Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait

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Summary

Although drought is an increasing problem in agriculture, the contribution of the root-associated bacterial microbiome to plant adaptation to water stress is poorly studied. We investigated if the culturable bacterial microbiome associated with five grapevine rootstocks and the grapevine cultivar Barbera may enhance plant growth under drought stress. Eight isolates, over 510 strains, were tested *in vivo* for their capacity to support grapevine growth under water stress. The selected strains exhibited a vast array of plant growth promoting (PGP) traits, and confocal microscopy observation of *gfp*-labelled *Acinetobacter* and *Pseudomonas* isolates showed their ability to adhere and colonize both the *Arabidopsis* and grapevine rhizoplane. Tests on pepper plants fertilized with the selected strains, under both optimal irrigation and drought conditions, showed that PGP activity was a stress-dependent and not a *per se* feature of the strains. The isolates were capable of increasing shoot and leaf biomass, shoot length, and photosynthetic activity of drought-challenged grapevines, with an enhanced effect in drought-sensitive rootstock. Three isolates were further assayed for PGP capacity under outdoor conditions, exhibiting the ability to increase grapevine root biomass. Overall, the results indicate that PGP bacteria contribute to improve plant adaptation to drought through a water stress-induced promotion ability.

Introduction

Drought is a major problem in agriculture worldwide. For example, Europe experienced an extreme drought event in 2003, exacerbated by high summer temperatures, which led to a dramatic reduction in primary productivity (Ciais *et al*., **2005**; Palliotti *et al*., **2009**; Olesen *et al*., **2011**). With the predicted increase in reduced rainfall and heat events due to global warming, plant productivity in temperate regions is threatened; water scarcity may lead to reduced plant development, leaf wilting, unbalanced fruit composition and seed maturation (Ciais *et al*., **2005**).

Plants respond to dry conditions in several ways, including modification of root architecture (shallow versus deep rooting) and leaf shape. Such responses can differ between perennial trees and annual plants, such as cereals (Vandeleur *et al*., **2009**; Alsina *et al*., **2011**). The former can potentially increase their resistance to drought by increased architectural plasticity of the root system that can explore deeper parts of the soil, while the latter exhibit a more limited plasticity due to a shorter life cycle (Gambetta *et al*., **2013**). From a community perspective, plants can even react through modification of the species composition in the biocoenosis, especially when there are long lasting episodes of climate change (Chaves *et al*., **2010**).

Irrigation supports plant growth during drought and is increasingly required even for plants that traditionally are not irrigated, such as grapevine in Northern Italy. In many wine-producing areas, vineyard irrigation is increasingly important to maintain wine yield and quality (Zhang *et al*., **2012**). This is leading to aquifer overexploitation with increased depletion of groundwater, threatening future crop production (Gleeson *et al*., **2012**; Scanlon *et al*., **2012**). Thus, it is urgent to develop sustainable agricultural practices to support productivity, minimizing the threat to water resources.

The beneficial microbiome associated with roots and plant tissues, including the so-called plant growth promoting (PGP) bacteria (Marasco *et al*., **2013a**), can contribute to alleviate plant stress by a variety of mechanisms (Hayat *et al*., **2010**; Mapelli *et al*., **2013**). Among them, PGP bacteria can directly enhance micronutrient uptake and affect phytohormone homeostasis, or indirectly stimulate the plant immune system against phytopathogens (Balloi *et al*., **2010**) and improve soil texture and structure (Mapelli *et al*., **2012**). For instance, some PGP bacteria are endowed with the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (ACCd) (Glick *et al*., **2007**) that can cleave the plant ethylene precursor ACC, thereby lowering the level of ethylene in developing or stressed plants (Glick, **2004**).

Relatively little information is available concerning grapevine-associated bacteria, although some researches have shown that reproductive organs, including seeds, flowers and berries, can be densely colonized by an endophytic microbiome, including *Pseudomonas*, *Burkholderia* and *Bacillus* spp. (Compant *et al*., **2011**). The endophyte *Burkholderia phytofirmans* strain PsJN was shown to colonize grapevine rhizosphere and spread to inflorescence tissues through the xylem (Compant *et al*., **2005; 2008**). This beneficial endophyte has a role in the biocontrol of *Botrytis cinerea* and *Pseudomonas syringae* (Barka *et al*., **2002**). Besides a priming activity, strain PsJN protects grapevines from chilling, both through scavenging activity against cold stress molecules and affecting plant carbohydrate metabolism (Barka *et al*., **2006**; Fernandez *et al*., **2012**; Theocharis *et al*., **2012**). Despite being a powerful eco-friendly solution to plant growth impairment under adverse conditions, relatively limited attention has been devoted to the search for microbes with multiple PGP traits that can contribute to improve plant response to drought, in particular for grapevine (Marasco *et al*., **2013a,b**).

The present study aimed to investigate the potential of the culturable bacterial microbiome associated with the root system of the grapevine cultivar Barbera, and different Barbera-grafted wild grapevine rootstocks, in alleviating water stress in plants. Our results showed that (i) bacteria protected grapevines from drought under greenhouse and outdoor field-like conditions, (ii) drought-induced resistance was dependent on the rootstock cultivar and bacterial types, (iii) bacteria efficiently colonized the grapevine root system, affecting the diversity of the root-associated bacterial microbiome, and (iv) plant growth promotion under drought was not a *per se* trait of the tested bacterial strains, but rather an effect enhanced under water stress.

Results

Selection of candidate PGP strains for *in vivo* tests

Through an ACC-deaminase enrichment procedure (Penrose and Glick, **2003**), we established a collection of 510 strains from the rhizosphere and endosphere of the wild grapevine rootstocks 420A, 157.11, 161.49 and SO4 grafted with the grapevine cultivar Barbera and ungrafted Barbera plants grown in the Oltrepò Pavese soil. The collection encompassed eight bacterial families, including *Bacillaceae*, *Paenibacillaceae*, *Brucellaceae*, *Alcaligenaceae*, *Comomonadaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae* and *Sphingobacteriaceae*. In order to include the largest taxonomic span of the bacterial collection in the study, three endophytes and five rhizobacteria were selected (Table S1). These promising strains were phylogenetically affiliated to the genera *Pseudomonas* (S1 and S3), *Acinetobacter* (S2), *Bacillus* (S4), *Delftia* (S5 and S8), *Sphingobacterium* (S6) and *Enterobacter* (S7). A *Bacillus* sp. strain T4, isolated from the endosphere of a grapevine from a Tunisian vineyard (Marasco *et al*., **2013b**), was used as an outgroup from a different soil (Table 1).

Table 1. PGP traits of the ACCd bacterial strains selected for the inoculation of pepper and grapevine plantlets in order to examine their ability to induce plant resistance to drought

Strain	Species (acc. N°, % of similarity)	**Plant growth promoting traitsa, b**											
		IAA (μg ml−1)c	**P.S.d**	**Sid. (mm)**	**EPS**	**NH3**	**Prot. (mm)**	**5% NaCl**	**8% NaCl**	**10% NaCl**	**20% PEG**	**4°C**	**30°C**
S1	*P. plecoglossicida* (AB009457, 100)	12.46 ± 0.32	+++	15.3 ± 0.6	N	Y	N	N	N	N	Y	Y	Y
S2	*A. calcoaceticus* (X81661, 99.8)	0.75 ± 0.21	+	N	N	N	N	N	N	N	Y	Y	Y
S3	*P. mandelii* (AF058286, 97.7)	13.51 ± 0.26	+++	9.7 ± 1.2	Y	N	N	N	N	N	Y	Y	Y
S4	*B. tequilensis* (HQ223107, 98.4)	N.D.	+	5.3 ± 1.2	Y	Y	11.5 ± 0.9	Y	Y	N	Y	Y	Y
S5	*D. tsuruhatensis* (AB075017, 100)	6.87 ± 0.53	+	10.3 ± 1.5	N	Y	N	N	N	N	Y	Y	Y
S6	*S. canadense* (AJ233434, 100)	4.433 ± 0.69	+	N	Y	N	N	N	N	N	Y	Y	Y
S7	*E. ludwigii* (AJ853891, 100)	41.52 ± 2.94	++	23.0 ± 2.0	Y	Y	N	Y	N	N	Y	Y	Y
S8	*D. tsuruhatensis* (AB075017, 100)	8.57 ± 0.37	+	N	N	Y	N	N	N	N	Y	Y	Y
T4	*B. tequilensis* (HQ223107, 99.8)	10.54 ± 1.85	+	4.3 ± 0.6	Y	N	N	N	N	N	Y	Y	Y

To decipher their potential in plant growth promotion, the selected strains were examined for an array of PGP abilities *in vitro*, focusing both on conventional and drought-related PGP traits (Table 1). Considering the complex interplay between the ACC synthesis machinery and auxin signalling pathway *in planta* during stressful conditions (Glick *et al*., **2007**; Stearns *et al*., **2012**), and taking into account the role of auxins in influencing root architecture and morphogenesis especially under abiotic stress (Saini *et al*., **2013**), we evaluated the ability of the selected strains to synthesize this phytohormone. Almost all the strains exhibited this ability and auxin production ranged from 0.75 ± 0.21 to 13.51 ± 0.26 µg ml−1.

During drought, unbalanced uptake of macro and micronutrients that may precipitate due to lack of water availability (Vassilev *et al*., **2012**; Qi and Zhao, **2013**) can dramatically exacerbate the already compromised health status of plants. We observed that the majority of strains had the potential to contribute to plant nutrition by siderophore release and solubilization of inorganic phosphate compounds (Table 1). Furthermore, the selected bacteria were widely able to secrete mucilaginous material, possibly positively affecting bacteria root adhesion and colonization (Table 1). Such an exopolymeric matrix contributes to soil stabilization through (i) an increase in the amount of root adhering soil, (ii) an improvement in water-holding capacity and reduced water loss during desiccation due to its hydrophilic properties, (iii) a stimulation of root exudation, and (iv) protection of roots from the mechanical effects of soil hardness (Alami *et al*., **2000**; Ramey *et al*., **2004**; Rinaudi and Giordano, **2010**; Rossi *et al*., **2012**; Xu *et al*., **2013**). One of the strains, *Bacillus* sp. S4, presented protease activity, a trait that may contribute to the control of fungal plant pathogens.

Drought imposes adaptive constraints on both plants and their associated microorganisms. Thus, the isolates were screened for resistance to abiotic stresses typically associated with drought, such as resistance to salinization, osmotic stress, and growth at high and low temperatures. Interestingly, all isolates were osmotolerant and showed the ability to grow under low moisture conditions, induced by the addition of poly-ethylene-glycol to the culture media. Halotolerance was not common in the selected strains, with the exception of strain S4 which grew under different salt stress conditions (5 and 8% NaCl). While at 42°C and 4°C almost all strains exhibited growth, none of the isolates grew at 50°C, except *Bacillus* strain T4 isolated from a Tunisian vineyard soil (Table 1). Thus, the assayed isolates displayed a wide variety of traits potentially involved in bacterial contribution to plant health under drought stress.

Rhizocompetence assay

An adhesion assay was performed on *Arabidopsis thaliana* roots to assess the ability of the selected bacteria to adhere and colonize the rhizoplane, a key trait for stable association with the plant. Two *gfp*-labelled strains, *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3, were used to track root colonization. After 1 h of exposure to the bacterial cells, epifluorescence microscopy analysis revealed the presence of *gfp*-expressing cells adhering to the *Arabidopsis* rhizoplane (Fig. 1A and B, upper panels). Re-isolation experiments from the *Arabidopsis* rhizoplane showed a microbial density of 9.42 × 106 ± 5.57 × 106 CFU g−1 and 2.49 × 107 ± 4.44 × 106 CFU g−1 for *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3 respectively. A longer incubation of 4 h revealed that the root surface was massively colonized by the *gfp*-tagged bacterial cells (Fig. 1A and B, lower panels), suggesting that both strains actively colonized the plant rhizoplane. The re-isolation counts after exposure for 4 h to the PGP strains increased to 2.41 × 107 ± 7.86 × 106 CFU g−1 and 6.24 × 107 ± 1.29 × 107 CFU g−1 for *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3 respectively. To obtain an insight into the colonization ability of the strains on their plant host, the *gfp*-labelled bacteria were inoculated on grapevine plantlets. After 7 and 21 days, confocal microscopy analysis revealed the presence of *Acinetobacter* sp. S2 fluorescent cells on the root surface, confirming the ability of this strain to efficiently colonize the grapevine root system (Fig. 1C and D).

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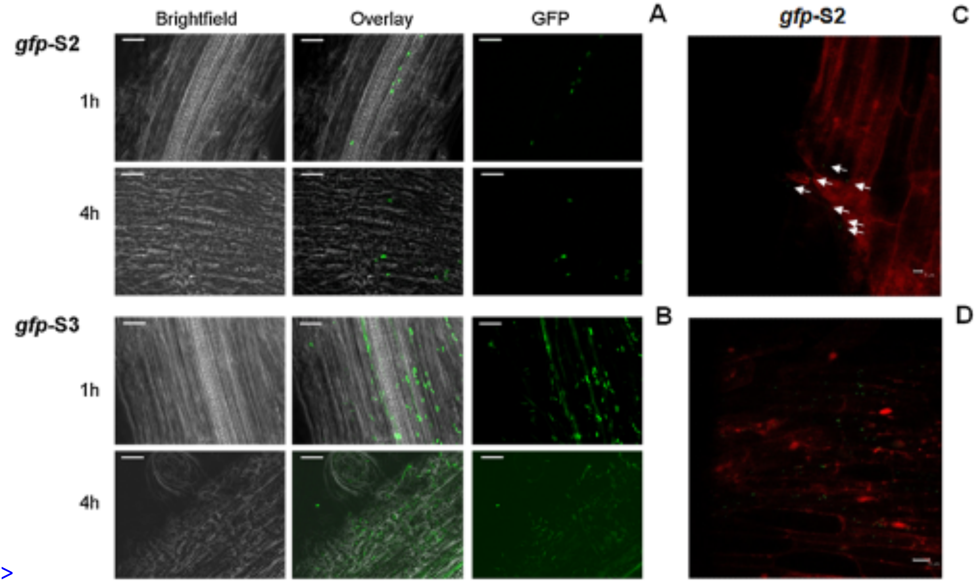

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Figure 1.

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Rhizocompetence of bacteria on *Arabidopsis thaliana* and grapevine roots. The plant root adhesion assay was performed using two *gfp*-labelled strains. A time-course experiment (1 h and 4 h) allowed us to monitor the adhesion profile of *gfp-Acinetobacter* sp. S2 (A) and *gfp-Pseudomonas* sp. S3 (B) on the *Arabidopsis* rhizoplane. For each image set, the first panel refers to phase contrast microscopy of Arabidopsis root; the second panel results from the merge of the phase contrast and the fluorescence images to visualize the adherence profile of the *gfp*-labelled cells; the third panel shows the corresponding image acquired under fluorescence light. (C) and (D) Confocal microscopy analysis of *gfp-Pseudomonas* sp. S3 strain colonizing the grapevine root surface 7 and 21 days after biofertilization with the selected strain. The red channel was used to acquire the root autofluorescence, providing information about its structure. Scale bars corresponds to 5 µm in A and B, and to 10 µm in C and D respectively. Arrows indicate GFP fluorescent bacteria along the root surface or root hair.

Is the plant growth promotion ability exerted by the bacteria a ‘*per se*’ trait rather than a drought-induced effect? The selected isolates were assayed in an *in vivo* promotion test both under irrigation and drought conditions, using *Capsicum annuum* as a model plant (Fig. 2). Under normal irrigation, no increase in root biomass was detected in plants exposed to the bacteria, while root biomass was higher under drought stress (Fig. 2). Plants treated with *Pseudomonas* sp. S1 and S3, *Acinetobacter* sp. S2, *Sphingobacterium* sp. S8, and consortia C2 and C3 showed an increase in root system weight specifically during drought, suggesting that the PGP promotion of these isolates is a water stress-dependent trait (Fig. 2).

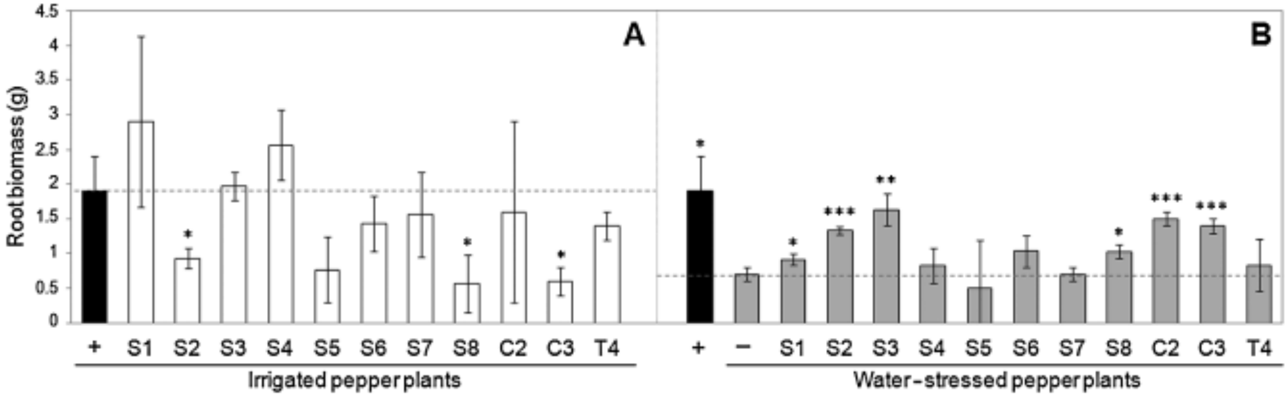

Figure 2.

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PGP bacteria were tested for pepper growth promotion under well-irrigated conditions (A) and drought stress (B). '+', abiotic control, irrigated at the water-holding capacity of the soil throughout the experiment; '−', abiotic control, subjected to drought. The graphs show the increase in root fresh biomass of pepper plants treated with the selected bacteria compared with the untreated plants. Data were subjected to statistical analysis using Student's *t*-test with significance reported as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

PGP bacteria promote grapevine plant resistance to water stress

On the basis of their intrinsic sensitivity to water stress, 1-year-old plantlets of Barbera grafted on SO4, Kober 5BB and 420A rootstocks were exposed to the selected PGP bacteria and further tested for growth performance during drought. A greenhouse experiment was performed in pots using the soil collected from 'Le Fracce' farm in order to simulate the specific conditions of water stress that grapevine plants experience under the original soil conditions (Table S2). Two-year-old grapevine plantlets inoculated with six selected isolates and two mixed bacterial consortia showed variable increases in fresh aerial biomass compared with drought-stressed uninoculated plants, under restricted irrigation to maintain 50% of the field capacity.

The highest increases were detected with the SO4 rootstock; SO4 is considered to be the most sensitive rootstock among those tested (Koundouras *et al*., **2008**), but inoculation with PGP bacteria strongly promoted growth despite water stress. In particular, in plants grafted on the SO4 rootstock, major increases were observed in the aerial fresh biomass, shoot length and leaf fresh weight (Fig. 3). Only *Acinetobacter* sp. S2 was able to promote an increase in SO4 aerial fresh biomass (Fig. 3A). SO4 plants supplemented with consortia C2 and C3, and *Sphingobacterium* sp. S6 showed an increase in shoot fresh biomass (Fig. 3B). In SO4 rootstock, leaf fresh biomass, strictly correlated to photosynthesis functionality, was significantly higher in plants treated with *Pseudomonas* sp. S1 and consortium C3 compared with the negative uninoculated control ($P \leq 0.05$) and enhanced further ($P \leq 0.01$) with *Acinetobacter* sp. S2 and *Sphingobacterium* sp. S6 (Fig. 3C). While no differences were observed in the number of shoots per plant, a concomitant increase in shoot length was recorded in SO4 rootstock (Fig. 3D). Untreated plants had a shoot length of 15.23 ± 6.11 cm, while SO4 plants fertilized with strains *Acinetobacter* sp. S2, *Sphingobacterium* sp. S6 and *Delftia* sp. S5 showed statistically significant increases in shoot length (Fig. 3D). Interestingly, *Sphingobacterium* sp. S6 caused a significant increase in the number of nodes per shoot (7.73 ± 1.42) compared with the negative control (3.93 ± 1.32, $P = 0.0023$). These results indicate that inoculation with PGP bacteria as single strains or consortia can aid SO4 plantlets to tolerate drought and increase the aerial biomass, contributing to enhance performance under stress (Fig. 3E).

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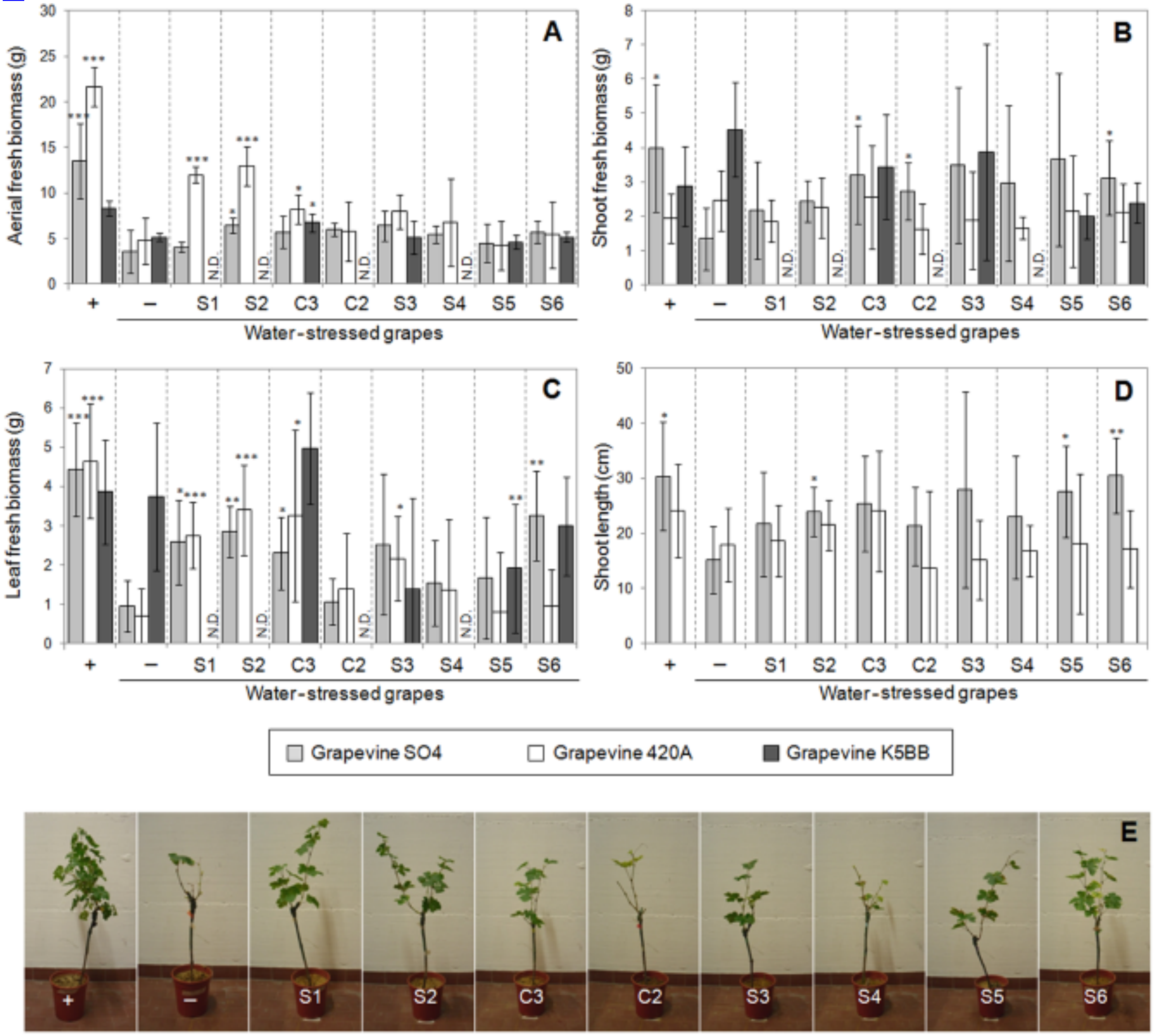

Figure 3.

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PGP bacteria improve grapevine resistance to drought. '+', abiotic control, irrigated at the water-holding capacity of the soil throughout the experiment; '–', abiotic control, subjected to drought. A, aerial fresh biomass; B, shoot fresh biomass; C, leaf fresh biomass; D, shoot length of grapevines exposed to PGP bacteria; (E) representative images of re-watered SO4 rootstock plants subjected to water stress for 30 days. Data were subjected to statistical analysis using the Student *t*-test, with significance reported as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. The mean values of each rootstock were compared separately because the genotypes differ in their growth characteristics.

Bacteria-exposed 420A plantlets exhibited a less pronounced promotion effect during drought. Nevertheless, an increase in average aerial fresh biomass was recorded for 420A plantlets treated with *Pseudomonas* sp. S1, *Acinetobacter* sp. S2 and consortium C3 compared with the uninoculated control (Fig. 3A). This was reflected in an increase in leaf fresh weight for grapevine plantlets treated with S1, S2, C3 and S3 strains (Fig. 3C).

Similarly, Kober 5BB rootstock inoculated with consortium C3 showed improved aerial fresh biomass compared with the uninoculated control (Fig. 3A).

In summary, strains *Pseudomonas* sp. S1 and S3, *Acinetobacter* sp. S2, and consortium C3 (composed of strains S6, S7 and S8: a *Sphingobacterium*, *Enterobacter* and *Delftia* sp., respectively) improved plant resistance to water stress, enhancing plant epigeous biomass and length. Moreover, the promotion mediated by the best performing strains was observed in all the assayed rootstocks, with enhanced effects on SO4 rootstock plants.

Assessment of PGP bacteria effects on plant physiology

In order to evaluate how bacteria alleviated water stress *in planta*, 30 days after drought induction, a series of physiological parameters were measured, including net photosynthesis (Pn), transpiration (E), stomatal conductance (Gs) and internal CO2 (Ci) (Fig. 4). Reduction to 50% of the water field capacity induced a stress condition that affected all the considered physiological parameters of all the exposed plants; nevertheless, some PGP bacteria caused an improvement in leaf physiological parameters compared with uninoculated plants (Fig. 4).

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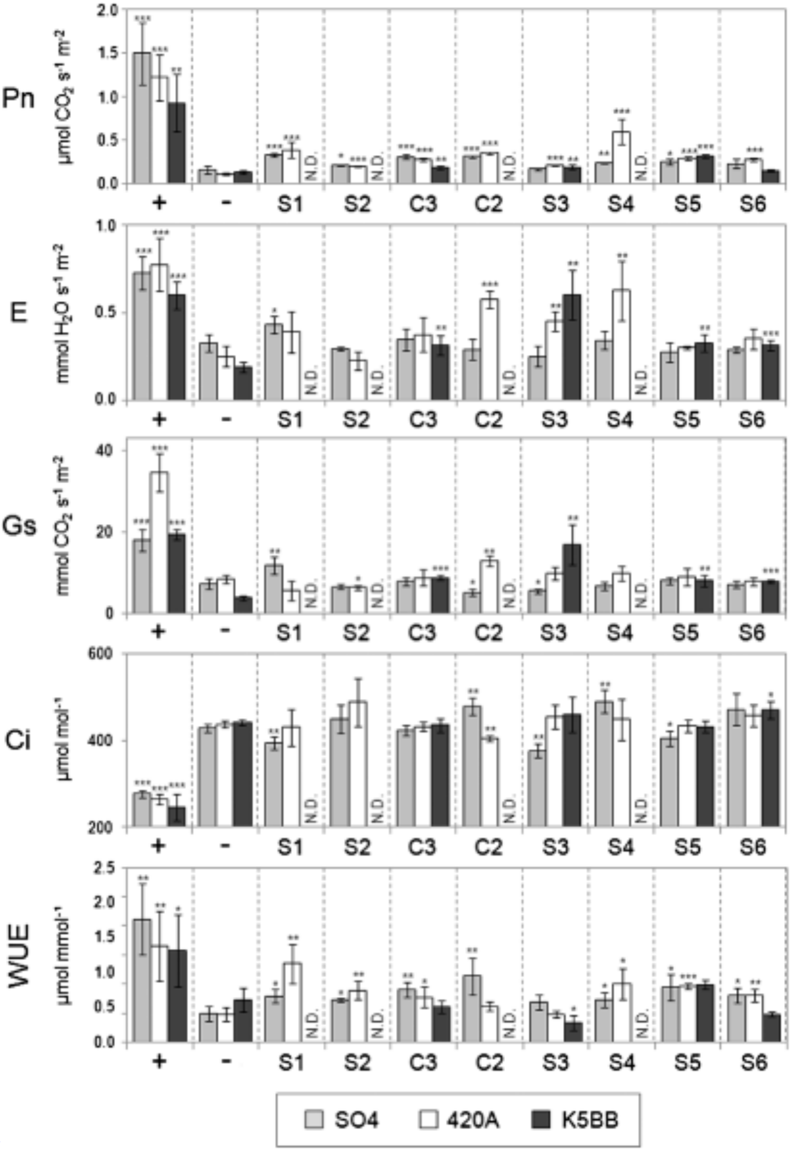

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Figure 4.

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PGP bacteria increased plant resistance to drought stress. '+', abiotic control, irrigated at the water-holding capacity of the soil throughout the experiment; '−', abiotic control, subjected to drought by interrupting watering for 30 days. Pn, net photosynthesis; E, evapotranspiration; Gs, stomatal conductance; Ci, internal carbon dioxide (CO2). Leaf physiological parameters and water use efficiency (WUE) values, determined as Pn/E ratio, in treated and untreated plants. Data were subjected to statistical analysis using the Student *t*-test, with significance reported as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. The data reported in the graphs are representative of three replicate plants.

Photosynthesis inhibition induced by drought was alleviated in SO4 rootstock plantlets inoculated with strains *Pseudomonas* sp. S1, *Acinetobacter* sp. S2, *Bacillus* sp. S4 and *Delftia* sp. S5, and consortia C2 and C3, compared with the relative uninoculated control plantlets (Fig. 4). Strains S1, S3 and S5 slightly decreased Ci.

Rootstock 420A inoculated with all strains and consortia tested displayed significantly improved Pn values compared with the uninoculated plants (Fig. 4). Only strains *Pseudomonas* sp. S3 and *Bacillus* sp. S4 and consortium C2 caused statistically higher E values in 420A rootstock under water stress compared with the uninoculated control (Fig. 4). Rootstock K5BB plantlets showed significantly higher values for Pn, E and Gs when inoculated with strains *Pseudomonas* sp. S3, *Delftia* sp. S5 and consortium C3 compared with the relative uninoculated control plants, while strain *Sphingobacterium* sp. S6 positively affected only E and Gs (Fig. 4).

To better evaluate the impact of drought alleviation on plant physiology, water use efficiency (WUE) was determined (Fig. 4). Strains *Pseudomonas* sp. S1, *Acinetobacter* sp. S2, *Bacillus* sp. S4, *Delftia* sp. S5 and *Sphingobacterium* sp. S6 and the consortium C3 significantly increased WUE in both SO4 and 420A rootstocks. Strain *Pseudomonas* sp. S3, ineffective in SO4 and 420A plants, increased WUE in K5BB inoculated plants (Fig. 4).

Grapevine growth promotion under outdoor conditions

One-year-old ungrafted Barbera plantlets were cultivated outdoors during summer (Table S3) in soil collected from the vineyard of origin (Table S2). Plants were inoculated with strains *Pseudomonas* sp. S1, *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3, three of the best performing strains in inducing drought tolerance based on the previous experiments (Figs 3 and 4). PGP strain *Bacillus* sp. T4 (Table 1), isolated from grapevine plants in a Tunisian soil (Marasco *et al*., **2013b**), was also included in the experiment as a non-autochthonous isolate. Forty-nine days after bacterial treatment, plants were harvested for root biomass and length analysis. Grapevine plants exposed to the Oltrepò Pavese native bacterial strains displayed an increase in plant biomass (Fig. 5A), attributed to a proliferation in root structure (Fig. 5B and C). Uninoculated control and strain T4-exposed plants displayed non-statistically different root biomasses (Fig. 5A–C). PGP bacteria promoted the formation of a more robust root system in plants inoculated with strains *Pseudomonas* sp. S1, *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3 (Fig. 5B). Similar results were observed for root dry weight, confirming the potential of PGP bacteria in stimulating grapevine growth (Fig. 5C). To link the beneficial effect mediated by bacteria *in planta* with bacterial persistence in the rhizosphere and the effect of strain inoculation on the structure of the overall microbial community, a 16S rRNA-based PCR-denaturing gel gradient electrophoresis (PCR-DGGE) analysis was performed (Fig. 5E). Cluster analysis of the PCR-DGGE profiles showed that fertilization of grapevine with the selected strains dramatically affected the structure of the rhizosphere community compared with the untreated control (Fig. 5F). Re-sequencing of DNA from DGGE bands confirmed the establishment of the inoculated strains in the rhizosphere and their efficient root colonization (Fig. 5E).

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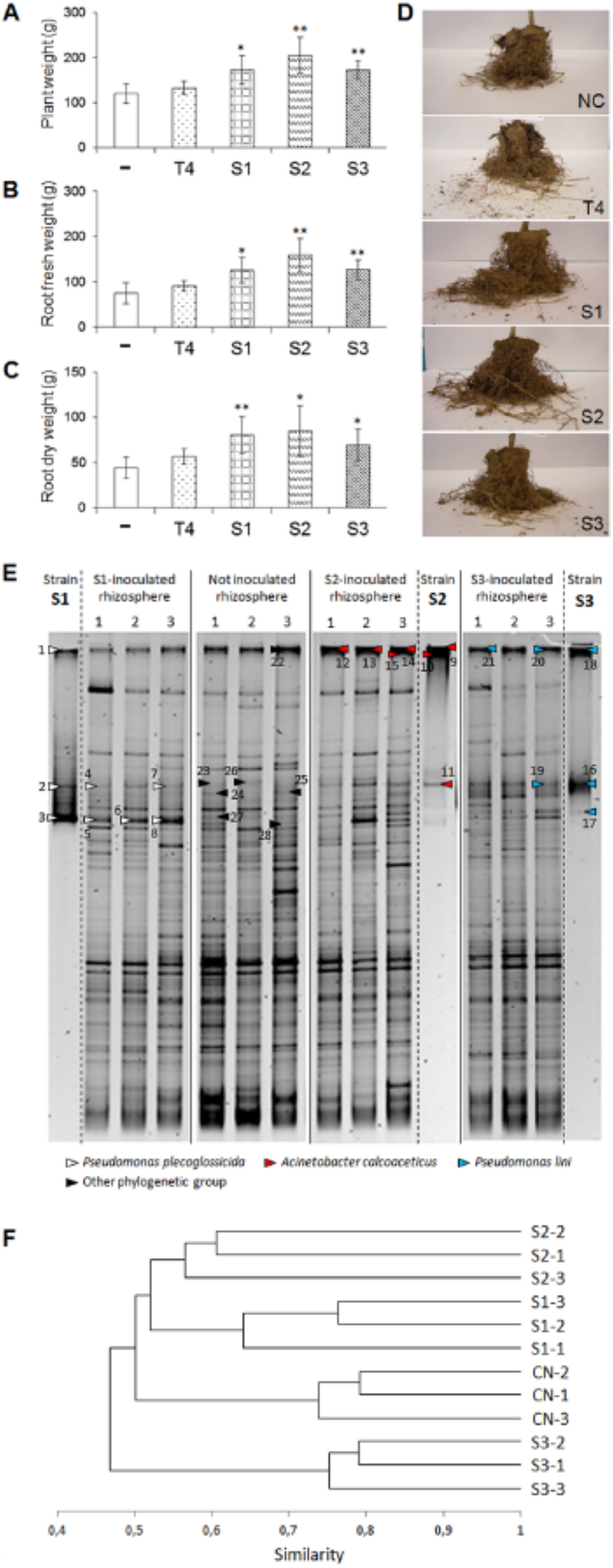

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Figure 5.

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PGP bacteria can efficiently improve plant growth under field-like conditions. '−', abiotic control. (A) Plant fresh biomass; (B) fresh root weight; (C) dry root weight of 1-year old ungrafted inoculated Barbera plantlets grown outdoors. (D) Representative images of the root architecture of grapevine plantlets exposed to the bacterial inoculants. (E) DGGE profiles based on the 16S rRNA gene of the rhizosphere bacterial communities associated with grapevine cultivated outdoors. DGGE profiles of *Pseudomonas* sp. S1 and S2 and *Acinetobacter* sp. S3 are included. Arrowheads indicate the bands that were excised from the gel, successfully amplified and sequenced. (F) Cluster analysis of the DGGE gel profiles of the rhizosphere 16S rRNA of grapevines exposed to S1, S2 and S3 isolates compared with uninoculated plants (−).

Discussion

Water stress is an environmental constraint that hinders grapevine rootstock growth and development, compromising the photosynthetic activity of the whole plant and negatively affecting fruit quality and yield (Chaves *et al*., **2010**). The rootstock plays a critical role in sensitivity to drought (Flexas *et al*., **1999**; Vandeleur *et al*., **2009**), and scion transpiration rate is controlled through different genetic architectures, thus implying different quantitative trait loci (Marguerit *et al*., **2012**).

Generally, there is still little knowledge concerning the contribution of root-associated microbes to plant adaptation to drought, particularly in grapevine. Indeed, under stressful conditions, plants favour the establishment of microorganisms in the rhizosphere and endosphere, which are able to improve the ecosystem services necessary to sustain plant growth (Koberl *et al*., **2011**; Timmusk *et al*., **2011**; Marasco *et al*., **2012**).

We investigated the potential of selected bacteria to alleviate drought stress in grapevine by addressing a series of questions: (i) Can root-associated bacteria enhance resistance to water stress in plants in general, and in particular in grapevine? (ii) Is the drought resistance effect dependent on the rootstock cultivar and bacterial type? (iii) What is the extent of root colonization of selected strains in a non-sterile root system? (iv) Is any PGP effect under water stress a *per se* trait of a bacterial strain or is it activated under stress?

The results of the study represent the end-point of an experimental approach for the selection of promising PGP strains that may support drought resistance in grapevine. This approach encompassed isolation of ACC-deaminase bacteria from the grapevine rhizosphere and endosphere, generating a collection of 510 strains. The genetic redundancy of the collection was reduced by a typing approach targeting the 16S-23S rRNA spacer region (Daffonchio *et al*., **1998a,b; 2000**) that allowed the selection, and further *in planta* testing, of eight different ribotypes, and the corresponding strains affiliated to the genera *Acinetobacter*, *Sphingobacterium*, *Enterobacter*, *Delftia*, *Bacillus* and *Pseudomonas*.

Certain species affiliated to some of the identified genera, such as *Delftia*, are opportunistic pathogens that thrive in drinkable water (Garcia *et al*., **2012**). Indeed, it is well demonstrated that potential opportunistic human pathogens inhabit the rhizosphere of crops (Berg *et al*., **2005**; **2011**). Little is known about their pathogenicity in comparison to their clinical counterparts; however, they are thought to possess differential genome expression profiles, different antibiotic resistance patterns and virulence activity (Mendes *et al*., **2013**). From an ecological perspective, according to their colonization strategy, potential human pathogenic bacteria survive in the soil and colonize plant tissues using plants as an intermediate host allowing them to reach the human gastrointestinal tract (Tyler and Triplett, **2008**).

We tested the root colonization capacity of two of the strains, *Acinetobacter* sp. S2 and *Pseudomonas* sp. S3, in more detail. They were both able to grow in the rhizosphere, as shown by bacterial counts after different time periods of contact with the root. They were also rhizocompetent and efficient root colonizers capable of adhering to the root surface and colonizing the rhizoplane of both *Arabidopsis* and grapevine, as revealed through the use of *gfp*-labelled strains.

To evaluate their PGP potential, the selected strains were screened *in planta* under simulated drought conditions. Under water deficit, plantlets fertilized with the bacteria showed improved development of the aerial portion, exhibiting significantly increased shoot and leaf fresh biomass and shoot length. In the case of SO4, the most sensitive to drought of the studied grapevine rootstocks, shoot biomass and length of drought-stressed plants treated with *Acinetobacter* sp. S2 were 81.2% and 57% higher, respectively, than in stressed but uninoculated plants. Similar promotion effects on shoot vegetation were observed in 1103P and 41B rootstocks fertilized with PGP strains from a microbial bank collection (Sabir *et al*., **2012**). The use of the vineyard soil could have played a role in facilitating the establishment of a stable relationship between bacteria and plants, considering that the isolates were re-inoculated in the soil from which they were isolated (Aballay *et al*., **2011**). Our soil sampling strategy minimized the potential toxic effects of soil copper on the plants. Vineyard soils contain high copper concentrations due to the use of treatments against fungal phytopathogens (Baize, **1997**). In our experiments, we used the original vineyard soil at a depth of more than 30 cm in order to reduce the toxic impact of copper on grapevine roots (Chopin *et al*., **2008**). We measured a total copper concentration of 42.3 ppm in the Ap2 horizon (below 30 cm), compared with 107.9 ppm detected in Ap1 (0–30 cm). Indeed, we observed no stress symptoms in the well-irrigated plants grown in the Ap2 soil.

Besides the growth promotion effect under drought stress, the measurement of *in vivo* physiological parameters showed that the inoculated plantlets displayed increased Pn and E values compared with the untreated plants. The effectiveness of PGP isolates in plant promotion under drought conditions appeared to be a robust trait, being confirmed in two rather different plant models, pepper and grapevine, with *Pseudomonas* sp. S1 and S3 and A*cinetobacter* sp. S2 being the best performing strains. Although ineffective in the promotion of pepper plantlets under well-irrigated conditions, the selected strains were specifically able to improve pepper root biomass under drought conditions, during the temporal window analysed. Our findings potentially indicate that the selected strains are specifically involved in drought resistance rather than promotion *per se*. Drought tolerance was demonstrated to be a habitat-adapted trait for fungal endophytes from rice, but these isolates promoted plant development even during irrigation (Redman *et al*., **2011**). Recent literature concerning plant–microbe interactions during drought is mainly focused on osmotic tolerant bacteria. Strains affiliated to *Bacillus* and *Ochrobactrum* sp., isolated from salinized soils, were able to delay wilting symptoms and induce an antioxidant response in wheat plants subjected to water stress (Chakraborty *et al*., **2013**).

In our study, bacteria-mediated PGP promotion was not only exhibited under greenhouse conditions, since the three most effective strains also supported plant growth under outdoor conditions. A larger root system was retrieved in bacteria-treated plantlets, compared with the untreated control. Barbera exposed to *Acinetobacter* sp. S2 showed a root biomass twofold higher than that of untreated plants (158 ± 37 versus 74.6 ± 23.3 g), supporting the potential beneficial effects of the strain even under 'field-like' conditions.

The root growth promotion effect was coupled with a dramatic change in the structure of the native microbial community of the soil following biofertilization with *Pseudomonas* sp. S1 and S3, and *Acinetobacter* sp. S2. This effect of PGP bacteria on rhizosphere microbial communities has not been previously reported in grapevine. In field-grown corn plants, the structure of the microbial community was primarily affected by plant developmental stage rather than PGP bacteria treatment (Piromyou *et al*., **2011**). The use of biofertilizer as a biocontrol agent affected the fungal rather than bacterial community structure, with a reduction of potentially dangerous phytopathogen species in tomato and sweet pepper (Schmidt *et al*., **2012**). All the three strains used in our study were clearly detectable in the 16S rRNA gene PCR-DGGE profiles of inoculated plants, but not in those of uninoculated plants. This supports a specific enrichment of the selected strains in the rhizosphere community and the establishment of a strong relationship with plant roots that, in turn, is associated with the observed growth promotion effect under water stress.

Under dry conditions, soil should experience a more aerobic condition with less denitrification and nitrogen loss (Hartmann *et al*., **2013**). A reduced amount of water should decrease soil pore clogging, resulting in reduced root cell death and an altered rhizodeposition pattern (Sanaullah *et al*., **2012**). The mode of action of bacteria in helping plants under drought stress remains largely elusive, and new hypotheses should consider the above-mentioned characteristics of soil-root-microbe systems under water shortage. We speculate that the capacity of our selected bacteria to interfere with auxin and ethylene homeostasis through synthesis of indole acetic acid and ACC-deaminase activity could have a role in the promotion of a more robust system (Mayak *et al*., **2004**) able to exploit a larger soil volume and improve water uptake. Changes in root morphology have also been documented for *Azospirillum* sp. endowed with ACC-deaminase activity and able to produce nitric oxide (Molina-Favero *et al*., **2008**).

Other compounds that were produced by the selected bacteria and potentially involved in supporting plant growth under drought conditions include siderophores; similar to organic acids or specific enzymes, they improve the bioavailability of minerals. Siderophore production could enhance the bioavailability of metal cofactors that may exhibit decreased solubility and availability to the plant during drought due to decreased water content in soil (Vassilev *et al*., **2012**; Qi and Zhao, **2013**).

The production of exopolymeric substances (EPS) by some of the selected strains may also have played a role in supporting plant growth under water stress, contributing to the formation of a hydrophilic biofilm around the roots acting as an additional sheath to protect the root system from soil hardness (Rossi *et al*., **2012**; Xu *et al*., **2013**). EPS on root tissues could also act as mild emulsifiers protecting biological membranes and contributing to scavenging reactive oxygen species, hence enhancing plant resistance to water stress (Llamas *et al*., **2012**; Dimitrova *et al*., **2013**).

In conclusion, the selected strains increased the potential fitness of grapevine plants under water stress by enhancing growth and biomass under greenhouse and field-like conditions. Thus, grapevine-associated bacteria represent a tool to alleviate the effects of drought in grapevine. Experiments with pepper plants showed that this plant growth promotion was not a *per se* trait of the strains but rather it was specifically activated under water stress conditions. It remains unknown how the host selects beneficial endophytes and rhizobacteria to increase fitness under drought conditions. Similarly, the molecular basis of the mechanisms that bacteria activate to stimulate grapevine resistance to water stress is yet to be determined.

Experimental procedures

Identification and handling of bacteria

The bacteria used in the present study were a subset of selected strains from a collection of 510 isolates from the rhizosphere (rhizobacteria) and root tissue (endophytes) of ungrafted grapevine plants (*V. vinifera* L., cv. Barbera), and Barbera grapevine rootstocks named 402A, 157.11, 161.49 and SO4 (*Vitis ripariae* × *Vitis berlandieri*). Grapevine plants were sampled at the end of July 2009 in a vineyard of 'Le Fracce' farm, located in the Oltrepò Pavese wine-producing region, which is an important area for Barbera wine production in the Lombardy region (Italy). The procedure adopted for isolation and de-replication of the bacterial collection was essentially as described by Marasco and colleagues (**2012**), using an ACC-deaminase enrichment culture as previously described (Penrose and Glick, **2003**). The partial 16S rRNA gene sequences of the nine selected strains (S1, S2, S3, S4, S5, S6, S7, S8 and T4) were deposited with the following accession numbers: HE610897, HE610896, HE610893, HE610894, HE610899, HE610898, HE610895, HF562860 and HF585069.

In vitro characterization of the PGP potential and abiotic stress tolerance of the selected strains

Auxin IAA (indol-3-acetic acid) production was assessed as previously described (Brick *et al*., **1991**). The presence of IAA in the culture supernatant was determined spectrophotometrically at 530 nm. Pure IAA (Sigma-Aldrich, Italy) was used as the standard and uninoculated media served as the control. The mineral P-solubilizing ability of the strains was determined on Pikovskaya's liquid medium amended with 0.5% tricalcium phosphate [Ca3(PO4)2] as inorganic P (Nautiyal, **1999**). The culture supernatant was used for qualitative spectrophotometer assays at 600 nm. The optical density (OD) values were used to classify the phosphate solubilizers into three groups on a qualitative basis (Mehta and Nautiyal, **2001**), fixing 0.3 OD as the threshold value for phosphate solubilization. Siderophore release was detected by the formation of orange halos on chrome azurol S (CAS) agar plates after incubation for 7 days at 30°C, as described elsewhere (Schwyn and Neilands, **1987**). Assays for exopolysaccharides release, protease and ammonia production, and resistance to abiotic stresses (temperatures, halotolerance and osmotic stress), were performed as previously described (Marasco *et al*., **2012**).

Bacteria transformation, *in vitro* rhizoplane adhesion, re-isolation experiments from axenic *Arabidopsis* roots and grapevine colonization

Bacteria transformation and the adhesion assay on *Arabidopsis* roots were performed essentially as described previously (Marasco *et al*., **2012**). *Arabidopsis* was selected as a model plant for the evaluation of root adherence ability, as already shown (Fan *et al*., **2012**). Root images were acquired using an epifluorescence microscope Leica DM 4000 B, and further analysed using the mbf ImageJ software. In order to re-isolate the colonizing strains, *Arabidopsis* plantlets were placed in a 96-well plate 3 days post-germination of surface-sterilized seeds. The plant roots were dipped in 108 cells ml−1 bacterial suspension for 1 h or 4 h before being gently washed to remove weakly bound bacteria. The short incubation time allowed us to evaluate how quickly bacteria interact with and adhere to the root system, which is considered to be the first stage of rhizosphere colonization and/or endosphere penetration (Barret *et al*., **2011**). After a washing procedure, the plant root was crushed, re-suspended in sterile physiological solution (9 g l−1 NaCl) and used for serial dilutions that were plated on tryptic soy agar medium (TSA).

A colonization assay was performed on the grapevine root system by dipping 'Black magic' grapevine plants (the root system and associated soil) in an *Acinetobacter* sp. S2 suspension concentrated at 108 cells ml−1 for 16 h. After biofertilization, plants were planted in pots, covered with aluminium foil and placed in the greenhouse. Plants were appropriately irrigated throughout the experiment. Seven and 21 days after biofertilization, root specimens were gently removed, washed with water and analysed by confocal laser microscopy (Leica TCSNT). Images were acquired using Leica Confocal Software, using a BP530/30 GFP filter (excitation at 488 nm) and LP590 TRITC filter (excitation at 568 nm).

Plant growth promotion of pepper plantlets in soil under well-irrigated and water stress conditions

Pepper seeds were sown in trays in wet agriperlite. After 1 week, uniform-sized seedlings were selected and planted in soil. The seedlings were maintained in a growth chamber at a day/night temperature of 25/20°C, with ∼100 µmol photons m−2 s−1 of light supplied for 12 h during the day. The selected strains used for the experiment were streaked on TSA plates to verify their purity and then inoculated in 300 ml of tryptic soy broth in a 1000 ml Erlenmeyer flask and incubated at 30°C on a shaker (150 r.p.m.) for 48 h. Bacteria were collected by centrifugation (4000 r.p.m., 15 min) and washed twice with physiological buffer (9 g l−1 NaCl). The pellet was re-suspended in sterilized water and used to inoculate pepper plantlets. During the second week, the seedlings were fertilized once with the bacterial suspensions of the selected PGP bacteria at a concentration of 108 cells g−1 of soil, while uninoculated plants were watered with tap water. Single bacterial cultures (*Pseudomonas* sp. S1, *Acinetobacter* sp. S2, *Pseudomonas* sp. S3, *Bacillus* sp. S4, *Delftia* sp. S5 and *Sphingobacterium* sp. S6) and two bacterial consortia (consortium C2 composed of equal cell numbers of strains *Acinetobacter* sp. S2 and *Bacillus* sp. S4; and consortium C3, prepared by mixing equal cell numbers of strains *Sphingobacterium* sp. S6, *Enterobacter* sp. S7 and *Delftia* sp. S8) were used as inocula. The consortia composition was determined by the assemblage of strains characterized by a similar growth rate (data not shown). Plants were divided into two groups: the former was properly irrigated throughout the experiment, while water was withheld for 7 days one week after bacteria inoculation in the latter group. After this induced drought, water irrigation was resumed for 2 days, and plants were harvested for biomass and length measurements. Statistical analysis was performed using the Student *t*-test with $P \leq 0.05$ considered statistically significant.

Induction of drought stress in grapevine plants

The bacteria cultures for the biofertilization of grapevine plantlets were prepared as described in the previous paragraph for pepper plants. The root systems of 1-year-old grapevine plantlets were dipped in a 108 cells ml−1 bacterial suspension in water in order to maximize the contact between bacteria and the roots. In the case of the uninoculated control, the plantlets were dipped directly in water. After 24 h, grapevine plantlets were planted in plastic pots (18 cm diameter) filled with 2.5 kg of soil collected from 'Le Fracce' vineyard (Table S2). Pots were placed in a greenhouse at 25/20°C (day/night temperature), with a 16 h photoperiod, under 500 µmol s−1 m−2 light irradiance and 70% relative humidity. A completely randomized design with five replicates was employed for each treatment for SO4 and 420A plantlets (*Vitis ripariae* × *Vitis berlandieri*), characterized respectively by low and medium drought adaptability (Sampaio and Vasconcelos, **2005**; Zsofi *et al*., **2008**). The treatments for SO4 and 420A plantlets were as follows: abiotic control watered at full field capacity (+), abiotic control subjected to drought (−), and plants fertilized with strains/consortia S1, S2, S3, S4, S5, S6, and consortia C2 and C3. The treatments for Kober 5BB plantlets (*Vitis ripariae* × *Vitis berlandieri*), with medium drought resistance (Sampaio and Vasconcelos, 2005), were as follows, using four replicates: abiotic control watered at full field capacity (+), abiotic control subjected to drought (−) and plants fertilized with strains/consortium S3, S5, S6 and C3. The soil samples used in the experimental tests were collected from the horizon Ap2. The soil profile was determined on a hilly summit 150 m a.s.l.; this profile best represented the silt loam pedotypes widely developed on the summit of the hill slopes, with a fragipan horizon in depth, developed from the loess. Soil for the experimental procedure was collected at depths of more than 30 cm in order to exclude the fraction of soil colonized by grasses and disturbed by mechanical means and pasturing. The collected fraction contains the lowest concentration of copper, which is used in vineyards as a fungicide against downy mildew (Komarek *et al*., **2010**). This soil, due to its lower available water capacity, is frequently subjected to drought in summer. Soil water content in the pots was maintained at the field capacity for 15 days; each day, pots were supplemented with the amount of water lost by evapotranspiration, which was determined by weighing each pot. After this acclimatization period, the soil water content was reduced to half of the field capacity for all inoculated plants. Uninoculated plants irrigated at half of the field capacity were used as the negative control (−). Uninoculated plants watered at field capacity were used as the positive control (+) to establish grapevine plantlet growth under an optimal water regime. Drought was maintained for 30 days, and plant water demand was monitored every 2–3 days by a ML2× Theta Probe soil moisture sensor (Delta-T Devices, Cambridge, UK). In order to characterize plant physiological status during drought, gas exchange measurements were taken on young, fully expanded, intact leaves of grapevine plants with a portable photosynthesis system (CIRAS-2, PP System, USA). Net CO2 assimilation rate (Pn), stomatal conductance (Gs) and transpiration (E) were assessed at a CO2 concentration of 400 µmol mol, 50% relative humidity, 28°C temperature, 500 µmol s−1 airflow and a photon flux density of 1500 µmol (m2)−1 s−1. The instrument was stabilized according to the manufacturer guidelines. The WUE was calculated as Pn divided by E. After the induced drought, all plants were re-watered at field capacity for 3 days and carefully harvested for the final analysis of biomass and length parameters.

Grapevine growth promotion under outdoor conditions

The three strains, *Pseudomonas* sp. S1 and S3 and *Acinetobacter* sp. S2, which performed best during drought induction in the greenhouse assay, were further characterized for their ability to support grapevine plant growth under field-like conditions. The bacterial cultures for grapevine plant inoculation were prepared as previously described, including strain T4, identified as *Bacillus subtilis* and isolated from a vineyard in Tunisia. One-year old grapevine plantlets of non-grafted Barbera were kindly provided by 'Pèpinières Guillaume' (Charcenne, France) and used in this experiment. Grapevine plants were dipped in a bacterial suspension of 108 cells g−1 of soil in water for 24 h. After this incubation period, the plants were planted in plastic pots filled with soil collected from 'Le Fracce' vineyard (Table S2). Pots were placed in the courtyard of the Department of Food, Environmental and Nutritional Sciences of the University of Milan. Plant growth was monitored for 55 days (22 July to 10 September 2010) under 'field-like' conditions; subsequently, plants were harvested for further analysis.

PCR-DGGE analysis of rhizosphere-associated bacterial communities of grapevine plants cultivated outdoors

DNA was extracted from rhizosphere soil using a MOBIO kit, following manufacturer instructions. Primers 907R and 357F with a GC-clamp were used for the amplification of bacterial 16S rRNA genes (Muyzer *et al*., **1993**). The polymerase chain reaction (PCR) was performed in 0.2 ml tubes with a 50 µl reaction volume. The reaction mixture contained the diluted buffer 1X, 1.5 mM MgCl2, 5% DMSO, 0.12 mM of a mixture of dNTPs, 0.3 µM of each primer, 1 U Taq polymerase and 10 ng of template. When necessary, DNA was properly diluted. Cycling conditions used to amplify the 16S rRNA gene fragment were as follows: 94°C for 4 min, followed by 10 cycles of 94°C for 0.5 min, 61°C for 1 min and 72°C for 1 min; followed by a further 20 cycles of 94°C for 0.5 min, 56°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. Two microlitres of the PCR product were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide prior to DGGE. For DGGE analysis, 100–150 ng of the PCR products generated from each sample was separated using polyacrylamide gel (8% of a 37:1 acrylamide–bisacrylamide mixture in a Tris acetate EDTA (TAE) 1X buffer, 0.75 mm thick, 16 × 10 cm) with a 45–60% denaturant gradient. Gel was run overnight at 90 V in TAE 1X buffer at 60°C in a DCode apparatus (Bio-Rad, Milan, Italy). The gel was stained with 1X Syber Green (Life Technologies) in TAE buffer and scanned with gel photo GS-800 system.

The DGGE bands were excised from the gel using a sterile cutter and eluted in 50 μl water at 37°C for 6 h. Re-amplification of DNA eluted from DGGE bands was performed using 907R and 357F primers without the GC-clamp, using the following protocol: 95°C for 5 min, 30 cycles of 95°C for 1 min, 61°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were checked by electrophoresis in 1% agarose gel. Fragment sequencing was performed by Macrogen (South Korea), and the obtained sequences were aligned in the EzTaxon database. The DGGE sequences were submitted under accession numbers from HG330198 to HG330225.

The band profile of fragments in the DGGE gel was converted into line plots with ImageJ software (Schneider *et al.*, **2012**), and the *x*/y values obtained were imported into an Excel file. The matrix of *x*/y values of 16S rRNA gene band profiles was subjected to cluster analysis using the Pearson correlation coefficient. The multivariate analyses were conducted using the xlstat software (vers. 7.5.2 Addinsoft, France).

Statistical analysis

A randomized block design was adopted to perform the experiments. The mean values of the measured parameters were compared separately to their internal negative control in order to avoid any effect of morphological or growth features due to the genotype. The growth parameter data were subjected to statistical analysis using the Student *t*-test with significance at $P \leq 0.05$.

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Ancillary

Supporting Information

Filename	Description
emi12439-sup-0001-si.docx 18K	**Table S1.** Details of the bacterial collection obtained from th Oltrepò Pavese soil. The numbers of total, rhizosphere (R) an endosphere (E) isolates obtained from each plant type are sh The results of isolate dereplication are shown as number of I types. The number of species identifying the different ITS typ the R and E fractions are also indicated. Species identification according to 16S rRNA gene sequences, of the eight isolates s for the *in vivo* experiments on grapevine plantlets are also sh **Table S2.** Physical-chemical characterization of the soil used greenhouse and outdoor experiments. **Table S3.** Environmental conditions during the outdoor expe performed during summer (22 July to 10 September 2012).

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