

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H⁺-pumping pyrophosphatase in pepper plants

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1681215> since 2018-11-15T09:31:11Z

Published version:

DOI:10.1111/1462-2920.14272

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

[Vigani G, Rolli E, Marasco R, Dell'Orto M, Michoud G, Soussi A, Raddadi N, Borin S, Sorlini C, Zocchi G, Daffonchio D., Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H⁺ - pumping pyrophosphatase in pepper plants, Environmental Microbiology ,2018, Epub ahead of print], doi: 10.1111/1462-2920.14272.]

The publisher's version is available at:

[inserire URL sito editoriale presa dal campo URL, cioè dc.identifier.url]

When citing, please refer to the published version.**Link to this full text:**

[inserire l'handle completa, preceduta da <https://onlinelibrary.wiley.com/doi/full/10.1111/1462-2920.14272>]

Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H⁺-pumping pyrophosphatase in pepper plants

Gianpiero Vigani

Department of Life Sciences and Systems Biology, University of Turin, Plant Physiology Unit, 10135, Turin, Italy

These authors contributed equally to this work. [Search for more papers by this author](#)

Eleonora Rolli

Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133, Milan, Italy

These authors contributed equally to this work. Present address: IPS2, Institute of Plant Sciences Paris-Saclay, 91405

Orsay, France. [Search for more papers by this author](#)

Ramona Marasco

Biological and Environmental Sciences and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

These authors contributed equally to this work. [Search for more papers by this author](#)

Marta Dell'Orto

Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DISAA), University of Milan, 20133, Milan, Italy

[Search for more papers by this author](#)

Grégoire Michoud

Biological and Environmental Sciences and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

[Search for more papers by this author](#)

Asma Soussi

Biological and Environmental Sciences and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

[Search for more papers by this author](#)

Noura Raddadi

Department of Civil, Alma Mater Studiorum University of Bologna, Chemical, Environmental and Materials Engineering (DICAM), Bologna, Italy

[Search for more papers by this author](#)

Sara Borin

Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133, Milan, Italy

[Search for more papers by this author](#)

Claudia Sorlini

Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133, Milan, Italy

[Search for more papers by this author](#)

Graziano Zocchi

Corresponding Author

E-mail address: graziano.zocchi@unimi.it

Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DISAA), University of Milan, 20133, Milan, Italy

For correspondence. E-mail

daniele.daffonchio@kaust.edu.sa

; Tel. +966 (2) 8082884; E-mail

graziano.zocchi@unimi.it

[Search for more papers by this author](#)

Daniele Daffonchio

iris-AperTO

University of Turin's Institutional Research Information System and Open Access Institutional Repository

Corresponding Author

E-mail address: daniele.daffonchio@kaust.edu.sa

<http://orcid.org/0000-0003-0947-925X>

Biological and Environmental Sciences and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

For correspondence. E-mail

daniele.daffonchio@kaust.edu.sa

; Tel. +966 (2) 8082884; E-mail

graziano.zocchi@unimi.it

First published: 22 May 2018

<https://doi.org/10.1111/1462-2920.14272>

Summary

It has been previously shown that the transgenic overexpression of the plant root vacuolar proton pumps H⁺-ATPase (V-ATPase) and H⁺-PPase (V-PPase) confer tolerance to drought. Since plant-root endophytic bacteria can also promote drought tolerance, we hypothesize that such promotion can be associated to the enhancement of the host vacuolar proton pumps expression and activity. To test this hypothesis, we selected two endophytic bacteria endowed with an array of *in vitro* plant growth promoting traits. Their genome sequences confirmed the presence of traits previously shown to confer drought resistance to plants, such as the synthesis of nitric oxide and of organic volatile organic compounds. We used the two strains on pepper (*Capsicum annuum* L.) because of its high sensitivity to drought. Under drought conditions, both strains stimulated a larger root system and enhanced the leaves' photosynthetic activity. By testing the expression and activity of the vacuolar proton pumps, H⁺-ATPase (V-ATPase) and H⁺-PPase (V-PPase), we found that bacterial colonization enhanced V-PPase only. We conclude that the enhanced expression and activity of V-PPase can be favoured by the colonization of drought-tolerance-inducing bacterial endophytes.

Introduction

Drought is one of the most severe abiotic plant stresses that strongly limits crop productivity (Calanca, 2017). Plants have evolved mechanisms both to cope with drought independently, including escape strategies, avoidance and tolerance (Jarzyniak and Jasiński, 2014), and to cooperate with beneficial plant growth promoting (PGP) microorganisms that provide the plant-host with ecosystem services and activities that mitigate the effects of several abiotic stresses (Lau and Lennon, 2011; 2012; Marasco *et al.*, 2013a 2013b; Chen *et al.*, 2017; Rolli *et al.*, 2017; Vergani *et al.*, 2017).

During periods of drought, plants tune their tissue turgor for low water potential by modulating osmotic adjustments, a phenomenon that must be tightly regulated to maintain cell homeostasis (Fang and Xiong, 2015). Perturbation of the osmotic balance involves changes in ion fluxes across the plasma membrane and tonoplast (Gaxiola *et al.*, 2007). The plant enzyme vacuolar proton pumps V-ATPase (H⁺-adenosine triphosphatase) and V-PPase (H⁺-pyrophosphatase) hydrolyse adenosine triphosphate (ATP) and pyrophosphate (PPi), respectively, to increase the ion concentration in the vacuoles by establishing the necessary electrochemical gradient across the tonoplast (Maeshima, 2001; Martinoia *et al.*, 2006). The resulting increase in the vacuolar osmotic pressure is coupled with a decrease in the cell water potential, which in turn facilitates water uptake from soil, alleviating the drought stress. The role of V-ATPase and V-PPase in plant tolerance to drought has been demonstrated by their overexpression in several plants, including *Arabidopsis thaliana* (Gaxiola *et al.*, 2001), tomato (Park *et al.*, 2005; Da-Gang *et al.*, 2012), rice (Zhang *et al.*, 2011), tobacco (Arif *et al.*, 2013), maize (Li *et al.*, 2008), barley (Schilling *et al.*, 2014), sugarcane (Kumar *et al.*, 2014; Raza *et al.*, 2016), watermelon rootstock (Park *et al.*, 2014), peanuts (Qin *et al.*, 2013), alfalfa (Bao *et al.*, 2016; Wang *et al.*, 2016b), and cotton under both laboratory and field conditions (Pasapula *et al.*, 2011).

In arid environments, desert farming favours the selection of drought-protecting microbial assemblages (Marasco *et al.*, 2012; TerHorst *et al.*, 2014; Soussi *et al.*, 2016) that are enriched and rearranged in the plant

iris-AperTO

University of Turin's Institutional Research Information System and Open Access Institutional Repository

rhizosphere and endosphere (Mapelli *et al.*, 2013; Marasco *et al.*, 2013a; 2016; Cherif *et al.*, 2015; Ferjani *et al.*, 2015; Santos-Medellín *et al.*, 2017). PGP microorganisms use several mechanisms to stimulate drought tolerance in plants (Vurukonda *et al.*, 2016; Etesami and Maheshwari, 2018): (i) the microbial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase contributes to control the concentration of the plant stress phytohormone ethylene, by degrading its precursor ACC (Glick, 2014); (ii) microorganisms contribute to modulate plant hormone homeostasis by producing auxin [i.e., indole-3-acetic acid (IAA)], cytokinins and gibberellins. These phytohormones are involved in a wide range of adaptive responses and may determine changes in plant root gene expression and root architecture (Egamberdieva *et al.*, 2017; Lim and Kim, 2013); (iii) by manipulating the plant antioxidant system microorganisms decrease the reactive oxygen species (ROS) concentration (Wang *et al.*, 2012); (iv) the microbial release of osmolytes acts synergistically with those produced by the plant and enhances resistance to water stress (Etesami and Maheshwari, 2018); (v) exopolysaccharides produced by microorganisms recondition the root microenvironment by favouring water retention and protecting plant roots against desiccation (Rossi *et al.*, 2012); (vi) microorganisms contribute to enhance the plant induced systemic tolerance to drought by altering the host physiology and the metabolic processes (Cho *et al.*, 2013); (vii) an indirect effect of microbial activity is the solubilization of poorly available nutrients such as iron and phosphorus (Pii *et al.*, 2015). Interestingly, evidences suggest that the bacteria-mediated protection is a drought-activated mechanism (Rolli *et al.*, 2015).

Considering the drought-protecting role of vacuolar proton pumps and the ability of root system-associated bacteria to enhance plant drought-tolerance, we hypothesize that such bacterial-mediated tolerance can be associated with an increment of expression and activity of those pumps. In this study, we selected two endophytic bacteria, denoted by E1 (*Bacillus subtilis*) and E3 (*Paenibacillus illinoisensis*), with plant growth promoting (PGP) phenotypes and genome traits that may influence the plants' response to drought. Hence, to test our hypothesis, we analysed the response to drought conditions of pepper (*Capsicum annuum* L.), chosen for its high sensitivity to water stress (Jaimez *et al.*, 2000), both with and without the endophytic bacteria, and assessed the bacterial effect on the expression of the vacuolar proton pumps, V-ATPase and V-PPase.

Results

Plant growth promoting potential of endophyte strains isolated from drought-resistant plants cultivated under desert farming

Through functional screenings for plant growth promoting (PGP) potential, including *in vitro* assays and a rhizocompetence test (Cherif *et al.*, 2015; Marasco *et al.*, 2012), we selected two root endophytes of pepper plants (*Capsicum annuum* L.) cultivated under desert farming conditions (Marasco *et al.*, 2012). The two endophytes, E1 and E3, were affiliated to *Bacillus subtilis* and *Paenibacillus illinoisensis* with 16S rRNA gene sequence identity of 100% and 99% respectively (Fig. 1A). Both E1 and E3 showed multiple PGP phenotypes that have the potential to favour plant tolerance to drought (Table 1). However, the two strains showed different tolerances to osmolytes. E1 tolerated higher concentrations of salt (NaCl and KCl), urea and sodium lactate (Table 1 and Supporting Information Fig. S1) than E3, and was also endowed with several traits that encourage plant root colonization, like the formation of a biofilm-like pellicle, swimming and swarming motility, cellulose degradation activity, and the production of biosurfactants involved in the reduction of root surface tension (Table 1 and Supporting Information Table S1). Both strains produced complex mixtures of volatile organic compounds (VOCs), including some capable of promoting plant growth or protecting against phytopathogens, for example, 2,3-butanediol, acetoin and carbon dioxide (among others Cho *et al.*, 2008; Cortes-Barco *et al.*, 2010; Supporting Information Table S2 and Fig. S2). The complex mixtures of VOCs produced by E1 and E3 promoted the formation of a larger plant biomass in

Arabidopsis thaliana plantlets grown under both normal conditions and osmotic stress induced by 100 mM mannitol (Supporting Information Fig. S3).

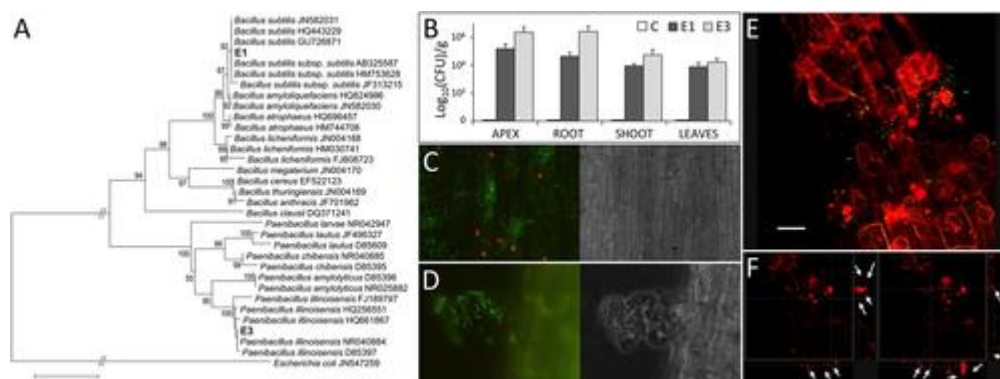


Figure 1

[Open in figure viewerPowerPoint](#)

Phylogenetic affiliation of E1 (*B. subtilis*) and E3 (*P. illinoensis*), and rhizocompetence/colonization ability.

A. Phylogenetic trees of E1 and E3 using the complete 16S rRNA gene sequence. Neighbour-joining phylogenetic tree-based 16S rRNA gene sequences from E1 and E3, and their closest phylogenetic neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position. B. Enumeration of Rif^R versions of the E1^{Rif} and E3^{Rif} strains after isolation on selective medium. C. Epifluorescence and phase contrast images of pepper roots densely colonized by E1-*gfp*. D. Epifluorescence and phase contrast images of E1-*gfp* cells entrapping root hairs. E. Confocal microscopy analysis of E1-*gfp* colonized roots. F. The boxes indicated by dashed lines show orthogonal views of a three-dimensional confocal image created from a z-stack of x/y-scans. White arrows indicate the potential endophytic colonization of a root cortex by E1-*gfp* cells that were found in the orthogonal views of the inner root tissue.

Caption

Table 1. *In vitro* plant growth promoting activities of bacterial endophytes E1 (*B. subtilis*) and E3 (*P. illinoensis*).

(A) Plant growth promoting traits	Bacterial strain		
	E1	E3	
Biostimulation	ACCd activity	No	No
	Auxin production	No	No
Biofertilization	Phosphate solubilization	Yes	Yes
	Siderophore production	Yes	No
	Exopolysaccharide release	Yes	No

(A) Plant growth promoting traits		Bacterial strain	
		E1	E3
VOCs*	Putative N ₂ fixation	Yes	No
	NH ₄ production	Yes	No
	VOCs mixture	Yes	Yes
	2,3 butandiol	Yes	No
Plant growth promotion by VOCs*	Normal condition	Yes	Yes
	Stress condition	Yes	Yes
Recolonization ability	Pellicule formation	Yes	Yes
	Cellulase	Yes	No
	Swimming ability	Yes	No
	Swarming ability	Yes	No
	Biosurfactant production*	Yes	No
(B) Resistance to different types of abiotic stresses		Bacterial strain	
		E1	E3
Salt stress	5% NaCl	Yes	No
	8% NaCl	Yes	No
	10% NaCl	Yes	No
Osmotic stress	10% PEG	Yes	Yes
	20% PEG	Yes	Yes

(A) Plant growth promoting traits		Bacterial strain		
		E1	E3	
Temperature	4°C	No	No	
	42°C	Yes	Yes	
	50°C	Yes	No	
	NaCl	3%	1%	
	Potassium chloride	3%	5%	
	Sodium sulfate	5%	5%	
	Ethylene glycol	20%	20%	
	Sodium formate	1%	1%	
	Osmolyte (+)*	Urea	2%	5%
		Sodium lactate	3%	4%
		Sodium phosphate	200 mM	200 mM
		Sodium benzoate	20 mM	20 mM
		Ammonium sulfate	100 mM	100 mM
		Sodium nitrate	80 mM	40 mM

- Presence or absence of (A) PGP traits and (B) abiotic stress tolerance were evaluated following the methods described in the experimental section. Additional details of the results marked with a star (*) are reported in Supporting Information Tables S1 and S2 and Supporting Information Figs. S1–S3. (+) Indicates the highest concentration of osmolytes (% w/v) tolerated by the strains.

The genomes of E1 and E3 have been sequenced (Supporting Information Table S3). A functional survey of the PGP traits in the genomes revealed that E1 and E3 were endowed with multiple metabolic properties that could contribute to the alleviation of drought-induced stress (Table 2). Gene pathways for the synthesis of VOCs were detected, those included the presence of the gene encoding for the butanediol dehydrogenase that

catalyse the reaction to produce the PGP-volatile 2,3-butanediol (i.e., Cho *et al.*, 2008). The genome analysis also showed the presence of genes encoding for the acetolactate synthase (*alsS*) and the acetolactate decarboxylase (*alsD*), which catalyse the two-step conversion from pyruvate to acetoin that can be further converted into 2,3-butanediol by the enzyme butanediol dehydrogenase, along with the genes for proline/glycine betaine transporters (Table 2). In E1, the *alsS*, *alsD*, and butanediol dehydrogenase gene showed a 100% similarity to the reference genes of *Bacillus subtilis* 168. In E3, the percentages of similarity to the sequenced *Paenibacillus* spp. genomes were very low for all three of these genes since no reference strain was available (Table 2 and Supporting Information Fig. S4). In both the E1 and E3 genomes, we detected genes encoding for a nitric oxide synthase, ROS detoxifying enzymes, and exopolysaccharide synthesis. Both the E1 and E3 genomes lacked genes known to be involved in the synthetic pathways of auxin derivatives and the ACC deaminase (*acdS*) (Table 2).

Table 2. Genomic screening of E1 (*B. subtilis*) and E3 (*P. illinoensis*) for PGP metabolic properties and functions possibly involved in plant drought-resistance.

Potential PGP traits involved in plant drought tolerance	Bacterial strain	
	E1	E3
Glycine betaine pathway	ABC transport enzymes of glycine, betaine/proline (<i>proX</i> , <i>proW</i> and <i>proV</i>)	Absent
Detoxification of ROS	Present	Present
Exopolysaccharide synthesis	<i>noeJ</i> and <i>noeL</i> genes	<i>noeJ</i> and <i>noeL</i> genes
ACC deaminase (<i>acdS</i>)	Absent	Absent
Butanediol dehydrogenase	Present	Present
	100% similarity <i>B. subtilis</i> 168	49.7% similarity <i>B. subtilis</i> 168
Acetolactate synthase (<i>alsS</i>)	Present	Present
	99% similarity <i>B. subtilis</i> 168	29% similarity <i>B. subtilis</i> 168
Acetolactate decarboxylase (<i>alsD</i>)	Present	Present
	100% similarity <i>B. subtilis</i> 168	Absent
Auxin production (<i>iaaM</i> , <i>iaaH</i> , <i>ipdC</i>)	Absent	Absent

Potential PGP traits involved in plant drought tolerance	Bacterial strain	
	E1	E3
Nitric oxide synthase	Present	Present

Recolonization ability has been also evaluated as bacterial essential trait to exert PGP functions. Quantification of the bacterial colonization of the plant tissues by the spontaneous rifampicin-resistant mutants E1^{Rif} and E3^{Rif} revealed that they established dense populations ranging from about 10⁵ to 10⁶ CFU g⁻¹ in the root tissue (considering both apex and cortical tissue) and from about 10³ to 10⁴ CFU g⁻¹ in the stem and leaf tissues (Fig. 1B). These relatively high bacterial counts were obtained after washing the plant organ surfaces with a physiological buffer several times to eliminate those cells not tightly associated with the plant. Thus, the strains were capable of efficiently colonizing the rhizoplane and the other investigated tissues. Fingerprinting profiles (Internal Transcribed Spacers, ITS-PCR) of randomly picked colonies were identical to those of E1 and E3, confirming that the colonies belonged to the supplemented E1^{Rif} and E3^{Rif} strains (Supporting Information Fig. S5). The *green fluorescent protein (gfp)*-derived E1 strain tightly adhered to the pepper root surface after 24 h (Fig. 1C). Several bacterial cells were found enveloped in the root hairs, suggesting that these plant appendages may represent sites of penetration into the root interior (Fig. 1D). *Gfp*-cells able to colonize the root surface were also detected within the outer cortex (Fig. 1E and F), suggesting that E1 endophytic lifestyle was rapidly established upon inoculation.

Bacterial endophytes promote resistance of pepper plants to drought under hydroponic conditions

The capacity of E1 and E3 to enhance plant drought tolerance was evaluated under hydroponic conditions by adding 20% polyethylene glycol (PEG) to the growth medium in order to modify the osmotic potential and induce a severe drought stress in a relatively controlled manner (Supporting Information Fig. S6). Neither the nutrient solution used to grow the pepper plants nor the PEG added to induce the drought stress supported bacterial growth (Supporting Information Fig. S7), indicating that the bacteria were sustained only by the root exudates of the plants. After 48 h of PEG treatment, the uninoculated plants were strongly affected, while the E1- and E3-inoculated plants did not show any visual symptoms of drought stress (Fig. 2A, top panels). Both the fresh and dry plant biomasses were significantly affected by the PEG treatment ($F_{1,17} = 67.91$, $p < 0.001$ and $F_{1,17} = 41.91$, $p < 0.001$, respectively) and the bacterial treatment ($F_{2,16} = 18.72$, $p < 0.001$ and $F_{2,16} = 8.93$, $p = 0.004$, respectively). Accordingly, the E1 and E3 strains positively affected both the fresh (+40%–50%) and the dry (+20%–30%) weights of the pepper plants, inducing a protection effect independent from the growth condition (–PEG/+PEG; Fig. 2B) compared to the uninoculated plants.

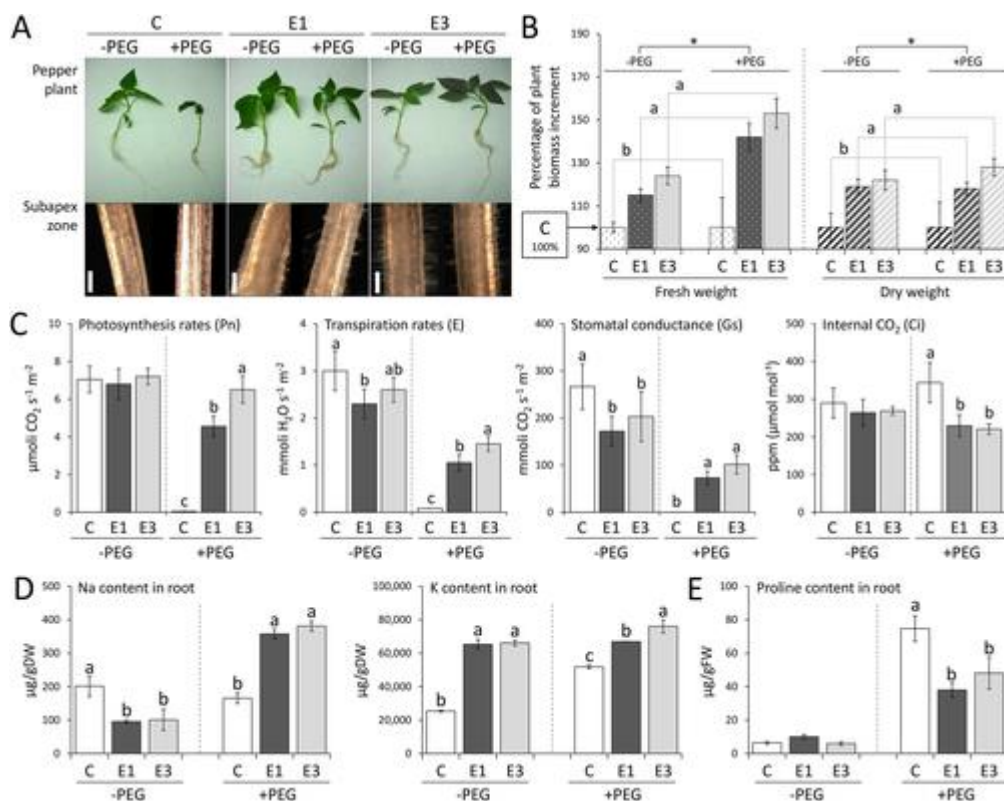


Figure 2

[Open in figure viewer](#)[PowerPoint](#)

Bacterial endophytes-induced drought resistance in pepper plants under hydroponic conditions.

A. (Upper panel) Endophyte strains E1 and E3 promoted the growth of pepper plants cultivated under hydroponic conditions and subjected to water stress (20% PEG). (Lower panel) Root sub-apical zone under normal (–PEG) and stress (+PEG) conditions. Bar size, 500 μm . B. Fresh and dry weight of uninoculated and inoculated pepper plants under drought stress expressed as a percentage of increment of inoculated plants compared to the control (i.e., uninoculated) plants (control values set to 100). ‘*’ denotes a significant ($p < 0.05$) difference between +PEG and –PEG roots independent of the bacterial treatment. Different letters denote significantly ($p < 0.05$) different means for uninoculated, E1- and E3-inoculated plants independent of the drought stress. C. Net photosynthesis (Pn), evapotranspiration (E), stomatal conductance (Gs), internal CO_2 (Ci) of inoculated and uninoculated pepper plants measured after 48 h of drought stress in both condition (–PEG and +PEG). Uninoculated control plants are indicated in white and marked with ‘C’ under the histograms, while inoculated plants are labelled either ‘E1’ (dark grey) or ‘E3’ (light grey). Data are relative to one of three independent experiments (four plants per inoculation treatment/stress level; $n = 24$). D. Na, K and (E) proline contents in pepper roots after 48 h of drought stress (three independent experiments; $n = 18$). Different letters indicate significant differences among treatments (C, E1 and E3) and drought stress (–PEG and +PEG).

Caption

A proliferation of the number and changes in the morphology of the root hairs was recorded in the bacteria-inoculated plants in both –PEG and +PEG plants (Fig. 2A). While the E3-inoculated roots developed thin and long root hairs, the E1-inoculated roots showed thick and short hairs (Fig. 2A, bottom panels). PEG, which significantly decreased the root-hair diameter and length in a previous study (Robin *et al.*, 2015), may have caused the lower number and shorter length of the hairs in the +PEG (stressed) plant roots.

Leaf-gas exchange measurements confirmed that E1 and E3 alleviated the effect of drought on the plant physiology (Fig. 2C). Under drought stress (+PEG) conditions, significantly higher values were measured for all the physiological parameters in the inoculated plants than in the uninoculated control group (Pn: E1 and

E3, $p < 0.001$; E: E1 and E3, $p < 0.001$; Gs: E1 and E3, $p < 0.001$; Ci: E1, $p = 0.019$ and E3, $p = 0.001$; Fig. 2C). The uninoculated stressed (+PEG) plants showed an impaired photosynthesis system, whereas the inoculated plants had a better physiological status. While both strains, E1 and E3, exerted a protective effect, the E3-inoculated plants maintained the highest net photosynthesis and transpiration rate values under drought stress (Fig. 2C). In the absence of stress (-PEG), no significant differences were found between inoculated and uninoculated plants in the net photosynthesis or the internal CO₂ (Sidak multiple comparisons $p > 0.05$); whereas, depending on the strain, the inoculated plants had significantly lower values of stomatal conductance and transpiration rate than the uninoculated plants (Gs: E1, $p = 0.002$ and E3, $p = 0.044$; E: E1, $p = 0.004$). Thus, the bacterial and PEG treatments caused significant effects that were observed in all the physiological parameters analysed (photosynthesis, Pn: $F_{2,22} = 57.80$, $p < 0.001$; transpiration rate, E: $F_{2,22} = 28.15$, $p < 0.001$; stomatal conductance, Gs: $F_{2,22} = 16.60$, $p < 0.001$; internal CO₂, Ci: $F_{2,22} = 5.41$, $p = 0.014$).

We also evaluated the osmolyte and ion contents in the root and leaf tissues of the plants (Fig. 2D and Supporting Information Fig. S8). The interaction between the bacterial and PEG treatments significantly affected the Na⁺ and K⁺ contents in particular ($F_{2,16} = 178.16$, $p < 0.001$ and $F_{2,16} = 52.93$, $p < 0.001$, respectively). Under drought stress, treatment with E1 and E3 strongly increased the accumulation of Na⁺ (E1 and E3, $p < 0.001$) and K⁺ (E1 and E3, $p < 0.001$) ions in the root tissues (Fig. 2D), which improved the cell turgor (Nieves-Cordones *et al.*, 2016). Proline content was increased in the roots of plants subjected to water stress compared to the control group, with a significant effect of the interaction between the bacterial and PEG treatments ($F_{2,16} = 21.11$, $p < 0.001$; Fig. 2E). No differences in proline content were observed between the inoculated and uninoculated plants under -PEG conditions (Sidak multiple comparisons $p > 0.05$). However, under +PEG stress conditions, the proline content was higher in the uninoculated plants than in the inoculated ones (E1 and E3, $p < 0.001$), though no significant differences were observed between the E1 and E3 treatments (Fig. 2E).

Bacterial endophytes promote pepper plant resistance to drought in soil

Strains E1 and E3 also conferred drought tolerance to the pepper plants cultivated in soil. The untreated plants were severely affected after 7 days without watering, whereas the treated plants showed turgid tissues and better development (Fig. 3A). Compared to the uninoculated plants, strains E1 and E3 significantly enhanced the pepper plant biomass under drought conditions in terms of both fresh (E1: +34% ± 9%, pair-wise C-,E1: $p = 0.042$; E3: +32% ± 8%, pair-wise C-,E3: $p = 0.028$) and dry weights (E1: +42% ± 9%, pair-wise C-,E1: $p = 0.041$; E3: +37% ± 4%, pair-wise C-,E3: $p = 0.043$; Fig. 3B). The stressed plants inoculated with E1 and E3 showed a similarly fresh and dry biomass not significantly different from that of irrigated plants (Monte Carlo multiple comparisons, $p > 0.05$; Fig. 3B).

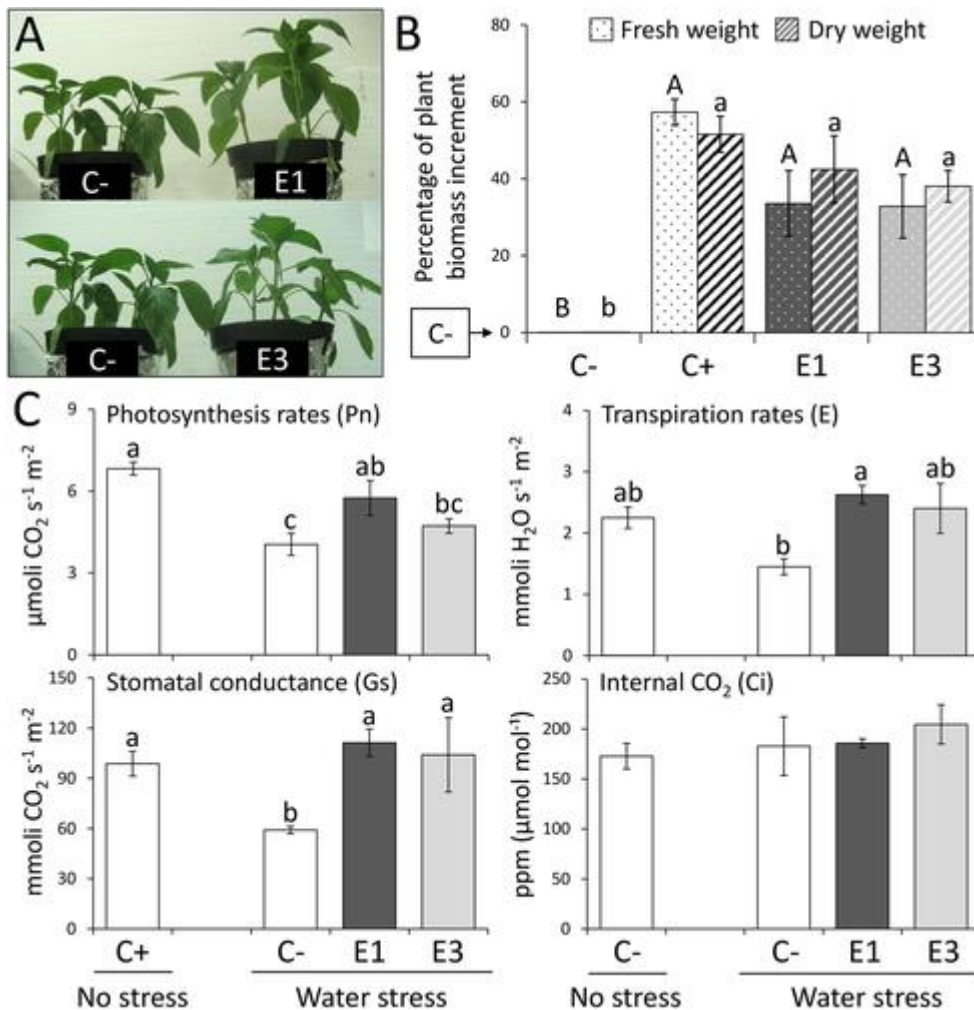


Figure 3

[Open in figure viewerPowerPoint](#)

Endophyte strains show PGP potential to promote drought resistance in pepper plants cultivated in soil.

A. E1- and E3-colonized (pots on the right) and untreated (pot on the left) pepper plants in a non-sterile soil. B. Fresh and dry weights of the plant roots. 'C+' plants properly irrigated. Values are reported as the percentage increase over the negative control, 'C-'. C. Physiological parameters of the inoculated (E1 and E3) and uninoculated (C+: irrigated; C-: drought stressed) pepper plants. Abbreviations are the same as in Fig. 2. Statistical analysis (one-way ANOVA, Tukey's Multiple Comparison Test when a significant *F*-test was obtained) is reported using different letters to indicate the means relative to the treated/untreated and stressed/unstressed plants ($p < 0.05$). The data reported in the graphs are relative to one experiment (three plants per inoculation treatment/stress; $n = 12$), which was representative of the three independent experiments.

Caption

The uninoculated and E3-treated plants under drought stress conditions were characterized by a significantly reduced net photosynthetic rate in the leaves (C-,C+: $p = 0.002$ and E3,C+: $p = 0.005$, respectively), unlike the E1-treated plants (E1,C+: $p = 0.21$). Stomatal conductance was significantly reduced in the uninoculated plants only (C-,C+: $p = 0.009$), and the transpiration rate was not affected at all (Fig. 3C). Comparable internal CO₂ contents were observed in both the inoculated stressed plants and the uninoculated unstressed control group ($p > 0.05$, in both cases), which were significantly higher than those of the uninoculated stressed plants ($p < 0.001$). Taken together, our results confirm that the endophytes E1 and E3 protected pepper plants from drought in soil as well as drought stress induced by PEG in a nutrient solution.

Bacterial endophytes affect pepper root morphology and the expression and activity of root V-PPase

Colonization by the bacterial endophytes also affected the pepper root morphology (Fig. 4A and B). There did not appear to be significant effects of the interaction between bacterial treatment and PEG treatment, thus the two factors have been taken in account separately. The bacterial treatment increased the size of the sub-apical root zone ($F_{2,22} = 14.448$, $p < 0.001$), with a large diameter observed in plants treated by either E1 or E3 compared to the untreated plants (E1: +52%, $p = 0.02$; E3: +90%, $p < 0.01$; Fig. 4B). Treatment with PEG, on the other hand, did not have any effect on root diameter ($F_{1,23} = 0.03$, $p > 0.05$; Fig. 4B).

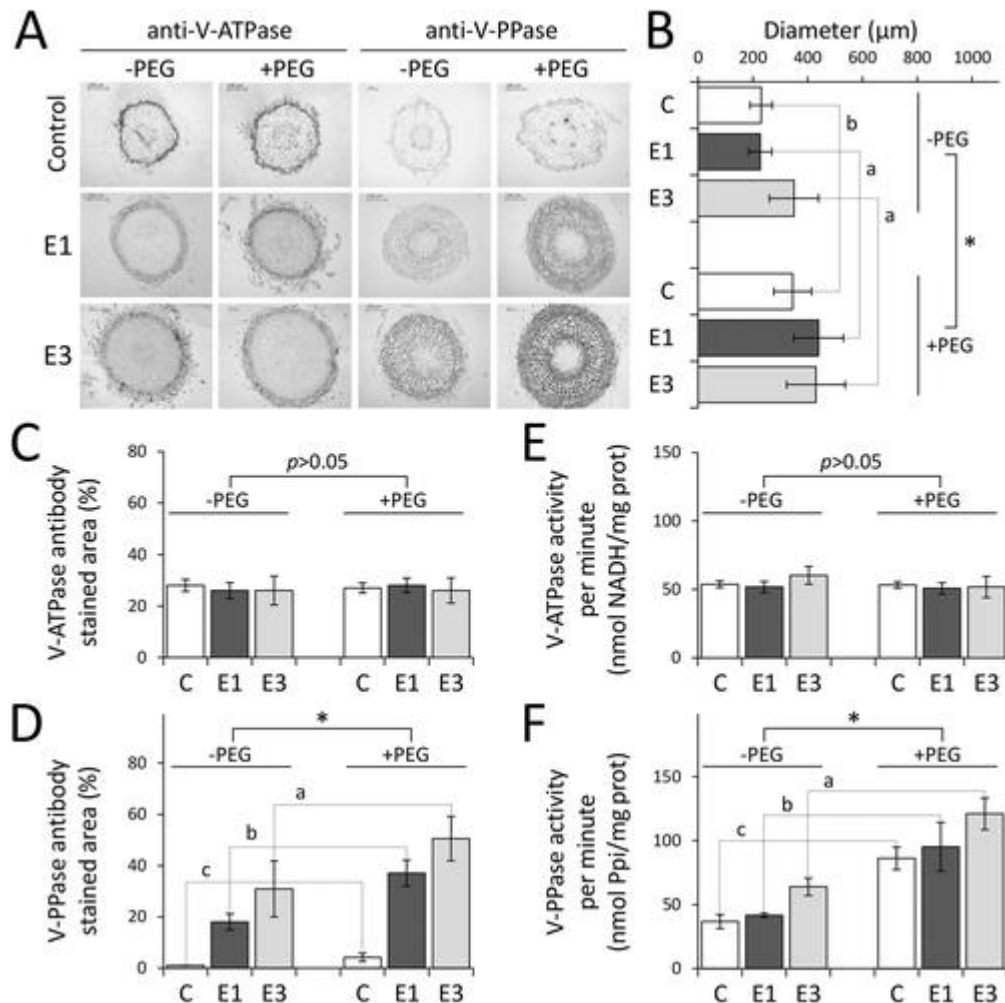


Figure 4

[Open in figure viewerPowerPoint](#)

Treatment with endophyte strains enhances V-PPase expression and activity in pepper roots.

A. Immunolocalization of V-ATPase and V-PPase in root cross sections (900–1000 µm from the tip) of E1- and E3-inoculated and uninoculated pepper plants treated with (+) or without (-) PEG. The images correspond to one experiment representative of three independent experiments. B. Root diameter (µm) measured from root cross sections at a distance of 900–1000 µm from the tip. Results expressed as mean diameter ± standard deviation of all sectioned roots ($n = 24$). C,D. Quantification of the immunoreactive areas of the vacuolar proton pumps, V-ATPase (C) and V-PPase (D), in the root cross sections. Results expressed as mean ± standard deviation of three independent experiments ($n = 18$). E,F. Activities of V-ATPase (E) and V-PPase (F) in the tonoplast-enriched fractions of roots of E1- and E3-inoculated and uninoculated pepper plants treated with (+) or without (-) PEG. Results expressed as mean ± standard

deviation of four independent experiments ($n = 24$). Statistical analysis (two-way ANOVA, REGWQ post hoc test, $p < 0.05$). ‘*’ denotes a significant difference between the drought-stressed (+PEG) and the unstressed roots independent of the bacterial treatment. Different letters denote significantly different means for uninoculated, E1- and E3-inoculated plants independent of the drought stress (REGWQ post hoc test, $p < 0.05$).

Caption

In the untreated plants, the root elongation zone (measured from the tip) was restricted to 350–700 μm , while it extended to 500–1500 μm in the treated plants. Immunostaining experiments performed on thin sections of root tissue from the pepper plants grown under hydroponic conditions revealed that exposure to the bacterial endophytes affected the expression of V-PPase under simulated drought conditions (+PEG; $F_{1,17} = 22.638$, $p < 0.001$; Fig. 4A and D), but not of V-ATPase ($F_{1,17} = 0.042$, $p < 0.05$; Fig. 4A and C). No protein signals were detected in the thin root sections in the absence of the specific primary antibody treatments against V-ATPase and V-PPase, indicating that non-specific reactions attributable to the secondary antibody or to the staining procedure did not occur (Supporting Information Fig. S9). No significant differences were detected in the accumulation or distribution of V-ATPase for bacterial treatment ($F_{2,16} = 0.259$, $p > 0.05$) or PEG treatment ($F_{1,17} = 0.042$, $p > 0.05$; Fig. 4C). The PEG treatment caused the V-PPase accumulation in the external cell layers and in the stele of the root segment corresponding to the elongation zone ($F_{1,17} = 22.638$, $p < 0.001$; Fig. 4A and D). In addition, the bacterial treatments significantly enhanced the accumulation of V-PPase in the cortex root layers ($F_{2,16} = 57.954$, $p < 0.001$) regardless of the PEG treatment, suggesting that colonization by both E1 and E3 is associated with a drought-independent V-PPase protein accumulation (Fig. 4A and D). In order to confirm such results at the enzymatic level, the activity of the two proton pumps, V-ATPase and V-PPase, was assayed on the tonoplast-enriched fraction of the whole root tissues (Fig. 4E and F). The proton pumps activities reflected the respective protein expression observed by immunostaining experiments but with lower increases induced by inoculation with E1 and E3. No significant difference was retrieved in the V-ATPase activity for either factor analysed (bacterial treatment: $F_{2,16} = 2.619$, $p > 0.05$; PEG treatment: $F_{1,17} = 1.728$, $p > 0.05$; Fig. 4E). A significant increase in V-PPase activity was detected in the drought stressed (+PEG) roots compared to the unstressed (–PEG) roots ($F_{1,17} = 16.646$, $p = 0.001$; Fig. 4F). Moreover, the V-PPase activity was significantly affected by the bacterial treatment ($F_{2,16} = 84.383$, $p < 0.001$), with the highest values observed in E3-inoculated plants (Fig. 4F).

Discussion

In this study, we investigated the relationship between the capacity of endophytic bacteria to increase plant drought tolerance and to activate the vacuolar proton pumps, V-ATPase and V-PPase, in the plant host. V-ATPase and V-PPase are key enzymes in plant response to drought, as it was demonstrated that its overexpression induces a strong drought tolerance in many plants (among others Bao *et al.*, 2016; Da-Gang *et al.*, 2012; Shen *et al.*, 2014). V-ATPase and V-PPase establish a proton gradient across the vacuolar membrane that allows the plant tissues to maintain cell turgor at a low soil-water potential (Gaxiola *et al.*, 2007, 2016). We found that the pepper plants became resistant to drought and enhanced the expression in the root of V-PPase only, when the plants were colonized by endophytic bacteria (E1 and E3). However, the induction of V-PPase activity in E1 and E3 inoculated plants under drought stress was lower than the protein expression enhancement detected by immunolocalization of the V-PPase under the same conditions. Such discrepancy might be due to a dilution effect. In fact, V-PPase activity was measured from the root tonoplast-enriched fraction obtained from the whole root system, while the V-PPase detection by immunohistochemistry was performed on root sections belonging to the elongation and differentiation zone. The latter is the root portion specifically involved in the water/nutrient uptake process (Barberon and Geldner, 2014), thus any change in expression/activity of proteins engaged in the water/nutrient uptake is expected to be concentrated in this limited root zone. Overall, our finding confirms the association of V-PPase expression with drought resistance and indicates that the endophytic bacteria may activate such expression.

The E1 and E3 strains rapidly adhered to the plant roots and were characterized by many beneficial PGP traits endowed with biopromotion (Patel and Saraf, 2017), biofertilization (Mapelli *et al.*, 2012) and

bioprotection against abiotic stresses (Dimkpa *et al.*, 2009). A combination of microscopy analysis and strain re-isolation tests showed that both strains had competence in colonizing the endosphere and translocating to the different organs of the pepper plants. High cell counts at the root (between 10^5 and 10^6 CFU g^{-1} of tissue) confirmed that the bacterial cells were tightly attached to the rhizoplane and possibly penetrated the root tissues to multiply in the root endosphere. Emerging lateral roots breaks are considered preferential sites for penetration of bacteria in the inner tissues and from there to the phloem and xylem vessels (Compant *et al.*, 2010). Driven by the plant transpiration flux, bacteria can be further translocated to the shoots and leaves (Compant *et al.*, 2010). For instance, the PGP strain *Paraburkholderia phytofirmans* PsJN colonizes root rhizodermis cells, internal tissues, internodes and leaves of grapevine (Compant *et al.*, 2008). Similarly, *Azoarcus* strain BH72 penetrates the rhizoplane in the elongation and differentiation zone of the root and systemically colonizes the rice plant tissues presumably by longitudinal spreading through vessels (Hurek *et al.*, 1994).

Different entry strategies to the root tissue are possible, including a controlled digestion of the root by lytic enzymes or passing through the natural breaks at elongated areas (Hardoim *et al.*, 2015). The two strains showed cellulase activity *in vitro*. The ability to produce cell wall-degrading enzymes such as cutinases, pectinases, cellulases, hemicellulases, proteases and lignin-peroxidases is a key strategy adopted by microorganisms to penetrate the cuticle and cell walls and thus to enter into plant tissues (Brader *et al.*, 2014). Furthermore, the capacity of E1 and E3 to produce biosurfactants and bioemulsifiers may have played a role in root colonization by changing the root surface wettability, similar to epiphytic bacteria exploiting these molecules to increase their survival times in the phyllosphere (Burch *et al.*, 2011). The versatility of bacteria, such as strains E1 and E3, in colonizing different plant tissues should favour the spread of the carried drought tolerance traits in the plant organs, thus potentially enhancing their beneficial effect on the whole plant.

VOC blends produced by strains E1 and E3 promoted the biomass growth of *Arabidopsis* plantlets under both normal and osmotic stress conditions. The characterization of such VOC blends showed that strain E1 produced 2,3-butanediol, a compound that was previously demonstrated to promote plant growth (i.e., Ryu *et al.*, 2003). Other bacteria, such as the *B. subtilis* strain GB03 and PGP strains including *Pseudomonas chlororaphis* strain O6, were observed to produce 2,3-butanediol (Ryu *et al.*, 2003; Cho *et al.*, 2008). The VOCs released by *B. subtilis* GB03 triggered an increased synthesis of auxin in the *Arabidopsis* leaves and translocation of the auxin to the roots at the lateral primordia, facilitating new root formation (Zhang *et al.*, 2007). Under drought conditions, *Arabidopsis* plants inoculated with *P. chlororaphis* O6 or exposed to 2,3-butanediol exhibited increased stress tolerance (Cho *et al.*, 2008).

Our survey of the two bacterial genomes did confirm the presence of tested PGP traits and did reveal metabolites that can positively affect plant growth. Strains E1 and E3 are endowed with genes for the synthesis of nitric oxide (NO), a key secondary messenger that triggers auxin-mediated rapid cellular and organ responses in plants (Schlicht *et al.*, 2013). NO plays a dominant role in the establishment of symbiosis between *Rhizobia*-like bacteria and leguminous plants (Hichri *et al.*, 2015), and the ability of *Azospirillum brasilense* to affect secondary and adventitious root formation in tomato plants was shown to be largely NO-dependent and auxin-independent (Creus *et al.*, 2005; Molina-Favero *et al.*, 2008). Notably, despite auxin (i.e., IAA) production is a widespread and conserved PGP trait in plant-associated bacterial communities (i.e., Marasco *et al.*, 2018), we did not detect such trait in E1 or E3. Both phenotypic assays and our search in their genomes for genes encoding the two major biosynthetic routes of bacterial IAA production, that is, pathways for indole pyruvic acid and indole-3-acetamide (Spaepen *et al.*, 2007), were negative. However, regardless of their ability to produce IAA, beneficial bacteria have been shown to manipulate plant auxin homeostasis, thus regulating the postembryonic development of the plant root system (Wang *et al.*, 2015).

The *in vivo* potential of the endophyte strains to enhance plant resistance to drought was evaluated in hydroponic conditions and confirmed in a non-sterile soil. Under drought stress conditions, induced either by PEG or by interruption of irrigation, the endophyte-treated plants presented a more robust root system, improved photosynthetic activity, and better physiology than the untreated plants. The plant response was similar to that of the switchgrass *Panicum virgatum* L. treated with the beneficial strain *Paraburkholderia*

phytofirmans strain PsJN, where the positively affected leaf physiology supported higher photosynthetic activity and promoted an increased production of siliques under different levels of drought stress (Wang *et al.*, 2016a). The improved drought resistance of plants colonized by E1 and E3 is associated with the enhanced expression and activity of V-PPase, but not with the V-ATPase that did not show significant change when exposed to bacteria/stress. Pepper plants treated with E1 and E3 had increased contents of Na⁺ and K⁺ in the root tissues compared to untreated plants. Similar effects were observed in *Arabidopsis* and tomato plants subjected to drought and were attributed to cooperation between V-PPase and Na⁺/H⁺ antiporter activity in vacuole osmotic adjustments (Park *et al.*, 2005; Brini *et al.*, 2006; Pasapula *et al.*, 2011). E1 and E3 colonization did not increase the root content of the osmoprotectant compound proline, suggesting that the drought tolerance was induced through a different mechanism from that observed with other beneficial bacteria (Cohen *et al.*, 2015).

We observed that the colonization of the two endophytes was associated with not only the enhanced expression and activity of the V-PPase, but also the formation of a larger root system, the proliferation of root hair, and thicker primary roots. These are all morphological changes previously associated with enhanced plant resistance to drought (Bardgett *et al.*, 2014). V-PPase activity has also been associated with the transport of auxin and both the abundance and distribution of PIN1, a major auxin translocator (Li *et al.*, 2005), in addition to its effect on vacuolar pH homeostasis. It has been demonstrated that plants overexpressing V-PPase showed an increase in the auxin-mediated cell division at organogenesis, resulting in a larger and more extended root system that may enhance plant performance during drought (Park *et al.*, 2005).

In conclusion, from the phenotypic and genomic survey of the two endophytes, we identified PGP traits and genes that may encourage the resistance to drought. Our data show that in drought-stressed pepper plants, endophytic bacteria efficiently colonize the root system, affect plant activity and response to environmental challenges, and support the expression and activity of the V-PPase an enzyme involved in the alleviation of drought stress.

Experimental procedures

Endophyte isolation, identification and in vitro characterization of the PGP potential

The two bacterial strains used in this study, E1 and E3, were isolated from the root tissues of pepper plants cultivated under desert farming (Marasco *et al.*, 2012). A phylogenetic tree based on an analysis of the entire 16S rRNA sequence was inferred by the neighbour-joining method, with 2000 replicates for a bootstrap test in the molecular evolutionary genetics analysis software, MEGA 4 (Kumar *et al.*, 2008).

A functional screening for PGP traits (described in Supporting Information Methods S1) was performed through a series of *in vitro* assays encompassing the (i) biostimulation and (ii) biofertilization activities possibly enhanced by bacteria. Briefly, the indole acetic acid (IAA) production and ACCd activity were measured to evaluate the biostimulation activity. The biofertilization trait was evaluated by measuring the capacity of our strains to solubilize mineral P, release siderophores, regulate atmospheric nitrogen and produce ammonia and exopolysaccharide (EPS).

Abiotic stress tolerance was tested in order to evaluate the ability of selected strains to grow in the presence of salt (NaCl 5%, 8% and 10%), polyethylene glycol (10% and 20%) and at different temperatures (4°C, 42°C and 50°C), as described in Marasco and colleagues (2012).

A phenotype microarray (PM) assay (Biolog) and a PM9 osmolytes microplate containing 96 different conditions (Supporting Information Fig. S1) were used to compare the cellular phenotypes of E1 and E3.

Different inoculating fluid (IF) solutions (proprietary formulation supplied by Biolog) were prepared and used to inoculate the PM plates, following the Biolog PM protocol for gram-positive bacteria (Supporting Information Method S2). The colour change of the redox potential indicator (Dye F, Biolog) due to the metabolic activity of bacterial cells was monitored and measured by PM technology (Biolog) using an OmniLog instrument set at 30°C. Reducing the dye caused the formation of a blue colour, which was recorded by a CCD camera every 15 min (Bochner *et al.*, 2001). The kinetic colorimetric data were stored in computer files and analysed using the Biolog software.

The biofilm formation was evaluated as described in Burton and colleagues (2006); the endoglucanase and endopolygalacturonase activity was evaluated according to Compant and colleagues (2008).

To analyse the VOCs produced by strains E1 and E3, 2 ml of MS media were poured in 40 ml airtight-seal glass vials stopped with a silicone PTFE septum and inoculated with 20 µl of 10⁸ bacterial cells. A solid-phase microextraction (SPME) manual holder (Supelco, Bellefonte, PA, USA), with a StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco), was inserted through the PTFE cap (Tait *et al.*, 2014). The SPME was exposed for 30 min to the bacterial culture headspace and detected using gas chromatography (GC, Agilent Technologies, USA) combined with a mass spectroscopy detector (MS, Agilent Technologies, USA), as described in Supporting Information Method S3. The plant growth promoting effect of bacterial VOCs was tested on *Arabidopsis*. Bi-plate sterile petri dishes (with septum) were used for plant exposure to bacterial VOCs according to previously described methods (Ryu *et al.*, 2003). Both sides of the petri dish contained half strength MS, Murashige and Skoog medium (added with 0.8% agar and 1.5% sucrose, adjusted to pH 5.7) for bacterial and plant growth. On the plant side 100 mM mannitol was added to simulate drought stress (Zhang *et al.*, 2010). *Arabidopsis* Col0 seeds were surface sterilized, placed on half strength MS plates and vernalized for 2 days at 4°C in the absence of light. The plates were then placed in a growth cabinet with a light and dark cycle of 16 and 8 h, respectively, at 200 µmol photons m⁻² s⁻¹ light, at 22°C temperature and 50% relative humidity, and germinating plantlets were let to grow for 2 days. Bacterial strains were inoculated onto PAF liquid medium and incubated at 28°C under shaking overnight. Bacterial cells were harvested, washed twice with 9 g l⁻¹ saline buffer and diluted to 10⁹ CFU ml⁻¹, as determined by optical density. Two-day-old *Arabidopsis* seedlings were transferred to new growth media with or without 100 mM mannitol in the first half of sterile bi-plate petri dishes. The other half of the plates was inoculated with 20 µl of bacterial suspension culture or supplemented with the same volume of sterile water. The bi-plates were accurately sealed with parafilm and placed in the growth cabinet for 15 days. At the end of the exposure period, the plants were removed from the agar and fresh weight was determined.

The E1 and E3 biosurfactant productions were evaluated by inoculating cells into a glucose mineral salts medium (GMSM: 10 g l⁻¹ glucose; 0.7 g l⁻¹ KH₂PO₄; 0.9 g l⁻¹ Na₂HPO₄; 2 g l⁻¹ NaNO₃; 0.4 g l⁻¹ MgSO₄ · 7H₂O; 0.1 g l⁻¹ CaCl₂ · 2H₂O; 2 ml of trace elements [per litre, 2 g FeSO₄ · 7H₂O, 1.5 g MnSO₄ · H₂O, 0.6 g (NH₄)₆Mo₇O₂ · 4H₂O]; pH = 6.72). The flasks were incubated overnight at 30°C on a rotary shaker (150 rpm). The surface activity of the cell-free supernatants was evaluated by measuring the emulsification index, the drop collapse assays, and the interfacial surface tension (IFT, mn m⁻¹), as reported in the Supporting Information Method S4.

Genome sequencing and assembly, and wide survey of PGP traits

Bacillus subtilis E1 draft genome was recently deposited under the accession number GCA_000724125.1. The functional annotation was performed using the RAST server (Moriya *et al.*, 2007). The strain *Paenibacillus illinoisensis* E3 was sequenced using Illumina HiSeq technology; the reads were assembled using velvet. The annotation was then performed by the Indigo Server (Alam *et al.*, 2013). The genome was deposited in NCBI under the Bioproject number PRJNA430863.

Genes involved in the PGP mechanisms (i.e., glycine betaine pathway, ROS and auxin production, exopolysaccharide synthesis, nitric oxide synthase, ACC deaminase activity and VOCs production) were selected using several published data (Yan *et al.*, 2008; Bertalan *et al.*, 2009; Ma *et al.*, 2011; Sant'Anna *et al.*, 2011; Weilharter *et al.*, 2011; Yu *et al.*, 2011; Taghavi and van der Lelie, 2013). Using the UniProt database (The UniProt Consortium, 2017) the KEGG orthology (KO) related to the selected PGP genes was extracted. Then the obtained PGP-KO were compared with those present in the two bacterial genomes, which were obtained using the KEGG Automatic Annotation Server (KAAS, Moriya *et al.*, 2007). The presence or absence of each PGP-KO in the bacterial genomes was evaluated for both strains.

Bacteria and plant growth conditions

Bacteria were grown in Pseudomonas Agar F (PAF) liquid medium. For plant treatment, a hydroponic solution with 2×10^7 cells ml⁻¹, supplemented with 20% PEG, was prepared from the bacteria after two washes in physiological buffer (9 g l⁻¹ NaCl). The ability of bacteria to thrive in the growth medium of hydroponic solution was tested by monitoring the growth curve of endophyte strains inoculated in two solutions, one with PEG and one without (Supporting Information Fig. S3).

Pepper seeds (*Capsicum annuum* L.) were sown in agro-perlite, watered with 0.1 mM CaSO₄, and incubated in the dark at 26°C for 6 days to allow plant germination. Plantlets were transferred into a nutrient solution composed of 2 mM Ca(NO₃)₂, 0.75 mM K₂SO₄, 0.65 mM MgSO₄, 0.5 mM KH₂PO₄, 10 μM H₃BO₃, 1 μM MnSO₄, 0.5 μM CuSO₄, 0.5 μM ZnSO₄, 0.05 μM (NH₄)₂MoO₇·O₂₄ and 0.1 mM Fe(III)-EDTA. The final pH was adjusted to 6.0–6.2 with NaOH. The hydroponic cultures were kept in a growth chamber and constantly aerated. Day and night regimes of 16 and 8 h at temperatures of 18°C and 24°C, respectively, and a photosynthetic photon flux density (PPFD) of 200 μmol m⁻² s⁻¹ at the plant level were applied.

Evaluation and quantification of bacterial recolonization ability by microscopy analysis and re-isolation procedures

The fluorescent phenotypes of the strains were obtained using a plasmid pGFP-ratiometric carrying the *gfp* gene under the control of P32 promoter. The adopted transformation protocol was described by Tamagnini and colleagues (2008). Only a single transformed colony was retrieved from the E1 transformation, and the E3 transformation was unsuccessful. The resulting strain, E1-*gfp*, was fluorescent under selective conditions and the plasmid-retaining stability was estimated to be 80% after 24 h of growth under non-selective conditions (Cutting and Vander Horn, 1990).

To evaluate the colonization capability of E1-*gfp*, pepper plants grown in hydroponic medium (prepared as described above) were inoculated overnight with 10⁷ cells ml⁻¹ by agitation. After 20–24 h, the plant roots were washed to remove the weakly bound bacteria and were further observed under an epifluorescent Leica microscope using the GFP filter (excitation at 488 nm). The acquired images were analysed by using the MBF-ImageJ. A confocal analysis was performed using a confocal laser scanning microscope, the Leica TCSNT, equipped with an Argon/Krypton (Ar/Kr) laser. GFP filters (excitation at 488 nm) were used to monitor E1-*gfp*, and dsRED filters (excitation at 558 nm) were used to acquire the root autofluorescence. The experiments were repeated twice on three replicate plants.

To obtain the spontaneous rifampicin mutants of the assayed endophytes, a protocol similar to that described by Dey and colleagues (2004) was adopted. Two isolates were grown overnight in PAF medium at 30°C. Then, the cell cultures were plated onto PAF_{Rif100} plates containing 100 μg ml⁻¹ rifampicin and incubated at 30°C for 2 days. Single colonies were purified and streaked at least twice on the PAF_{Rif100} medium before being plated on PAF_{Rif200} plates (containing 200 μg ml⁻¹ rifampicin). Colonies demonstrating the ability to grow at this rifampicin concentration were used for further analysis. To evaluate the ability of the spontaneous rifampicin mutants (E1 and E3) to colonize pepper plants, the same protocol used to induce drought stress was applied, unless plants were processed to isolate bacteria. The plant organs were carefully washed in sterile physiological solution to prevent the bacterial cell from actively colonizing the plant surface. One gram of apical (0–3 cm) and subapical (>3 cm) root segments, stems, and leaves were smashed

in a mortar. After 20–30 min, serial dilutions of the samples were plated on PAF plates containing 200 $\mu\text{g ml}^{-1}$ rifampicin. After 2–4 days of incubation at 30°C, the single colonies were counted and at least three colonies per fraction were checked by ITS-PCR to reconfirm bacteria identity (Daffonchio *et al.*, 2000). The results are the average of three independent experiments using two replicate plants for each experiment.

Evaluation of the PGP potential of endophyte strains to promote drought resistance of pepper plants in a hydroponic system

We used a hydroponic system to deeply study whether the plant–microbe interaction affected the activity and expression of V-PPase and V-ATPase. Preliminary studies indicated that 20% PEG was necessary to rapidly induce a severe water-stress event (Supporting Information Fig. S2). After germination, the pepper plants were grown in a nutrient solution over 21 days. Then, the plants were inoculated using 10⁸ CFU ml⁻¹ nutrient solution and were maintained in contact with bacteria for 24 h. Half of the plants were transferred to a complete nutrient solution, while the other half were transferred to a nutrient solution supplemented with 20% PEG6000. The plants were harvested for analysis after 48 h, and the fresh weights of both the shoot and root biomasses were determined. After drying at 65°C in an oven to reach a constant weight, the dry weights of both the shoot and root biomass were also determined. Some freshly collected roots were observed with a LEICA DM R optical microscope, and images were acquired with a Leica EC3 camera and LAS V4.1 software. Statistical analyses were conducted with SSP software. A two-way ANOVA ($p < 0.05$) was used to analyse the independent and interdependent effects of the two factors, ‘Bacterial treatment’ and ‘PEG treatment’, and of their interaction. In the presence of a significant *F*-test for interaction, pairwise comparisons were carried out by applying Sidak's correction ($p < 0.05$). In the absence of a significant *F*-test for interaction, the effect of the inoculation treatment was analysed independently on the stress level by comparing the mean to the REGWQ post hoc test ($p < 0.05$).

Evaluation of bacterial effect on physiological parameters of plants under drought stress in a hydroponic system

After 48 h, leaf-gas exchange measurements of the net photosynthesis (Pn), transpiration (E), stomatal conductance (gs) and internal CO₂ (Ci) were performed according to Marasco and colleagues (2012). Analyses of the osmolytes and osmoprotectants were conducted as described in the Supporting Information Methods S5 and S6. For the immunolocalization of the vacuolar proton pumps in the root tissue, the five largest root apical segments were sampled from two plants for each treatment and for three experiments. These samples were fixed in 4% paraformaldehyde (w/v), dehydrated, and then embedded in paraffin as described by (Dell'Orto *et al.*, 2002). A microtome was used to cut serial sections of 6 μm and up to 1500 μm from the tip. The sections were mounted on polylysine-treated slides, deparaffinized in xylene and rehydrated through an ethanol series. Immunological detection was performed, as in Dell'Orto and colleagues (2013), by raising polyclonal antibodies against two peptides corresponding to V-ATPase and V-PPase from *Arabidopsis* (Maeshima and Yoshida, 1989; Maeshima, 2001; Kobae *et al.*, 2004). A biotinylated secondary antibody, the ExtrAvidin-Peroxidase system (ExtrAvidin Peroxidase Staining Kit, Sigma) catalysing the staining reaction between 3-amino-9-ethylcarbazole (AEC) and H₂O₂ was also used. Root sections were observed with a LEICA DM R optical microscope, and images were acquired with a Leica EC3 camera and LAS V4.1 software. The stained area (%) was quantified using ImageJ software. The cross-section diameters were measured at a distance of 900–1000 μm from the tip.

Isolation of the tonoplast-enriched fraction from roots and determination of V-ATPase and V-PPase activity were performed according to the protocols described in Dell'Orto and colleagues (2013). The immunoreactive bands were quantified as a percentage of the stained area by ImageJ software. Statistical analyses were conducted with SSP software. Two-way ANOVA ($p < 0.05$) was used to analyse the effects of the bacterial treatment, the PEG treatment and their interaction. In the presence of a significant *F*-test for their interaction, pairwise comparisons were carried out by applying Sidak's correction ($p < 0.05$). In the

absence of a significant *F*-test for their interaction, the effect of the inoculation treatment was analysed independently on the stress level by comparing the mean to the REGWQ post hoc test ($p < 0.05$).

Evaluation of the PGP potential of endophyte strains to promote pepper resistance to drought in soil

Pepper seeds (*Capsicum annuum* L.) were spread on wet agro-perlite. After 7 days, three seedlings of the same size were selected and transferred to a plastic pot (14 cm diameter) containing commercial soil. The seedlings were grown in a growth chamber at day and night temperatures of 25°C and 20°C, respectively, with approximately 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light supplied for 12 h during the daytime. During the second week, the plantlets were inoculated with a bacterial suspension at a concentration of 10^8 cells g^{-1} soil, while the uninoculated plants were watered with tap water. Starting 7 days after inoculation, water was withheld for 12 days. The ‘positive control’ plants were properly irrigated. After 7 days of withholding water, the physiological parameters were measured as described above. Statistical analysis (one-way ANOVA) was conducted with SPSS software and, when a significant *F*-test was obtained, the means were compared using Tukey's test ($p < 0.05$). After the 12-day drought period, water irrigation was resumed for 3 days before the plants were harvested for biomass measurements. Three independent experiments with three replicate plants per treatments were conducted. Statistical analysis (one-way ANOVA) was used to compare treatments using the Monte Carlo test in Primer 6 software (999 permutations).

Acknowledgements

The authors declare an absence of competing financial interests. This research was supported by funding from the Italian MIUR FIRB project no. RBIN047MBH ‘Strategy to improve crop productivity under water stress’, the EU project BIODESERT (European Community's Seventh Framework Programme CSA-SA REGPOT-2008-2) under grant agreement no. 245746, and King Abdullah University of Science and Technology (KAUST) baseline research funds to DD. ER acknowledges support from Università degli Studi di Milano, DeFENS, Regione Lombardia (contract ‘Dote Ricerca’). The authors thank (i) Dr. Vasco Meneghini for support with the confocal microscopy analysis and interpretation, and a critical reading of the manuscript; (ii) Dr. Umberto Fascio at the Centro Interdipartimentale di Microscopia Applicata of the University of Milan for technical support with the confocal microscope; (iii) Dr. M. Maeshima of Nagoya University, Nagoya, Japan, for the kind gift of antibodies against V-ATPase and V-PPase; (iv) Dr. Bessem Chouaia of the University of Milan for the help in the bacterial genome assembly and (v) Dr. Alessia Perego and Dr. Patrizia Zaccheo of the University of Milan and (vi) Dr. Marco Fusi of King Abdullah University of Science and Technology for assistance with the statistical analysis; (vii) Francesco Della Valle of King Abdullah University of Science and Technology for help in critical revision of the manuscript.

References

- Alam, I., Antunes, A., Kamau, A. A., Ba alawi, W., Kalkatawi, M., Stingl, U., and Bajic, V. B. (2013) INDIGO – INtegrated data warehouse of MIcrobial GenOMes with examples from the Red Sea extremophiles *PLoS One*. 8: e82210.
- Arif, A., Zafar, Y., Arif, M., and Blumwald, E. (2013) Improved growth, drought tolerance, and ultrastructural evidence of increased turgidity in tobacco plants overexpressing *Arabidopsis* vacuolar pyrophosphatase (AVP1) *Mol Biotechnol*. 54: 379–392.
- Bao, A.-K., Du, B.-Q., Touil, L., Kang, P., Wang, Q.-L., and Wang, S.-M. (2016) Co-expression of tonoplast cation/H⁺ antiporter and H⁺ –pyrophosphatase from xerophyte *Zygophyllum xanthoxylum* improves alfalfa plant growth under salinity, drought and field conditions *Plant Biotechnol J*. 14: 964–975.
- Barberon, M., and Geldner, N. (2014) Radial transport of nutrients: the plant root as a polarized epithelium *Plant Physiol*. 166: 528–537.
- Bardgett, R. D., Mommer, L., and De Vries, F. T. (2014) Going underground: root traits as drivers of ecosystem processes *Trends Ecol Evol*. 29: 692–699.

- Bertalan, M., Albano, R., de Pádua, V., Rouws, L., Rojas, C., Hemerly, A., *et al.* (2009) Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5 *BMC Genom.* 10: 450.
- Bochner, B. R., Gadzinski, P., and Panomitros, E. (2001) Phenotype microarrays for high-throughput phenotypic testing and assay of gene function *Genome Res.* 11: 1246–1255.
- Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014) Metabolic potential of endophytic bacteria *Curr Opin Biotechnol.* 27: 30–37.
- Brini, F., Hanin, M., Mezghani, I., Berkowitz, G. A., and Masmoudi, K. (2006) Overexpression of wheat Na⁺/H⁺ antiporter TNHx1 and H⁺-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants *J Exp Bot.* 58: 301–308.
- Burch, A. Y., Browne, P. J., Dunlap, C. A., Price, N. P., and Lindow, S. E. (2011) Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production *Environ Microbiol.* 13: 2681–2691.
- Burton, E., Yakandawala, N., LoVetri, K., and Madhyastha, M. S. (2006) A microplate spectrofluorometric assay for bacterial biofilms *J Ind Microbiol Biotechnol.* 34: 1–4.
- Calanca, P. P. (2017) Effects of abiotic stress in crop production. In *Quantification of Climate Variability, Adaptation and Mitigation for Agricultural Sustainability*, Ahmed, M., and Stockle, C. O. (eds). Cham: Springer International Publishing, pp. 165–180.
- Chen, C., Xin, K., Liu, H., Cheng, J., Shen, X., Wang, Y., and Zhang, L. (2017) *Pantoea alhagi*, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat *Sci Rep.* 7: 41564.
- Cherif, H., Marasco, R., Rolli, E., Ferjani, R., Fusi, M., Soussi, A., *et al.* (2015) Oasis desert farming selects environment-specific date palm root endophytic communities and cultivable bacteria that promote resistance to drought *Environ Microbiol Rep.* 7: 668–678.
- Cho, S. M., Kang, B. R., Han, S. H., Anderson, A. J., Park, J.-Y., Lee, Y.-H., *et al.* (2008) 2R,3R-butanediol, a bacterial volatile produced by *Pseudomonas chlororaphis* O6, is involved in induction of systemic tolerance to drought in *Arabidopsis thaliana* *Mol Plant-Microbe Interact.* 21: 1067–1075.
- Cho, S., Kim, Y. H., Anderson, A. J., and Kim, Y. C. (2013) Nitric oxide and hydrogen peroxide production are involved in systemic drought tolerance induced by 2R,3R-butanediol in *Arabidopsis thaliana* *Plant Pathol J.* 29: 427–434.
- Cohen, A. C., Bottini, R. R., Pontin, M., Berli, F. J., Moreno, D., Boccanlandro, H. H., *et al.* (2015) *Azospirillum brasilense* ameliorates the response of *Arabidopsis thaliana* to drought mainly via enhancement of ABA levels *Physiol Plant.* 153: 79–90.
- Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Ait Barka, E., and Clement, C. (2008) Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues *FEMS Microbiol Ecol.* 63: 84–93.
- Compant, S., Clément, C., and Sessitsch, A. (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization *Soil Biol Biochem.* 42: 669–678.
- Cortes-Barco, A. M., Hsiang, T., and Goodwin, P. H. (2010) Induced systemic resistance against three foliar diseases of *Agrostis stolonifera* by (2R,3R)-butanediol or an isoparaffin mixture *Ann Appl Biol.* 157: 179–189.
- Creus, C. M., Graziano, M., Casanovas, E. M., Pereyra, M. A., Simontacchi, M., Puntarulo, S., *et al.* (2005) Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato *Planta.* 221: 297–303.
- Cutting, S. M., and Vander Horn, P. B. (1990) Genetic analysis. In *Molecular Biological Methods for Bacillus*, Harwood, C. R., and Cutting, S. M. (eds). Chichester: John Wiley, pp. 27–74.
- Da-Gang, H., Wang, S.-H., Luo, H., Ma, Q.-J., Yao, Y.-X., You, C.-X., and Hao, Y.-J. (2012) Overexpression of MdVHA-B, a V-ATPase gene from apple, confers tolerance to drought in transgenic tomato *Sci Hortic (Amsterdam).* 145: 94–101.
- 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: 5460–5468.
- Dell’Orto, M., Pirovano, L., Villalba, J. M., González-Reyes, J. A., and Zocchi, G. (2002) Localization of the plasma membrane H⁺-ATPase in Fe-deficient cucumber roots by immunodetection *Plant Soil.* 241: 11–17.

- Dell'Orto, M., De Nisi, P., Vigani, G., and Zocchi, G. (2013) Fe deficiency differentially affects the vacuolar proton pumps in cucumber and soybean roots *Front Plant Sci.* 4: 326.
- Dey, R., Pal, K. K., Bhatt, D. M., and Chauhan, S. M. (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria *Microbiol Res.* 159: 371–394.
- Dimkpa, C., Weinand, T., and Asch, F. (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions *Plant Cell Environ.* 32: 1682–1694.
- Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd-Allah, E. F., and Hashem, A. (2017) Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness *Front Microbiol.* 8: 1–14.
- Etesami, H., and Maheshwari, D. K. (2018) Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects *Ecotoxicol Environ Saf.* 156: 225–246.
- Fang, Y., and Xiong, L. (2015) General mechanisms of drought response and their application in drought resistance improvement in plants *Cell Mol Life Sci.* 72: 673–689.
- Ferjani, R., Marasco, R., Rolli, E., Cherif, H., Cherif, A., Gtari, M., *et al.* (2015) The date palm tree rhizosphere is a niche for plant growth promoting bacteria in the oasis ecosystem *Biomed Res Int.* 2015: 1–10.
- Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L., and Fink, G. R. (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H⁺-pump *Proc Natl Acad Sci U S A.* 98: 11444–11449.
- Gaxiola, R. A., Palmgren, M. G., and Schumacher, K. (2007) Plant proton pumps *FEBS Lett.* 581: 2204–2214.
- Gaxiola, R. A., Regmi, K., Paez-Valencia, J., Pizzio, G., and Zhang, S. (2016) Plant H⁺-PPases: reversible enzymes with contrasting functions dependent on membrane environment *Mol Plant.* 9: 317–319.
- Glick, B. R. (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world *Microbiol Res.* 169: 30–39.
- Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., *et al.* (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes *Microbiol Mol Biol Rev.* 79: 293–320.
- Hichri, I., Boscari, A., Castella, C., Rovere, M., Puppo, A., and Brouquisse, R. (2015) Nitric oxide: a multifaceted regulator of the nitrogen-fixing symbiosis *J Exp Bot.* 66: 2877–2887.
- Hurek, T., Reinhold-Hurek, B., Van Montagu, M., and Kellenberger, E. (1994) Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses *J Bacteriol.* 176: 1913–1923.
- Jaimez, R. E., Vielma, O., Rada, F., and Garcia-Nunez, C. (2000) Effects of water deficit on the dynamics of flowering and fruit production in *Capsicum chinense* jacq in a tropical semiarid region of Venezuela *J Agron Crop Sci.* 185: 113–119.
- Jarzyniak, K. M., and Jasiński, M. (2014) Membrane transporters and drought resistance - a complex issue *Front Plant Sci.* 5: 687.
- Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T., Nakagawa, T., and Maeshima, M. (2004) Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis *Plant Cell Physiol.* 45: 1749–1758.
- Kumar, S., Nei, M., Dudley, J., and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences *Brief Bioinform.* 9: 299–306.
- Kumar, T., Uzma, K., Khan, M. R., Abbas, Z., and Ali, G. M. (2014) Genetic improvement of sugarcane for drought and salinity stress tolerance using *Arabidopsis* vacuolar pyrophosphatase (AVP1) gene *Mol Biotechnol.* 56: 199–209.
- Lau, J. A., and Lennon, J. T. (2011) Evolutionary ecology of plant-microbe interactions: soil microbial structure alters selection on plant traits *New Phytol.* 192: 215–224.
- Lau, J. A., and Lennon, J. T. (2012) Rapid responses of soil microorganisms improve plant fitness in novel environments *Proc Natl Acad Sci.* 109: 14058–14062.
- Li, J., Yang, H., Ann Peer, W., Richter, G., Blakeslee, J., Bandyopadhyay, A., *et al.* (2005) *Arabidopsis* H⁺-PPase AVP1 regulates auxin-mediated organ development *Science.* 310: 121–125.
- Li, B., Wei, A., Song, C., Li, N., and Zhang, J. (2008) Heterologous expression of the TsVP gene improves the drought resistance of maize *Plant Biotechnol J.* 6: 146–159.

- Lim, J. H., and Kim, S. D. (2013) Induction of drought stress resistance by multi-functional PGPR *Bacillus licheniformis* K11 in pepper *Plant Pathol J.* 29: 201–208.
- Ma, Y., Prasad, M. N. V., Rajkumar, M., and Freitas, H. (2011) Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils *Biotechnol Adv.* 29: 248–258.
- Maeshima, M. (2001) Tonoplast transporters: organization and function *Annu Rev Plant Physiol Plant Mol Biol.* 52: 469–497.
- Maeshima, M., and Yoshida, S. (1989) Purification and properties of vacuolar membrane proton translocating inorganic pyrophosphatase from mung bean *J Biol Chem.* 264: 20068–20073.
- Mapelli, F., Marasco, R., Balloi, A., Rolli, E., Cappitelli, F., Daffonchio, D., and Borin, S. (2012) Mineral–microbe interactions: biotechnological potential of bioweathering *J Biotechnol.* 157: 473–481.
- Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., *et al.* (2013) Potential for plant growth promotion of rhizobacteria associated with *Salicornia* growing in Tunisian hypersaline soils *Biomed Res Int.* 2013: 1–13.
- Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., *et al.* (2012) A drought resistance-promoting microbiome is selected by root system under desert farming *PLoS One.* 7: e48479.
- Marasco, R., Rolli, E., Fusi, M., Cherif, A., Abou-Hadid, A., El-Bahairy, U., *et al.* (2013a) Plant growth promotion potential is equally represented in diverse grapevine root-associated bacterial communities from different biopedoclimatic environments *Biomed Res Int.* 2013: 1–17.
- Marasco, R., Rolli, E., Vigani, G., Borin, S., Sorlini, C., Ouzari, H., *et al.* (2013b) Are drought-resistance promoting bacteria cross-compatible with different plant models? *Plant Signal Behav.* 8: e26741.
- Marasco, R., Mapelli, F., Rolli, E., Mosqueira, M. J., Fusi, M., Bariselli, P., *et al.* (2016) *Salicornia strobilacea* (synonym of *Halocnemum strobilaceum*) grown under different tidal regimes selects rhizosphere bacteria capable of promoting plant growth *Front Microbiol.* 7: 1286.
- Marasco, R., Rolli, E., Fusi, M., Michoud, G., and Daffonchio, D. (2018) Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality *Microbiome.* 6: 3.
- Martinoia, E., Maeshima, M., and Neuhaus, H. E. (2006) Vacuolar transporters and their essential role in plant metabolism *J Exp Bot.* 58: 83–102.
- Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L. (2008) Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato *Mol Plant-Microbe Interact.* 21: 1001–1009.
- Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. C., and Kanehisa, M. (2007) KAAS: an automatic genome annotation and pathway reconstruction server *Nucleic Acids Res.* 35: W182–W185.
- Nieves-Cordones, M., Ródenas, R., Chavanieu, A., Rivero, R. M., Martínez, V., Gaillard, I., and Rubio, F. (2016) Uneven HAK/KUP/KT protein diversity among angiosperms: species distribution and perspectives *Front Plant Sci.* 7: 1–7.
- Park, S., Li, J., Pittman, J. K., Berkowitz, G. a., Yang, H., Undurraga, S., *et al.* (2005) Up-regulation of a H⁺-pyrophosphatase (H⁺-PPase) as a strategy to engineer drought-resistant crop plants *Proc Natl Acad Sci.* 102: 18830–18835.
- Park, M., Han, J., Ahn, Y., Kim, J., Lee, H., Jang, Y., *et al.* (2014) Ectopic expression of *Arabidopsis* H⁺-pyrophosphatase AVP1 enhances drought resistance in bottle gourd (*Lagenaria siceraria* Standl.) *Plant Cell Tissue Organ Cult.* 118: 383–389.
- Pasapula, V., Shen, G., Kuppu, S., Paez-Valencia, J., Mendoza, M., Hou, P., *et al.* (2011) Expression of an *Arabidopsis* vacuolar H⁺-pyrophosphatase gene (AVP1) in cotton improves drought- and salt tolerance and increases fibre yield in the field conditions *Plant Biotechnol J.* 9: 88–99.
- Patel, T., and Saraf, M. (2017) Biosynthesis of phytohormones from novel rhizobacterial isolates and their in vitro plant growth-promoting efficacy *J Plant Interact.* 12: 480–487.
- Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review *Biol Fertil Soils.* 51: 403–415.
- Qin, H., Gu, Q., Kuppu, S., Sun, L., Zhu, X., Mishra, N., *et al.* (2013) Expression of the *Arabidopsis* vacuolar H⁺-pyrophosphatase gene AVP1 in peanut to improve drought and salt tolerance *Plant Biotechnol Rep.* 7: 345–355.
- Raza, G., Ali, K., Asraf, M. Y., Mansoor, S., Javid, M., and Asad, S. (2016) Overexpression of an H⁺-PPase gene from *Arabidopsis* in sugarcane improves drought tolerance, plant growth, and photosynthetic responses *Turkish J Biol.* 40: 109–119.

- Robin, A., Uddin, M., and Bayazid, K. (2015) Polyethylene glycol (PEG)-treated hydroponic culture reduces length and diameter of root hairs of wheat varieties *Agronomy*. 5: 506–518.
- Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M. L., *et al.* (2015) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait *Environ Microbiol*. 17: 316–331.
- Rolli, E., Marasco, R., Saderi, S., Corretto, E., Mapelli, F., Cherif, A., *et al.* (2017) Root-associated bacteria promote grapevine growth: from the laboratory to the field *Plant Soil*. 410: 369–382.
- Rossi, F., Potrafka, R. M., Pichel, F. G., and De Philippis, R. (2012) The role of the exopolysaccharides in enhancing hydraulic conductivity of biological soil crusts *Soil Biol Biochem*. 46: 33–40.
- Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Wei, H.-X., Pare, P. W., and Kloepper, J. W. (2003) Bacterial volatiles promote growth in *Arabidopsis* *Proc Natl Acad Sci*. 100: 4927–4932.
- Sant'Anna, F. H., Almeida, L. G., Cecagno, R., Reolon, L. A., Siqueira, F. M., Machado, M. R., *et al.* (2011) Genomic insights into the versatility of the plant growth-promoting bacterium *Azospirillum amazonense* *BMC Genom*. 12: 409.
- Santos-Medellín, C., Edwards, J., Liechty, Z., Nguyen, B., and Sundaresan, V. (2017) Drought stress results in a compartment-specific restructuring of the rice root-associated microbiomes *MBio*. 8: e00764–e00717.
- Schilling, R. K., Marschner, P., Shavrukov, Y., Berger, B., Tester, M., Roy, S. J., and Plett, D. C. (2014) Expression of the *Arabidopsis* vacuolar H⁺–pyrophosphatase gene (AVP1) improves the shoot biomass of transgenic barley and increases grain yield in a saline field *Plant Biotechnol J*. 12: 378–386.
- Schlicht, M., Ludwig-Müller, J., Burbach, C., Volkmann, D., and Baluska, F. (2013) Indole-3-butyric acid induces lateral root formation via peroxisome-derived indole-3-acetic acid and nitric oxide *New Phytol*. 200: 473–482.
- Shen, G., Wei, J., Qiu, X., Hu, R., Kuppu, S., Auld, D., *et al.* (2014) Co-overexpression of AVP1 and AtNHX1 in cotton further improves drought and salt tolerance in transgenic cotton plants *Plant Mol Biol Rep*. 33: 167–177.
- Soussi, A., Ferjani, R., Marasco, R., Guesmi, A., Cherif, H., Rolli, E., *et al.* (2016) Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential *Plant Soil*. 405: 357–370.
- Spaepen, S., Vanderleyden, J., and Remans, R. (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling *FEMS Microbiol Rev*. 31: 425–448.
- Taghavi, S., and van der Lelie, D. (2013) Genome sequence of the plant growth-promoting endophytic bacterium *Enterobacter* sp. 638. In *Molecular Microbial Ecology of the Rhizosphere*, de Bruijn, F. J. (ed). Hoboken, NJ: John Wiley & Sons, Inc., pp. 899–908.
- Tait, E., Perry, J. D., Stanforth, S. P., and Dean, J. R. (2014) Identification of volatile organic compounds produced by bacteria using HS-SPME-GC-MS *J Chromatogr Sci*. 52: 363–373.
- Tamagnini, I., Guglielmetti, S., Mora, D., Parini, C., Canzi, E., and Karp, M. (2008) Generation and comparison of bioluminescent and fluorescent *Bacillus licheniformis* *Curr Microbiol*. 57: 245–250.
- TerHorst, C. P., Lennon, J. T., and Lau, J. A. (2014) The relative importance of rapid evolution for plant-microbe interactions depends on ecological context *Proc R Soc B Biol Sci*. 281: 20140028.
- The UniProt Consortium. (2017) UniProt: the universal protein knowledgebase *Nucleic Acids Res*. 45: D158–D169.
- Vergani, L., Mapelli, F., Marasco, R., Crotti, E., Fusi, M., Di Guardo, A., *et al.* (2017) Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. *Front Microbiol* 8: Article 1385.
- Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., and SkZ, A. (2016) Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria *Microbiol Res*. 184: 13–24.
- Wang, C.-J., Yang, W., Wang, C., Gu, C., Niu, D.-D., Liu, H.-X., *et al.* (2012) Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting *Rhizobacterium* strains *PLoS One*. 7: e52565.
- Wang, J., Zhang, Y., Li, Y., Wang, X., Nan, W., Hu, Y., *et al.* (2015) Endophytic microbes *Bacillus* sp. LZR216-regulated root development is dependent on polar auxin transport in *Arabidopsis* seedlings *Plant Cell Rep*. 34: 1075–1087.
- Wang, B., Seiler, J. R., and Mei, C. (2016a) A microbial endophyte enhanced growth of switchgrass under two drought cycles improving leaf level physiology and leaf development *Environ Exp Bot*. 122: 100–108.

- Wang, F., Wang, C., Sun, Y., Wang, N., Li, X., Dong, Y., *et al.* (2016b) Overexpression of vacuolar proton pump ATPase (V-H⁺-ATPase) subunits B, C and H confers tolerance to salt and saline-alkali stresses in transgenic alfalfa (*Medicago sativa* L.) *J Integr Agric.* 15: 2279–2289.
- Weilharter, A., Mitter, B., Shin, M. V., Chain, P. S. G., Nowak, J., and Sessitsch, A. (2011) Complete genome sequence of the plant growth-promoting endophyte *Burkholderia phytofirmans* strain PsJN *J Bacteriol.* 193: 3383–3384.
- Yan, Y., Yang, J., Dou, Y., Chen, M., Ping, S., Peng, J., *et al.* (2008) Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501 *Proc Natl Acad Sci.* 105: 7564–7569.
- Yu, H., Yuan, M., Lu, W., Yang, J., Dai, S., Li, Q., *et al.* (2011) Complete genome sequence of the nitrogen-fixing and rhizosphere-associated bacterium *Pseudomonas stutzeri* strain DSM4166 *J Bacteriol.* 193: 3422–3423.
- Zhang, H., Kim, M.-S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., *et al.* (2007) Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis* *Planta.* 226: 839–851.
- Zhang, H., Murzello, C., Sun, Y., Kim, M.-S., Xie, X., Jeter, R. M., *et al.* (2010) Choline and osmotic-stress tolerance induced in *Arabidopsis* by the soil microbe *Bacillus subtilis* (GB03) *Mol Plant-Microbe Interact.* 23: 1097–1104.
- Zhang, J., Li, J., Wang, X., and Chen, J. (2011) OVP1, a vacuolar H⁺-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance *Plant Physiol Biochem.* 49: 33–38.

Notes :

- 1 These authors contributed equally to this work.
- 2 Present address: IPS2, Institute of Plant Sciences Paris-Saclay, 91405 Orsay, France.
- Presence or absence of (A) PGP traits and (B) abiotic stress tolerance were evaluated following the methods described in the experimental section. Additional details of the results marked with a star (*) are reported in Supporting Information Tables S1 and S2 and Supporting Information Figs. S1–S3. (+) Indicates the highest concentration of osmolytes (% w/v) tolerated by the strains.

This full text was downloaded from iris-AperTO: <https://iris.unito.it/>