460-2461.

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N.K., Bhatnagar, S., Eisen, J.A. and Sundaresan, V., 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences*, *112*(8), pp.E911-E920.

Egamberdieva, D., Kucharova, Z., Davranov, K., Berg, G., Makarova, N., Azarova, T., Chebotar, V., Tikhonovich, I., Kamilova, F., Validov, S.Z. and Lugtenberg, B., 2011. Bacteria able to control foot and root rot and to promote growth of cucumber in salinated soils. *Biology and fertility of soils*, *47*(2), pp.197-20.

Egamberdieva, D., Wirth, S., Behrendt, U., Abd_Allah, E.F. and Berg, G., 2016. Biochar treatment resulted in a combined effect on soybean growth promotion and a shift in plant growth promoting rhizobacteria. Frontiers in microbiology, 7, p.209.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., Rizzi, A., Urso, R., Brusetti, L., Borin, S., Mora, D., Scuppa, P., Pasqualini, L., Clementi, E., Genchi, M., Corona, S., Negri, I., Grandi, G., Alma, A., Kramer, L., Esposito, F., Bandi, C., Sacchi, L., Daffonchio, D., 2007. Bacteria of the genus Asaia stably associate with Anopheles stephensi, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences, 104(21)*, pp. 9047-9051.

Fierer, N., Jackson, J.A., Vilgalys, R. and Jackson, R.B., 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.*, *71*(7), pp.4117-4120.

Frascari, D., Zanaroli, G., Motaleb, M.A., Annen, G., Belguith, K., Borin, S., Choukr-Allah, R., Gibert, C., Jaouani, A., Kalogerakis, N. and Karajeh, F., 2018. Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt, Morocco, and Tunisia. *Integrated environmental assessment and management*, *14*(4), pp.447-462.

Fu, X., Guo, D., Wang, H., Dai, X., Li, M. and Chen, F., 2017. Differentiating between root- and leaf-litter controls on the structure and stability of soil micro-food webs. *Soil Biology and Biochemistry*, *113*, pp.192-200.

Hammer, Ø., Webb, K.E. and Depreiter, D., 2009. Numerical simulation of upwelling currents in pockmarks, and data from the Inner Oslofjord, Norway. *Geo-Marine Letters*, *29*(4), pp.269-275.

Hardoim, P.R., van Overbeek, L.S. and van Elsas, J.D., 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends in microbiology*, *16*(10), pp.463-471.

Jha, B., Gontia, I. and Hartmann, A., 2012. The roots of the halophyte Salicornia brachiata are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. *Plant and Soil*, *356*(1-2), pp.265-277.

Khallouki, F., Haubner, R., Ricarte, I., Erben, G., Klika, K., Ulrich, C.M. and Owen, R.W., 2015. Identification of polyphenolic compounds in the flesh of Argan (Morocco) fruits. *Food chemistry*, *179*, pp.191-198.

Kumar, M., Brader, G., Sessitsch, A., Mäki, A., van Elsas, J.D. and Nissinen, R., 2017. Plants assemble species specific bacterial communities from common core taxa in three arcto-alpine climate zones. *Frontiers in microbiology*, *8*, p.12.

Kumar, M., Männistö, M.K., van Elsas, J.D. and Nissinen, R.M., 2016. Plants impact structure and function of bacterial communities in Arctic soils. *Plant and soil*, *399*(1-2), pp.319-332.

Kumar, S., Karan, R., Kapoor, S., Singh, S.P. and Khare, S.K., 2012. Screening and isolation of halophilic bacteria producing industrially important enzymes. *Brazilian Journal of Microbiology*, *43*(4), pp.1595-1603.

Louati, M., Khouja, A., Abdelkrim, A.B., Hannachi, A.S. and Baraket, G., 2019. Adaptation of Argania spinosa L. in Northern Tunisia: Soil analysis and morphological traits variability. *Scientia Horticulturae*, *255*, pp.220-230.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Del Rio, T.G. and Edgar, R.C., 2012. Defining the core Arabidopsis thaliana root microbiome. *Nature*, *488*(7409), p.86.

Magid, J., De Neergaard, A. and Brandt, M., 2006. Heterogeneous distribution may substantially decrease initial decomposition, long-term microbial growth and N-immobilization from high C-to-N ratio resources. *European Journal of Soil Science*, *57*(4), pp.517-529.

Mantelin, S., Fischer-Le Saux, M., Zakhia, F., Béna, G., Bonneau, S., Jeder, H., de Lajudie, P., Cleyet-Marel J-C. 2006. Emended description of the genus Phyllobacterium and description of four novel species associated with plant roots: Phyllobacterium

bourgognense sp. nov., Phyllobacterium ifriqiyense sp. nov., Phyllobacterium leguminum sp. nov. and Phyllobacterium brassicacearum sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 56*, pp. 827–839

Manzanera, M., García-Fontana, C., Vílchez, J.I., Narváez-Reinaldo, J.J., González-López, J., 2015. Genome Sequence of Microbacterium sp. Strain 3J1, a Highly Desiccation-Tolerant Bacterium That Promotes Plant Growth. *Genome Announcements 3(4*), e00713-15.

Mapelli, F., Marasco, R., Balloi, A., Rolli, E., Cappitelli, F., Daffonchio, D. and Borin, S., 2012. Mineral–microbe interactions: biotechnological potential of bioweathering. *Journal of Biotechnology*, *157*(4), pp.473-481.

Mapelli, F., Marasco, R., Fusi, M., Scaglia, B., Tsiamis, G., Rolli, E., Fodelianakis, S., Bourtzis, K., Ventura, S., Tambone, F. and Adani, F., 2018. The stage of soil development modulates rhizosphere effect along a High Arctic desert chronosequence. *The ISME journal*, *12*(5), p.1188.

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D. and Borin, S., 2013. Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *BioMed research international*, *2013*.

Marasco, R., Mapelli, F., Rolli, E., Mosqueira, M.J., Fusi, M., Bariselli, P., Reddy, M., Cherif, A., Tsiamis, G., Borin, S. and Daffonchio, D., 2016. Salicornia strobilacea (synonym of Halocnemum strobilaceum) grown under different tidal regimes selects rhizosphere bacteria capable of promoting plant growth. *Frontiers in microbiology*, *7*, p.1286.

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., Abou-Hadid, A.F., El-Behairy, U.A., Sorlini, C., Cherif, A. and Zocchi, G., 2012. A drought resistance-promoting microbiome is selected by root system under desert farming. *PloS one*, *7*(10), p.e48479.

Marasco, R., Rolli, E., Fusi, M., Michoud, G. and Daffonchio, D., 2018 (a). Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality. *Microbiome*, *6*(1), p.3.

Marasco, R., Mosqueira, M.J., Fusi, M., Ramond, J.B., Merlino, G., Booth, J.M., Maggs-Kölling, G., Cowan, D.A. and Daffonchio, D., 2018 (b). Rhizosheath microbial community assembly of sympatric desert speargrasses is independent of the plant host. *Microbiome*, *6*(1), p.215.

Mina, D., Pereira, J.A., Lino-Neto, T., Baptista, P. 2019. Exploring the Phyllosphere Bacterial Community for Improving Tree Crop Protection. In Microbiome in Plant Health and Disease. Challenges and Opportunities. pp. 35-52. *Springer Nature Singapore. ISBN* 978-981-13-8495-0

Miniaci, C., Bunge, M., Duc, L., Edwards, I., Bürgmann, H. and Zeyer, J., 2007. Effects of pioneering plants on microbial structures and functions in a glacier forefield. *Biology and Fertility of Soils*, *44*(2), pp.289-297.

Nouaim, R. and Chaussod, R., 1994 (a). Mycorrhizal dependency of micropropagated argan tree (Argania spinosa): I. Growth and biomass production. *Agroforestry systems*, *27*(1), pp.53-65.

Nouaim, R., Lineres, M., Esvan, J.M. and Chaussod, R., 1994 (b). Mycorrhizal dependency of micropropagated argan tree (Argania spinosa): II. Mineral nutrition. *Agroforestry systems*, *27*(1), pp.67-77.

Pampurova, S. and Van Dijck, P., 2014. The desiccation tolerant secrets of Selaginella lepidophylla: What we have learned so far?. *Plant Physiology and Biochemistry*, *80*, pp.285-290.

Pampurova, S., Verschooten, K., Avonce, N. and Van Dijck, P., 2014. Functional screening of a cDNA library from the desiccation-tolerant plant Selaginella lepidophylla in yeast mutants identifies trehalose biosynthesis genes of plant and microbial origin. *Journal of plant research*, *127*(6), pp.803-813.

Price, M.N., Dehal, P.S. and Arkin, A.P., 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS one*, *5*(3), p.e9490.

Puente, M.E., Li, C.Y. and Bashan, Y., 2009. Endophytic bacteria in cacti seeds can improve the development of cactus seedlings. *Environmental and Experimental Botany*, *66*(3), pp.402-408.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(D1), pp.D590-D596.

R Core Team, 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Raddadi, N., Giacomucci, L., Marasco, R., Daffonchio, D., Cherif, A., Fava, F., 2018. Bacterial polyextremotolerant bioemulsifiers from arid soils improve water retention capacity and humidity uptake in sandy soil. *Microbial Cell Factories*, pp. 17:83.

Reis, F., Soares-Castro, P., Costa, D., Tavares, R.M., Baptista, P., Santos, P.M., Lino-Neto, T. 2019. Climatic impacts on the bacterial community profiles of cork oak soils. *Applied Soil Ecology 143*, pp. 89–97.

Riva, V., Terzaghi, E., Vergani, L., Mapelli, F., Zanardini, E., Morosini, C., Raspa, G., Di Guardo, A. and Borin, S., 2019. Exploitation of Rhizosphere Microbiome Services. In *Methods in Rhizosphere Biology Research* (pp. 105-132). Springer, Singapore.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*, p.e2584.

Rolli, E., Marasco, R., Saderi, S., Corretto, E., Mapelli, F., Cherif, A., Borin, S., Valenti, L., Sorlini, C. and Daffonchio, D., 2017. Root-associated bacteria promote grapevine growth: from the laboratory to the field. *Plant and Soil*, *410*(1-2), pp.369-382.

Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M.L., Gandolfi, C., Casati, E., Previtali, F., Gerbino, R. and Pierotti Cei, F., 2015. Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. *Environmental microbiology*, *17*(2), pp.316-331.

Ruas, M.P., Ros, J., Terral, J.F., Ivorra, S., Andrianarinosy, H., Ettahiri, A.S., Fili, A. and Van Staëvel, J.P., 2016. History and archaeology of the emblematic argan tree in the medieval Anti-Atlas Mountains (Morocco). *Quaternary international*, *404*, pp.114-136.

SantaCruz-Calvo, L., González-López, J., Manzanera, M., 2013. Arthrobacter siccitolerans sp. nov., a highly desiccation-tolerant, xeroprotectant-producing strain isolated from dry soil. International Journal of Systematic and Evolutionary Microbiology, 63, pp. 4174–4180.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J. and Sahl, J.W., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, *75*(23), pp.7537-7541.

Sgroy, V., Cassán, F., Masciarelli, O., Del Papa, M.F., Lagares, A. and Luna, V., 2009. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte Prosopis strombulifera. *ApplIed microbiology and Biotechnology*, *85*(2), pp.371-381.

Soldan, R., Mapelli, F., Crotti, E., Schnell, S., Daffonchio, D., Marasco, R., Fusi, M., Borin, S. and Cardinale, M., 2019. Bacterial endophytes of mangrove propagules elicit early establishment of the natural host and promote growth of cereal crops under salt stress. *Microbiological research*, *223*, pp.33-43.

Soussi, A., Ferjani, R., Marasco, R., Guesmi, A., Cherif, H., Rolli, E., Mapelli, F., Ouzari, H.I., Daffonchio, D. and Cherif, A., 2016. Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. *Plant and Soil*, *405*(1-2), pp.357-370.

Terzaghi, E., Vergani, L., Mapelli, F., Borin, S., Raspa, G., Zanardini, E., Morosini, C., Anelli, S., Nastasio, P., Sale, V.M. and Armiraglio, S., 2019. Rhizoremediation of weathered PCBs in a heavily contaminated agricultural soil: Results of a biostimulation trial in semi field conditions. *Science of the Total Environment*, *686*, pp.484-496.

Theis, K.R., Dheilly, N.M., Klassen, J.L., Brucker, R.M., Baines, J.F., Bosch, T.C., Cryan, J.F., Gilbert, S.F., Goodnight, C.J., Lloyd, E.A. and Sapp, J., 2016. Getting the hologenome concept right: an eco-evolutionary framework for hosts and their microbiomes. *Msystems*, *1*(2), pp.e00028-16.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research*, *25*(24), pp.4876-4882.

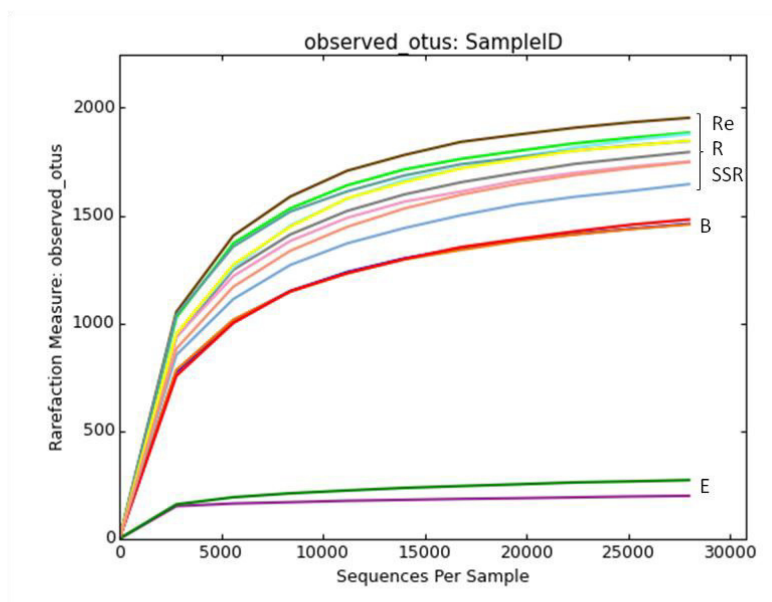
Tiwari, S., Singh, P., Tiwari, R., Meena, K.K., Yandigeri, M., Singh, D.P. and Arora, D.K., 2011. Salt-tolerant rhizobacteria-mediated induced tolerance in wheat (Triticum aestivum) and chemical diversity in rhizosphere enhance plant growth. *Biology and Fertility of soils*, *47*(8), p.907.

Vacheron, J., Desbrosses, G., Bouffaud, M.L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Legendre, L., Wisniewski-Dyé, F. and Prigent-Combaret, C., 2013. Plant growth-promoting rhizobacteria and root system functioning. *Frontiers in plant science*, *4*, p.356.

Vilchez, S., Manzanera, M., 2011. Biotechnological uses of desiccation-tolerant microorganisms for the rhizoremediation of soils subjected to seasonal drought. *Applied Microbiology and Biotechnology, 91*, pp.1297–1304.

Supplementary figure

Figure S1. Rarefaction curve of the 16S rRNA Illumina libraries. Rarefaction curves were calculated for each sample. E: endosphere; R: rhizosphere; SSR: root surrounding soil; B: bulk soil; Re: residuesphere.



Supplementary table

Supplementary Table 1. Phylogenetic identification of the bacterial isolates. List of the bacterial strains isolated from each fraction, identified by 16S rRNA gene sequencing (one representative strain for each ITS group has been sequenced) and blast search in the NCBI public database. The column 'n° isolates/ITS' reports the number of isolates corresponding to each representative of the ITS groups.

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
E	A-8E29	1	*Arthrobacter polychromogenes*	KR085878	99	886/892
E	A-8E62	3	*Arthrobacter oryzae*	KR233762	99	939/950
E	A-8E73	1	*Bacillus simplex*	CP017704	100	999/999
E	A-8E26	4	*Flavobacterium piscis*	NR_133746	98	883/900
E	A-8E16	1	*Phyllobacterium ifriqiyense*	KF844047	99	898/899
E	A-8E1	56	*Pseudomonas fluorescens*	KR187014	99	886/892
E	A-8E31	2	*Pseudomonas frederiksbergensis*	HF584798	100	849/849
E	A-8E54	2	*Stenotrophomonas tumulicola*	LC066089	100	879/879
E	A-8E2	8	*Variovorax paradoxus*	KU258286	99	895/896
E	A-8E57	1	*Variovorax paradoxus*	MF101074	99	907/914
E	A-8E74	1	*Variovorax paradoxus*	MF101074	99	846/848
R	A-8R65	2	*Acinetobacter johnsonii*	KP236312	99	699/701
R	A-8R7	1	*Arthrobacter pascens*	KF515608	99	899/900
R	A-8R9	2	*Arthrobacter polychromogenes*	KR085878	99	844/850
R	A-8R10	1	*Paenarthrobacter nitroguajacolicus*	KX036598.1	100	949/949

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
R	A-8R12	3	*Arthrobacter oxydans*	LN774368	100	898/898
R	A-8R14	4	*Paenarthrobacter nitroguajacolicus*	MF796802	100	549/549
R	A-8R21	2	*Arthrobacter aurescens*	JX293327	100	849/849
R	A-8R22	1	*Arthrobacter aurescens*	JX293327	99	783/786
R	A-8R37	1	*Arthrobacter oxydans*	FR877676	99	696/700
R	A-8R38	2	*Paenarthrobacter nitroguajacolicus*	KX036598	100	899/899
R	A-8R44	2	*Arthrobacter oxydans*	JF496330	99	798/799
R	A-8R54	1	*Arthrobacter oxydans*	JF496330	100	799/799
R	A-8R60	3	*Arthrobacter pascens*	KY933458	99	695/700
R	A-8R69	1	*Arthrobacter pascens*	KC934839	99	698/702
R	A-8R4	1	*Brevibacterium frigoritolerans*	KU877654	100	798/798
R	A-8R20	1	*Bacillus thuringiensis*	KM269287	99	748/749
R	A-8R43A	1	*Bacillus deserti*	KT720271	99	844/847
R	A-8R5	1	*Paenibacillus xylanilyticus*	KT003257	97	624/645
R	A-8R2	1	*Chryseobacterium arachidis*	NR_133723	98	877/899
R	A-8R30	1	*Sinorhizobium meliloti*	KC172024	100	849/849
R	A-8R1	2	*Flavobacterium aquidurense*	NR_042470	98	982/999
R	A-8R13	1	*Flavobacterium johnsoniae*	AM921621	98	783/799
R	A-8R23	1	*Flavobacterium pectinovorum*	AM934674	97	767/793
R	A-8R28	1	*Flavobacterium saccharophilum*	KR085800	98	590/601
R	A-8R32	1	*Flavobacterium pectinovorum*	NR_104717	97	756/789
R	A-8R52	1	*Flavobacterium saccharophilum*	KR085800	97	763/784
R	A-8R64	1	*Flavobacterium resistens*	NR_044292	97	672/693
R	A-8R41	1	*Microbacterium flavescens*	JF778699	99	837/849
R	A-8R53	1	*Microbacterium flavescens*	HQ530520	99	692/702
R	A-8R78	1	*Microbacterium arthrosphaerae*	KF876895	99	798/801
R	A-8R17	1	*Pseudomonas putida*	DQ133506	99	595/597
R	A-8R18	1	*Pseudomonas frederiksbergensis*	KT634069	99	798/799
R	A-8R45	1	*Pseudomonas frederiksbergensis*	KT634069	100	798/798
R	A-8R48	1	*Pseudomonas frederiksbergensis*	KT634069	99	678/687
R	A-8R50B	2	*Pseudomonas frederiksbergensis*	KT634069	100	799/799
R	A-8R51	1	*Pseudomonas frederiksbergensis*	KT634069	99	643/647
R	A-8R67	2	*Pseudomonas brassicacearum*	KT997466	100	1000/1000
R	A-8R58	1	*Pseudomonas brassicacearum*	KY203804	100	844/844
R	A-8R11	2	*Streptomyces drozdowiczii*	KX510091	100	700/700
R	A-8R36	1	*Streptomyces tuirus*	KX055838	99	798/800
R	A-8R6	3	*Variovorax paradoxus*	MF101074	100	798/798
R	A-8R29	1	*Variovorax paradoxus*	KX150839	99	787/792
R	A-8R33	2	*Variovorax paradoxus*	KX036593	100	669/669
R	A-8R40	1	*Variovorax paradoxus*	EU169160	99	737/740
R	A-8R57	1	*Variovorax paradoxus*	FR877674	99	787/792
R	A-8R61	2	*Variovorax paradoxus*	FR877674	99	795/797
R	A-8R77	1	*Variovorax paradoxus*	MF101074	99	849/850
R	A-8R24	2	*Xanthomonas sp. RP-B14*	FM997990	99	599/600
R	A-8R47	1	*Flavobacterium resistens*	NR_044292	98	976/996
SSR	A-8S73	1	*Arthrobacter globiformis*	JF496439	100	850/850
SSR	A-8S42	1	*Arthrobacter crystallopoietes*	KJ542129	100	800/800
SSR	A-8S61	1	*Arthrobacter crystallopoietes*	KJ542129	100	800/800
SSR	A-8S21	1	*Bacillus niacini*	KC511548	100	800/800
SSR	A-8S31	2	*Bacillus simplex*	KY285289	99	696/699

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
SSR	A-8S36	1	*Bacillus niacini*	KC511548	99	797/801
SSR	A-8S41	1	*Brevibacterium frigoritolerans*	MF800956	100	800/800
SSR	A-8S1	1	*Bacillus firmus*	KC355265	98	782/802
SSR	A-8S8	1	*Bacillus niacini*	JF496441	99	749/750
SSR	A-8S24	1	*Bacillus deserti*	NR_117383	99	793/800
SSR	A-8S16	2	*Brevibacterium frigoritolerans*	MF800956	100	800/800
SSR	A-8S51	1	*Bacillus deserti*	KT720271	99	846/849
SSR	A-8S45	1	*Bacillus niacini*	JF682054	98	719/731
SSR	A-8S48	1	*Bacillus niacini*	HQ284935	99	799/800
SSR	A-8S55	1	*Brevibacterium frigoritolerans*	KU877654	100	750/750
SSR	A-8S57	1	*Bacillus niacini*	HF584943	99	791/800
SSR	A-8S39	1	*Bacillus deserti*	KT720271	99	794/799
SSR	A-8S59	1	*Brevibacterium frigoritolerans*	MF800956	100	800/800
SSR	A-8S63	1	*Brevibacterium frigoritolerans*	KU877654	98	798/800
SSR	A-8S79	1	*Bacillus deserti*	KT720271	99	696/700
SSR	A-8S65	1	*Bacillus niacini*	HQ284935	99	796/801
SSR	A-8S67	1	*Brevibacterium frigoritolerans*	MF101183	99	798/800
SSR	A-8S68	1	*Bacillus simplex*	KY285289	98	726/742
SSR	A-8S80	2	*Brevibacterium frigoritolerans*	MF062983	99	799/800
SSR	A-8S23	1	*Bacillus niacini*	HF585023	99	797/799
SSR	A-8S72	1	*Brevibacterium frigoritolerans*	MF101183	99	646/647
SSR	A-8S78	1	*Brevibacterium frigoritolerans*	KY127309	99	697/698
SSR	A-8S27	1	*Chryseobacterium indoltheticum*	KX249602	99	556/563
SSR	A-8S40	1	*Chryseobacterium kwangjuense*	NR_108175	98	684/697
SSR	A-8S4	1	*Chryseobacterium ginsenosidimutans*	NR_108691	98	695/709
SSR	A-8S15	1	*Flavobacterium xueshanense*	KR085801	98	682/696
SSR	A-8S26	1	*Dyella yeojuensis*	FN796854	99	686/692
SSR	A-8S14	1	*Lysobacter antibioticus*	KU613064	99	696/701
SSR	A-8S6	2	*Arthrobacter crystallopoietes*	HM480372	98	690/705
SSR	A-8S47	1	*Paenibacillus lautus*	JF496308	99	898/900
SSR	A-8S2	1	*Paenibacillus lautus*	KU955657	99	848/850
SSR	A-8S11	1	*Pseudoxanthomonas indica*	JQ659735	99	697/700
SSR	A-8S9	1	*Sphingomonas ginsengisoli*	NR_132664	99	694/703
SSR	A-8S62	1	*Stenotrophomonas rhizophila*	JN700143	99	696/700
SSR	A-8S76	1	*Stenotrophomonas rhizophila*	JN700143	100	750/750
SSR	A-8S3	1	*Streptomyces flavotricini*	EU593749	100	700/700
SSR	A-8S10	2	*Streptomyces caeruleatus*	KY908443	100	800/800
SSR	A-8S35	1	*Streptomyces ostreogriseus*	AB184392	99	800/802
SSR	A-8S43	2	*Streptomyces caeruleatus*	KY908443	100	850/850
SSR	A-8S56	1	*Streptomyces bobili*	KP338792	100	850/850
SSR	A-8S44	1	*Streptomyces caeruleatus*	KF876897	100	800/800
SSR	A-8S34	1	*Bacteroidetes bacterium ONC1*	FN554385	98	887/905
SSR	A-8S74	1	*Terribacillus saccharophilus*	KC764977	100	700/700
SSR	A-8S7	1	*Variovorax paradoxus*	EU169160	99	796/799
SSR	A-8S12	2	*Variovorax paradoxus*	EU169160	100	748/748
SSR	A-8S25	3	*Variovorax paradoxus*	KU522201	99	697/699
SSR	A-8S32	4	*Variovorax boronicumulans*	MF796742	99	688/694
SSR	A-8S60	1	*Variovorax boronicumulans*	MF796710	99	699/700
SSR	A-8S64	1	*Variovorax paradoxus*	FR877674	99	794/798
SSR	A-8S13	1	*Acinetobacter lwoffii*	MF988732	100	782/782

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
SSR	A-8S18	1	*Arthrobacter globiformis*	MF620067	99	840/842
SSR	A-8S29	1	*Variovorax boronicumulans*	CP023284	99	876/877
B	A-8B73	1	*Agromyces iriomotensis*	KY753307	99	692/698
B	A-8B1	1	*Arthrobacter oxydans*	HF585024	99	791/802
B	A-8B26	4	*Arthrobacter oxydans*	KR085876	99	600/606
B	A-8B78	2	*Pseudarthrobacter siccitolerans*	KY218890	99	697/701
B	A-8B48	1	*Arthrobacter oxydans*	KC128918	100	798/798
B	A-8B57	1	*Arthrobacter humicola*	KT382249	99	898/900
B	A-8B64	1	*Arthrobacter oxydans*	KR085876	99	799/800
B	A-8B65	1	*Pseudarthrobacter siccitolerans*	KY218890	99	898/901
B	A-8B66	1	*Arthrobacter oxydans*	JF496330	99	850/851
B	A-8B3	2	*Bacillus wiedmannii*	MF988727	99	645/650
B	A-8B5	2	*Bacillus thuringiensis*	KY419155	100	749/749
B	A-8B7	1	*Bacillus cereus*	KY312802	99	799/800
B	A-8B8	1	*Bacillus massiliosenegalensis*	NR_125590	98	784/796
B	A-8B22	1	*Brevibacterium frigoritolerans*	KU877654	100	800/800
B	A-8B28	1	*Bacillus deserti*	KT720271	99	847/850
B	A-8B29	1	*Bacillus cereus*	KY312801	99	799/801
B	A-8B20	3	*Bacillus cereus*	KY312801	99	849/850
B	A-8B35	2	*Bacillus niacini*	HQ284935	99	699/700
B	A-8B76	2	*Bacillus niacini*	KC511548	99	647/650
B	A-8B80	4	*Bacillus niacini*	KC315764	100	950/950
B	A-8B51	1	*Bacillus niacini*	HQ284935	99	699/700
B	A-8B56	2	*Bacillus niacini*	KT720170	99	795/796
B	A-8B62	1	*Bacillus badius*	KY950621	97	635/654
B	A-8B72	1	*Bacillus niacini*	HQ284935	99	647/650
B	A-8B2	3	*Bacillus cereus*	KM977832	99	798/800
B	A-8B36B	1	*Bacillus cereus*	KM977832	99	509/513
B	A-8B70	1	*Massilia niastensis*	JF496359	99	816/824
B	A-8B46	2	*Microbacterium hydrocarbonoxydans*	EU714352	99	799/801
B	A-8B11	1	*Arthrobacter humicola*	KT382249	99	716/725
B	A-8B67	1	*Staphylococcus warneri*	MG027625	100	845/845
B	A-8B6	2	*Streptomyces fragilis*	EU841657	99	848/850
B	A-8B13	1	*Streptomyces gobitricini*	KU884395	99	798/800
B	A-8B16	1	*Streptomyces fragilis*	KM186631	99	848/850
B	A-8B18	1	*Streptomyces venezuelae*	KY007173	99	899/900
B	A-8B24	1	*Streptomyces fragilis*	KX352763	99	898/900
B	A-8B25	1	*Streptomyces bottropensis*	KY753283	99	702/706
B	A-8B31	3	*Streptomyces fragilis*	KM186631	99	899/901
B	A-8B34	2	*Streptomyces tumescens*	KU647226	100	851/851
B	A-8B47	1	*Streptomyces nojiriensis*	KX380878	100	900/900
B	A-8B54	3	*Streptomyces fragilis*	KM186631	99	899/901
B	A-8B43	1	*Streptomyces fragilis*	KM186631	99	894/898
B	A-8B41	1	*Streptomyces flavovariabilis*	FJ792572	99	845/852
B	A-8B79	2	*Streptomyces flavovariabilis*	FJ792572	99	801/808
B	A-8B49	1	*Streptomyces luteoverticillatus*	KU310977	99	896/900
B	A-8B50	1	*Streptomyces hawaiiensis*	EU624140	100	900/900
B	A-8B60	1	*Streptomyces tumescens*	KU647226	100	902/902
B	A-8B69	1	*Streptomyces cinnamocastaneus*	AB184588	99	698/699
B	A-8B71	1	*Streptomyces ciscaucasicus*	KY753363	99	662/663

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
B	A-8B74	1	*Streptomyces scabiei*	KP718596	99	646/650
B	A-8B12	1	*Variovorax paradoxus*	MF101074	99	746/747
B	A-8B63	1	*Streptomyces atroolivaceus*	KY744653	100	605/605
Re	A-8SB13	1	*Streptomyces flavovariabilis*	FJ792572	98	903/926
Re	A-8SB20	1	*Streptomyces tumescens*	KU647226	98	786/805
Re	A-8SB18	1	*Arthrobacter globiformis*	KY859819	99	897/902
Re	A-8SB41	1	*Arthrobacter crystallopoietes*	KY753214	98	935/950
Re	A-8SB2	1	*Bacillus megaterium*	KT764108	98	877/893
Re	A-8SB3	3	*Bacillus megaterium*	FJ174652	99	960/961
Re	A-8SB5	7	*Bacillus simplex*	KF478196	99	859/865
Re	A-8SB9	1	*Brevibacterium frigoritolerans*	MF620091	100	596/596
Re	A-8SB12	2	*Bacillus niacini*	KT720170	99	891/893
Re	A-8SB14	1	*Bacillus simplex*	KY646079	99	893/899
Re	A-8SB25	1	*Bacillus megaterium*	KC764961	100	989/989
Re	A-8SB29	2	*Bacillus cereus*	KP411923	100	899/899
Re	A-8SB43	1	*Bacillus simplex*	JF496503	99	753/764
Re	A-8SB22	1	*Variovorax paradoxus*	FR877674	100	848/848
Re	A-8SB4	3	*Chryseobacterium ginsenosidimutans*	KU924004	97	831/858
Re	A-8SB73	1	*Chryseobacterium ginsenosidimutans*	KU924004	97	823/846
Re	A-8SB6	1	*Flavobacterium xueshanense*	KR085801	98	842/861
Re	A-8SB7	1	*Flavobacterium spartansii*	NR_133748	98	782/799
Re	A-8SB8	1	*Flavobacterium aquidurense*	NR_042470	98	932/949
Re	A-8SB15	1	*Flavobacterium gyeonganense*	NR_134035	98	857/874
Re	A-8SB16	1	*Flavobacterium pectinovorum*	NR_114994	97	882/912
Re	A-8SB19	1	*Flavobacterium xueshanense*	KR085801	98	975/995
Re	A-8SB21	1	*Flavobacterium xueshanense*	KR085801	98	877/897
Re	A-8SB42	1	*Flavobacterium pectinovorum*	NR_114994	98	983/1003
Re	A-8SB57	1	*Flavobacterium chungangense*	NR_044581	98	872/890
Re	A-8SB59	2	*Flavobacterium chungangense*	NR_044581	98	872/893
Re	A-8SB77	1	*Flavobacterium resistens*	NR_044292	98	780/799
Re	A-8SB70	1	*Flavobacterium chungangense*	NR_044581	97	803/824
Re	A-8SB69	1	*Flavobacterium aquidurense*	NR_042470	98	781/797
Re	A-8SB33	1	*Flavobacterium procerum*	NR_136820	97	772/801
Re	A-8SB45	1	*Flavobacterium arsenitoxidans*	LN995689	97	871/900
Re	A-8SB49	1	*Lysobacter antibioticus*	CP011129	99	995/999
Re	A-8SB26	4	*Lysobacter antibioticus*	CP011129	99	797/798
Re	A-8SB48	1	*Massilia niastensis*	JF496359	98	987/1003
Re	A-8SB53	1	*Microbacterium oxydans*	KR085856	99	996/1000
Re	A-8SB75	1	*Microbacterium invictum*	NR_042708	98	793/806
Re	A-8SB54	1	*Microbacterium flavescens*	EU714363	99	693/694
Re	A-8SB34	1	*Paracoccus yeei*	CP020442	100	849/849
Re	A-8SB40	1	*Pseudomonas helmanticensis*	KY457748	99	898/899
Re	A-8SB66	1	*Pseudomonas alcaligenes*	JF915337	99	939/944
Re	A-8SB1	2	*Streptomyces spororaveus*	KX035075	99	949/959
Re	A-8SB23	1	*Streptomyces nojiriensis*	KX380878	99	900/901
Re	A-8SB24	1	*Streptomyces ambofaciens*	KT363055	100	916/916
Re	A-8SB32	1	*Streptomyces pluricolorescens*	KU324442	99	906/912
Re	A-8SB31	2	*Streptomyces flavovariabilis*	FJ792572	99	891/899
Re	A-8SB35	1	*Streptomyces netropsis*	KP339302	99	749/760
Re	A-8SB36	1	*Streptomyces ambofaciens*	EU841656	99	912/919

Fraction	Isolate	n° isolates/ ITS	Closest described relative (BLAST)	acc.n° (NCBI)	Id %	base
Re	A-8SB51	1	*Streptomyces gardneri*	JN999909	98	1075/1094
Re	A-8SB52	1	*Streptomyces venezuelae*	KY007173	97	647/664
Re	A-8SB58	1	*Streptomyces rectiviolaceus*	KJ081286	99	744/749
Re	A-8SB63	1	*Streptomyces ambofaciens*	EU593561	100	849/849
Re	A-8SB44	1	*Streptomyces virginiae*	KU317910	100	949/949
Re	A-8SB55	1	*Variovorax paradoxus*	KX150846	97	952/980
Re	A-8SB72	1	*Variovorax paradoxus*	FR877674	99	882/893
Re	A-8SB80	1	*Variovorax paradoxus*	KX036607	100	749/749
Re	A-8SB10	1	*Xanthomonas translucens*	GU902285	99	796/799
Re	A-8SB11	1	*Xanthomonas translucens*	GU902285	99	896/899
Re	A-8SB38	1	*Xanthomonas translucens*	GU902285	99	750/759
Re	A-8SB71	1	*Flavobacterium ginsengisoli*	KY077156	97	834/863

Supplementary Table 2. Plant growth promotion traits of the cultivable bacteria isolated from the root apparatus of *A. spinosa* (E, R, SSR), bulk soil (B) and residuesphere (Re). The table includes the results of the screening performed to characterize the *in vitro* direct and indirect PGP potential of the bacterial strains. Sid: siderophore production; PO_4: phosphate solubilization activity; NH_3: ammonia production; EPS: exopolysaccharides.

Strain	PO_4 sol.	Sid	NH_3	Protease	Esterase	EPS
A-8E1	1	0	1	1	0	0
A-8E16	0	0	1	0	0	1
A-8E2	0	0	1	0	0	0
A-8E26	0	0	1	1	0	0
A-8E29	0	0	1	1	0	0
A-8E31	1	0	1	0	0	0
A-8E54	0	0	1	1	0	0
A-8E57	0	0	1	0	0	0
A-8E62	0	0	1	1	0	0
A-8E73	0	0	1	0	1	0
A-8E74	0	0	0	0	0	0
A-8R01	0	0	1	1	0	0
A-8R02	0	0	1	1	0	0
A-8R04	0	0	1	0	1	0
A-8R05	0	0	0	1	0	0
A-8R06	0	0	1	0	0	0
A-8R07	0	0	1	1	0	0
A-8R09	0	0	1	1	0	0
A-8R10	0	0	1	1	0	0
A-8R11	0	0	0	1	0	0
A-8R12	0	0	1	0	0	0
A-8R13	0	0	0	0	0	0
A-8R14	0	0	1	1	0	0
A-8R17	0	1	1	0	0	0
A-8R18	1	1	1	0	0	0
A-8R20	0	0	1	0	0	0
A-8R21	0	0	1	1	0	0
A-8R22	0	0	1	1	0	0
A-8R23	0	0	1	1	0	0
A-8R24	0	0	1	1	1	0
A-8R28	0	0	1	1	0	0
A-8R29	0	0	1	0	0	0
A-8R30	0	0	1	0	0	0
A-8R32	0	0	1	1	0	0
A-8R33	0	0	1	0	1	0
A-8R36	0	0	0	0	0	0

Strain	PO_4 sol.	Sid	NH_3	Protease	Esterase	EPS
A-8R37	0	0	0	1	0	0
A-8R38	0	0	1	1	1	0
A-8R40	0	0	1	0	0	0
A-8R41	0	0	1	0	1	0
A-8R43A	0	0	1	0	0	0
A-8R44	0	0	1	1	0	0
A-8R45	1	1	1	0	0	0
A-8R47	1	1	1	0	0	0
A-8R48	1	1	1	0	0	0
A-8R50B	0	0	1	0	0	0
A-8R51	0	0	1	1	0	0
A-8R52	0	0	1	0	0	0
A-8R53	0	1	1	0	0	0
A-8R54	0	0	1	1	0	0
A-8R57	0	0	1	0	0	0
A-8R58	1	1	1	0	1	0
A-8R60	0	0	0	1	0	0
A-8R61	0	0	1	0	0	0
A-8R64	0	0	0	0	0	0
A-8R65	0	0	0	0	1	0
A-8R67	0	1	0	1	0	0
A-8R69	0	0	1	0	0	0
A-8R77	0	0	0	0	0	0
A-8R78	0	0	1	0	0	0
A-8S01	0	0	1	0	0	0
A-8S02	0	0	0	0	0	0
A-8S03	1	1	1	0	0	0
A-8S04	0	1	1	1	0	0
A-8S06	1	1	1	0	0	0
A-8S07	1	0	1	1	0	0
A-8S08	0	1	1	1	0	0
A-8S09	0	0	1	0	0	0
A-8S10	1	1	1	1	0	0
A-8S11	0	1	1	1	0	0
A-8S12	0	1	1	0	1	0
A-8S13	1	1	1	0	0	0
A-8S14	0	1	1	0	0	0
A-8S15	0	1	1	1	0	0
A-8S16	0	0	1	1	0	0
A-8S18	1	0	1	1	0	0
A-8S21	1	1	1	0	0	0
A-8S23	0	1	1	0	0	0
A-8S24	0	1	0	0	1	0
A-8S25	1	0	1	0	1	0
A-8S26	0	0	1	1	0	0
A-8S27	0	1	1	1	0	0
A-8S29	1	0	1	1	0	0
A-8S31	0	0	1	0	0	0
A-8S32	1	0	1	0	0	0
A-8S34	0	1	1	0	1	0
A-8S35	0	0	1	0	0	0
A-8S36	0	1	1	0	0	0
A-8S39	0	0	1	0	0	0
A-8S40	0	1	1	1	0	0
A-8S41	0	1	1	1	0	0
A-8S42	1	0	1	0	0	0
A-8S43	0	0	1	0	0	0
A-8S44	0	1	0	1	0	0
A-8S45	0	0	1	1	0	0
A-8S47	0	0	0	0	0	0
A-8S48	0	0	1	0	0	0
A-8S51	0	0	1	0	0	0

Strain	PO_4 sol.	Sid	NH_3	Protease	Esterase	EPS
A-8S55	0	0	1	1	0	0
A-8S56	0	1	1	0	0	0
A-8S57	0	0	1	1	0	0
A-8S59	1	0	1	0	0	0
A-8S60	1	1	1	0	0	0
A-8S61	0	0	1	1	0	0
A-8S62	0	0	1	1	0	0
A-8S63	0	0	1	1	0	0
A-8S64	1	0	1	1	0	0
A-8S65	0	0	1	0	0	0
A-8S67	0	0	1	1	0	0
A-8S68	0	1	1	1	0	0
A-8S72	0	0	1	1	0	0
A-8S73	0	1	1	0	0	0
A-8S74	0	0	1	0	0	0
A-8S76	0	0	1	1	0	0
A-8S78	1	0	1	1	0	0
A-8S79	0	0	1	0	0	0
A-8S80	0	1	1	1	0	0
A-8B01	0	0	1	1	0	0
A-8B02	0	0	1	1	0	0
A-8B03	0	1	1	0	0	0
A-8B05	0	0	1	1	0	0
A-8B06	0	0	1	0	0	0
A-8B07	0	0	1	0	0	0
A-8B08	0	0	1	1	1	0
A-8B11	0	1	1	0	0	0
A-8B12	0	0	0	0	0	0
A-8B13	0	0	1	0	0	0
A-8B16	0	0	1	0	0	0
A-8B18	0	0	1	0	0	0
A-8B20	0	1	1	0	0	0
A-8B22	0	0	1	0	0	0
A-8B24	0	0	1	1	0	0
A-8B25	0	0	1	0	0	0
A-8B26	0	0	1	1	0	0
A-8B28	0	1	1	1	0	0
A-8B29	0	0	1	1	0	0
A-8B31	0	0	0	0	0	0
A-8B34	0	0	1	0	0	0
A-8B36B	0	0	1	1	0	0
A-8B41	0	1	1	1	0	0
A-8B43	0	0	1	1	0	0
A-8B46	0	0	1	1	0	0
A-8B47	0	1	1	0	1	0
A-8B48	0	0	1	0	0	0
A-8B49	0	0	1	1	0	0
A-8B50	0	0	0	0	0	0
A-8B51	0	0	1	1	0	0
A-8B54	0	1	1	1	0	0
A-8B56	0	0	1	0	0	0
A-8B57	0	1	1	0	0	0
A-8B60	0	0	1	1	0	0
A-8B62	0	0	1	0	0	0
A-8B63	0	0	1	1	0	0
A-8B64	0	1	1	1	0	0
A-8B65	0	0	1	1	0	0
A-8B66	0	0	1	1	0	0
A-8B67	0	1	1	0	0	0
A-8B69	0	1	1	0	1	0
A-8B70	0	1	1	0	1	0
A-8B71	0	1	1	0	1	0

Strain	PO_4 sol.	Sid	NH_3	Protease	Esterase	EPS
A-8B72	0	1	1	0	1	0
A-8B73	0	0	1	0	0	0
A-8B74	0	0	1	1	0	0
A-8B76	0	0	1	1	0	0
A-8B78	0	0	1	1	0	0
A-8B80	0	0	0	0	0	0
A-8SB01	1	0	1	0	1	0
A-8SB02	1	0	0	1	1	0
A-8SB03	1	0	1	1	1	0
A-8SB04	0	0	1	1	0	0
A-8SB05	0	0	1	0	1	0
A-8SB06	0	0	1	1	0	0
A-8SB07	0	0	1	1	0	0
A-8SB08	0	0	1	1	0	0
A-8SB09	0	0	1	1	1	0
A-8SB10	0	0	1	1	1	0
A-8SB11	0	0	1	1	0	0
A-8SB12	0	0	1	1	0	0
A-8SB14	1	1	1	1	0	0
A-8SB15	0	0	1	1	0	0
A-8SB16	0	0	1	1	0	0
A-8SB18	0	0	1	0	0	0
A-8SB19	0	0	1	1	0	0
A-8SB21	0	0	1	1	0	0
A-8SB22	0	1	1	1	0	0
A-8SB23	0	1	1	1	0	0
A-8SB24	0	1	1	0	1	0
A-8SB25	0	0	1	1	1	0
A-8SB29	0	1	1	1	1	0
A-8SB32	0	1	1	1	1	0
A-8SB33	0	0	1	0	1	0
A-8SB34	0	0	1	0	1	0
A-8SB35	0	1	1	0	1	0
A-8SB36	0	0	1	0	1	0
A-8SB38	0	0	1	1	1	0
A-8SB40	0	0	1	0	0	0
A-8SB41	0	1	1	0	1	0
A-8SB42	0	0	1	0	0	0
A-8SB43	0	0	1	0	1	0
A-8SB44	0	1	1	0	0	0
A-8SB45	0	0	1	1	0	0
A-8SB48	0	0	1	1	0	0
A-8SB49	0	1	1	1	1	0
A-8SB51	0	0	1	0	0	0
A-8SB52	0	0	1	1	0	0
A-8SB53	0	1	1	1	1	0
A-8SB55	1	0	1	0	0	0
A-8SB57	0	0	1	1	0	0
A-8SB58	0	0	1	0	0	0
A-8SB59	0	0	1	1	0	0
A-8SB63	0	1	1	1	0	0
A-8SB69	0	0	1	1	0	0
A-8SB70	0	1	1	0	0	0
A-8SB71	0	0	1	1	0	0
A-8SB72	0	1	1	1	1	0
A-8SB73	0	0	1	1	1	0
A-8SB75	0	0	1	0	0	0
A-8SB77	0	0	1	0	0	0
A-8SB80	0	1	1	0	0	0

Chapter V

Plant growth promoting effect of bacteria inocula on tomato plants cultivated under different water conditions during long-term greenhouse experiment

Abstract

Nowadays plant-associated bacteria are receiving growing attention as promising tool to improve crop resistance to water stress, potentially reducing drought-related production losses. In this work, five plant growth promoting (PGP) bacteria, previously isolated from root apparatus of extremophilic plants, were selected for the inoculation of tomato plants cultivated under three different water conditions. Physiological parameters and plant productivity were evaluated to estimate the effect of PGP inocula on plants growth. The most promising strains *Micrococcus yunnanensis* KMP123-M1 and *Pseudomonas stutzeri* SR7-77 were selected for a further *in vivo* experimentation performed on a higher plant replicates in order to obtain statistically significant results. Both isolates significantly promoted plant physiological parameters, root biomass and soil hydrology under strong water stress conditions. Plant productivity showed to increase in bacterized plants even if the result is lacking statistical significance. The results of this study underlined the importance of performing long-term experimentation to have a complete panorama of PGP inocula effect on plants during all the growing season.

1. Introduction

A major challenge facing agriculture in the 21st century is the need to increase the productivity of cultivated land in emerging conditions of limited water availability (Falkenmark, 2013). To cope with drought stress and avoid dehydration, plants may assume different adaptive measures, the most important being the change in root architecture and resource allocation (Smith et al., 2012; Hasibeder et al., 2015). Nevertheless, climate changes are further making the situation worse, projecting the global water demand to increase by 55% by 2050 (Ercin et al., 2014), thus making urgent the need to develop crop plants with improved drought tolerance. In this scenario, there is growing interest in plant-associated microorganisms as potential sustainable resource to increase water use efficiency in agriculture and

minimize water footprint (Santos-Medellin et al., 2017; Kaushal et al., 2016; Soussi et al., 2016). Plants have evolved with complex bacterial communities having important roles for nutrition, health, and overall fitness of their plant hosts (Compant et al., 2019). In adverse environmental conditions and in particular, under drought, it has been found that plant microbiota is enriched in microorganisms able to thrive under abiotic stresses, by developing several adaptations required to counteract water shortage and mitigate its effect on host plant (Soussi et al., 2016). These tolerant microorganisms can confer a certain level of stress tolerance to the host and can contribute to alleviate plant stress by a variety of mechanisms. (Marasco et al., 2012; Mapelli et al., 2013). The beneficial microbiome associated to plants growing in arid environment, including the so-called plant growth promoting (PGP) bacteria, have the potential to impart drought tolerance by several mechanisms like producing the phytohormone indole-3-acetic acid (IAA) and exopolysaccharides (EPS), activating the 1-aminocyclopropane-1-carboxylate (ACC) deaminase, inducing accumulation of osmolytes and antioxidants and regulating different stress responsive genes (Vurukonda et al., 2016). Therefore, the ecological services provided by the microbiota can be considered as an extended plant trait, and as such could be targeted in crop improvement. Plants adapted to extreme growth conditions and their associated microbial communities thus represent a source of bacterial inocula which could potentially help other plant species, including agricultural crops, to cope with limited water resource availability (Chen et al., 2017; Yuan et al., 2016).

The present study is part of the European project MADFORWATER (https://www.madforwater.eu/) aimed to develop technological strategies for reducing water vulnerability in North African Countries (Egypt, Morocco and Tunisia). In this frame, the aim of this work was to find microbial resources, isolated from extremophilic plants, able to help tomato plants (*Lycopersicon esculentum*), a water stress-sensitive plant (Dodds et al., 1997), facing water shortage conditions and able to improve growth and productivity of the

plants. Ngumbi and Kloepper (2016) reviewed several researches concerning bacterial-mediated drought tolerance to crops, including tomato crop. For instance, *Achromobacter piechaudii* ARV8 demonstrated to increase water use efficiency of tomato plants in dry salty soil (Mayak et al., 2004a). The bacterial inoculum increased also the plant biomass and reduced the production of ethylene by tomato seedlings exposed to transient water stress thus potentially decreasing detrimental plant stress responses (Mayak et al., 2004b). Other recent studies demonstrated the ability of PGP bacteria to i) improve biomass and physiological parameters (Eke et al., 2019) and ii) increase water use efficiency and reduce oxidative stress enzymes (Brilli et al., 2019) in tomato plants subjected to drought stress. However, the effect of PGP inocula on tomato plants subjected to drought stress has been rarely analyzed for the entire plant life cycle, up to the plant production stage (Le et al. 2018). To evaluate the effective potential of PGP selected bacteria in improving crop productivity under conditions of water shortage in dry lands, it is nevertheless fundamental the elucidation of plants response to PGP inocula, not only in terms of biomass improvement in the first plant life stage but also as crop production in long-term *in vivo* experiments. Aim of this work was therefore to test the plant response to water shortage in plants bacterized with selected strains previously isolated from the roots of plants growing in extremely arid lands. Bacterized tomato plants were cultivated in greenhouse for their entire life cycle, up to fruit production, and compared with bacterized plants maintained in identical conditions.

2. Material and methods

2.1. Selection and characterization of the bacterial strains

The bacterization of tomato plants were performed with five bacteria isolated in previous works from root apparatus of different extremophilic plants and were selected according to their PGP abilities.

Micrococcus yunnanensis KMP123-M1 was isolated from the endosphere of V*icennia marina* mangrove propagules sampled along the central Red Sea (Soldan et al., 2019): mangroves have been defined as “true extremophiles” because they are among the most salt tolerant plants known (Parida and Jha, 2010). The endophytic strain *Bacillus simplex* RP-26 was isolated from the resurrection plant *Selaginella* sp., a plant species that can survive extreme dehydration (VanBuren et al., 2018). *Pseudomonas stutzeri* SR7-77 was isolated from the rhizopshere of *Salicornia strobilacea*, a halophilic plant collected in the coastal area of Ras Lamsa, Tunisia (Marasco et al., 2016). Lastly, *Arthrobacter nitroguajacolicus* 2-50 and *Arthrobacter aurescens* 2-30 were isolated from the rhizosphere of *Centaurea nitrescens* collected in the SIN Caffaro, a site located in Northern Italy (Brescia municipality) extremely polluted by chlorinated compounds: both these strains significantly promoted tomato plant growth compared to the non-inoculated control during a greenhouse experiment (Vergani et al., 2017).

In order to select the two most promising PGP strains to be use in a second-round *in vivo* experiment with tomato plants, further in vitro tests were performed on the five bacterial strains used in the first experiment, as described below (Paragraph 2.3).

2.2. Plant growth promotion of tomato plants in soil under well-irrigated and water stress conditions (first experiment)

2.2.1. Experimental design and setup

Long-term experiment was conducted in a semi-controlled greenhouse of the Mediterranean Agronomic Institute of Bari (IAMB) and was applied to an overall of 180 tomato plants (var. Camone) until the end of their life cycle. The whole plants were subdivided in ten replicates for three different water regimes and six inoculation treatments. The diverse water regimes applied during the experiment consisted in 100%, 75% and 50% of plant water requirement, which simulated an optimal irrigation state and a moderate and

a strong water stress condition, respectively. Pots were irrigated every two days compensating the water lost by evapotranspiration and considering the water soil content. Evapotranspiration fluxes were monitored using an evaporation pan Class A and the soil water content was measured by a Time Domain Reflectometry (TDR) applied to one pot per each water regime conditions and by weighting 90 pots with an electronic balance. Evapotranspiration readings were collected every day at 6.00 pm.

The five bacterial strains described in the previous paragraph were applied as inocula to tomato plants. The sixth treatment is referred to non-bacterized plants considered as negative control of the experiment, which received only sterile water.

2.2.2. Plant and bacteria materials

Tomato seeds were germinated in polystyrene plug tray filled with commercial soil. After 40 days, the most uniform-sized seedlings were planted in 10 L randomized pots with 9,5 kg of soil and 0,5 kg of pumice placed on the bottom to facilitate water drainage. Bacterial inocula were applied twice to the plants: a first time one week after transplantation and a second time in the middle of tomato growing season, 120 days after transplantation. The bacterial suspension of *M. yunnanensis* KMP123-M1, *B. simplex* RP-26, *P. stutzeri* SR7-77, *A. nitroguajacolicus* 2-50 and *A. aurescens* 2-30 were prepared in sterilized distilled water at the concentration of 10^7 cells/g of soil. For the preparation of the inocula, bacterial isolates were grown in Tryptic Soy Broth (TSB) for 24 h at 30°C, the bacterial cells were centrifuged at 4000 rpm for 15 min and the pellet was re-suspended with sterile distilled water to obtain a bacterial concentration of 10^7 cells/g of soil. The inocula were applied to the soil surrounding the collar of potted tomato plantlets. The non-inoculated seedlings were watered with sterilized distilled water. One week after the first bacterial inoculation, the application of the differentiated water treatments started.

2.2.3. Plant growth and yield measurement

Four measurement campaigns, corresponding to different growth stage of tomato plants, were organized to monitor physiological parameters of the plants. Leaf photosynthesis, conductance and transpiration were thus measured for five out of ten plant replicates by LI-COR portable photosynthesis system. The index of water use effectiveness in the photosynthesis process (water use efficiency, WUE) was evaluated as the photosynthesis/transpiration ratio.
At the end of the experiment root and shoot length, collar diameter and fresh and dry root weight were measured to determine the plant growth of 5 replicates. Five different harvest campaigns were performed and the diameter and weight of ripe fruits produced by all ten replicates for a total of 180 plants were recorded and the tomato yield per plant was calculated. Moreover, the water productivity (WP) index, defined as the ratio of yield/volume of water consumed by the plant (evapotranspiration) was calculated.

2.3. In vitro characterization of the bacterial strains

2.3.1. Rhizocompetence potential

The biofilm production capacity of the isolates was evaluated by a colorimetric assay based on crystal violet staining, as described in Chapter VI of the thesis: the OD at 610 nm of crystal violet stained cells was determined and defined as “adhesion unit”. The OD value of the bacterial strains were compared to the adhesion unit of *E. coli* 172ATCC25404 used as reference strain.

2.3.2. Tolerance and degradation of micropollutants

The five bacterial strains were analysed for their ability to tolerate micropollutants that could be present in treated wastewaters: these tests were performed considering the aim of the overall MADFORWATER project,

i.e. the development and application of technological solutions for wastewaters treatment and efficient reuse in agriculture tailored to the needs of Mediterranean African Countries. In this frame, since treated wastewaters will be use for irrigation purpose, the bacterial strains candidates to crop inoculation should be tolerant to micropollutants eventually present in treated wastewaters. The five bacterial strains were indeed firstly tested for their ability to grow in the presence of metals mixture composed with $ZnCl_2$, $NiCl_2$, $CdCl_2$ at a concentration of 20 mg/l, 2 mg/l and 0,1 mg/l, respectively, and successively with a concentration ten times higher. The test was performed in a 96 wells-microtiter with 180 µl of TSB medium (with and without metals) and 20 µl of bacterial culture grown until the late exponential phase. Each strain was tested in triplicate and the OD_{610} in metal containing medium was statistically compared with the OD_{610} reached in TSB without metals. The absorbance at 610 nm was measured with a microtiter-plate reader (Tecan InfiniteF200Pro) at time zero and after 24 and 48 hours of incubation at 30°C.

An antibiotic susceptibility test was performed with a disk-diffusion assay using cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg) (CONDA Pronadisa, Madrid). Bacteria were classified as sensitive or resistant after 24 h of incubation at 30°C, comparing the results with the clinical and QC parameters provided by LABORATORIOS CONDA S.A. using *E. coli* ATCC 25922 as reference strain.

Finally, the dye decolorization potential of the bacteria was studied *in vitro* using the azo dye Reactive Black 5, as model molecule, and two dyes used by a textile company located in Tunisia, Tubantin blue and Blue S-2G. The test followed the same procedure described in Chapter VI of this thesis.

2.3.3. Vitality test in tap water and urban treated wastewater

In order to verify the survival in urban treated wastewater (TWW) and tap water of the two selected strains, the bacterial inocula were prepared with a

concentration of 10^8 cell/ml and were suspended in sterilized tap water or sterilized TWW collected from one of the Milan's municipal treatment plants. The bacterial suspensions were placed for 40 days at 30°C to simulate North African countries' temperature. The bacterial vitality was periodically verified by plate counting. The bacterial suspensions were serially diluted and 10 µl of the dilutions were spotted in triplicate on Tryptic Soy Agar (TSA) medium. After 48 hours of incubation at 30°C the number of colony-forming units (cfu) per ml of bacterial suspension was determined.

2.4. Plant growth promotion of tomato plants in soil under well-irrigated and water stress conditions (second experiment)

2.4.1. Experimental design and setup

In the semi-controlled greenhouse located in IAMB, 252 tomato plants (var. Camone) were distributed according to a randomized complete block design and were subjected to three water regimes (100%, 75% and 50% of plant water requirement) and three inoculation treatments (2 bacterial strains and the non-bacterized control) considering 28 plants as replicates for each thesis.

2.4.2. Plant and bacterial materials

Pot preparation followed the procedure of the first greenhouse experiment. Bacterial inocula were applied to the plants one week after transplantation. The bacterial suspensions of *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77, obtained in TSB medium for 24 hours, were prepared by harvesting the cells by centrifugation following resuspension in sterilized distilled water at the concentration of 10^7 cells/g of soil. The non-inoculated seedlings were watered with sterilized distilled water. One week after bacterial inoculation, the application of the differentiated water treatments started.

2.4.3. Parameters measurement

The physiological parameters, leaf photosynthesis, conductance and transpiration, of 14 plant replicates were monitored by LI-COR instrument during three measurement campaigns and WUE was calculated as described for the first experiment. During the last campaign also soil permeability was indirectly measured according with Darcy's low that describes the flow of a fluid (water) through a porous medium (soil). At the end of the experiment root and shoot dry weights, collar diameter and shoot length were measured to determine the plant growth of 14 replicates for each thesis. Moreover, the root surface density was analysed through the image analysis software, ImageJ. The diameter and weight of ripe fruits of 252 plants were recorded and tomato yield and WP index were calculated.

2.5. Statistical analyses

For the first greenhouse experiment data were compared with t-student test while for the second greenhouse experiment statistical analyses were performed with Minitab 16 statistical software and data were analyzed by two-way ANOVA, using "bacteria" and "water regimes" as factors and "block" as random factor.

3. Results and discussion

3.1. PGP bacteria promotion of tomato plant growth and productivity under different water conditions

Bacteria associated with plants naturally adapted to adverse environmental conditions are known to be stress resilient due to the development of several strategies and potentially able to confer stress tolerance to the plant host (Soussi et al., 2016). From a wide collection of bacterial strains isolated from the root apparatus of extremophilic plants, five bacterial strains were indeed selected basing on the characterization of their PGP potential *in vitro* and *in vivo*. *In vivo* PGP characterization had been, however, performed only in

short term assays (30 days from planting). Pure cultures of the strains were applied as inoculants to the soil surrounding the collar of potted tomato plantlets in greenhouse. The plants were subsequently cultivated for the entire life cycle, up to fruit production, under three different water conditions: optimal irrigation state (100%), moderate water stress condition (75%) and strong water shortage condition (50%). During plant growth and at the end of the experiment growth and productivity of the plant was monitored. The ability of the five selected bacteria to increase growth of tomato plants was evaluated firstly measuring net leaves photosynthesis, transpiration rate and stomatal conductance during four different measurement campaigns, corresponding to vegetative, flowering, fruit setting stages and at the end of the season of tomato plants. As showed in Table 1, the five PGP strains showed to significantly increase several plant physiological parameters in comparison to the non-bacterized plants. Notably, most of the bacteria seemed to have a better effect under water stress condition with the exception of *A. aurescens* 2-30, which significantly promoted plant photosynthesis, transpiration and conductance only under full irrigation condition. Previous works showed that PGP bacteria can induce a beneficial effect on plants growth depending on different factors related to plant, bacterial strain and the environmental conditions. For instance, Rolli and co-workers demonstrated on grapevine plants that PGP activity can be either stress-dependent and -independent (Rolli et al., 2015). In other works PGP bacteria were demonstrated to be specifically able to enhance plant growth i) under drought conditions, being ineffective in plant growth promotion under optimal irrigation conditions or ii) vice versa (Rolli et al., 2015; Chen et al., 2017) or iii) they improved plant growth under both non-stressed and drought conditions (Gagnè-Bourque et al., 2016). These completely different results claim an unpredictability of the beneficial effect of plant bacterization, which needs to be tested in each specific combination of plant, bacteria and environmental condition. Plant physiology improvement was generally more

evident in bacterized plants at flowering and fruit setting stages, when tomato plants need a higher amount of nutrient for growing.

Table 1. Effect of bacterial inocula on the physiological parameters (P=photosynthesis; T=transpiration; C=conductance; WUE=water use efficiency) measured during the experiment at four different tomato plant growth stages: 26 DAT (day after transplantation)=vegetative stage; 46 DAT=early flowering stage; 81 DAT=late flowering/fruit setting stage; 108 DAT=end of the season. MY=*Micrococcus yunnanensis* KMP123-M1; BS=*Bacillus simplex* RP-26; PS=*Pseudomonas stutzeri* SR7-77; AA=*Arthrobacter aurescens* 2-30; AN=*Arthrobacter nitroguajacolicus* 2-50. 100%=optimal irrigation state; 75% moderate water stress; 50% strong water stress. The * indicates a significant improvement in bacterized plants compared to the non-inoculated plants.

Parameter	DAT	MY			BS			PS			AA			AN		
		100%	**75%**	**50%**	**100%**	**75%**	**50%**	**100%**	**75%**	**50%**	**100%**	**75%**	**50%**	**100%**	**75%**	**50%**
P	26															
	46				*					*	*			*		*
	81								*							*
	108															
T	26			*												
	46			*						*	*					
	81															
	108						*				*					
C	26			*												
	46									*	*					
	81							*								
	108						*				*					
WUE	26							*								
	46	*			*			*	*		*			*	*	
	81	*						*	*							
	108															

During these growing stages indeed tomato plants need fertilizers so it is possible to speculate that the tested bacteria have a role in improving plant

nutrition. Transpiration and stomatal conductance were also significantly enhanced during the vegetative stage by *M. yunnanensis* KMP123-M1 under drought condition and during the end of the season by *B. simplex* RP-26 under water shortage conditions and *A. aurescens* 2-30 under full irrigation condition.

Leaf water use efficiency of tomato plants was calculated as the photosynthesis/transpiration ratio and the results showed that PGP effects were more significant in plants growing under optimal irrigation condition, especially at flowering stage. All the strains were able to enhance water use efficiency 46 days after plant transplantation when tomato plants were irrigated with 100% of water needs. In particular *P. stutzeri* SR7-77 showed the best result, being able to significantly enhance the efficiency of leaf water use under full irrigation condition from the vegetative stage to the fruit setting and also under moderate drought stress condition from flowering to fruit setting stages.

None of the strains showed to improve plant biomass at the end of the growing season. No significant results were obtained with the five PGP bacteria in comparison to the non-inoculated plants, even if the strains and in particular *A. nitroguajacolicus* 2-50 and *A. aurescens* 2-30 previously demonstrated the ability to promote biomass in short term experiments on 30 days grown tomato seedlings (Vergani et al., 2017). In the literature there are many *in vivo* PGP experiments showing a significant effect of selected PGP bacteria in improving plant biomass. However, these studies are often performed as short-term evaluation test and, as a consequence, they do not necessarily have a relevant impact in a field scenario (Compant et al., 2019). For this reason, long-term *in vivo* experiments are important in order to obtain a complete and exhaustive perspective of the effects of PGP bacteria on cultivated plants.

Considering that for tomato crop the productivity is fundamentally related to the fruit yield, more interesting results were obtained in this work by investigating the amount of fruit obtained by bacterized and non-bacterized

plants. Ripe fruits were harvested during five campaigns (92, 99, 106, 118 and 130 day after transplantation) according to visual inspection. Ripening of the fruits was confirmed by the measurement of Brix degrees and pH on a subset of tomatoes. Brix degrees were always higher than 5, which is the commodity-related value to be commercialized as category A, and pH was always around 4. Figure 1 shows the overall fruits production of tomato plants inoculated with the five PGP bacteria and non-bacterized plants. Positive results were obtained in particular for plants optimally irrigated (100%) and plants subjected to a strong water stress (50%). Even if these results were not supported by statistical significance, some potential indications could lead to i) speculate that with a different experimental design (e.g. more replicated plants) the bacterization could result in an improvement of fruit yield and the mitigation of losses due to drought stress, ii) evaluate the most promising strains to be tested in a second round of experiments. Under both water conditions, 100% and 50%, bacterized plants produced, on the average, a higher number and total mass of fruits in comparison to non-inoculated control plants, consequently potentially proving a higher productivity (Figure 1). At full irrigation condition, in particular, 10 replicated plants inoculated with *M. yunnanensis* KMP123-M1 produced globally 62 tomatoes (1607 g in total) compared to 51 tomatoes (1395 g in total) produced by the 10 non bacterized control plants showing 22% increase on the number and 15% increase on the total fruit mass (Fig. 1). Under strong drought stress condition, moreover, the results were even more interesting showing a higher yield improvement, in particular in plants bacterized with *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77. Plants bacterized with *M. yunnanensis* KMP123-M1 produced 15 tomatoes (239 grams in total) compared to 9 tomatoes (131 gram in total) produced by non-inoculated plants showing 82% increase (Figure 1). Plants bacterized with *P. stutzeri* SR7-77 produced 14 tomatoes (261 grams), a double-yield compared to the negative control plants: as shown in Figure 2, the average

weight of each tomato produced by plants inoculated with *P. stutzeri* SR7-77 was indeed higher compared to the fruits produced by non-inoculated plants

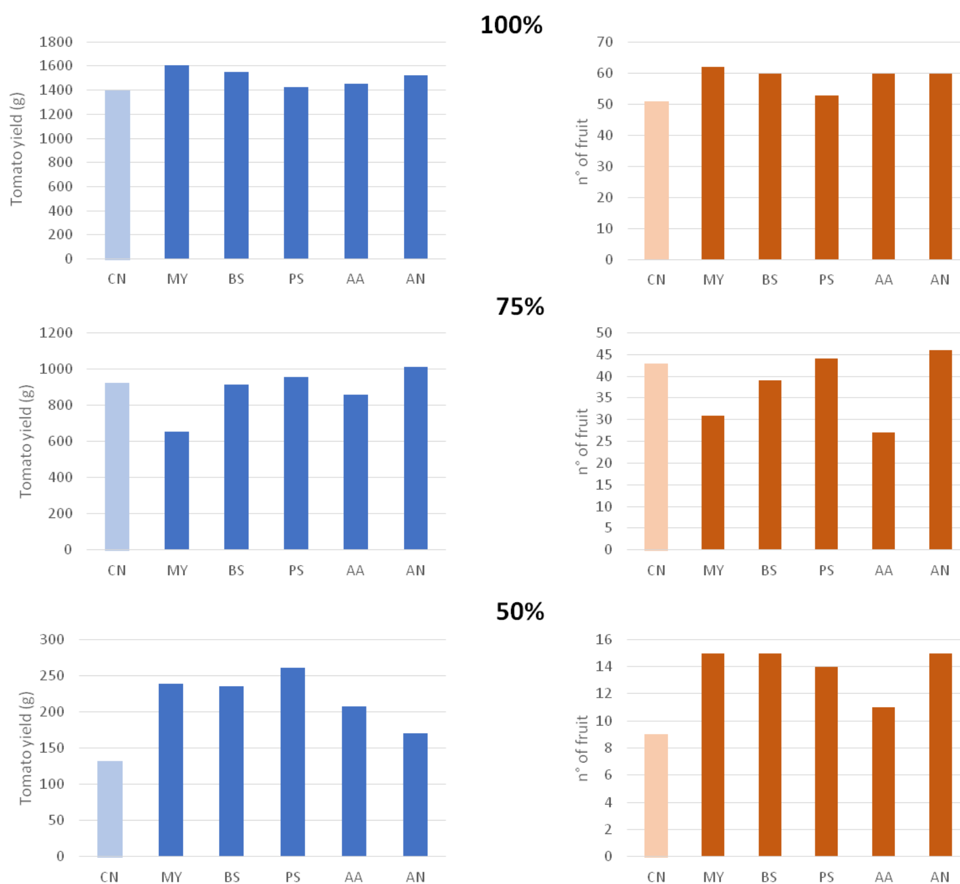


Figure 1. Fruits production of ten tomato plants for each treatment (CN=negative control, non-bacterized plant; plants inoculated with: MY= *Micrococcus yunnanensis* KMP123-M1; BS=*Bacillus simplex* RP-26; PS=*Pseudomonas stutzeri* SR7-77; AA=*Arthrobacter aurescens* 2-30; AN=*Arthrobacter nitroguajacolicus* 2-50). Histograms on the left show fruit yield of ten plants expressed in grams; histograms on the right show fruit production expressed in number of fruits produced by ten plants. Results are showed separately for the three different irrigation treatment: 100%=optimal irrigation state; 75% moderate water stress; 50% strong water stress.

and also by plants inoculated with *M. yunnanensis* KMP123-M1. All the PGP strains under strong water stress condition promoted the number of tomatoes produced by the plants and on average the weight of single tomato, with the exception of *A. nitroguajacolicus* 2-50 that produced almost

the double number of tomatoes but with a reduced weight compared to the non-inoculated plants (Figure 1, Figure 2). Moreover, tomato plants inoculated with PGP bacteria and irrigated with 50% of water needed, produced ripe fruits earlier than non-bacterized plants (Figure 3), accordingly to the significant effects observed on plant physiology. Earlier fruits production can thus represent a very important advantage especially considering the restrictive irrigation conditions in drought-affected countries.

Figure 2. Average weight (g) of each tomato produced under strong water stress condition (50%) by non-inoculated plant (CN) and plants inoculated with MY= *Micrococcus yunnanensis* KMP123-M1; BS=*Bacillus simplex* RP-26; PS=*Pseudomonas stutzeri* SR7-77; AA=*Arthrobacter aurescens* 2-30; AN=*Arthrobacter nitroguajacolicus* 2-50.

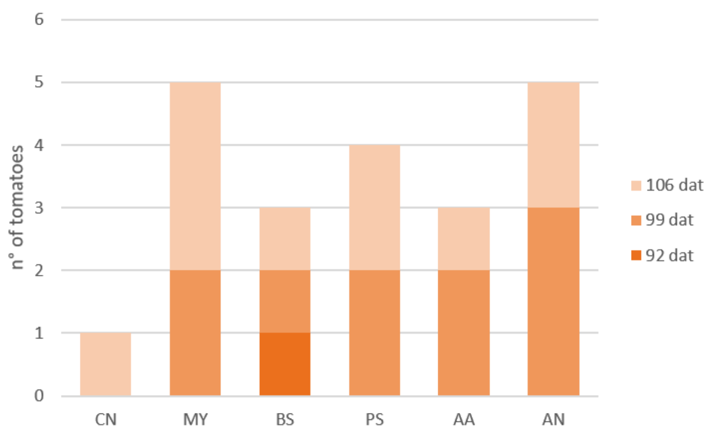


Figure 3. Number of ripe tomatoes produced under strong water stress (50%) during the first three harvest campaigns (92 day after transplantation (dat), 99 dat and 106 dat) by non-inoculated plants (CN) and plants bacterized with: MY= Micrococcus yunnanensis KMP123-M1; BS=Bacillus simplex *RP-26*; PS=Pseudomonas stutzeri *SR7-77*; AA=Arthrobacter aurescens 2-30; AN=Arthrobacter nitroguajacolicus 2-50.

To better evaluate the influence of PGP bacteria on tomato plants production under different water conditions, plant water productivity was calculated as the ratio of fruit yield/volume of water consumed by the plant (evapotranspiration). Bacterial inocula improved the water productivity of tomato plants both under optimal irrigation condition and under strong water stress, meaning that with the same volume of consumed water, plants were able to produce higher amount, in terms of gram, of tomatoes. The improvement in water productivity was statistically significant, supporting the speculation that the lack of significance obtained in evaluating only plant fruit yield could be overcome with a strongest experimental effort.

More specifically, at full irrigation condition, water productivity was significantly influenced by *M. yunnanensis* KMP123-M1, *B. simplex* RP-26 and *A. nitroguajacolicus* 2-50: these PGP treatments thus increased water productivity respectively by 28%, 42% and 43%. Under strong drought stress condition, significantly results were obtained with *B. simplex* RP-26 (81% of increase) and *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77 that increased water productivity by 100% (Figure 4). Bacteria able to promote water productivity are thus able to optimize plant water use efficiency and this result is of particular interests in countries threatened by water shortage conditions.

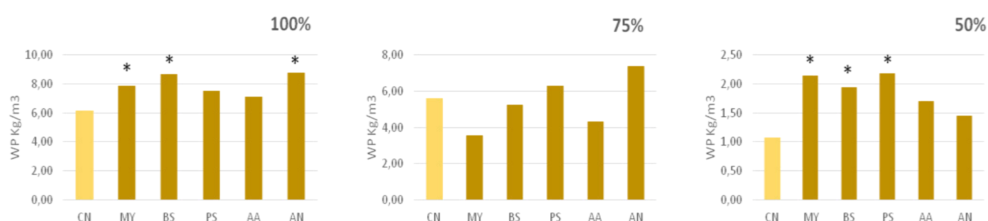


Figure 4. Effect of PGP bacteria inocula on water productivity (WP) of tomato plants under the three different water conditions (100%=optimal irrigation state; 75% moderate water stress; 50% strong water stress). CN=negative control, non-bacterized plant; plants inoculated with: MY= *Micrococcus yunnanensis* KMP123-M1; BS=*Bacillus simplex* RP-26; PS=*Pseudomonas stutzeri* SR7-77; AA=*Arthrobacter aurescens* 2-30; AN=*Arthrobacter nitroguajacolicus* 2-50. *$p<0.05$ vs negative control.

Basing on the obtained data, the two strains *M. yunnanensis* and *P. stutzeri* were considered most promising in improving plant growth and productivity especially under drought stress, and had been thus selected for a second in vivo long-term experimentation. Yield data were complemented by further characterization for additional traits of interest in the particular context of promoting agriculture in MAC regions by using treated wastewater (TWW) for irrigation.

3.2. Selection of the most promising strains for the second greenhouse experiment

The strains were firstly evaluated for their biofilm formation ability, a trait that could enhance the adhesion of the bacterial inocula to the plant root apparatus, thus in principle improving their colonization capacity (Ramey et al., 2004). As shown in Table 2, *M. yunnanensis* KMP123-M1 demonstrated a strong ability of surface adhesion (1.67 adhesion unit) compared to the *E. coli* ATCC25404 reference strain (0.50 adhesion unit). On the contrary, according to the classification proposed by Stepanovic et al. (2007), *P. stutzeri* SR7-77 and *B. simplex* RP-26 can be considered as weak biofilm producers (0.27 and 0.17 adhesion units respectively), while the two *Arthrobacter* strains seemed to have no adhesion competence (Table 2). The biofilm producer strains, *M. yunnanensis* KMP123-M1, *P. stutzeri* SR7-77 and *B. simplex* RP-26 should be favoured in initiate and maintain contact with the root apparatus of the host, thus improving the possibility to provide beneficial activity to the plant.

Since it is estimated that at least 20 million hectares of farmlands are globally irrigated with wastewaters (treated or, in a worst scenario, untreated) (Bouaroudj et al., 2019) as non-conventional water resources to alleviate water shortage, the five bacterial strains were further subjected to *in vitro* tests that characterized their ability to tolerate/degrade micropollutants that could be found in treated wastewaters (TWW). Several micropollutants

are frequently present at trace concentrations in wastewater because common wastewater treatment plants are not tailored for their removal (Gorito et al., 2017). In particular, in the present study we tested the bacterial ability to tolerate a mixture of metals and to degrade different dyes as micropollutants commonly present in urban and industrial wastewaters (Noreen et al., 2017, Zahran et al., 2019). All the five strains were able to grow on a rich medium containing zinc, nickel and cadmium at low concentrations while with at ten times more concentrated metal mixture, *P. stutzeri* SR7-77 and *A. nitroguajacolicus* 2-50 resulted sensible to the pollutants (Table 2). This high metal concentration nevertheless is highly exceeding the average concentrations found in wastewaters (Zn=5 mg/l, Ni=4 mg/l and Cd=0.2 mg/l; Kurniawan et al. 2006).
The ability to decolorize the model dye RB5 was not spread among the tested strains, since all the decolorization efficiencies were under 20% as showed in Table 2. On the contrary, the blue dyes used by a textile Tunisian company where well decolorized by all the strains, with the exception of *A. nitroguajacolicus* 2-50 that presented very low percentage of decolorization (0% for Tubantin blue and 12% of efficiency for S2-G blue). In particular *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77 showed a high decolorization percentage (> 40%) for both Tubantin blue and Blue-S2G (Table 2).
Furthermore, other emerging organic pollutants commonly found in wastewaters, that in the last decade are receiving growing attention, are antibiotic molecules (Berglund et al., 2014; Huang et al., 2017). Since they are not completely metabolized by human and animal's bodies, antibiotics are discharged in municipal wastewaters in their original or transformed species: since wastewater treatment plants are not specifically designed to remove antibiotics, they often escape the treatments and are release into the environment (Luo et al., 2014). Antibiotic resistance could be useful for the survival of bacteria used as inoculum for plant irrigated with TWW, however, the release of antibiotic resistant bacteria in the environment must be

avoided considering the increasing diffusion of antibiotic resistance genes and bacteria (Rizzo et al., 2013; Liang et al., 2018; Yan et al., 2018).

Table 2. Characterization of the five bacterial strains used as inoculants in greenhouse experiment. In vitro ability to bind the root apparatus: Biofilm = colorimetric assay for biofilm formation (adhesion unit of *E. coli* 172ATCC25404 = 0.504); Metal tolerance to: MIX1 = $ZnCl_2$ 20 mg/l, $NiCl_2$ 2 mg/l and $CdCl_2$ 0.1 mg/l and MIX2 = $ZnCl_2$ 200 mg/l, $NiCl_2$ 20 mg/l and $CdCl_2$ 1 mg/l; in vitro decolorization ability of RB5 = reactive black 5; T-blue = tubantin blue; S2-G blue = Blue-S2G at 100 mg/l; antibiotic resistance to RA = rifampicin, VA = vancomycin , CIP = ciprofloxacin; KF = cephalotin, TE = tetracycline, C = chloramphenicol. The "+"/"-" indicate that the bacterium is positive/negative to the test. R indicates the resistance to the antibiotic; S indicates the susceptibility to the antibiotics.

STRAIN	Biofilm (adeshion unit)	Metals tolerance		Dyes degradation			Antibiotic resistance					
		MIX1	MIX2	RB5	T-blue	S2-G blue	RA	VA	CIP	KF	TE	C
KMP123-M1	1,67	+	+	19%	45%	60%	S	S	S	S	S	S
RP-26	0,17	+	+	16%	13%	57%	S	S	S	S	S	S
SR7-77	0,27	+	-	18%	92%	43%	S	R	S	R	S	S
2-50	0,02	+	-	0%	1%	27%	S	S	R	S	S	S
2-30	0,00	+	+	17%	23%	64%	S	S	R	S	S	S

In the case that resistant bacteria are chosen as inoculum for in field application, further analysis must be performed to verify their ability to transfer genetic information to other bacteria of soil/plant community. The five bacteria considered in the experiment were thus analysed for their antibiotic susceptibility or resistance. *M. yunnanensis* KMP123-M1 and *B. simplex* RP-26 resulted susceptible to all the tested antibiotics while *A. nitroguajacolicus* 2-50 and *A. aurescens* 2-30 showed resistance to ciprofloxacin (5 µg) and *P. stutzeri* SR7-77 was resistant to vancomycin (30 µg) and cephalotin (30 µg) (Table 2).

According to the results obtained with *in vivo* experiment performed on tomato plants and the additional *in vitro* tested traits, the two PGP strains

selected for the second greenhouse *in vivo* experimentation were *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77. Both bacteria indeed demonstrated to significantly promote some physiological parameters of tomato plants under optimal irrigation condition and, particularly under strong water shortage condition (Table 1). At water regime 50%, *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77 showed the best results in terms of productivity: plants inoculated with these two strains showed the highest fruit yield in comparison to all the other bacterized and non-bacterized plants and a water productivity significantly promoted compared to the non-bacterized control plants. *M. yunnanensis* KMP123-M1, in particular, was the best strain in terms of additional traits like biofilm formation and dye decolorization, thus having the better probability to interact with the plant by adhering to the root apparatus and to survive in TWW containing micropollutants, by testing dyes as model contaminant. This strain resulted, moreover, tolerant to a metals mix even at high concentration and was sensitive to all the tested antibiotics, with strong advantage in terms of environmental safety. *P. stutzeri* SR7-77 showed resistance to two over six tested antibiotics, to make proper safety evaluations nevertheless further analysis are need to study i) the location of the genes in mobile genetic elements which could sustain their transferability, and ii) the ability of the strain to horizontally transfer this genetic information to other bacteria.

3.3. PGP bacterial survival in tap water and TWW

The two selected bacteria, *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77, were in addition tested for their ability to survive in tap water and in urban TWW for 40 days, in order to demonstrate their applicability in field whenever the bacterial inoculum would be distributed to crops through the irrigation system. Suitable formulation for bioinoculant are, in this case, needed to ensure long term viability of cells during storage (Compant et al., 2019) that can be restricted, for instance in liquid formulation, because of the lack of carrier protection (Herrmann and Lesueur, 2013). The strain survival

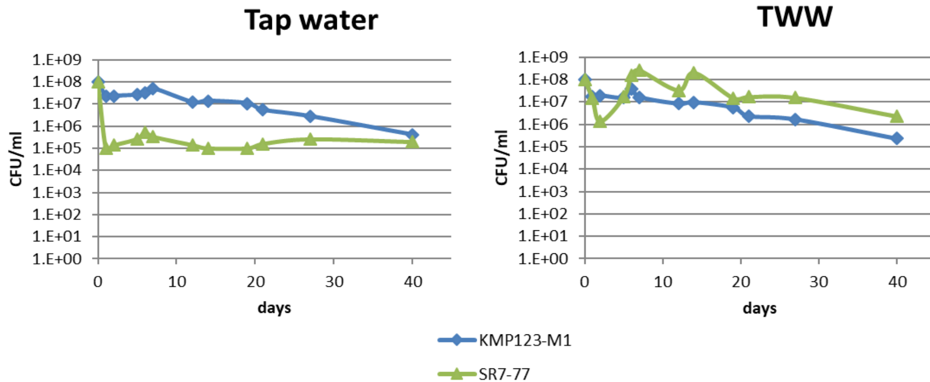


Figure 5. Bacterial cell counts of *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77 incubated in sterile tap water and urban TWW

over time would guarantee that the bacteria, when applied with irrigation water, would survive in the storage and distribution devices, arriving alive to the crops. According to the results, the two analyzed strains showed good ability to survive in both types of water: at the end of the incubation time the strains were present with a concentration of almost 10^5 cell/ml if inoculated in tap water and in urban wastewater (Figure 5). *M. yunnanensis* KMP123-M1 presented the same behavior in tap water and in urban TWW: in both cases its concentration gradually decreased from 10^8 cell/ml till almost 10^5 cell/ml after 40 days of experiment. For *P. stutzeri* SR7-77, a different behaviour was observed in tap water and in urban TWW. The bacteria resuspended in tap water drastically decreased after one day from 10^8 cell/ml to 10^5 cell/ml and maintained this concentration up to 40 days of incubation. When the strain was resuspended in urban TWW, again its concentration decreased from 10^8 cell/ml to 10^6 cell/ml in two days but during the following days it grew, achieving the starting concentration. Finally, the concentration gradually decreased till 10^6 cell/ml. The survival of *P. stutzeri* SR7-77 was better when the bacterial cells were suspended in municipal TWW rather than in tap water, hypothetically due to the presence of nutrient or other undefined preserving agents.

3.4. *M. yunnanensis KMP123-M1 and P. stutzeri* **SR7-77** *promotion of tomato plants under water shortage conditions*

M. yunnanensis KMP123-M1 and *P. stutzeri* SR7-77 strains have been further tested in a second round of *in vivo* experiment on tomato plants cultivated under optimal and water shortage irrigations. Physiological parameters of inoculated and non-inoculated control plants were measured at three different times corresponding to vegetative stage, early flowering stage and fruit setting stage. PGP bacteria had effect on plant physiology only during the fruit setting stage of plants cultivated with strong water stress condition (Figure 6). *M. yunnanensis* KMP123-M1 promoted leaf photosynthesis (45%), stomatal conductance (49%) and transpiration (36%) of tomato plants compared to the non-bacterized plants, but only data regarding stomatal conductance were supported by the statistical analysis ($p<0.05$). *P. stutzeri* SR7-77 increased significantly all the three physiological parameters by 94%, 120% and 73% respectively, as shown in Figure 6. Both strains revealed the capability to improve tomato plant physiology under strong water stress condition also during the first experiment, in particular during the flowering stage (Table 1). The comparison of results obtained in the two experimental trials demonstrated that inoculation with both the tested bacteria resulted in the improvement of plant physiological parameters, especially under drought stress conditions. In the different experiments the beneficial effect was nevertheless significant in different plant growing stages of the plants in the two different experiments, probably due to a difference in the experimental settings which was due to organizational constraint. The bacterization of tomato plants was indeed carried out 7 days after transplantation (dat) during the first experiment and 16 dat during the second one, so it is possible speculate that a delay of bacteria effect corresponded to a delay of plant treatment. Moreover, plants of the first experiment were bacterized a second time, at 75 dat, even if this treatment

seemed not to positively affect plant physiological parameters in the subsequent stage (Table 1).

The improvement of water use efficiency (WUE), calculated as the ratio of photosynthesis/transpiration, under full irrigation state and moderate water stress condition that was promoted during the first experiment, was not improved during the second one. The improvement (10%) of WUE was however visible in plants bacterized with *P. stutzeri* SR7-77 under strong water shortage conditions during fruits setting stage, but the data was not statistically significant (Figure 6).

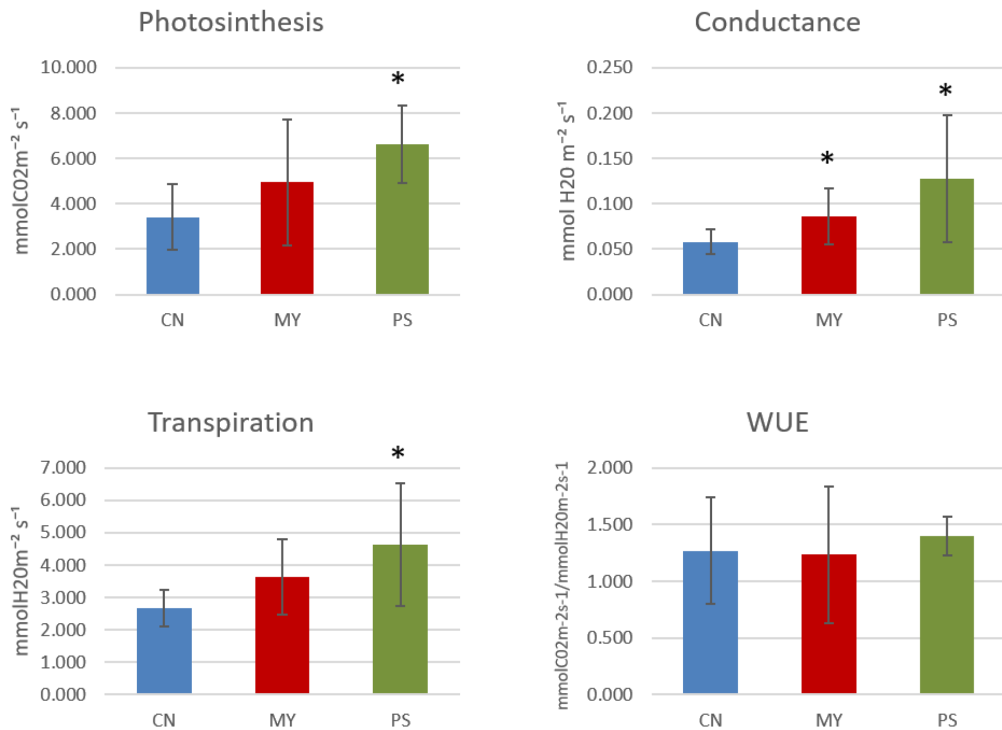


Figure 6. Effect of PGP bacteria on physiological parameters (leaf photosynthesis, stomatal conductance, transpiration) and on the measured plant water use efficiency (=WUE) on plant irrigated with strong water shortage (50%) at the third measuring campaign (fruit setting stage). CN= non-bacterized negative control; MY= *M. yunnanensis* KMP123-M1; PS=*P. stutzeri* SR7-77. * $p<0.05$ vs negative control.

To better investigate the effect of PGP bacteria on plants cultivated under strong drought stress during the second experimental trial, soil permeability

was measured at the end of the experiment as soil hydraulic property. According to statistical analysis, the permeability of the soil was strongly affected by both bacteria (Figure 7).

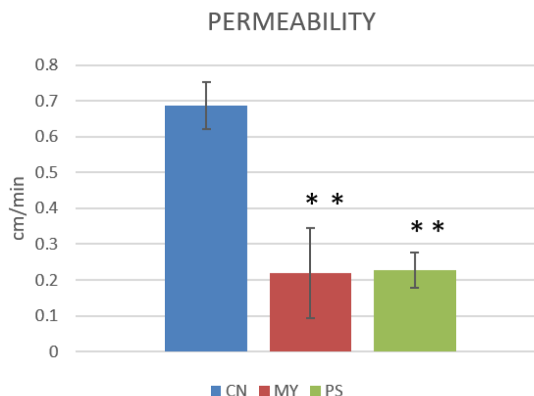


Figure 7. Effect of PGP bacteria on soil permeability under strong water stress (50%). CN= soil of non-bacterized plants; MY= soil of plants bacterized with *M. yunnanensis* KMP123-M1 (MY) and PS= soil of plats bacterized with *P. stutzeri* SR7-77 (PS). **$p<0.01$ vs negative control.

This parameter was clearly reduced (68%) in the soil surrounding the roots (SSR) of bacterized plants: from 0.7 cm/min in non-bacterized plant SSR to 0.2 in SSR plants inoculated with both strains. The decrease in soil permeability could due to the ability of PGP bacteria to produce exopolysaccharides (EPS) that increase soil aggregation and maintain higher water potential around the roots (Vurukonda et al., 2016): in this condition plants can thus uptake more water, nutrients and counteract drought stress. The ability of members of the species *M. yunnanensis* and *P. stutzeri* to produce EPS was indeed reported by several studies (Franzetti et al., 2011; Maaley et al., 2016; Onbasli and Aslim 2009; Sandhya et al., 2010). At the end of the experiment, plant growth parameters were measured and the results showed a promotion of root apparatus mass in plants inoculated with *P. stutzeri* SR7-77. The measured root dry weight was not improved by the presence of this strain, as we observed in the first experiment: this could mean that the effect of the bacterization changes the root architecture, thus affecting RSD, rather than improve the root biomass.

Root surface density (RSD) of plants bacterized with *P. stutzeri* SR7-77 were indeed significantly improved (85%) under drought stress conditions (75% and 50% treatment) in comparison to non-bacterized plants and were comparable to RSD of plants inoculated with the same bacteria and plant non-inoculated cultivated under optimal irrigation condition (Figure 8). These results could demonstrate that this PGP strain has the potential to alleviate drought stress in tomato plants.

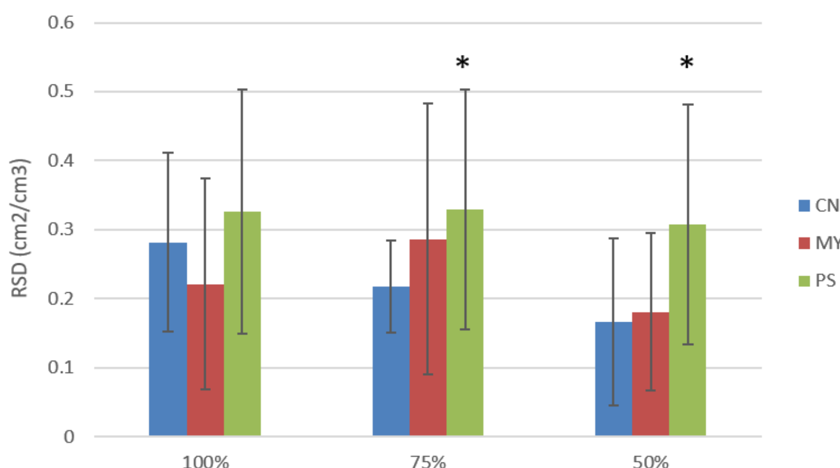


Figure 8. Root surface density (RSD) of tomato plants under different water conditions (100%=optimal irrigation state; 75% moderate water stress; 50% strong water stress) inoculated with *M. yunnanensis* KMP123-M1 (MY) and *P. stutzeri* SR7-77 (PS) and non-inoculated (CN). *p<0.05 vs negative control.

Plant productivity was also evaluated as tomato yield and water productivity. Under both conditions of drought stress the yield of plants inoculated with *M. yunnanensis* KMP123-M1 were higher (7% of increase at moderate water stress condition and 14% of increase at strong water shortage condition) than non-bacterized control plants (Figure 9). Both the strains increased also WP in bacterized plants compared to non-bacterized ones by 6% and 18%, respectively, under moderate water stress condition and by 20% and 12%, respectively, under strong water shortage (Figure 9). Data on tomato yield and WP were nevertheless not supported by statistical significance (Figure 9), leading to speculate that the strains could potentially improve plant

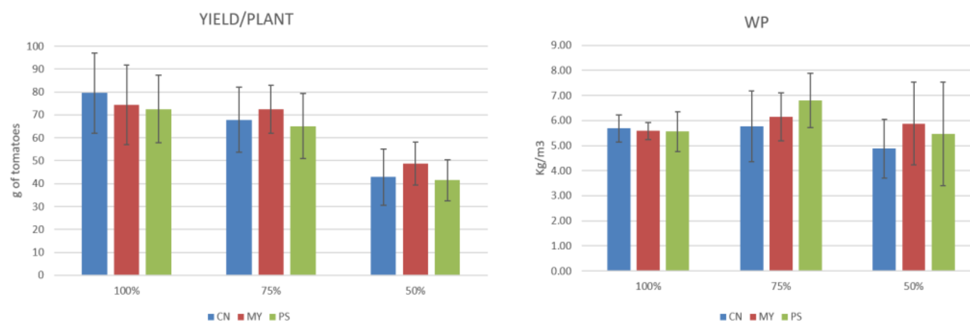


Figure 9. Fruit productivity of non-bacterized plants (CN) and plants inoculated with *M. yunnanensis* KMP123-M1 (MY) and *P. stutzeri* SR7-77 (PS) cultivated under optimal irrigation state (100%), moderate water stress (75%) and strong water stress (50%). The graph on the left represent yield expressed in grams of tomato per plant; the graph on the right represent water productivity (WP) calculated as the ratio yield/evapotranspiration.

productivity and water use efficiency in drought conditions, but the results would need further confirmation with supplementary long-term experimentations. It is necessary underline that the two experiments run in the present PhD thesis work were not performed with the same conditions because of several factors. Intrinsic environmental variations were due to the fact that the experiments were conducted in a semi-controlled greenhouse without temperature control, and during the first experiment run in 2017 the temperatures were particularly higher than in the same season in 2018. Moreover, other technical factors had been due to i) overall project needs and constraints, e.g. the repeated inoculation in the first experiment had been judged not applicable in the frame of the set up-of a low-cost technology, ii) externalization of the greenhouse related activities to other project partners with personnel constrains that resulted in a delay in plantlet transplant and bacterization.

4. Conclusion

Microbiota associated to root apparatus of extremophilic plants is gaining attention as source of bioinoculants for crop dealing with limited water availability. However, numerous studies performed short-term PGP experimentation on seedlings, hence there is a knowledge gap in the

evaluation of the effect of PGP bacteria on plant productivity: beneficial effects denoted in the first plant life stages do not necessarily become improvements in biomass and fruit productivity. With this study, performed with tomato plants subjected to different water regime conditions up to the end of their life cycle, we revealed that different PGP bacteria selected from plants adapted to extreme drought conditions or to very contaminated soil, positively affected tomato plants in physiological parameters, biomass, fruit productivity and water use efficiency. The effect was nevertheless i) different between the PGP strains, ii) differentially exerted under different water regimes, iii) exerted only in specific plant life stages, iv) highly dependent upon environmental conditions and experimental settings, v) not always statistically significant.

Even if a positive effect could be speculated, plants productivity was not significantly improved by none of the tested PGP isolates, which nevertheless showed a significant influence on of i) specific plant physiological parameters, i.e. photosynthesis, transpiration and stomatal conductance ii) root apparatus biomass and architecture iii) soil hydrology parameters. The beneficial effects were in particular more significant under water shortage conditions, indicating the potential capacity of the PGP bacteria to alleviate plant water stress.

The best performing strains were *M. yunnanensis* KMP123-M1 and *P. stutzeri* SR7-77 which demonstrated at some extent to improve plant physiology and productivity. Both the strains significantly promoted stomatal conductance of tomato plants cultivated under strong water deficit conditions and significantly improved soil hydrology by reducing its permeability. Furthermore, *P. stutzeri* SR7-77 significantly promoted leaf photosynthesis and transpiration of tomato plants and improved root biomass under strong water stress conditions. Through *in vitro* tests we showed, moreover, that these strains have additional traits that could make them promising candidates for the application to crops irrigated with recycled water like treated wastewaters. The strains demonstrated a good ability in biofilm

production, important bacterial trait for adhering to plant root apparatus, and showed tolerance to different micropollutants typically found in urban and industrial wastewaters and recalcitrant to traditional wastewater treatments.

The results obtained in this work underline the importance of performing long-term *in vivo* experiments to obtain reliable and applicable data about the effects that PGP inocula can exert on crop productivity. The obtained results were nevertheless strongly dependent upon the experimental setting, claiming further experimentation efforts.

References

Berglund, B., Khan, G. A., Weisner, S. E., Ehde, P. M., Fick, J., Lindgren, P. E., 2014. Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. Science of the total environment, 476, 29-37.

Bouaroudj, S., Menad, A., Bounamous, A., Ali-Khodja, H., Gherib, A., Weigel, D. E., Chenchouni, H., 2019. Assessment of water quality at the largest dam in Algeria (Beni Haroun Dam) and effects of irrigation on soil characteristics of agricultural lands. Chemosphere, 219, 76-88.

Brilli, F., Pollastri, S., Raio, A., Baraldi, R., Neri, L., Bartolini, P., Podda, A., Loreto, F., Maserti, B.E., Balestrini, R., 2019. Root colonization by Pseudomonas chlororaphis primes tomato (*Lycopersicum esculentum*) plants for enhanced tolerance to water stress. Journal of plant physiology, 232, 82-93.

Chen, C., Xin, K., Liu, H., Cheng, J., Shen, X., Wang, Y., Zhang, L. 2017. Pantoea alhagi, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat. Scientific Reports, 7, 41564.

Compant, S., Samad, A., Faist, H., & Sessitsch, A. 2019. A review on the plant microbiome: Ecology, functions and emerging trends in microbial application. *Journal of Advanced Research*.

Dodds, G.T., Trenholm, L., Rajabipour, A., Madramotoo C., Norris E. 1997. Yield and quality of tomato fruit under water table management. J. Am. Soc. Hortic. Sci. 122, 491–498

Eke, P., Kumar, A., Sahu, K. P., Wakam, L. N., Sheoran, N., Ashajyothi, M., Patel, A., Fekam, F. B. 2019. Endophytic bacteria of desert cactus (Euphorbia trigonas

Mill) confer drought tolerance and induce growth promotion in tomato (*Solanum lycopersicum L.*). Microbiological research, 228, 126302.

Ercin, A. E., Hoekstra, A. Y. 2014. Water footprint scenarios for 2050: A global analysis. *Environment international*, *64*, 71-82.

Falkenmark, M. 2013. Growing water scarcity in agriculture: future challenge to global water security. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, *371*(2002), 20120410.

Franzetti, A., Gandolfi, I., Bertolini, V., Raimondi, C., Piscitello, M., Papacchini, M., Bestetti, G. 2011. Phylogenetic characterization of bioemulsifier-producing bacteria. International biodeterioration & biodegradation, 65(7), 1095-1099.

Gagné-Bourque, F., Bertrand, A., Claessens, A., Aliferis, K. A., Jabaji, S. 2016. Alleviation of drought stress and metabolic changes in timothy (Phleum pratense L.) colonized with Bacillus subtilis B26. Frontiers in plant science, 7, 584.

Gorito, A. M., Ribeiro, A. R., Almeida, C. M. R., Silva, A. M. 2017. A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation. Environmental pollution, 227, 428-443.

Hasibeder, R., Fuchslueger, L., Richter, A., Bahn M. 2015. Summer drought alters carbon allocation to roots and root respiration in mountain grassland. New Phytol 205:1117–1127.

Herrmann, L. and Lesueur, D., 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. Applied microbiology and biotechnology, 97(20), pp.8859-8873.

Huang, X., Zheng, J., Liu, C., Liu, L., Liu, Y., Fan, H. 2017. Removal of antibiotics and resistance genes from swine wastewater using vertical flow constructed wetlands: effect of hydraulic flow direction and substrate type. Chemical Engineering Journal, 308, 692-699.

Kaushal, M., Wani, S. P. 2016. Plant-growth-promoting rhizobacteria: drought stress alleviators to ameliorate crop production in drylands. *Annals of microbiology*, *66*(1), 35-42.

Kurniawan, T.A., Chan, G.Y., Lo, W.H. and Babel, S., 2006. Physico–chemical treatment techniques for wastewater laden with heavy metals. Chemical engineering journal, 118(1-2), pp.83-98.

Le, T. A., Pék, Z., Takács, S., Neményi, A., Daood, H. G., Helyes, L. 2018. The effect of plant growth promoting rhizobacteria on the water-yield relationship and carotenoid production of processing tomatoes. HortScience, 53(6), 816-822.

Liang, Y., Zhu, H., Bañuelos, G., Shutes, B., Yan, B., Cheng, X. 2018. Removal of sulfamethoxazole from salt-laden wastewater in constructed wetlands affected by plant species, salinity levels and co-existing contaminants. Chemical Engineering Journal, 341, 462-470.

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang S., Wang, X. C. 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of the total environment, 473, 619-641

Maalej, H., Hmidet, N., Boisset, C., Bayma, E., Heyraud, A., Nasri, M. 2016. Rheological and emulsifying properties of a gel-like exopolysaccharide produced by Pseudomonas stutzeri AS22. Food hydrocolloids, 52, 634-647.

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D., Borin, S. (2013). Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *BioMed research international*, *2013*.

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., Abou-Hadid, A., El-Behairy, U., Sorlini, C., Cherif, A., Zocchi, G., Daffonchio, D. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PloS one*, *7*(10), e48479.

Marasco, R., Mapelli, F., Rolli, E., Mosqueira, M. J., Fusi, M., Bariselli, P., Reddy, M., Cherif, A., Tsiamis, G., Borin, S., Daffonchio, D. (2016). Salicornia strobilacea (synonym of Halocnemum strobilaceum) grown under different tidal regimes selects rhizosphere bacteria capable of promoting plant growth. *Frontiers in microbiology*, *7*, 1286.

Mayak, S., Tirosh, T., Glick, B. R. 2004a. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant physiology and Biochemistry*, *42*(6), 565-572.

Mayak, S., Tirosh, T., Glick, B. R. 2004b. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Science*, *166*(2), 525-530.

Ngumbi, E., & Kloepper, J. 2016. Bacterial-mediated drought tolerance: current and future prospects. *Applied Soil Ecology*, *105*, 109-125.

Noreen, M., Shahid, M., Iqbal, M., Nisar, J. 2017. Measurement of cytotoxicity and heavy metal load in drains water receiving textile effluents and drinking water in vicinity of drains. *Measurement*, *109*, 88-99.

Onbasli, D., & Aslim, B. 2009. Effects of some organic pollutants on the exopolysaccharides (EPSs) produced by some Pseudomonas spp. strains. Journal of hazardous materials, 168(1), 64-67.

Parida, A. K., & Jha, B. 2010. Salt tolerance mechanisms in mangroves: a review. *Trees*, *24*(2), 199-217.

Ramey, B. E., Koutsoudis, M., von Bodman, S. B., Fuqua, C. 2004. Biofilm formation in plant–microbe associations. Current opinion in microbiology, 7(6), 602-609.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., Fatta-Kassinos, D. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Science of the total environment, 447, 345-360.

Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M. L., Gandolfi, C., Casati, E., Previtali, F., Gerbino, R., Pierotti Cei, F., Borin, S., Sorlini, C., Zocchi, G., Daffonchio, D. 2015. Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environmental microbiology, 17(2), 316-331.

Sandhya, V., Ali, S. Z., Venkateswarlu, B., Reddy, G., Grover, M. 2010. Effect of osmotic stress on plant growth promoting Pseudomonas spp. Archives of microbiology, 192(10), 867-876.

Santos-Medellín, C., Edwards, J., Liechty, Z., Nguyen, B., Sundaresan, V. 2017. Drought stress results in a compartment-specific restructuring of the rice root-associated microbiomes. *MBio*, *8*(4), e00764-17.

Smith, S., and De Smet, I. 2012. Root system architecture: insights from Arabidopsis and cereal crops. Philos Trans R Soc Lond B Biol Sci 367: 1441–1452.

Soldan, R., Mapelli, F., Crotti, E., Schnell, S., Daffonchio, D., Marasco, R., Fusi, M., Borin, S., Cardinale, M. 2019. Bacterial endophytes of mangrove propagules elicit early establishment of the natural host and promote growth of cereal crops under salt stress. Microbiological research, 223, 33-43.

Soussi, A., Ferjani, R., Marasco, R., Guesmi, A., Cherif, H., Rolli, E., Mapelli, F., Ouzari, H.I., Daffonchio, D., Cherif, A. (2016). Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. *Plant and Soil*, *405*(1-2), 357-370.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., Ruzicka, F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis, 115(8), 891-899.

VanBuren, R., Wai, C. M., Ou, S., Pardo, J., Bryant, D., Jiang, N., Mockler, T., Edger, P., Michael, T. P. 2018. Extreme haplotype variation in the desiccation-tolerant clubmoss Selaginella lepidophylla. *Nature communications*, *9*(1), 13.

Vergani, L., Mapelli, F., Marasco, R., Crotti, E., Fusi, M., Di Guardo, A., Armiraglio, S., Daffonchio, D., Borin, S. (2017). Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. *Frontiers in microbiology*, *8*, 1385.

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., SkZ, A. 2016. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiological research*, *184*, 13-24.

Yan, W., Xiao, Y., Yan, W., Ding, R., Wang, S., Zhao, F. 2018. The effect of bioelectrochemical systems on antibiotics removal and antibiotic resistance genes: a review. Chemical Engineering Journal.

Yuan, Z., Druzhinina, I. S., Labbé, J., Redman, R., Qin, Y., Rodriguez, R., Zhang, C., Tuskan, G., Lin, F. 2016. Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. Scientific reports, 6, 32467.

Zahran, S. A., Ali-Tammam, M., Hashem, A. M., Aziz, R. K., Ali, A. E. 2019. Azoreductase activity of dye-decolorizing bacteria isolated from the human gut microbiota. Scientific reports, 9(1), 5508.

Chapter VI

Root Bacteria Recruited by *Phragmites australis* in Constructed Wetlands Have the Potential to Enhance Azo-Dye Phytodepuration

[Published in *Microorganisms*, *7(10), p.384.* Riva, V., Mapelli, F., Syranidou, E., Crotti, E., Choukrallah, R., Kalogerakis, N. and Borin, S., 2019. Root Bacteria Recruited by Phragmites australis in Constructed Wetlands Have the Potential to Enhance Azo-Dye Phytodepuration.]

Abstract

The microbiome associated with plants used in phytodepuration systems can boost plant growth and services, especially in ecosystems dealing with recalcitrant compounds, hardly removed via traditional wastewater (WW) treatments, such as azo-dyes used in textile industry. In this context, we aimed to study the cultivable microbiome selected by *Phragmites australis* plants in a Constructed Wetland (CW) in Morocco, in order to obtain candidate inoculants for the phytodepuration of azo-dye contaminated WW. A collection of 152 rhizospheric and endophytic bacteria was established. The strains were phylogenetically identified and characterized for traits of interest in the phytodepuration context. All strains showed Plant Growth Promotion potential in vitro and 67% of them significantly improved the growth of a model plant in vivo compared to the non bacterized control plants. Moreover, most of the isolates were able to grow in presence of several model micropollutants typically found in WW, indicating their potential use in phytodepuration of a wide spectrum of effluents. The six most promising strains of the collection were tested in CW microcosms alone or as consortium: the consortium and two single inocula demonstrated to significantly increase the removal of the model azo-dye Reactive Black 5 compared to the non bacterized controls.

1. Introduction

The textile industry is one of the major producers of liquid effluent pollutants, due to the large quantity of water used in its production processes (up to 150 L of water to dye 1 kg of cotton) [1]. In fact, during the dyeing process, a significant fraction of dyes does not bind to the fibers and is released as effluent into the textile wastewaters. Azo-dyes, which are the most used dyes in the textile manufacturing, are xenobiotic compounds highly recalcitrant to degradation processes: their improper discharge in aqueous ecosystems leads to a reduction in water sunlight penetration, which decreases photosynthetic activity and dissolved oxygen concentration, besides having

toxic effects on aquatic flora and fauna [2]. These synthetic molecules, which are also applied in other commercial sectors such as printing, cosmetics and food industries [3,4], represent a relevant environmental and health issue, in particular in those countries where wastewater is used for irrigation purposes without prior proper treatments [3]. Reactive azo-dyes are recalcitrant to conventional wastewater treatment processes and in some cases up to 90% of these molecules could remain unprocessed after activated sludge treatment [5]. Moreover, the specific treatment required to implement the removal of azo-dyes in wastewaters [6–8] are in most cases expensive and cannot be incorporated in conventional treatment processes [9].

In the Middle East and North Africa (MENA) region, the textile industry represents a relevant sector for the local economy: textile production provides 6% of Gross Domestic Product (GDP) in Egypt and 7% in Morocco and Tunisia [10]. Since this region suffers water scarcity, the use of wastewater treatment plant final effluent for irrigation purposes is a common practice that is expected to increase in parallel to the population and economy growth [10]. However, the quality of treated wastewater is still poor in the MENA countries and many pollutants are insufficiently removed because of the lack of tertiary treatments and the poor plant maintenance: as a result, sewages are often directly discharged into the rivers [11,12]. A promising solution, especially for low-income countries, is represented by Constructed Wetland (CW) systems that exploit biological treatments for the depuration of wastewaters. Compared to the common physicochemical processes used for the removal of azo-dyes from textile wastewater effluents [13], CWs are low cost systems that can be easily operated and maintained because they do not require additional energy and chemicals [14].

CWs are engineered systems that take advantage of purifying processes naturally occurring in wetlands where chemical, physical and biological processes may spontaneously take place at the same time, thanks to the different components of the system and their interactions [15–17]. Soil/sediments and plant root apparatus can improve water quality by

sorption, sedimentation, phytodegradation and plant uptake of organic and inorganic compounds. Moreover, these matrices provide a large surface area for the growth of complex and diverse microbial communities that play a pivotal role in wetland biological processes [18–20].

CWs demonstrated to efficiently decrease the concentration of conventional wastewater components such as organic carbon (chemical and biochemical oxygen demand), total suspended solid (TSS) and nutrients (e.g., ammonia and phosphorus) [14,15]. Recent studies showed that micropollutants, including emerging organic contaminants (EOCs) like pharmaceuticals and personal care products, plasticizers and heavy metals can be removed in CWs [21–23]. The biological removal of synthetic dyes was recently showed in floating treatment wetland mesocosms carrying *Phragmites australis* in combination with three dye-degrading bacteria [24]. Furthermore, a *Bacillus* strain, isolated from the rhizosphere of sorghum plants grown in textile wastewater contaminated soil, efficiently decolorized different azo dyes and was indicated as a powerful inoculant for the bioremediation of textile wastewaters [2].

In CW systems, although plants play a significant role in direct uptake of pollutants from wastewaters, the processes of transformation and mineralization of nutrient and organic pollutants greatly depend on the microbial communities associated to their root systems [18]. The root associated microbiome could also counteract the effects on plants (i.e., lower growth and performance) caused by the stressful environmental conditions occurring in phytodepuration systems, mainly due to pollutants′ presence. In this context, the exploitation of Plant Growth Promoting (PGP) bacteria can be a promising strategy for the improvement of CW services. Syranidou and coworkers [25] demonstrated that the bacterial community of *Juncus acutus* plants in a pilot CW study treating bisphenol A (BPA)-contaminated groundwater was enriched in strains with PGP traits that showed tolerance to high concentration of metals and ability to degrade different organic compounds comprising BPA.

The identification and functional characterization of the microorganisms associated to plants commonly used in CW systems is therefore fundamental, not only to predict the degradation potential of the process, but also to find new powerful microbial inoculants that could ameliorate plant services and the overall technology efficiency [22]. In this work, we studied the cultivable fraction of the bacterial community associated to the root system of *Phragmites australis* (common reed), which is widely used for phytodepuration given its ability to grow in different freshwater environments, including the most polluted ones, and to absorb many contaminants thanks to its high lignin and cellulose content [20]. *P. australis* samples collected in a CW plant as a tertiary treatment of municipal WWs were used to establish a collection of bacterial isolates that has been identified and characterized for the ability i) to promote plant growth, ii) to decolorize dyes and iii) to tolerate model micropollutants (heavy metals, bisphenol-A). Basing on the screening results, the most promising strains were selected for a microcosm-scale CW experiment to test their suitability as candidate inoculants for the specific removal of the azo-dye Reactive Black-5 from synthetic textile wastewaters.

2. Materials and Methods

2.1. Sample Collection

Phragmites australis root systems were sampled from triplicate plants growing in a Constructed Wetland plant located in Drarga (Souss-Massa region, southern Morocco) used as municipal wastewater tertiary treatment.

Samples were collected using sterile tools and transferred to the laboratories of the University of Milan within 48 hours. The rhizosphere soil — defined as soil particles tightly adhering to roots (1–3 mm) — was separated from the root tissues in sterile conditions according to the procedure described by Marasco et al. [26].

2.2. Bacteria Collection Establishment

The replicated samples (n = 3) of *P. australis* roots and rhizospheric soil were pooled and homogenized prior to endophytic (from surface sterilized roots) and rhizospheric (from rhizosphere) bacteria isolation.

To obtain the endophytic bacterial collection, roots collected from 3 plant specimens (around 1 g/specimen) were vigorously washed in physiological solution (0.9% NaCl) for 10 min and surface-sterilized in 70% ethanol for 3 min, 1% sodium hypochlorite for 5 min, 70% ethanol for 30 s and rinsed four times for 2 min in sterile distilled water before a final washing step in sterile distilled water for 30 min. A 100 µL sample of the last rinsing water was plated on the same medium subsequently used for bacteria isolation, 1:10 869 medium [27], to confirm root surface sterility. Finally, roots were smashed with sterile mortar and pestle. One gram of the resulting root tissue homogenate was suspended in 9 mL of physiological solution, serially diluted and plated in triplicate on 1:10 869 medium prepared by using the 0.22 µm pore size filter-sterilized treated wastewater (TWW) obtained from the largest municipal wastewater treatment plant of Milan municipality (Milano-Nosedo, Northern Italy) and supplemented with cycloheximide 0.1 g/L to prevent fungal growth. Rhizosphere soil samples collected from the same specimens were pooled (1 g/specimen) and 1 gram of homogenized soil (fresh weight) was subjected to the same protocol described for the root tissue homogenate. After 48 hours of incubation at 30 °C, the number of colony-forming units (cfu) per gram of roots/soil was determined and, for each sample, bacteria colonies were randomly picked and spread three times on the same medium in order to obtain pure bacterial cultures [28]. A collection of 80 endophytic and 72 rhizospheric bacteria was established and cryopreserved in 25% glycerol stocks at −80 °C. The isolates were labeled with codes including sampling site (′CWM′ for Constructed Wetland in Morocco), plants species (′P′ for *P. australis*), medium used for the isolation (′8′ for 1:10 869) and the fraction (′R′/′E′ for rhizosphere/endosphere) followed by progressive numbers.

2.3. Bacteria Genotyping and Identification

The genomic DNA of each isolate was extracted through boiling cell lysis [29]. The bacteria collection was de-replicated by fingerprinting analyses of the 16S–23S rRNA Intergenic Transcribed Spacer (ITS) region performing the ITS-PCR protocol described by Mapelli et al. [30]. The PCR products were separated on 1.5% agarose gel and ITS-fingerprinting profiles were visualized using Gel Doc system (Bio-Rad, Milan, Italy). Isolates which showed the same ITS band pattern were grouped in ITS clusters. At least one representative strain per each ITS cluster has been selected for subsequent physiological characterizations and for taxonomic identification through 16S rRNA gene amplification using the universal primers 27F and 1492R as described by Mapelli et al. [30]. Partial 16S rRNA sequences were obtained from Macrogen, Rep. of South Korea. Nucleotide sequences were edited in Chromas Lite 2.01 and compared with those deposited in the GenBank database, using the BLAST suite. The partial 16S rRNA gene sequences of the bacterial isolates were deposited in the EBI database under the accession numbers LS991341-LS991401.

2.4. In Vitro Screening of Plant Growth Promoting Activities and Rhizocompetence Potential

The screening of Plant Growth Promoting (PGP) traits in the bacterial isolates was focused on biostimulation and rhizocompetence-related activities. For biostimulation indolacetic acid (IAA) production was assessed following the protocol described by Bric et al. [31] and the ACC-deaminase (ACC-d) activity was determined by the method of Penrose and Glick [32] using Salt Mineral medium supplemented with ACC as unique N source. Protease activity was determined from clearing zones in skimmed milk agar according to Nielsen and Sørensen [33]. The rhizocompetence potential of the isolates was established by applying different in vitro analyses. First, the production of exopolysaccharides (EPS) was evaluated according to Santaella et al. [34] using modified Weaver mineral media enriched with sucrose. Swimming and

swarming lifestyles were considered to evaluate bacterial mobile pattern using solid medium prepared with 0.3% and 0.5% of agar according to Mi et al. [35]. The biofilm production capacity of the isolates was evaluated by a colorimetric assay based on crystal violet staining, as described below [36]. Two-hundred µl of bacterial suspension at 10^6 cell/ml were added in 96-wells microtiter and after 24 h of incubation the optical density (OD) was measured at 610 nm using a microtiter-plate reader (Tecan Infinite F200Pro). The supernatant was removed, the wells were washed twice with 200 µL of PBS and let dry for 15 min before the staining with 200 µL of crystal violet (diluted 1:10 with EtOH) for 15 min. The wells were then washed twice with distilled water and let dry for 15 min. Crystal violet was re-suspended with EtOH 97% and OD was measured at 610 nm. The OD measure was compared with the absorbance of *E. coli* ATCC25404 used as positive control and the quantity of formed biofilm was expressed as percentage. Each assay was performed in triplicate. The non inoculated medium was stained as well with crystal violet and used as negative control to determine background OD.

2.5. Plant Growth Promotion of Model Plants Under Liquid Substrate Culture and of Juncus acutus Plants in So il

Bacterial isolates were grown in Tryptic Soy Broth (TSB) for 24 h at 30 °C, the bacterial cells were centrifuged at 4000 rpm for 15 min and the pellet was re-suspended with 10mM $MgSO_4$ to obtain a bacterial concentration of 10^8 cell/mL. *Lycopersicon esculentum* seeds, chosen as model plant, were sterilized with 70% ethanol for 3 min and then 5% sodium hypochlorite for 10 min followed by five rinsing steps in sterile distilled water. Surface-sterilized seeds were soaked for 1 h in the bacterial suspension at a concentration of 10^8 cell/ml; non-inoculated seeds were watered with sterile distilled water as negative control. Treated seeds were transferred to germination pouches (CYG Seed Germination Pouches, Mega International, Minneapolis) under sterile condition (seven seeds per pouch). Five pouches per each bacterial strain/negative control were established. The pouches were watered with 20

mL of sterile tap water and placed under greenhouse conditions. After 20 days, different morphometric parameters of plants were recorded: seed germination, root length, shoot length, secondary root development and vigor index (i.e., % of germination X seedling length) of the seedlings.

Plant growth promoting test was also performed with *J. acutus* plants in soil under greenhouse conditions. Four seedlings of the same weight were bacterized with a mixture of the six most promising strains (10^8 cell/mL) and four non-inoculated plants were used as negative control. After 45 days of growth, root and shoot lengths, root and shoot fresh and dry biomasses were measured; chlorophyll leaf content was measured according to Sharma et al. [37] method. All original data related to the in vivo PGP tests reported in this work are available within the Dataverse ′madforwater-wp2′ created by the University of Milan at the following link: https://doi.org/10.13130/RD_UNIMI/VXKHSF.

2.6. Bacterial Tolerance to Metals and Emerging Organic Pollutants (EOP)

The isolates were tested for their ability to grow in the presence of increasing concentration of $CdCl_2$ (0.05, 0.5 and 1 mM), $ZnCl_2$ and $NiCl_2$ (0.5, 1 and 2 mM) and all the three metals mixed together at two different concentrations ($CdCl_2$-$ZnCl_2$-$NiCl_2$: 0.1 µM-30 µM-1.7 µM; 0.5 µM-150 µM-8.5 µM). The bacterial ability to tolerate metals was investigated in a 96 wells-microtiter with 180 µL of TSB medium (with and without metals) and 20 µL of bacterial culture grown until the late exponential phase. Each strain was tested in triplicate. The absorbance at 610 nm was measured with a microtiter-plate reader (Tecan InfiniteF200Pro) at time zero and after 24 and 48 h of incubation at 30 °C. Bacterial tolerance to Bisphenol-A (BPA) was also evaluated. After bacterial cultivation in TSB medium, 1 mL of culture broth was centrifuged at 4000 rpm for 15 min. The cell pellet was washed three times with sterile 10 mM $MgSO_4$ and 100 µL of bacterial culture were spread on 284 solid medium [25] with or without the supplement of 100 µL of 100 mg/l BPA spread on the

plate as a sole carbon source. The plates were incubated at 30 °C for 7 days. Antibiotic susceptibility test of the strains was performed with a disk-diffusion assay using 6 different antibiotics (the molecules were selected to span several mechanisms of action): cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg) (LABORATORIOS CONDA S.A., Madrid, Spain). After 24 h of incubation at 30 °C, bacteria were classified as sensitive or resistant according to the interpretative standards provided by LABORATORIOS CONDA S.A. using *E. coli* ATCC 25,922 as reference strain.

2.7. In Vitro Study of Bacterial Dye Decolorization Potential

Reactive Black 5 (dye content ≥ 50%, Sigma-Aldrich, RB5) is the commonly used azo-dye chosen as model molecule to examine bacterial decolorization capability [3]. Rhizospheric strains were thus tested for RB5 decolorization capacity, while a selection of these rhizospheric strains was also tested for the capacity to decolorize different dyes used by a textile company located in Tunisia: Bezactive rouge S-Matrix, Tubantin blue and Blue S-2G. Bacterial inocula were added to TSB liquid medium containing 100 mg/l of dye. At time zero and after 72 hours of incubation at 30 °C, 1 mL of the culture broth was centrifuged at 8000× *g* for 5 min and the absorbance of the supernatant was measured using a spectrophotometer (597 nm for RB5, 522 nm for Bezactive rouge S-Matrix, 589 nm for Tubantin blue and 615 nm for Blue S-2G). Each strain was tested in triplicate and a non-inoculated negative control was assessed. Decolorization efficiency was calculated, according to Wang et al. [4], as:

$$\text{Decolorization efficiency (\%)} = \frac{\mathbf{OD_0 - OD_1}}{\mathbf{OD_0}} \times \mathbf{100}$$

where OD_0 referred to the initial absorbance and OD_1 referred to the absorbance after 72 h of incubation. The absorbance value was then related to dye concentration according to the standard curve obtained with known dye concentrations ranging from 2.5 to 150 mg/L.

2.8. Bioaugmentation of Juncus acutus Microcosms for the Treatment of Mixed Contamination

Plants of *J. acutus* were collected from Souda bay (Chania, Crete, Greece) and washed with freshwater. The ability of *J. acutus* to grow in presence of single and mixed contamination was estimated under greenhouse conditions in glass vessels covered with aluminum foil and filled with 950 g of gravel to support the plant (around 25 g). During this first experiment, four conditions were setup in 350 ml-replicate vessels (n = 3), differing in the liquid phase content: (a) RB5 50 mg/L; (b) RB5 10 mg/L + metals mixture ($CdCl_2$-$ZnCl_2$-$NiCl_2$: 0.5 µM-150 µM-8.5 µM); (c) RB5 5 mg/L + metals mixture ($CdCl_2$-$ZnCl_2$-$NiCl_2$: 0.5 µM-150 µM-8.5 µM); (d) tap water as control. After three weeks of growth, the health status of the plants irrigated with the contaminated solutions was compared to that of the control plants, assessing the capacity of *J. acutus* to cope with the dye and metal presence.

After this procedure, a new experiment was setup using six bacterial strains as single inoculum (10^8 cell/mL) and as a consortium with *J. acutus* plants (around 19 g) growing in gravel as substrate (390 g) in modified beakers (Figure 1). Three replicated microcosms per each bacterial treatment were prepared. The control microcosms were setup with *J. acutus* plants without bacterial inoculation. Each microcosm was filled with 150 mL of a solution containing RB5 10 mg/L and metals ($CdCl_2$-$ZnCl_2$-$NiCl_2$: 0.5 µM-150 µM-8.5 µM). In order to simulate the operational conditions of a constructed wetland system, every day the microcosms were irrigated with 25 mL of the solution described above, freshly prepared, and the effluents were collected measuring the concentration of RB5 (as explained above). After 27 days of operation the plants were harvested and the fresh and dry weight of *J. acutus* roots and shoots were measured to determine the in vivo PGP ability of the strains.

Figure 1. Schematic representation of the CW-microcosm with *J. acutus* plant and gravel as substrate. The pipette on the left allows to introduce the contaminated irrigation solution into the beaker and the tube on the right allows to collect the microcosm effluent for further analyses.

3. Results

3.1. Cultivable Bacteria Associated to Phragmites australis in Constructed Wetlands Treating Municipal Wastewater

A collection of 80 endophytic and 72 rhizospheric bacteria was obtained from *P. australis* surface-sterilized roots and rhizospheric soil, respectively. The abundance of cultivable bacteria was similar in the rhizosphere and in the endosphere fractions: $2.5 \times 10^8 \pm 4.6 \times 10^7$ and $1.2 \times 10^8 \pm 4.4 \times 10^7$ cfu per gram of fresh soil/root respectively.

The ITS fingerprinting analysis was applied to de-replicate the bacterial collection and reduce its phylogenetic redundancy. Forty-five ITS profiles were recognized within the rhizospheric isolates and 16 ITS profiles within the endospheric ones. The strains, identified by partial 16S rRNA sequencing, belonged to 4 phyla, namely *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Supplementary Table S1). The most abundant genera in the overall collection were *Bacillus* and *Pseudomonas*, representing 41% and 18% of the strains, respectively. Among the two fractions of the *P. australis* root system, endosphere and rhizosphere, we nevertheless observed a different distribution of the bacterial phylogenetic groups (Figure 2A). The

endophytic strains showed reduced phylogenetic diversity, belonging only to six genera. 78% of the endophytic strains were assigned to the genera *Bacillus* and *Pseudomonas*: 89% of the *Bacillus* isolates belonged to *B. pumilus* and the 71% of the *Pseudomonas* isolates was identified as *P. thivervalensis.* Thus, a limited and peculiar community colonized the endosphere fraction of *P. australis*. In contrast, the rhizosphere fraction showed a higher bacterial diversity. 57% of the rhizosphere strains were grouped within the *Firmicutes* phylum while the other isolate strains of the rhizosphere were equally distributed among *Actinobacteria* (14%), *Bacteroidetes* (14%) and *Proteobacteria* (15%) phyla (Figure 2B). Apart from the species *B. thuringiensis*, that included the 24% of the rhizobacteria isolates, the other species present in the rhizosphere were represented by few or single isolates, which explains the high bacterial variability into this fraction. Only 8 endophytic isolates were considered as potentially pathogens for humans and plants according to their taxonomy and to a reference document provided by the German Committee on Biological Agents that classifies Prokaryotes into Risk Groups [38]: these potentially pathogens isolates belonged to the species *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* (Supplementary Table S1) and were excluded from the collection given their low suitability for future applications in the field linked to safety issues.

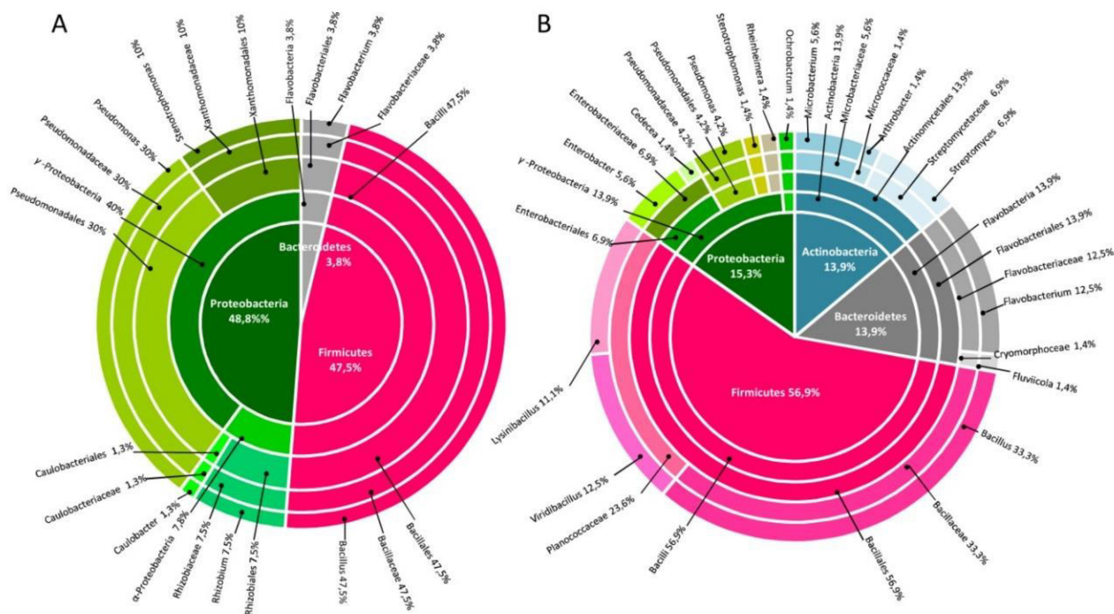


Figure 2. Taxonomic distribution of 16S rRNA sequences of culturable endophytic (**A**) and rhizospheric (**B**) bacteria isolated from *P. australis* plants. The inner pie graph shows the strain taxonomic identification according to phylum and each outer ring represents their identity at the class, order, family and genus level.

3.2. PGP Potential of P. australis Associated Bacteria

The potential of the bacterial strains associated to *P. australis* to sustain plant growth was assessed by screening in vitro the PGP traits potentially involved in root development, a key aspect for CW plants, which are threatened by different abiotic stresses [39]. In particular, we assessed whether the isolates were able to produce indolacetic acid (IAA), a phytohormone that promotes the root apparatus growth [40], and whether they displayed ACC-deaminase

activity that potentially reduces the deleterious effect of ethylene, the plant stress-related hormone, by degrading its precursor ACC [41]. 72% of the strains were able to produce IAA and 67% showed ACC-deaminase activity, while 53% showed to have both the activities. Despite the difference in phylogenetic composition, these two PGP-related traits resulted similarly represented among endosphere and rhizosphere isolates (Figure 3). Moreover, the isolate collection was tested for additional traits that could sustain the capacity of the strains to degrade WW organic material and to move into the environment and adhere to the root surface. In total, 41% of the strains were positive to protease activity test and 14% of the isolates produced EPS, mainly represented by endophytes. The swimming and swarming lifestyle (Figure 3) was shown by 26% and 24% of the isolates, respectively, most of which belonging to the *Firmicutes* phylum, and it was more widespread among the rhizosphere strains. *Bacillus*, *Lysibacillus* and *Pseudomonas* showed to predominantly hold these PGP traits among the different genera (Supplementary Table S2).

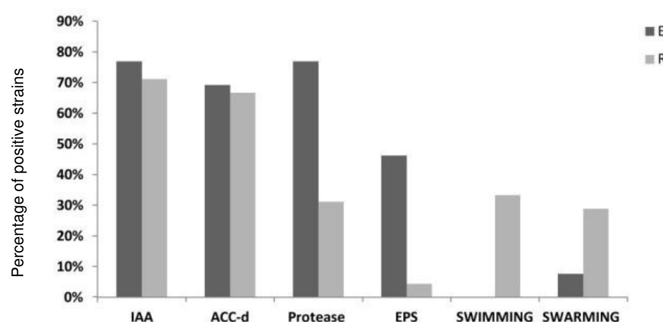


Figure 3. Characterization of the bacteria collection for plant growth promotion (PGP) traits. Percentage of strains isolated from endosphere (E, black) and rhizosphere (R, gray) fractions that resulted positive to PGP assays. Among PGP activities: IAA = indole-3-acetic acid production, ACC-d = ACC deaminase activity, Protease = protease production, EPS = exopolysaccharides production, SWIMMING = swimming lifestyle, SWARMING = swarming lifestyle.

The PGP capability of *P. australis*-associated bacteria was also tested in vivo under liquid substrate culture using *L. esculentum* as model plant bacterized on seeds (Supplementary Table S4). Thirty-nine of the 58 tested isolates (3 endophytic and 36 rhizospheric strains) showed significant growth promotion in tomato inoculated plants in comparison to non-inoculated ones. Among the different measured parameters, the most positively affected by the bacteria inoculation were the shoot length (Supplementary Figure S1) and the vigor index of the seedling: 31 bacteria significantly increased shoot length and 23 bacteria improved the seedling vigor index. Moreover, 18 strains improved root length, 7 different rhizobacteria enhanced the root dry weight and other 5 strains enhanced the number of secondary roots. Finally, 5 strains increased the percentage of germination. Most of the bacteria with PGP capability on tomato plants improved at least two physiological parameters and the strains *Viridibacillus arenosi* CWMP-8R10 and *Bacillus megaterium* CWMP-8R7 enhanced five out of the six assessed parameters.

3.3. Metal and Organic Pollutant Tolerance and Degradation Potential of the Isolated Bacteria

The bacterial collection proved to include a high number of metal-resistance strains. Among the tested isolates, 15 different metal-resistance phenotypes were detected (Supplementary Table S3). All the strains were able to grow on rich medium containing the three metals in micromolar concentrations supplemented simultaneously ($ZnCl_2$, $NiCl_2$ and $CdCl_2$, 30 µM, 1.7 µM and 0.1 µM respectively) (Figure 4) and 12% of the isolates were tolerant to the three metals (Cd, Ni and Zn) at all the tested concentrations, up to 1–2 mM. The large majority of the isolates resulted tolerant to Zn and Ni, while on the contrary, the sensitivity to Cd was very high and only 30% of the strains was able to grow in a medium containing this metal at the lowest concentration (0.5 mM $CdCl_2$) (Figure 4). The tolerance to $CdCl_2$ was further assessed at a

concentration lower than 0.5 mM, allowing the identification of strains tolerant to 0.05 mM $CdCl_2$ (74% of the collection).

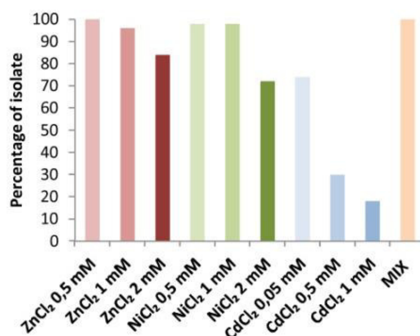


Figure 4. Characterization of the bacteria collection for metal tolerance. Percentage of strains tolerant to $ZnCl_2$, $NiCl_2$ and $CdCl_2$ at three different concentrations and to the MIX of metals (30 µM $ZnCl_2$, 1.7 µM $NiCl_2$ and 0.1 µM $CdCl_2$).

The ability of the strains to tolerate organic pollutants was assessed on model micropollutants typically found in municipal and industrial wastewaters like BPA and antibiotics. All the rhizospheric bacteria and 77% of the endophytic bacteria showed tolerance to 100 mg/L BPA supplemented as unique carbon source to the growth medium (Supplementary Table S4, Table 2). However, these bacteria grew also on control plates with mineral medium and no addition of BPA, so probably they tolerated BPA presence, but they do not have the ability to use it as carbon source. Antibiotic resistance was also widespread within the bacterial collection, since only 24% of the isolates were inhibited by all the tested antibiotics. None of the strains were resistant to rifampicin, but the resistance to the other antibiotics was detected in the majority of the strains. In particular, the resistance to ciprofloxacin, cephalotin and vancomycin was observed in 57%, 47% and 45% of the strains among the bacterial collection, respectively (Figure 5). Most of the isolates showed multi antibiotic-resistance phenotype and among the tested isolates, 17

different antibiotic-resistance phenotypes were detected: as shown is Table 1, 2% of the bacterial isolates was resistant to five of the tested six antibiotics and 19% of the isolates showed four or three resistance simultaneously.

Table 1. Antibiotic-resistance phenotypes. The code of the isolates is simplified and includes only the fraction of origin (R = rhizosphere; E = endosphere) and the progressive number. The percentage in the last column is referred to the strains belonging to each phenotype. CIP = ciprofloxacin; C = chloramphenicol; KF = cephalotin; TET = tetracycline; VA = vancomycin; RA = rifampicin. 'R': refers to the strain resistance to the antibiotic at the indicated concentration.

		Antibiotic Resistance (R)							
Phenotype	**Code of the Isolates**	**CIP**	**C**	**KF**	**TET**	**VA**	**RA**	**N of R**	**% of Isolates**
1	E13	R	R	R	R	R		5	2
2	R34, R52	R	R	R	R			4	19
3	E2, E8, E14, E28, E33, R32	R	R	R		R			
4	R12	R	R		R	R			
5	R71	R		R	R	R			
6	R20		R	R	R	R			
7	R25	R		R	R			3	19
8	E16, E42, E73, R65, R76, R79	R		R		R			
9	E6, E21, R4, R69		R	R		R			
10	R3	R		R				2	14
11	R67	R			R				
12	R17, R78	R				R			
13	R6, R28		R	R					
14	R22, R26			R		R			
15	E15, R2, R16, R23, R33, R39, R49, R50, R64, R75, R80	R						1	22
16	R8, R19					R			
17	E27, R1, R7, R9, R10, R15, R31, R38, R40, R47, R57, R68, R72, R77							0	24

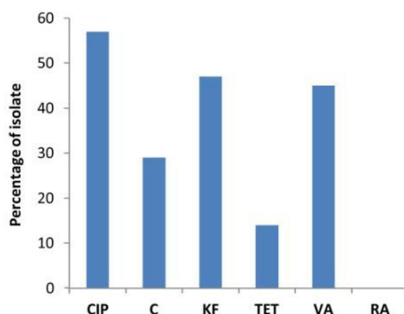


Figure 5. Characterization of the bacteria collection for antibiotic resistance. Percentage of strains resistant to CIP = ciprofloxacin (5 µg); C = chloramphenicol (30 µg); KF = cephalotin (30 µg); TET = tetracycline (30 µg); VA = vancomycin (30 µg); RA = rifampicin (5 µg).

The rhizobacteria collection (n = 36) was tested for the ability to decolorize the model azo-dye molecule Reactive Black 5 (Supplementary Table S4; Table S2). We tested specifically rhizobacteria for this activity, considering that the decolorization of azo-dye contaminated wastewaters in CW systems should be more significant in plant rhizosphere rather than in the endosphere. The results of the decolorization assay clustered the isolates in three groups according to their dye decolorization efficiency that was higher than 50% (17% of the tested strains), comprised between 20–50% (33% of the tested strains) and lower than 20% (50% of the tested strains).

3.4. Selection and Characterization of the Most Promising Strains for Bacterial Enhanced Phytodepuration

Within the bacteria collection, six strains were identified as the most promising for future application in phytodepuration systems, basing on the in vitro and in vivo characterization in terms of PGP activity, BPA tolerance and RB5 decolorization activity (Supplementary Table S4; Table 2). For the bacterial selection we also attempted to choose bacteria that presented resistance to

few antibiotic molecules, as resulted by disk-diffusion assays. Given that the majority of the isolates of the collection was resistant at least to one of the tested antibiotics under our experimental conditions, the selection was forced to include, among the bacteria with best PGP properties, BPA and metal tolerance and RB5 decolorization ability, those resistant to few antibiotics. The physiological features of the six selected strains (*Pseudomonas fluorescens* CWMP-8R25, *Microbacterium oxydans* CWMP-8R34, *Microbacterium maritypicum* CWMP-8R67, *Flavobacterium johnsoniae* CWMP-8R71, *Lysinibacillus fusiformis* CWMP-8R75 and *Enterobacter ludwigii* CWMP-8R78) are summarized in Table 2. These bacteria were further analyzed for other abilities important for the possible exploitation in bioaugmentation approaches, such as the capability to form biofilm and adhere to the plant root apparatus and their versatility in terms of tolerance/degradation of pollutants. The six strains were tested for the biofilm formation ability and 3 of them showed a biofilm formation higher than 50% compared to the *E. coli* ATCC25404 reference strain, one strain had a percentage of 44% and the remaining 2 strains showed a percentage lower than 20% (Table 2). All the strains were tolerant to a mixed solution of metals ($CdCl_2$-$ZnCl_2$-$NiCl_2$: 0.5 µM-150 µM-8.5 µM) five times more concentrated than that used to screen the whole bacteria collection. Finally, the decolorization of additional synthetic dyes (Bezactive rouge S-Matrix, Tubantin blue and Blue S-2G) was assessed and although most of the bacteria showed a low dye decolorization efficiency (< 20%), the two strains *F. johnsoniae* CWMP-8R71 and *E. ludwigii* CWMP-8R78 revealed a high efficiency of decolorization for all the tested dyes (Table 2).

Table 2. Characterization of the six most promising bacteria used as inoculants in CW microcosm-scale experiment. (**A**) In vitro plant growth promotion (PGP) tests: IAA = IAA production; ACC-d = ACC deaminase activity; Prot. = protease production. In vivo PGP test under hydroponic condition with L. esculentum plants: % germin. = germination percentage; root-l = root length; shoot-l = shoot length; 2ary roots = production of secondary roots; SVI = seedling vigor index; root dw = root dry weight. Ability to bind the root apparatus: Biof = colorimetric assay for biofilm formation using E. coli ATCC25404 as positive control; Swi = swimming lifestyle; Swa = swarming lifestyle; EPS exopolysaccharides release. (**B**) BPA tolerance. Antibiotic resistance to CIP = ciprofloxacin; C = chloramphenicol; KF = cephalotin; TET = tetracycline; VA = vancomycin; RA = rifampicin. Metal tolerance to: CdCl2 0.05, 0.5 and 1 mM; NiCl2 0.5, 1 and 2 mM; ZnCl2 0.5, 1 and 2 mM; MIX1 = ZnCl2 30µM, NiCl2 1.7µM and CdCl2 0.1 µM; MIX2 = ZnCl2 150 µM, NiCl2 8.5 µM and CdCl2 0.5µM. In vitro decolorization ability of RB5 = reactive black 5; BR = bezactive rouge S-matrix; TB = tubantin blue; BS2-G = Blue S2G at 100 mg/l. The + indicates that the bacterium is positive to the test. The stars indicate statistically significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). R indicates the resistance to the antibiotic.

(**A**)

Strain	Closest Described Relative	IAA	ACC-d	Prot.	Hydroponic Experiment						Biof	Swi	Swa	EPS
					% Germin.	Root-l	Shoot-l	2ary Roots	SVI	Root-dw				
CWMP-8R25	*Pseudomonas fluorescens*	+	+			*	**	**	*		44%			
CWMP-8R34	*Microbacterium oxydans*	+	+				**	*			84%			
CWMP-8R67	*Microbacterium maritypicum*	+	+	+			***		*		52%			
CWMP-8R71	*Flavobacterium johnsoniae*					***	***		***		6%			
CWMP-8R75	*Lysinibacillus fusiformis*	+					***				66%	+	+	
CWMP-8R78	*Enterobacter ludwigii*	+	+			***	***		***		21%	+		

(**B**)

Strain	**Closest Described Relative**	**BPA**	**Antibiotic Resistance**						**Metal Tolerance (mM)**											**Decolorization**			
			CIP	**C**	**KF**	**TET**	**VA**	**RA**	**$CdCl_2$**			**$NiCl_2$**			**$ZnCl_2$**			**MIX1**	**MIX2**	**RB5**	**BR**	**TB**	**BS2-G**
									0.05	**0.5**	**1**	**0.5**	**1**	**2**	**0.5**	**1**	**2**						
CWMP-8R25	*Pseudomonas fluorescens*	+	R		R	R			+	+	+	+	+	+	+	+	+	+	+	68%	10%	0%	22%
CWMP-8R34	*Microbacterium oxydans*	+	R	R	R	R			+	+	+	+	+	+	+	+	+	+	+	71%	20%	7%	25%
CWMP-8R67	*Microbacterium maritypicum*	+	R			R			+	+	+	+	+	+	+	+	+	+	+	64%	21%	4%	13%
CWMP-8R71	*Flavobacterium johnsoniae*	+	R		R	R	R		+			+	+	+	+	+	+	+	+	74%	85%	58%	15%
CWMP-8R75	*Lysinibacillus fusiformis*	+	R						+			+	+	+	+	+	+	+	+	74%	39%	29%	15%
CWMP-8R78	*Enterobacter ludwigii*	+	R				R		+	+	+	+	+		+	+	+	+	+	27%	22%	35%	45%

3.5. Evaluation of the Bacterial Contribution to Azo-Dye Removal by Juncus acutus in Microcosm-Scale CWs

A preliminary greenhouse experiment was setup to assess if *J. acutus* plants grown in a solution containing the azo-dye RB5, alone or in combination with metals, were able to tolerate the contaminants. The plants were able to grow under the setup conditions without showing any visible inhibition in comparison with control plants irrigated with tap water. A similar biomass amount of *J. acutus* plants was subsequently used to set up microcosms bacterized and artificially irrigated with a solution containing the RB5 azo-dye with the presence of metals, aiming to provide a stress factor to the plants (Figure 1). Triplicate microcosms were set up for each assay, represented by the single inoculum of the six selected strains, and a consortium including all the six bacteria together in equal concentrations. The amount of RB5, added daily to the microcosms with 25 mL of inflow containing 10 mg/l of dye, was monitored in the microcosm effluents for an overall period of 27 days and at the end of the experiment plant biomass was measured. None of the strains showed the promotion of root and shoot growth of *J. acutus* plants (data not shown) while positive results were obtained for two strains and the six-bacteria consortium in terms of RB5 decolorization (Figure 6). The RB5 concentration in the effluent of all the microcosms showed high fluctuations, which decreased over time. After 19 days the systems reached a stability condition, since the percentage of variation of RB5 concentration in the effluents in consecutive days was reduced to ± 10%. The results of the CW bioaugmentation experiment revealed that the RB5 concentration decreased also in the effluents of the non-inoculated *J. acutus* microcosms (Figure 6) indicating the depuration potential of the plant itself. However, the use of *F. johnsoniae* CWMP-8R71, *E. ludwigii* CWMP-8R78 and the consortium of the six bacteria (MIX) proved to be effective as inoculum for the bioaugmentation of *J. acutus*, given the higher decolorization efficiency observed in the treated CW microcosms compared to the control microcosms which did not received

the bacteria inoculum (Figure 6A–C). We considered that the bacterization significantly improved RB5 removal when the difference in its concentration between bacterized and not bacterized microcosms was statistically significant in more than 85% of the days in the stability time frame (i.e., after day 19). In particular, we observed a rapid response of the consortium, which, two days after the bacterization of *J. acutus* plants, was already able to significantly improve the decolorization of RB5 in comparison to the non-inoculated plants. Moreover, the positive result showed by the consortium, was more stable throughout the whole experimental time, significant in 100% of the days after system stabilization, while the six different single inocula showed higher fluctuations. Even if in microcosm conditions the consortium did not significantly affect *J. acutus* growth, in the soil experiment it showed a significant promotion of root biomasses and chlorophyll leaf content (Supplementary Table S5).

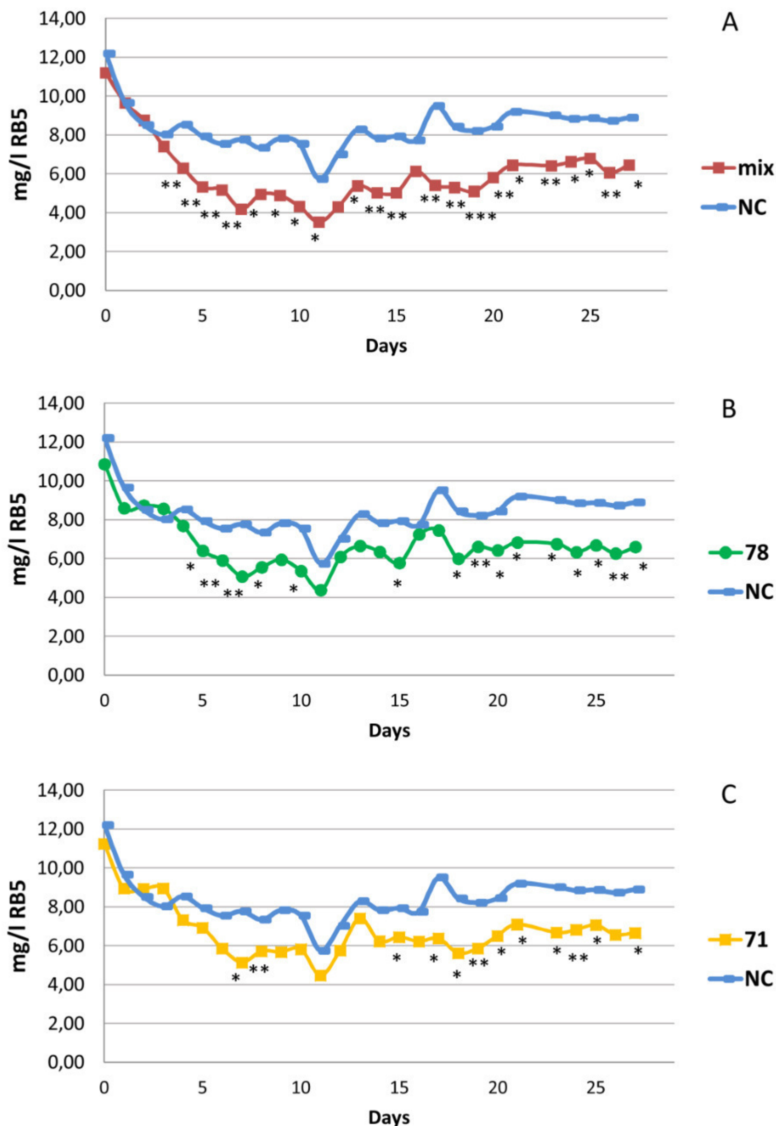


Figure 6. Concentration of RB5 daily measured from microcosm effluents over the experimental period (27 days). Values are reported as a means of the replicated CW microcosms (n = 3). RB5 concentration was compared between (**A**) NC = negative control, non-bacterized *J. acutus* plants, and plant inoculated using a consortium of the six isolates (mix); (**B**) NC and plants inoculated with 78 = *E. ludwigii* CWMP-8R78; (**C**) NC and plants inoculated with 71 = *F. jonhsoniae* CWMP-8R71. The stars indicate the days were statistically significant differences were observed (* $p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$).

4. Discussion

The selection of bacterial strains exploitable to improve phytodepuration should be the result of a broad screening that, starting from a large bacteria collection isolated from the root apparatus of the plant of interest, considers: i) the phylogenetic classification of the strain, in order to discharge possible human, animal and plant pathogens, ii) the characterization of the PGP potential of the isolates in vitro and in vivo on model plants, iii) the evaluation of the bacterial tolerance to different micropollutants which are typically found in WWs and could, in principle, contribute to create a stressful environment for the plant and the associated bacteria, and iv) the evaluation of the bacterial ability to degrade the pollutant of interest. In this frame, we applied a cultivation approach to isolate bacteria from root endosphere and rhizosphere of *P. australis*, the plant species most exploited in phytodepuration processes [20]. A high diversity in terms of bacterial taxonomy and abundance was found between the rhizosphere and endosphere fractions, as already reported in other studies and in different plant species [26,42]. Plants recruit in the rhizosphere a specific microbiome from the soil microbial community through the release of rhizodepositions, and only a selected number of microbes are able to overcome the root barrier and establish in the endosphere [43]. The results of this work, even if obtained on a single composite sample and only on the cultivable fraction of the bacterial community, demonstrated clearly this selection effect. Rhizosphere and endosphere collections differed substantially in terms of diversity with 14 genera isolated in the rhizosphere and only 6 in the endosphere. The two plant niches shared 4 bacterial genera, which were present nevertheless in different percentages. The bacteria collection obtained from *P. australis* endosphere was mainly represented by the genera *Bacillus* (48%) and *Pseudomonas* (30%), in agreement with previous reports. Shehzadi et al. [44] showed the abundance of the genus *Bacillus* in the endosphere of different wetland plant species, whereas several

studies confirmed the *Pseudomonas* prevalence in the endosphere of *P. australis* [45–47]. The *Bacillus* and *Pseudomonas* genera were commonly found associated to the root apparatus of plants growing under adverse environmental condition [24,48], since they are able to use a wide range of substrates as energy and carbon sources and are often tolerant to toxic compounds [46,49]. The strain collection obtained from *P. australis* rhizosphere in this study was dominated by *Firmicutes* (57% of the isolates), with a lower percentage of *Proteobacteria* and *Bacteroidetes,* that found a more suitable habitat in the plant endosphere, and *Actinobacteria*, that conversely were not isolated from the endosphere fraction. Previous studies, in contrast with our results, by applying cultivation-independent Next Generation Sequencing approaches retrieved in the rhizosphere of *P. australis* the dominance of the phylum *Proteobacteria* [50–52]. The dominance of *Firmicutes* in our collection could be a cultivation-related bias, although we have to consider that the plant species is not the only factor that modulates the bacterial community structure in the root apparatus, acting together with different abiotic factors [53] and making reasonable that the rhizosphere community of *P. australis* collected from CW systems treating different wastewaters hosts specific peculiar gram positive taxa. A further study of the bacterial community structure evaluated by metataxonomic approaches would lead to obtain a clearer picture about the existence of a core phylogenetic composition in the *P. australis* microbiome. Bacterial isolates are nevertheless necessary in order to exploit them for CW bioagumentation.

The root apparatus of *P. australis* growing in a CW treating municipal wastewaters may harbor different pathogenic microorganisms, unsuitable for their future exploitation as inoculants. Differently from Calheiros et al. [45], which isolated a high number of putative human pathogens from the endosphere of *Canna flaccida* plants grown in a CW system, basing on strain phylogenetic identification we found in our collection only few putative animal/human pathogenic strains, phylogenetically related to the *P.*

aeruginosa species and present only in the plant endosphere. It is known that one of the services provided by CW depuration systems is the high removal rates of enteric and fecal indicators [54], but the efficiency may depend on different operational parameters, including also plant species and plant permeability, justifying the differences observed in the two studies. The strains identified as the human potential pathogen *P. aeruginosa*, together with the plant potential pathogen *A. tumefaciens*, were no longer taken into account for the following analysis aimed at selecting bacterial strains exploitable in the improvement of CW performances.

In order to identify putative inoculants to be used in CW systems, we investigated our bacterial collection for the ability to promote plant growth. The PGP potential of the strains was assessed in vitro focusing on biostimulation related traits (i.e., ACC-deaminase activity and IAA production), relevant to sustain plant growth under the abiotic stresses that plants have to deal with in CWs, like saline waters, organic load fluctuation, presence of micropollutants [55,56]. Bacterial mediated modulation of plant hormonal balance has the potential to reduce the plant perception of stress conditions and to promote the development of the root apparatus [40,41]. Root biomass improvement is of paramount importance for the macrophytes used in CW systems since it has the potential to result in an improved filtration and reduced flow velocity of wastewaters, besides offering a higher surface and more extended habitat for the colonizing microbial community [57]. As shown in other studies performed on *P. australis*, a high percentage of bacteria isolated from both endosphere and rhizosphere was able to produce IAA and exhibited ACC deaminase activity [47,52], showing a significant potential of *P. australis* microbiome in sustaining plant adaptation and growth under the stressed conditions of a CW system.

Forty-one percent of the isolates exhibited, moreover, protease activity, an interesting trait for bacteria involved in the process of wastewater phytodepuration by catalyzing proteolysis that is the first degradation step of organic nitrogen compounds. Navarro-Torre and coworkers [58] discussed

about the importance of the enzymatic activity potential and showed that protease activity was one of the most common enzymatic activities within the bacterial collection isolated from a halophyte plant naturally growing in marshes and potentially useful for phytoremediation purposes because of its heavy metal-tolerance.

The PGP potential of the isolates was additionally tested in vivo under hydroponic conditions using tomato as model plant. Madhaiyan et al. [59] used the same system to prove the PGP potential of two bacteria isolated from rice, resulting able to promote the root elongation of *L. esculentum*. Our results confirmed the high PGP potential of strains recruited by *P. australis*, in particular the rhizospheric ones, which resulted more phylogenetically diverse and more active in the promotion of growth traits since 80% were able to promote at least one of the measured parameters. The results obtained in this work on *P. australis* microbiome confirm previous studies that demonstrated that plants adapted to stressed and contaminated environments represent an excellent reservoir of bacterial strains with multiple PGP capabilities able to enhance the host plant stress-tolerance and growth [44,48,60,61].

All the rhizospheric strains have been tested for their ability to decolorize RB5 azo-dye: the best RB5-performing ones were then screened for the decolorization of other additional colorants commonly used by the textile industry (Bezactive rouge S-Matrix, Tubantin blue and Blue S-2G). Azo-dyes are the most common synthetic organic dyes used in textile industry and, being released through the textile industry effluents, they represent wide spread xenobiotic pollutants. Even if we isolated the bacteria from plants collected in a municipal wastewater CW not specifically polluted by textile effluents, we found that 17% of the tested isolates showed significant RB5-decolorization with efficiencies higher than 50%, while 33% had decolorization efficiency between 20 and 50%.

The ability of the strains to tolerate different model micropollutants completed the strain selection for the subsequent microcosm-based tests, given the high variety of chemical compounds that can be found in sewages and could

constitute stress factors for plants and bacteria. We focused in particular on BPA, employed as a primary raw material for the production of poly-carbonate plastics but also for flame retardants used in textile industry [62] and used as intermediate chemical in the manufacture of antioxidants and dyes [63]; moreover we looked for metal tolerance since they are always found into the textile industry effluent as micropollutants [1,64,65]. Ninety-five percent of the isolates were tolerant to 100 mg/L BPA present in the growth medium. A similar result was reported by Syranidou et al. [25] studying a collection of endophytic bacteria isolated from *J. acutus* roots and leaves. A high percentage of the strains associated to *P. australis* possessed also high tolerance to metals like Zn, Ni and Cd, both supplemented singularly or in mixture in the growth medium. Metal tolerance is a useful trait in CW macrophyte plant associated bacteria since they could enhance the plant metal uptake and their translocation to the aboveground tissues [66].

Syranidou et al. [67], working with endophytic bacteria isolated from *J. acutus*, observed that the leaf isolates were particularly dominated by metal tolerant strains, although this plant shows a tendency to accumulate metals in the belowground tissues. Our results demonstrated that in *P. australis* metal tolerant strains are present both in root rhizosphere and endosphere. According to Syranidou et al. [67] among the tested metals the tolerance to cadmium was less common than to zinc and nickel, in particular among the endophytic bacteria, where the majority of the strains were tolerant to cadmium only at low concentration. This could be related to a differential phytoextraction ability of this plant species that should be further evaluated.

To identify the most promising isolates for the inoculation of macrophytes in a CW plant, we screened the collection also for the swimming/swarming ability and EPS production, which are important traits improving the competitiveness of inoculants and thus indicating a root colonizing competence. Specifically, only 26% and 24% of the tested isolates showed swimming and swarming lifestyle, respectively, likely because these motility lifestyles are typically dependent on stringent growth conditions [68] which cannot be generally

found in wastewaters. Moreover, swarming motility has been currently verified in *Gamma/Alpha-Proteobacteria* and *Firmicutes* phyla [69]: the majority of our strains with swarming motility were indeed identified as *Firmicutes*. The few bacteria of our collection that produced EPS were all isolated from the plant endosphere even if other works showed that also rhizobacteria have this ability, e.g., bacteria isolated from *Salicornia strobilacea* rhizosphere [48] and from *P. australis* rhizosphere growing in Cu-polluted conditions [70].

Antibiotics are widespread micropollutants in the human-impacted environments, especially in municipal WWs [71]. Most of the *P. australis* isolates showed to tolerate the presence of one or more antibiotics when supplemented in the growth medium, demonstrating that they have the potential to be active even in CWs receiving antibiotic contaminated influents. Our selection of the most promising bacteria to be exploited as CW inoculants took nevertheless into account the strain antibiotic resistance profile, selecting among the PGP, azo-dye degrading and micropollutant tolerant isolates, those strains with the lowest levels of antibiotic resistance. This selection was made in compliance with the 'One-Health' approach aiming to minimize the environmental spread of antibiotic resistance genes. Antibiotic resistance was a highly common trait in our bacterial collection and, in agreement with the results of Mahfouz et al. [72], our isolates showed greater resistance to antibiotics that have been available for longer in the environment (e.g., ciprofloxacin, cephalotin and vancomycin).

Considering all the mentioned features and the ability of the isolates to decolorize RB5 *in vitro*, six strains (Table 2) were identified as the most promising CW inoculants aimed to the depuration of textile wastewaters. The isolates were used to inoculate *J. acutus*, a halophyte plant species exploited for wastewater treatment in constructed wetlands and able to tolerate a wide range of contaminants [73], which we demonstrated to cope with the presence of RB5 and metals. The phytodepuration of textile wastewaters polluted with azo-dyes was tested with other macrophyte genera like *Phragmites* and *Typha* [74–77] but never with *Juncus*. Among azo-dyes, RB5 is the most used

and there are several studies focused on its removal from aqueous solutions through advanced methods such as electrochemical and anodic oxidation [77,78]. On the contrary, studies focused on the removal of RB5 with cheaper technologies like bacterial enhanced-phytodepuration have been mainly performed in vitro [2,4,79]. The bacterial performance to improve RB5 decolorization was tested for the first time in dynamic systems [56,67] by using as microcosm influent a solution containing metals and RB5 aiming to simulate the continuous flow conditions of a CW treating real textile wastewaters. Even if, during the experiment, the microcosms showed high fluctuations in terms of RB5 concentration in the effluent, putatively due to environmental variations (e.g., temperature, irradiation, humidity) that can daily alter water evaporation in the microcosms and plant evapotranspiration, the strains *Flavobacterium johnsoniae* CWMP-8R71, *Enterobacter ludwigii* CWMP-8R78 and the consortium of all the six inoculants significantly enhanced the phytodepuration potential of *J. acutus* plants when the system reached the removal stability. Both *F. johnsoniae* and *E. ludwigii* are recognized as efficient hydrocarbon degraders, plant growth promoters and good root colonizers [80–82], but no information about their azo-dye decolorization potential was provided by the literature. The six strains, when applied as pure culture to the plants did not improve the growth of *J. acutus* in CW microcosms during a 27 days treatment. However, the six-strain consortium showed appreciable PGP activity by significantly increasing root biomasses and the chlorophyll content of the leaves when applied for a longer period to potted soil *J. acutus* plants. The influence of the bacterial inoculation on the detoxification of textile effluent in a CW reactor planted with *Typha domingensis* was previously evaluated by Shehzadi et al. [83], proving the decolorization enhancement implemented by *Microbacterium arborescens* and *Bacillus pumilus* inocula. The effect of the inoculation of endophytic bacteria on the phytoremediation potential of *J. acutus* was also proved by Syranidou and coworkers [67] that showed the beneficial effect of

bioaugmentation with *Sphingomonas* sp., *Bacillus* sp. and *Ochrobactrum* sp. in the removal of EOCs and metals from the liquid phase of the system.
The results of our study highlighted that the synergistic relationship between specific bacteria inoculants and plants enhanced *J. acutus* growth and CW services and could be exploited to design specific WWTPs oriented at the degradation of textile dyes, recalcitrant organic pollutants that are not degraded by other conventional aerobic water treatment plants [84].

5. Conclusions

This study reveals that the root apparatus of *P. australis* plants growing in CWs is naturally associated to a beneficial microbiome able to promote plant growth and to tolerate the presence of different classes of micropollutants commonly detected in municipal and industrial wastewaters. The obtained results allowed to identify in particular six most promising strains which, tested in CW microcosm-scale experiment, enhanced the azo-dye phytodepuration capacity of *J. acutus* plants. Even if the application of these strains did not result in a complete dye removal, they could anyway be further exploited for improving the treatment of textile industry effluents, with low cost sustainable systems of particular interest for the MENA region.

References

1. Jadhav, J.P.; Kalyani, D.C.; Telke, A.A.; Phugare, S.S.; Govindwar, S.P. Evaluation of the efficacy of a bacterial consortium for the removal of color, reduction of heavy metals, and toxicity from textile dye effluent. *Bioresour Technol* **2010**, *101*,165-73. https://doi.org/10.1016/j.biortech.2009.08.027.
2. Mahmood, F.; Shahid, M.; Hussain, S.; Shahzad, T.; Tahir, M.; Ijaz, M.; Hussain, A.; Mahmood, K.; Imran, M.; Babar, S.A.K. Potential plant growth-promoting strain Bacillus sp. SR-2-1/1 decolorized azo dyes through NADH-ubiquinone:oxidoreductase activity. *Bioresour. Technol.* **2017**, *235*, 176–184.
3. Shafqat, M.; Khalid, A.; Mahmood, T.; Siddique, M.T.; Han, J.I.; Habteselassie, M.Y. Evaluation of bacteria isolated from textile wastewater and rhizosphere to simultaneously degrade azo dyes and promote plant growth. *J. Chem. Technol. Biotechnol.* **2017**, *92*, 2760-2768.

4. Wang, Z.W.; Liang, J.S.; Liang, Y. Decolorization of Reactive Black 5 by a newly isolated bacterium Bacillus sp. YZU1. *Int. Biodeterior. Biodegrad.* **2013**, *76*, 41–48. https://doi.org/10.1016/j.ibiod.2012.06.023.
5. Lucas, M.S.; Amaral, C.; Sampaio, A.; Peres, J.A.; Dias, A.A. Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of Candida oleophila. *Enzym. Microb. Technol.* **2006**, *39*, 51–55.
6. Khalid, A.; Arshad, M.; Crowley, D. *Bioaugmentation of azo dyes. Biodegradation of Azo Dyes*. Springer: Berlin; Heidelberg, Germany 2010, pp. 1–37.
7. Van der Zee, F.P.; Villaverde, S. Combined anaerobic–aerobic treatment of azo dyes—a short review of bioreactor studies. *Water Res*. **2005**, *39*, 1425-1440. https://doi.org/10.1016/j.watres.2005.03.007.
8. Shah, M.P. Azo Dye Removal Technologies. *Austin J. Biotechnol. Bioeng.* **2018**, *5*, 1090.
9. Chaukura, N.; Gwenzi, W.; Tavengwa, N.; Manyuchi, M.M. Biosorbents for the removal of synthetic organics and emerging pollutants, opportunities and challenges for developing countries. *Env. Dev.* **2016**, *19*, 84–89. https://doi.org/10.1016/j.envdev.2016.05.002.
10. Frascari, D.; Zanaroli, G.; Motaleb, M.A.; Annen, G.; Belguith, K.; Borin, S.; Choukr-Allah, R.; Gibert, C.; Jaouani, A.; Kalogerakis, N.; Karajeh, F.; et al. Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt; Morocco; and Tunisia. *Integr. Env. Assess. Manag.* **2018**, *14*, 447–462. https://doi.org/10.1002/ieam.4045.
11. Giorgetti, L.; Talouizte, H.; Merzouki, M.; Caltavuturo, L.; Geri, C.; Frassinetti, S. Genotoxicity evaluation of effluents from textile industries of the region Fez-Boulmane; Morocco, a case study. *Ecotoxicol. Env. Saf.* **2011**, https://doi.org/10.1016/j.ecoenv.2011.08.002.
12. Garner, E.; Zhu, N.; Strom, L.; Edwards, M.; Pruden, A. A human exposome framework for guiding risk management and holistic assessment of recycled water quality. *Env. Sci.: Water Res. Technol.* **2016**, *2,* 580–598.
13. Saratale, R.G.; Saratale, G.D.; Chang, J.S.; Govindwar, S.P. Bacterial decolorization and degradation of azo dyes, a review. *J. Taiwan Inst. Chem. Eng.* **2011**, *42*, 138–157. https://doi.org/10.1016/j.jtice.2010.06.006.
14. Vymazal, J. Constructed wetlands for wastewater treatment. *Water* **2010,** *2*, 530–549.
15. Wang, J.; Tai, Y.; Man, Y.; Wang, R.; Feng, X.; Yang, Y.; Fan, J.; Guo, J.J.; Tao, R.; Yang, Y.; Chen, Z.; et al. Capacity of various single-stage constructed wetlands to treat domestic sewage under optimal temperature in Guangzhou City; South China. *Ecol. Eng.* **2018**, *115*, 35–44. https://doi.org/10.1016/j.ecoleng.2018.02.008.
16. Wu, S.; Wallace, S.; Brix, H.; Kuschk, P.; Kirui, W.K.; Masi, F.; Dong, R. Treatment of industrial effluents in constructed wetlands, challenges; operational strategies and overall

performance. *Env. Poll* **2015**, *201*, 107–120. https://doi.org/10.1016/j.envpol.2015.03.006.

17. Gottschall, N.; Boutin, C.; Crolla, A.; Kinsley, C.; Champagne, P. The role of plants in the removal of nutrients at a constructed wetland treating agricultural (dairy) wastewater; Ontario; Canada. *Ecol. Eng.* **2007**, *29*, 154–163. https://doi.org/10.1016/j.ecoleng.2006.06.004.
18. Li, Y.; Zhu, G.; Ng, W.J.; Tan, S.K. A review on removing pharmaceutical contaminants from wastewater by constructed wetlands, design; performance and mechanism. *Sci. Total Env.* **2014**, *468*, 908–932. https://doi.org/10.1016/j.scitotenv.2013.09.018.
19. Faulwetter, JL.; Gagnon, V.; Sundberg, C.; Chazarenc, F.; Burr, MD.; Brisson, J.; Camper, AK.; Stein, OR. Microbial processes influencing performance of treatment wetlands: A review. *Ecol. Eng.* **2009**, *35,* 987–1004. https://doi.org/10.1016/j.ecoleng.2008.12.030.
20. Borruso, L.; Esposito, A.; Bani, A.; Ciccazzo, S.; Papa, M.; Zerbe, S.; Brusetti, L. Ecological diversity of sediment rhizobacteria associated with *Phragmites australis* along a drainage canal in the Yellow River watershed. *J. Soils Sediments* **2017**, *17*, 253–265. https://doi.org/10.1007/s11368-016-1498-y.
21. Luo, Y.; Guo, W.; Ngo, H.H.; Nghiem, L.D.; Hai, F.I.; Zhang, J.; Liang, S.; Wang, X.C. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Env.* **2014**, *473*, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065.
22. Zhang, D.; Luo, J.; Lee, Z.M.P.; Maspolim, Y.; Gersberg, R.M.; Liu, Y.; Tan, S.K.; Ng, W.J. Characterization of bacterial communities in wetland mesocosms receiving pharmaceutical-enriched wastewater. *Ecol. Eng.* **2016**, *90*, 215–224. https://doi.org/10.1016/j.ecoleng.2015.12.043.
23. Dimitroula, H.; Syranidou, E.; Manousaki, E.; Nikolaidis, N.P.; Karatzas, G.P.; Kalogerakis, N. Mitigation measures for chromium-VI contaminated groundwater–The role of endophytic bacteria in rhizofiltration. *J. Hazard. Mater.* **2015**, *281*, 114–120. https://doi.org/10.1016/j.jhazmat.2014.08.005.
24. Tara, N.; Arslan, M.; Hussain, Z.; Iqbal, M.; Khan, Q.M.; Afzal, M. On-site performance of floating treatment wetland macrocosms augmented with dye-degrading bacteria for the remediation of textile industry wastewater. *J. Clean Prod.* **2019**, *217*, 541–548. https://doi.org/10.1016/j.jclepro.2019.01.258.
25. Syranidou, E.; Christofilopoulos, S.; Politi, M.; Weyens, N.; Venieri, D.; Vangronsveld, J.; Kalogerakis, N. Bisphenol-A removal by the halophyte *Juncus acutus* in a phytoremediation pilot, Characterization and potential role of the endophytic community. *J. Hazard. Mater.* **2017**, *323*, 350–358.

26. Marasco, R.; Rolli, E.; Ettoumi, B.; Vigani, G.; Mapelli, F.; Borin, S.; Abou-Hadid, A.F.; El-Behairy, U.A.; Sorlini, C.; Cherif, A.; Zocchi, G.; et al. A drought resistance-promoting microbiome is selected by root system under desert farming. *PLoS ONE* **2012**, *7*, e48479. https://doi.org/10.1371/journal.pone.0048479.
27. Barac, T.; Taghavi, S.; Borremans, B.; Provoost, A.; Oeyen, L.; Colpaert, J.V.; Vangrosvelt, J.; Van Der Lelie, D. Engineered endophytic bacteria improve phytoremediation of water-soluble; volatile; organic pollutants. *Nat. Biotechnol.* **2004**, *22*, 583. https://doi.org/10.1038/nbt960.
28. Vergani, L.; Mapelli, F.; Marasco, R.; Crotti, E.; Fusi, M.; Di Guardo, A.; Armiraglio, S.; Daffonchio, D.; Borin; S. Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. *Front. Microbiol.* **2017**, *8*, 1385.
29. Ferjani, R.; Marasco, R.; Rolli, E.; Cherif, H.; Cherif, A.; Gtari, M.; Boudabous, A.; Daffonchio, D.; Ouzari, H.I. The date palm tree rhizosphere is a niche for plant growth promoting bacteria in the oasis ecosystem. *Biomed. Res. Int.* **2015**, 153851. http://dx.doi.org/10.1155/2015/153851.
30. Mapelli, F.; Marasco, R.; Rolli, E.; Barbato, M.; Cherif, H.; Guesmi, A.; Ouzari, I.; Daffonchio, D.; Borin, S. Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *Biomed. Res. Int.* **2013**, 248078. http://dx.doi.org/10.1155/2013/248078.
31. Bric, J.M.; Bostock, R.M.; Silverstone, S.E. Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *J. Appl. Env. Microbiol.* **1991**, *57*, 535–538.
32. Penrose, D.M.; Glick, B.R. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant* **2003**, *118*, 10–15
33. Nielsen, P.; Sørensen, J. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in Paenibacillus polymyxa and Bacillus pumilus strains from barley rhizosphere. *FEMS Microbiol. Ecol.* **1997**, *22*, 183–192.
34. Santaella, C.; Schue, M.; Berge, O.; Heulin, T.; Achouak, W. The exopolysaccharide of Rhizobium sp. YAS34 is not necessary for biofilm formation on Arabidopsis thaliana and Brassica napus roots but contributes to root colonization. *Env. Microbiol.* **2008,** *10*, 2150–2163. https://doi.org/10.1111/j.1462-2920.2008.01650.x.
35. Mi, Z.H.; Yu, Z.C.; Su, H.N.; Wang, L.; Chen, X.L.; Pang, X.; Qin, Q.L.; Xie, B.B.; Zhang, X.Y.; Zhou, B.C.; Zhang, Y.Z. Physiological and genetic analyses reveal a mechanistic insight into the multifaceted lifestyles of P seudoalteromonas sp. SM 9913 adapted to the deep-sea sediment. *Env. Microbiol.* **2015,** *17*, 3795–3806.

36. Burton, E.; Yakandawala, N.; LoVetri, K.; Madhyastha, M.S. A microplate spectrofluorometric assay for bacterial biofilms. *J. Ind. Microbiol. Biotechnol.* **2007,** *34*, 1–4. https://doi.org/10.1007/s10295-006-0086-3.
37. Sharma, A.; Johri, B.N.; Sharma, A.K.; Glick, B.R. Plant growth-promoting bacterium Pseudomonas sp. strain GRP3 influences iron acquisition in mung bean (Vigna radiata L. Wilzeck). *Soil Biol. Biochem.* **2003,** *35*, 887–894. https://doi.org/10.1016/S0038-071700119-6.
38. Committee on Biological Agents (ABAS). Available online: https://www.baua.de/EN/Tasks/Committee-administration/ABAS/ABAS_node.html
39. Soussi, A.; Ferjani, R.; Marasco, R.; Guesmi, A.; Cherif, H.; Rolli, E.; Mapelli, F.; Ouzari, HI.; Daffonchio, D.; Cherif, A. Plant-associated microbiomes in arid lands: Diversity, ecology and biotechnological potential. *Plant Soil* **2016**, https://doi.org/10.1007/s11104-015-2650-y.
40. Backer, R.; Rokem, JS.; Ilangumaran, G.; Lamont, J.; Praslickova, D.; Ricci, E.; Subramanian, S.; Smith, DL. Plant Growth-Promoting Rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant. Sci.* **2018**, *9*,1473.
41. Nascimento, F.X.; Rossi, M.J.; Glick, B.R. Ethylene and 1-Aminocyclopropane-1-carboxylate (ACC) in plant–bacterial interactions. *Front. Plant Sci.* **2018**, 9:114.
42. Hardoim, P.R.; van Overbeek, L.S.; van Elsas, J.D. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* **2008**, *16*, 463–471. https://doi.org/10.1016/j.tim.2008.07.008.
43. Reinhold-Hurek B.; Bünger W.; Burbano CS.; Sabale M.; Hurek, T. Roots shaping their microbiome, global hotspots for microbial activity. *Annu. Rev. Phytopathol.* **2015**, *53*, 403–424. https://doi.org/10.1146/annurev-phyto-082712-102342.
44. Shehzadi, M.; Fatima, K.; Imran, A.; Mirza, M.S.; Khan, Q.M.; Afzal, M. Ecology of bacterial endophytes associated with wetland plants growing in textile effluent for pollutant-degradation and plant growth-promotion potentials. *Plant. Biosyst.* **2016,** *150*, 1261–1270. https://doi.org/10.1080/11263504.2015.1022238.
45. Calheiros, C.S.C.; Pereira, S.I.A.; Brix, H.; Rangel, A.O.S.S.; Castro, P.M.L. Assessment of culturable bacterial endophytic communities colonizing Canna flaccida inhabiting a wastewater treatment constructed wetland. *Ecol. Eng.* **2017**, *98*, 418–426. https://doi.org/10.1016/j.ecoleng.2016.04.002.
46. Li, Y.H.; Zhu, J.N.; Zhai, Z.H.; Zhang, Q. Endophytic bacterial diversity in roots of *Phragmites australis* in constructed Beijing Cuihu Wetland (China). *FEMS Microbiol. Lett.* **2010,** *309*, 84–93.

47. Sauvêtre, A.; Schröder, P. Uptake of carbamazepine by rhizomes and endophytic bacteria of *Phragmites australis*. *Front. Plant Sci.* **2015**, *6*, 83. https://doi.org/10.3389/fpls.2015.00083.
48. Marasco, R.; Mapelli, F.; Rolli, E.; Mosqueira, M.J.; Fusi, M.; Bariselli, P.; Reddy, M.; Cherif, A.; Tsiami, G.; Borin, S.; Daffonchio, D. Salicornia strobilacea (synonym of Halocnemum strobilaceum) grown under different tidal regimes selects rhizosphere bacteria capable of promoting plant growth. *Front. Microbiol.* **2016**, *7*, 1286. https://doi.org/10.3389/fmicb.2016.01286.
49. Vergani, L.; Mapelli, F.; Zanardini, E.; Terzaghi, E.; Di Guardo, A.; Morosini, C.; Raspa, G.; Borin, S. Phyto-rhizoremediation of polychlorinated biphenyl contaminated soils, an outlook on plant-microbe beneficial interactions. *Sci. Total Env.* **2017**, *575*, 1395–1406. https://doi.org/10.1016/j.scitotenv.2016.09.218.
50. Borruso, L.; Bacci, G.; Mengoni, A.; De Philippis, R.; Brusetti, L. Rhizosphere effect and salinity competing to shape microbial communities in *Phragmites australis* (Cav.) Trin. ex-Steud. *FEMS Microbiol. Lett.* **2014**, *359*, 193–200. https://doi.org/10.1111/1574-6968.12565.
51. Behera, P.; Mohapatra, M.; Adhya, T.K.; Suar, M.; Pattnaik, A.K.; Rastogi, G. Structural and metabolic diversity of rhizosphere microbial communities of Phragmites karka in a tropical coastal lagoon. *Appl. Soil Ecol.* **2018**, *125*, 202–212. https://doi.org/10.1016/j.apsoil.2017.12.023.
52. Abed, R.M.; Al-Kharusi, S.; Gkorezis, P.; Prigent, S.; Headley, T. Bacterial communities in the rhizosphere of *Phragmites australis* from an oil-polluted wetland. *Arch. Agron. Soil Sci.* **2018,** *64*, 360–370.
53. Berg, G.; Grube, M.; Schloter, M.; Smalla, K. Unraveling the plant microbiome, looking back and future perspectives. *Front. Microbiol.* **2014**, *5*, 148. https://doi.org/10.3389/fmicb.2014.00148.
54. Wu, S.; Carvalho, P.N.; Müller, J.A.; Manoj, V.R.; Dong, R. Sanitation in constructed wetlands, a review on the removal of human pathogens and fecal indicators. *Sci. Total Env.* **2016**, *541*, 8–22.
55. Rehman, K.; Imran, A.; Amin, I.; Afzal, M. Inoculation with bacteria in floating treatment wetlands positively modulates the phytoremediation of oil field wastewater. *J. Hazard. Mater.* **2018**, *349*, 242–251. https://doi.org/10.1016/j.jhazmat.2018.02.013.
56. Ijaz, A.; Shabir, G.; Khan, Q.M.; Afzal, M. Enhanced remediation of sewage effluent by endophyte-assisted floating treatment wetlands. *Ecol. Engin.* **2015**, *84*, 58–66. https://doi.org/10.1016/j.ecoleng.2015.07.025.
57. Fountoulakis, M.S.; Daskalakis, G.; Papadaki, A.; Kalogerakis, N.; Manios, T. Use of halophytes in pilot-scale horizontal flow constructed wetland treating domestic

wastewater. *Env. Sci. Pollut. Res.* **2017,** *24,* 16682–16689. https://doi.org/10.1007/s11356-017-9295-8.

58. Navarro-Torre, S.; Mateos-Naranjo, E.; Caviedes, M.A.; Pajuelo, E.; Rodríguez-Llorente, I.D. Isolation of plant-growth-promoting and metal-resistant cultivable bacteria from Arthrocnemum macrostachyum in the Odiel marshes with potential use in phytoremediation. *Mar. Poll. Bull.* **2016,** *110*, 133–142.
59. Madhaiyan, M.; Poonguzhali, S.; Sa, T. Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (Lycopersicon esculentum L.). *Chemosphere* **2007,** *69*, 220–228. https://doi.org/10.1016/j.chemosphere.2007.04.017.
60. Baoune, H.; El Hadj-Khelil, A.O.; Pucci, G.; Sineli, P.; Loucif, L.; Polti, M.A. Petroleum degradation by endophytic Streptomyces spp. isolated from plants grown in contaminated soil of southern Algeria. *Ecotoxicol. Env. Saf.* **2018**, *147*, 602–609. https://doi.org/10.1016/j.ecoenv.2017.09.013.
61. Mapelli, F.; Marasco, R.; Balloi, A.; Rolli, E.; Cappitelli, F.; Daffonchio, D.; Borin, S. Mineral–microbe interactions, biotechnological potential of bioweathering. *J. Biotechnol* **2012,** *157*, 473–481.
62. Loos, R.; Hanke, G.; Umlauf, G.; Eisenreich, S.J. LC–MS–MS analysis and occurrence of octyl-and nonylphenol; their ethoxylates and their carboxylates in Belgian and Italian textile industry; waste water treatment plant effluents and surface waters. *Chemosphere* **2007,** *66*, 690–699.
63. Xue, J.; Liu, W.; Kannan, K. Bisphenols; benzophenones; and bisphenol A diglycidyl ethers in textiles and infant clothing. *Env. Sci. Technol.* **2017,** *51*, 5279–5286. https://doi.org/10.1021/acs.est.7b00701.
64. Noreen, M.; Shahid, M.; Iqbal, M.; Nisar, J. Measurement of cytotoxicity and heavy metal load in drains water receiving textile effluents and drinking water in vicinity of drains. *Measurement* **2017**, *109*, 88–99. https://doi.org/10.1016/j.measurement.2017.05.030.
65. Hussain, Z.; Arslan, M.; Malik, M.H.; Mohsin, M.; Iqbal, S.; Afzal, M. Integrated perspectives on the use of bacterial endophytes in horizontal flow constructed wetlands for the treatment of liquid textile effluent, phytoremediation advances in the field. *J. Env. Manag.* **2018**, *224*, 387–395.
66. Rajkumar, M.; Sandhya, S.; Prasad, M.N.V.; Freitas, H. Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnol Adv* **2012**, *30*, 1562–1574.
67. Syranidou, E.; Christofilopoulos, S.; Gkavrou, G.; Thijs, S.; Weyens, N.; Vangronsveld, J.; Kalogerakis, N. Exploitation of endophytic bacteria to enhance the phytoremediation potential of the wetland helophyte *Juncus acutus*. *Front. Microbiol.* **2016**, *7*, 1016. https://doi.org/10.3389/fmicb.2016.01016.

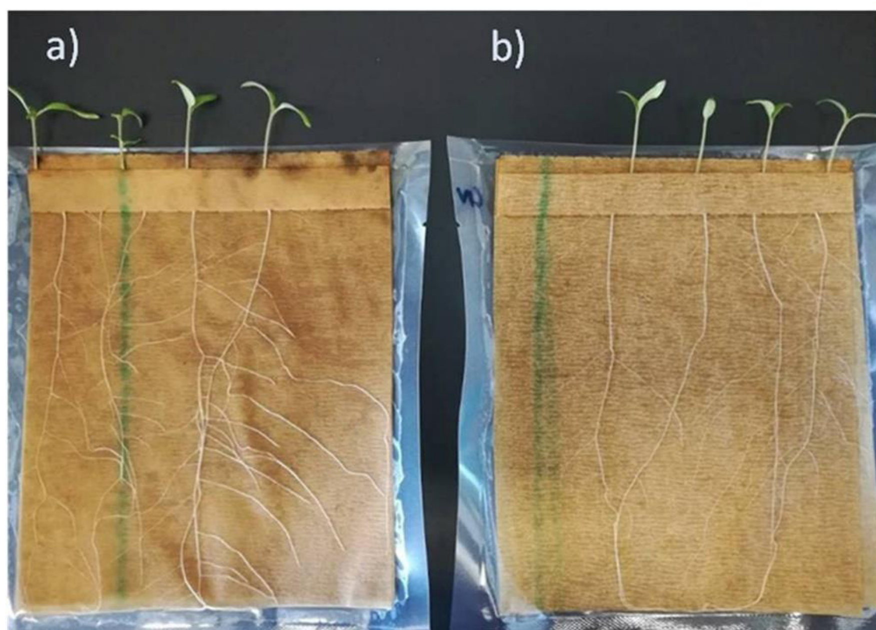
68. Sun, E.; Liu, S.; Hancock, R.E. Surfing motility, a conserved yet diverse adaptation among motile bacteria. *J. Bacteriol* **2018**, doi: 10.1128/JB.00394-18.
69. Kearns, D.B. A field guide to bacterial swarming motility. *Nat. Rev. Microbiol.* **2010,** *8*, 634.
70. Kunito, T.; Saeki, K.; Nagaoka, K.; Oyaizu, H.; Matsumoto, S. Characterization of copper-resistant bacterial community in rhizosphere of highly copper-contaminated soil. *Eu J. Soil Bio* **2001,** *37*, 95–102.
71. Rizzo, L.; Manaia, C.; Merlin, C.; Schwartz, T.; Dagot, C.; Ploy, M.C.; Michael, I.; Fatta-Kassinos, D. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment, a review. *Sci. Total Env.* **2013**, *447*, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032.
72. Mahfouz, N.; Caucci, S.; Achatz, E.; Semmler, T.; Guenther, S.; Berendonk, T.U.; Schroeder, M. High genomic diversity of multi-drug resistant wastewater Escherichia coli. *Sci. Rep.* **2018,** *8*, 8928.
73. Syranidou, E.; Christofilopoulos, S.; Kalogerakis, N. Juncus spp.—The helophyte for all (phyto) remediation purposes? *N Biotechnol.* **2017**, *38*, 43–55. https://doi.org/10.1016/j.nbt.2016.12.005.
74. Vymazal, J. Constructed wetlands for treatment of industrial wastewaters, a review. *Ecol. Eng.* **2014**, *73*, 724–751. https://doi.org/10.1016/j.ecoleng.2014.09.034.
75. Davies, L.C.; Carias, C.C.; Novais, J.M.; Martins-Dias, S. Phytoremediation of textile effluents containing azo dye by using *Phragmites australis* in a vertical flow intermittent feeding constructed wetland. *Ecol. Eng.* **2005**, *25*, 594–605. https://doi.org/10.1016/j.ecoleng.2005.07.003.
76. Mbuligwe, S.E. Comparative treatment of dye-rich wastewater in engineered wetland systems (EWSs) vegetated with different plants. *Water Res.* **2005**, *39,* 271–280. https://doi.org/10.1016/j.watres.2004.09.022.
77. Jager, D.; Kupka, D.; Vaclavikova, M.; Ivanicova, L.; Gallios, G. Degradation of Reactive Black 5 by electrochemical oxidation. *Chemosphere* **2018**, *190*, 405–416.
78. Zaidi, S.Z.J.; Harito, C.; Walsh, F.C.; de León, C.P. Decolourisation of reactive black-5 at an RVC substrate decorated with PbO 2/TiO 2 nanosheets prepared by anodic electrodeposition. *J. Solid State Electr.* **2018,** 1–12. https://doi.org/10.1007/s10008-018-3992-1.
79. Wang, H.; Zheng, X.W.; Su, J.Q.; Tian, Y.; Xiong, X.J.; Zheng, T.L. Biological decolorization of the reactive dyes Reactive Black 5 by a novel isolated bacterial strain Enterobacter sp. EC3. *J. Hazard. Mater.* **2009,** *171*, 654–659. https://doi.org/10.1016/j.jhazmat.2009.06.050.
80. Abdelwahab, R.A.I.; Cherif, A.; Cristina, C.R.U.Z.; Nabti, E. Extracts from Marine Macroalgae and Opuntia ficus-indica Cladodes Enhance Halotolerance and Enzymatic

Potential of Diazotrophic Rhizobacteria and Their Impact on Wheat Germination Under Salt Stress. *Pedosphere* **2018,** *28*, 241–254.

81. Liste, H.H.; Felgentreu, D. Crop growth; culturable bacteria; and degradation of petrol hydrocarbons (PHCs) in a long-term contaminated field soil. *Appl. Soil Ecol.* **2006,** *31,* 43–52.
82. Yousaf, S.; Afzal, M.; Reichenauer, T.G.; Brady, C.L.; Sessitsch, A. Hydrocarbon degradation; plant colonization and gene expression of alkane degradation genes by endophytic Enterobacter ludwigii strains. *Env. Poll.* **2011**, *159*, 2675–2683. https://doi.org/10.1016/j.envpol.2011.05.031.
83. Shehzadi, M.; Afzal, M.; Khan, M.U.; Islam, E.; Mobin, A.; Anwar, S.; Khan, Q.M. Enhanced degradation of textile effluent in constructed wetland system using Typha domingensis and textile effluent-degrading endophytic bacteria. *Water Res.* **2014**, *58*, 152–159. https://doi.org/10.1016/j.watres.2014.03.064.
84. Gottlieb, A.; Shaw, C.; Smith, A.; Wheatley, A.; Forsythe, S. The toxicity of textile reactive azo dyes after hydrolysis and decolourisation. *J. Biotechnol.* **2003,** *101*, 49–56. https://doi.org/10.1016/S0168-165600302-4.

Supplementary Figure

Supplementary Figure 1. Difference in root apparatus development of (a) bacterized and (b) non bacterized tomato seedlings.



Supplementary Tables

Supplementary table 1. Distribution of cultivable bacteria isolated from *P. australis* roots and rhizosphere according to the phylogenetic classification at the phylum, genus and species level.

Phylum	Genus	E (80)	R (72)	Species	E (80)	R (72)
Actinobacteria	*Arthrobacter*		1	*A. ureafaciens*		1
	Microbacterium		4	*M. kitamiense*		2
				M. oxydans		1
				M. maritypicum		1
	Streptomyces		5	*S. pluricolorescens*		2
				S. althioticus		1
				S. acidiscabies		1
				S. caeruleatus		1

Bacteroidetes	*Flavobacterium*	3	9	*F. gyeonganense*	2	3
				F. johnsoniae	1	5
				F. oncorhynchi		1
	Fluviicola		1	*F. taffensis*		1
Firmicutes	*Bacillus*	38	24	*B. aryabhattai*	2	
				B. horneckiae		1
				B. litoralis		1
				B. marisflavi		1
				B. megaterium		1
				B. niacini		1
				B. oceanisediminis		2
				B. pumilus	34	
				B. thuringiensis	2	17
	Lysinibacillus		8	*L. fusiformis*		6
				L. parviboronicapiens		1
				L. varians		1
	Viridibacillus		9	*V. arenosi*		9
Proteobacteria	*Caulobacter*	1		*C. segnis*	1	
	Cedecea		1	*C. neteri*		1
	Enterobacter		4	*E. cancerogenus*		2
				E. cloacae		1
				E. ludwigii		1
	Ochrobactrum		1	*O. anthropi*		1
	Pseudomonas	24	3	*P. aeruginosa*	2	
				P. brassicacearum	5	
				P. fluorescens		1
				P. plecoglossicida		1
				P. stutzeri		1
				P. thivervalensis	17	
	Rheinheimera		1	*R. chironomi*		1
	Rhizobium	6		*A. tumefaciens*	6	
	Stenotrophomonas	8	1	*S. rhizophila*		1
				S. chelatiphaga	8	

Supplementary Table 2. Percentage distribution of plant growth promoting activities according to the bacterial genera isolated from the root system of *P. australis*. IAA = 3-indoleacetic acid production, ACC-d = ACC deaminase activity, Protease = protease production, EPS = exopolysaccharides production, SWIMMING =swimming lifestyle, SWARMING = swarming lifestyle.

GENUS	n° strain	IAA	ACC-d	Protease	EPS	SWIMMING	SWARMING
Arthrobacter	1	0	100	100	0	0	0
Microbacterium	4	50	100	25	25	0	0
Streptomyces	4	50	50	0	0	0	0
Flavobacterium	10	20	50	20	20	0	0
Fluviicola	1	0	0	0	0	100	100
Bacillus	12	83	58	75	8	17	33
Lysinibacillus	7	100	57	57	0	86	86
Viridibacillus	2	100	100	0	0	100	100
Caulobacter	1	100	100	0	0	0	0
Cedecea	1	100	100	0	0	0	0
Enterobacter	4	100	100	0	0	75	25
Ochrobactrum	1	100	100	0	0	0	0
Pseudomonas	6	100	83	50	50	17	0
Rheinheimera	1	100	100	100	0	0	0
Stenotrophomonas	3	100	33	100	33	0	0

Supplementary Table 3 (next page). Metal-tolerance phenotypes. The code of the isolates is simplified and includes only the fraction of origin (R=rhizosphere; E=endosphere) and the progressive number. The percentage in the last column is referred to the strains belonging to each detected phenotype. **"+"**: refers to the strain tolerance to the metal at the indicated concentration.

		Metal Tolerance									
		$ZnCl_2$ (mM)			$NiCl_2$ (mM)			$CdCl_2$ (mM)			
Phenotype	**Code of the isolates**	**0.5**	**1**	**2**	**0.5**	**1**	**2**	**0.05**	**0.5**	**1**	**% of isolates**
1	R8, R25, R31, R32, R34, R67	+	+	+	+	+	+	+	+	+	12
2	R17, R20, R78	+	+	+	+	+		+	+	+	6
3	R3, R6, R50	+	+	+	+	+	+	+	+		6
4	R15	+	+		+	+	+	+	+		2
5	R22, R68	+	+	+	+	+		+	+		4
6	E2, E6, E8, E14, E16, E21, E28, E33, E73, R4, R28, R65, R71, R75	+	+	+	+	+	+	+			28
7	E13, R76, R79	+	+	+	+	+		+			6
8	R26, R69	+	+		+	+	+	+			4
9	R2	+	+		+	+		+			2
10	R16, R23, R38, R39, R47, R49, R52, R64	+	+	+	+	+	+				16
11	E15, R10, R40	+	+	+	+	+					6
12	R33	+	+		+	+	+				2
13	R7	+			+	+	+	+			2
14	E27	+	+		+	+					2
15	R77	+						+			2

Supplementary Table 4 (next page). Characterization of the bacterial collection.
In vitro plant growth promotion (PGP) tests: IAA = IAA production; ACC-d = ACC deaminase activity; Prot. = protease production. *In vivo* PGP test under hydroponic condition with *L. esculentum* plants: %germin. = germination percentage; root-l = root length; shoot-l = shoot length; 2ary roots = production of secondary roots; SVI = seedling vigor index; root dw = root dry weight. Ability to bind the root apparatus: Swimming = swimming lifestyle; Swarming = swarming lifestyle; EPS exopolysaccharides release. BPA tolerance. *In vitro* decolorization ability of RB5 = Reactive Black 5.
The + indicates that the bacterium is positive to the test. The stars indicate statistically significant differences (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$). R indicates the resistance to the antibiotic.

Strain	Closest described relative	IAA	ACC-d	Prot.	Hydroponic experiment						Swimming	Swarming	EPS	BPA	RB5
					%germin.	root-l	shoot-l	2ary roots	SVI	root-dw					
CWMP-8E2	*Pseudomonas thivervalensis*	+	+	+									+	+	
CWMP-8E6	*Flavobacterium johnsoniae*	+	+	+									+	+	
CWMP-8E8	*Stenotrophomonas chelatiphaga*	+	+	+									+	+	
CWMP-8E13	*Bacillus thuringiensis*			+								+		+	
CWMP-8E14	*Flavobacterium gyeonganense*		+		*			**						+	
CWMP-8E15	*Bacillus aryabhattai*	+	+	+											
CWMP-8E16	*Flavobacterium gyeonganense*													+	
CWMP-8E21	*Pseudomonas thivervalensis*	+	+	+				**					+	+	
CWMP-8E27	*Bacillus aryabhattai*	+	+	+										+	
CWMP-8E28	*Pseudomonas brassicacearum*	+	+	+									+	+	
CWMP-8E33	*Bacillus pumilus*	+		+									+		
CWMP-8E42	*Caulobacter segnis*	+	+					**						+	
CWMP-8E73	*Stenotrophomonas chelatiphaga*	+		+											
CWMP-8R1	*Bacillus niacini*	+												+	
CWMP-8R2	*Microbacterium kitamiense*		+				**		*	*				+	1%
CWMP-8R3	*Bacillus thuringiensis*	+	+	+							+	+		+	24%
CWMP-8R4	*Flavobacterium oncorhynchi*		+							*				+	17%
CWMP-8R6	*Flavobacterium johnsoniae*		+							**				+	7%

CWMP-8R7	*Bacillus megaterium*	+	+	+	**	*	***		***	*				+	14%
CWMP-8R8	*Enterobacter cancerogenus*	+	+			*	***	**	**	*	+	+		+	32%
CWMP-8R9	*Streptomyces althioticus*						***							+	
CWMP-8R10	*Viridibacillus arenosi*	+	+		*	**	***		***	**	+	+		+	9%
CWMP-8R12	*Streptomyces acidiscabies*		+			***	***		***					+	
CWMP-8R15	*Bacillus horneckiae*		+	+			**		*	*	+	+			29%
CWMP-8R16	*Lysinibacillus fusiformis*	+	+				**				+	+		+	11%
CWMP-8R17	*Enterobacter cloacae*	+	+								+	+		+	37%
CWMP-8R19	*Bacillus oceanisediminis*	+	+	+			*	*						+	
CWMP-8R20	*Cedecea neteri*	+	+			*	*		*					+	10%
CWMP-8R22	*Enterobacter cancerogenus*	+	+											+	35%
CWMP-8R23	*Lysinibacillus fusiformis*	+		+		***	***	**	*		+	+		+	18%
CWMP-8R26	*Flavobacterium gyeonganense*	+	+											+	13%
CWMP-8R28	*Flavobacterium johnsoniae*				*	*								+	5%
CWMP-8R31	*Microbacterium kitamiense*		+				*							+	6%
CWMP-8R32	*Ochrobactrum anthrop*	+	+					*	*					+	27%
CWMP-8R33	*Arthrobacter ureafaciens*		+	+										+	50%
CWMP-8R38	*Lysinibacillus varians*	+	+	+										+	22%
CWMP-8R39	*Lysinibacillus parviboronicapiens*	+		+		**			*		+	+		+	19%
CWMP-8R40	*Viridibacillus arenosi*	+	+			**	***		**		+	+		+	7%
CWMP-8R47	*Fluviicola taffensis*						***							+	20%

CWMP-8R49	*Lysinibacillus fusiformis*	+	+	+			***	*	+	+	+	18%
CWMP-8R50	*Pseudomonas plecoglossicida*	+	+	+							+	12%
CWMP-8R52	*Streptomyces pluricolorescens*	+			*		***	***			+	
CWMP-8R57	*Rheinheimera chironomi*	+	+	+		***	***	***			+	
CWMP-8R64	*Lysinibacillus fusiformis*	+	+				***		+	+	+	74%
CWMP-8R65	*Flavobacterium johnsoniae*						***				+	22%
CWMP-8R68	*Bacillus marisflavi*	+		+		*	***	***		+	+	10%
CWMP-8R69	*Pseudomonas stutzeri*	+				*	***	**			+	30%
CWMP-8R72	*Bacillus litoralis*	+	+								+	
CWMP-8R76	*Flavobacterium gyeonganense*			+		***	***	***			+	16%
CWMP-8R77	*Bacillus oceanisediminis*	+				*	***	***			+	
CWMP-8R79	*Stenotrophomonas rhizophila*	+		+		**	***	**			+	32%
CWMP-8R80	*Streptomyces caeruleatus*	+	+				**	*			+	

Supplementary Table 5. Effect of bacterial consortium on *Juncus acutus* plants grown in soil. The stars indicate statistically significant differences in comparison to the non bacterized plants (*= 0.01 < p < 0.05).

Treatment	ROOT			SHOOT			
	Length (cm)	Fresh weight (g)	Dry Weight (g)	Length (cm)	Fresh weight (g)	Dry Weight (g)	Chlorophyll (mg/g of leaf)
NC	22	0,78	0,24	35	3,30	0,85	0,23
MIX	23	1,37 *	0,32 *	36	4,45	1,10	0,36 *

GENERAL CONCLUSION

Competition over freshwater resources has been increasing during decades due to a growing population, economic development and increased demand for agricultural products (Garner et al., 2016). Today's problems related to water scarcity are estimated to be aggravated in the future due to climate changes, with a significant increase in demand for water and a decrease in its availability and quality (Ercin and Hoekstra, 2014). Water deficiency constitutes an insecurity that has to be overcome in the process of socio-economic development, especially in countries already characterized by high levels of water shortage such as the Mediterranean African Countries (MACs). In these countries, the consumption of freshwater in agriculture amounts to 85%, and the rapid urbanization growth together with the political instability are making the improvement of food security a hard challenge (Frascari et al., 2018). The European project named MADFORWATER (https://www.madforwater.eu/), which this PhD thesis is a part, is aimed to develop and apply technological and management solutions for reducing water vulnerability in MACs, in particular in Morocco, Tunisia and Egypt. Among several possible strategies to fight water crisis, the two solutions proposed with this thesis involved plant-associated bacteria and foresaw the increase of i) crop resistance to water stress thus potentially reducing drought-related production losses and ii) efficiency of phytodepuration systems, sustaining, as a consequence, water quality improvement and wastewaters reuse in agriculture.

A high number of bacteria were isolated from rhizosphere and endosphere of five plants of different species and habitats collected in MACs and of one plant sampled in a polluted Italian river. The obtained large bacterial collection was taxonomically identified and functionally characterized in order to identify the best promising strains for *in vivo* experimentations,

aiming to exploit them as bioinoculants for crops and for phytodepuration systems.

The collection showed a high phylogenetic diversity, being composed of 681 bacterial strains, belonging to 22 families and 36 genera. The diversity, at family level, of the isolate collection has been compared to a composed dataset of plant-associated bacterial taxa identified in the literature with molecular methods, independent from the cultivation bias. Aim of the comparison was to evaluate the entity of the cultivation bias, and to prove on the plant biome, a new concept recently expressed by Martiny (2019) on other environmental biomes, claiming that members of almost all the bacterial species identified by culture-independent methods could be obtained in culture. Our culture collection collectively represented 20% of the total bacterial families identified with culture-independent techniques meaning that the old established paradigm of only 1% of cultivable bacteria in environmental samples (Amann et al. 1995; Brock et al., 1987) was an underestimation. This result, on the other side, does not fully support the conclusion of Martiny (2019), putatively because of the high dependence of plant-associated bacterial communities on plant genotype and environmental factors, and due to the intrinsic limitation of a small set of culturing conditions applied in this study. In fact, if we look at the microbiome associated to the plant species *A. spinosa* analyzed in the present thesis by applying both culture-dependent and independent techniques on the same rhizosphere and endosphere samples, it is possible notice at phylum level a similar relative abundance between isolated and uncultured bacterial families. This result confirms that the plant genotype is a strong selector factor toward the composition of the plant microbiota, but within the same species members most of the relevant bacterial families can be brought into culture. Noteworthy, nevertheless, the overall plant isolate collection obtained in this work comprised members of the core plant microbiome together with members of satellite taxa. The cultivated diversity could therefore allow to better understand the function of both i) the core plant microbiome that may

have an important role for plant health since it is recurrent in different plant genotypes and environments but also ii) the satellite taxa that can specifically sustain the growth of particular plant species under specific environmental conditions.

The *A. spinosa*-associated microbiome was deeper analyzed in this work for the first time. Argan associated microbiome showed a different distribution of phylogenetic groups according to the relationship of the sampled niche with the plant: residuesphere, soil surrounding roots, bulk, rhizosphere and endosphere. The study revealed, moreover, the PGP potential of the isolate collection with a particular abundance of PGP activities in strains isolated from the residuesphere fraction, potentially providing a scientific explanation to the tradition widespread among farmers in South Western Morocco of using the litter collected under the trees and composed by argan leaves as agricultural soil amendment.

In order to evaluate the potential of plant-associated bacteria to improve drought tolerance in crop, a long-term greenhouse experiment was conducted with tomato plants cultivated under three different water regime conditions and bacterized with five different PGP bacteria isolated from the root apparatus of extremophilic plants and selected on the basis of preliminary short in vivo test assays. The results underlined the importance of performing long-term in vivo experiments to obtain reliable and applicable data about the effects that PGP inocula can exert on crop productivity. The beneficial effects of PGP bacteria, denoted in the first plant life stages in numerous short-term experimentations, indeed, do not necessarily become improvements in plant biomass and fruit productivity. In our study, even if a positive effect could be speculated, plant productivity was not statistically improved by none of the tested PGP strains, which nevertheless showed a significant influence on specific plant physiological parameters, root apparatus biomass and architecture, and soil hydrology parameters. These positive effects were in particular more significant under water shortage conditions, indicating the potential capacity of the isolates to alleviate plant

water stress. The best performing strains were *M. yunnanensis KMP123-M1 and P. stutzeri SR7-77* which also demonstrated, through *in vitro* tests, to have additional traits (i.e. ability in biofilm production and tolerance to micropollutants) that could make them promising candidates for the application to crops irrigated with recycled water.

In a second part of the work the potential ability of plant-associated bacteria to improve wastewater phytodepuration allowing the use of recycled water for irrigation, was tested on the bacterial collection specifically isolated from *P. australis* plants collected from a CW system in Morocco. The study revealed that the root apparatus of *P. australis* was naturally associated to a beneficial microbiome potentially able to promote plant growth and to tolerate the presence of different classes of micropollutants commonly found in urban and industrial wastewaters. The results obtained through different *in vitro* and *in vivo* tests, allowed to select six most promising isolates, which, tested in CW microcosm-scale experimentation, enhanced the decolorization of the model azo dye Reactive Black-5 in combination with *Juncus acutus* plants.

As a general conclusion, this PhD thesis confirmed that plants adapted to live under unconventional environmental conditions are naturally associated to a potentially beneficial microbiome. In particular, the microbiome associated to *A. spinosa* plants was described for the first time.

Several members of the plant microbiota were obtained in culture, despite the limited culturing conditions adopted, partially confirming recent theories about the culturability of environmental taxa.

The *in vivo* and/or *in vitro* functional characterization of the isolates demonstrated a PGP potential, which could be exploited as future sustainable tools for agriculture in dry lands. Moreover, the results underlined the importance of long-term *in vivo* experiment to successful exploit PGP bacteria as bioinoculant for crops growing in arid lands. In fact, further experimentation is needed to confirm the first evidences obtained in this thesis. However, several strains were selected for their potential to i)

improve the quality of waters treated with phytodepuration systems, that represent low cost plants especially suited for developing countries like MACs and ii) reduce drought stress in crop plants.

- Amann, R.I., Ludwig, W. and Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.*, *59*(1), pp.143-169.
- Brock, T.D., 1987. The study of microorganisms in situ: progress and problems. In *Symp. Soc. Gen. Microbiol.* (Vol. 41, pp. 1-17).
- Ercin, A.E. and Hoekstra, A.Y., 2014. Water footprint scenarios for 2050: A global analysis. *Environment international*, *64*, pp.71-82
- Frascari, D., Zanaroli, G., Motaleb, M.A., Annen, G., Belguith, K., Borin, S., Choukr-Allah, R., Gibert, C., Jaouani, A., Kalogerakis, N. and Karajeh, F., 2018. Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt, Morocco, and Tunisia. *Integrated environmental assessment and management*, *14*(4), pp.447-462.
- Garner, E., Zhu, N., Strom, L., Edwards, M. and Pruden, A., 2016. A human exposome framework for guiding risk management and holistic assessment of recycled water quality. *Environmental Science: Water Research & Technology*, *2*(4), pp.580-598.
- Martiny, A.C., 2019. High proportions of bacteria are culturable across major biomes. *The ISME Journal*, p.1.

IMPLICATIONS AND FUTURE DIRECTIONS

During my PhD thesis I established and identified a large bacterial collection isolated from the root apparatus of different plant species from which I selected promising bacteria used *in vivo* experimentations and which represents a source of PGP bacteria that could be applied in future tests. This isolate collection allowed also to partially confirm on the plant biome, a new and relevant concept in environmental microbiology, which is the possibility to obtain in culture a significant part of the microbial diversity of a certain biome. Using a limited set of culturing conditions, we were able to isolate the 20% of the bacterial families associated to root plants identified with culture-independent methods, meaning that for the plant microbiome the percentage of the overlap between cultured and uncultured microbial diversity could be higher than previously considered. We found also that the large isolate collection obtained included members of both core and satellite taxa, allowing future studies about the possibility to understand the role and function of important core members of the plants microbiota and also of minor taxa that can specifically help plants under peculiar environmental conditions.

For the first time, with this PhD thesis, the bacterial community associated to *A. spinosa*, a relevant tree for the economy and the ecology of Morocco, was identified and described. Moreover, the PGP potential of bacterial strains selected by this plant adapted to extreme drought conditions, claimed further investigation on the PGP traits of bacteria present in the plant residuesphere. This fraction, traditionally used as soil amendment in Morocco, could be indeed exploited as a source of beneficial bacteria that could be used as inoculant in other crops of interest.

In order to find sustainable solutions for reducing the water footprint in agriculture in developing countries of North Africa, two solutions that exploit plant-associated bacteria were proposed in this PhD thesis. The strategies

planned to improve i) drought tolerance in crop and ii) treated wastewater quality to be use for irrigation purposes.

The *in vivo* greenhouse experiment performed with tomato plants cultivated under different water regime conditions and bacterized with PGP bacteria, demonstrated the importance of performing long-term experiments to obtain reliable and applicable data about the PGP effects on crop productivity. The PGP inocula indeed showed to have potential capacity to alleviate plant water stress, improving different physiological parameters and soil hydrology properties under water shortage conditions, but no statistically significant effect on plant productivity was obtained. Further studies will need to be applied on larger experimental settings, in order to evaluate if the significant observed effects on plant growth and physiology induced by the bacterization, would have also a significant effect on fruit yield and water use efficiency.

Selected PGP strains isolated from plants used in constructed wetlands demonstrated, at CW microcosm-scale, to be able to enhance the azo-dye phytodepuration capacity of *J. acutus* plants, potentially improving the quality of treated wastewaters. The risk of antibiotic resistance spread into the environment through these systems should be nevertheless deeper understood. Antibiotic resistance is nowadays one major concern threatening human and animal health due to the emergence of multi-drug resistant pathogens and it is known that WWT plants and the rhizosphere niche are hot spots of HGT events that could improve the diffusion of antibiotic resistance genes that are frequently linked to mobile genetic elements. Efforts should be therefore oriented to i) study the presence and mobility of antibiotic resistance genes from wastewaters through phytodepuration plants to their effluents and subsequent irrigated soils and ii) improve the design of phytodepuration systems to maximize the quality and safety of the treated effluents in compliance to the “One Health” concept.

Publications

- **Riva, V.**, Terzaghi, E., Vergani, L., Mapelli, F., Zanardini, E., Morosini, C., Raspa, G., Di Guardo, A. and Borin, S., 2019. Exploitation of Rhizosphere Microbiome Services. *Methods in Rhizosphere Biology Research* (pp. 105-132). Springer, Singapore.
- **Riva V**, Riva F, Vergani L, Crotti E, Borin S, Mapelli F. "Microbial assisted phytodepuration for water reclamation: environmental benefits and threats". Chemosphere.
- **Riva V**, Mapelli F, Syranidou E, Crotti E, Choukrallah R, Kalogerakis N, Borin S. “Root bacteria recruited by Phragmites australis in Constructed Wetlands have the potential to enhance azo-dye phytodepuration”. Microorganisms.

National and international conferences

Oral presentations:

- **V. Riva**, F. Mapelli, E. Syranidou, N. Kalogerakis, R. Choukrallah, S. Borin “Plant growth promoting bacteria in phytodepuration systems: a promising tool to minimize water footprint in agriculture”. 15th International Phytotechnology Conference Phytotechnologies and Forestry: Sustainable Approaches to Mitigating the Environmental Consequences of Climate Change**.** Novi Sad 1-5 October 2018.
- **V. Riva**, S. Borin, F. Mapelli, M. Brochetti, E. Crotti, A. Cherif, H. Cherif, B. Bejaoui, R. Choukr-Allah, A. Rashed, M. El Fahl, N. La Maddalena “Plant growth promoting bacteria: a sustainable tool to minimize water footprint in agriculture in arid and semi-arid zones”. miCROPe: microbe-assisted crop production – opportunities, challenges & needs; Vienna 4-7 December 2017.

Poster presentations:

- **V. Riva**, F. Mapelli, E. Syranidou, N. Kalagerakis, R. Choukr-Allah, S. Borin. “Plant growth promoting bacteria: a promising tool in phytodepuration or a risk for antibiotic resistance spread?” 8th congress of Europeans Microbiologists-FEMS, Glasgow, 7-11/07/19
- **V. Riva**, F. Mapelli, S. Borin. “Plant growth promoting bacteria: a promising tool in phytodepuration or a risk for antibiotic resistance spread?” Microbiology 2019, XXXIII SIMGBM, Firenze, 19-22/06/19
- **V. Riva**. “Bacterial-mediated plant growth promotion: a strategy to reduce water footprint in agriculture”. XXII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Bolzano, 20-22/09/17

Training courses and seminars

- 26/11-30/11/18: “Translational aspects of plant microbiome research” ICGEB, Trieste.
- 4-8/09/17: “Computational analysis from genomic diversity to ecosystem structure”. Organized by SIMTREA. Firenze.
- “Il microbiota delle piante e il futuro dell’agricoltura” organized by Georgofili association – Florence, 26/01/17

Acknowledgements

At the end of these three intensive years of research and training, I wish to thank, once again, Professor Sara Borin and Dr. Francesca Mapelli for trusting me and guiding me during all my educational and professional journey: thanks for your motivation and knowledge you shared with me and for giving me the chance to reach great professional results.
Thanks to the BEaM LAB, especially to Elena always ready to shared her advices and enthusiasm, and to my office mates Lorenzo and Francesco for sharing with me innumerable moments of discussion and high-science, poetry-mails and icy jokes: thanks for your friendship, I will always be behind you crunching food!
Thanks to all the students that worked with me who unwittingly allowed me to growth at both professional and personal level.
I wish to thank Professor Nicolas Kalogerakis who gave me the opportunity to perform part of my experiments in his laboratories at the Technical University of Crete and thanks to Evi and Lena for their help and support. A special and warm thank to my lab mates and Greek friends that made "zoara" this short but beautiful parenthesis of my life: efharisto poli filous!
A super hug and thanks to my family, even greater and lovelier, that unconditionally support me in all my choices. Thanks to my artist consultants, Sonia and Serena, and heartfelt thanks to Simone who always shows me the world from a different and genial point of view: "and, what about mass?"
Finally I would like to thanks the most anarchic creatures that I've ever known which made my days unpredictable: thank you bacteria!