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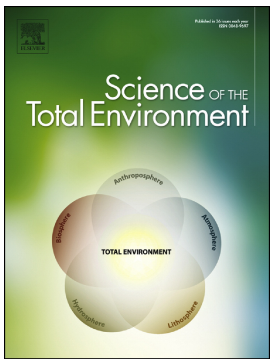

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Alginate as a feature of osmotolerance differentiation among soil bacteria isolated from wild legumes growing in Portugal

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Abstract

Plants are naturally colonized by bacteria that can exert beneficial effects on growth and stress tolerance. These bacteria can be used as inoculants to boost crop productivity and plants resilience, and can be especially interesting if they are able to survive to abiotic stresses, such as drought. Herein we report the mechanisms that soil bacteria resort to tolerate drought and we also explore the influence of each mechanism to the level of drought tolerance exhibited, in order to test the hypothesis that different levels of tolerance displayed by bacteria are linked to differential efficiency of osmotolerance mechanisms. For this, the biochemical and physiological responses of bacterial strains of different genera and displaying different levels of tolerance to osmotic stress (sensitive, moderately tolerant and tolerant) induced by polyethylene glycol-6000 (PEG) were studied. Betaine, trehalose and alginate content increased in the majority of the strains exposed to PEG. Betaine was the osmolyte with higher increases, evidencing the important role of this compound in the tolerance of bacteria to drought. However, betaine and trehalose levels were not significantly different among bacteria with different osmotolerance levels. Several biochemical endpoints (protein content, superoxide dismutase, catalase, glutathione-S-transferases) related to oxidative stress were assessed, since although oxidative damage has been reported in drought conditions, little information is available. The oxidative stress parameters were not sufficient to explain differences in osmotolerance observed for the tested strains. In contrast, alginate showed significant differences among the three levels of osmotolerance, linking the level of osmotolerance with the ability of soil bacteria to synthesize and accumulate alginate intracellularly for the first time. Moreover, our results show that this ability is present in different bacteria genera. Thus, evaluating the ability to

synthesize alginate might be an important cue when considering bacterial inoculants for osmotically stressful conditions.

Keywords

Plant growth promoting bacteria, drought, osmolytes, alginate, superoxide dismutase, catalase

1. Introduction

Drought is a common environmental stress that can severely affect root-associated bacterial communities (Naylor and Coleman-Derr, 2018). One third of the Earth's surface is arid, semi-arid, or seasonally arid (Gurevitch et al., 2002). In Mediterranean ecosystems, surface soils frequently experience long dry periods followed by a relatively rapid wetting (Fierer and Schimel, 2002). Moreover, an increase in intensity or frequency of droughts in some regions might be attributed to global warming (IPCC, 2018). Several programs have been launched to increase crop production in drought affected areas, for instance United Nations Development Programme (UNDP), European Union joint Programme to Combat Desertification in 2007, USDA Drought Resilience Partnership, India's Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad and the All India Coordinated Research Projects on Agri-Meteorology and Dryland Agriculture (AICRPAM and AICRPDA) and FAO - Coping with water scarcity.

Soil microorganisms constitute less than 0.5% (w/w) of the soil mass, but they play a key role in soil properties and are essential to maintain a productive and valuable soil system (Yan et al., 2015). Disturbance of the soil environment can shift microbial communities and can have detrimental effects on soil nutrient cycling (French et al., 2009). The osmotic

strength of environments is one of the physical parameters that determines the ability of organisms to proliferate in a given habitat (Patel et al., 2017). Evidence suggests that the regulation of cytoplasmic composition and hydration is a key feature of cellular homeostasis (Wood, 2011) and small differences in osmotolerance may reflect subtle differences in osmoadaptation mechanisms, with these differences influencing the relative fitness of individual species and strains (Freeman et al., 2013).The increase of specific solutes, which are compatible with cellular processes even at high concentrations is an efficient mechanism of tolerance to drought (Brown and Simpson, 1972). Compatible solutes are similar among phylogenetically distant organisms and comprise inorganic ions and few classes of organic osmolytes (Yancey et al., 1982). However, uptake and synthesis of osmolytes require large amounts of energy, resulting in reduced growth and activity (Oren, 1999; Schimel et al., 2007; Wichern et al., 2006). Measures (1975) found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. Yaakop et al. (Yaakop et al., 2016) described proline to be a critical osmoprotectant for *Jeotgalibacillus malaysiensis*. On the other hand, Perroud and LeRudulier (Perroud and Le Rudulier, 1985) found that the intracellular concentrations of glycinebetaine maintained by *E. coli* were proportional to the osmolarity of the medium. Trehalose has been found to be synthesized in a number of bacteria (Galinski and Truper, 1982; Larsen et al., 1987; Mackay et al., 1984; Reed, 1986), in response to osmotic stress and mutations which resulted in accumulation impairment of trehalose increased sensitivity to osmotic stress (Giaever et al., 1988). Accumulation of compatible solutes such as proline, glycinebetaine and trehalose protect proteins from denaturation (Schobert, 1977), scavenging hydroxyl radicals (Marulanda et al., 2009), and regulating the NAD/NADH ratio (Marulanda et al., 2009).

Other mechanisms that help bacteria to cope with water stress include production of extracellular polymeric substances such as alginate (Freeman et al., 2013; Ngumbi and Kloepper, 2016). Alginate is hygroscopic, can hold several times its weight in water thereby keeping cells hydrated (Robyt, 1998; Sutherland, 2001). Moreover, alginate production was reported to clearly provide a competitive advantage for bacteria in water-limited environments, leading to increased ecological success (Chang et al., 2007), and helping maintain membrane integrity (Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007; Welsh, 2000). Besides osmotic effects, drought stress also results in the accumulation of reactive oxygen species (ROS) (Benabdellah et al., 2011; Ngumbi and Kloepper, 2016). However, there is very limited information about the oxidative response of soil microorganisms to drought (Benabdellah et al., 2011). Oxidative damage is caused by increased concentrations of ROS, that can react with a large variety of biomolecules causing irreversible damage (Kim et al., 2008; Rivero et al., 2007; Wu and Ni, 2015) . Effects include changes in protein conformation, protein denaturation, restricted enzyme efficiency, changes in electron transport chains (Bérard et al., 2015; Vriezen et al., 2007) and lipid peroxidation (Potts, 1999). However, living organisms are equipped with antioxidant mechanisms to regulate intracellular ROS concentrations. ROS scavenging mechanisms include the enzymes superoxide dismutases (SOD), catalase (CAT) and glutathione-S-transferases (GSTs).

Since drought is a recurrent problem and it is projected to worsen (IPCC, 2018), understanding the mechanisms behind bacteria tolerance to drought is a subject deserving attention. This will contribute to mitigate drought effects in soil bacterial communities and to improve strategies for the use of beneficial bacteria in mediating drought tolerance in

other inhabitants of drought impacted ecosystems, namely plants. Indigenous bacterial populations may have adapted to stress conditions and evolved the capacity to survive in stressed soils (Marulanda et al., 2009), thus selection of drought tolerant strains isolated from stressed ecosystems can be a more effective strategy in reducing plants susceptibility to drought. With this in mind, the central focus of this study is to untangle the intracellular osmolytes used by phylogenetically diverse soil bacteria isolated from different edaphoclimatic conditions, and to understand the determinants driving the level of osmotolerance. To achieve this goal, representatives of all genera of bacteria previously isolated from wild legumes growing at different edaphoclimatic environments were used (Cardoso et al., 2018). Furthermore, strains with different osmotolerance levels from the most represented genera (*Pseudomonas* and *Flavobacterium*) were also studied. Altogether the 20 strains were grown in non-stressed (control) and drought stressed (PEG % inducting 50% growth inhibition, based on osmotolerance determination by Cardoso et al. (2018)) conditions and osmotic (proline, glycinebetaine, trehalose, alginate), antioxidant (SOD, CAT), biotransformation (GSTs) and damage (lipid peroxidation) endpoints were studied in order to test the hypothesis that different levels of tolerance displayed by bacteria are linked to a differential efficiences of osmotolerant mechanisms.

2. Material and methods

2.1. Bacterial strains

Bacteria were isolated from the nodules of plants from different wild legume species (*Ornithopus compressus* L., *Medicago lupulina* L., *Scorpiurus vermiculatus* L., *Vicia sativa* subsp. *sativa* L., *O. sativus* subsp. *sativus* Brot., *V. benghalensis* L., *O. pinnatus* (Miller) Druce, *Lotus corniculatus* L. and *Medicago* sp.) harvested from four sites in continental

Portugal in spring 2015, as described by Cardoso et al. (2018). These sites were Murtosa - MT (40 46 28.907 N 008 38 51.865 W - bioclimate inferior humid and inferior mesomediterranic (ambrotype/thermotype); Vale de Cambra - VC (40 51 09.113 N 008 18 32.222 W - bioclimate inferior hyperhumid and inferior mesotemperate); Alvito - AV (38 16 20.447 N 008 00 08.377 W - bioclimate superior dry and inferior mesomediterranean); Aljustrel - AT (37 55 49.127 N 8 06 26.485 W - bioclimate inferior dry and superior termomediterranean). Bioclimatic classification of the sites was done according to Mesquita (Mesquita, 2005) in Cardoso *et al*. (2018). Molecular typing of bacterial isolates identified 100 distinct profiles, belonging to 11 genera, with 85% of strains belonging to *Pseudomonas* and *Flavobacterium* and displaying different plant growth promotion (PGP) abilities and different tolerance levels to politethylene glycol (PEG) (Cardoso et al., 2018).

From this set twenty strains were selected using biodiversity and tolerance to PEG as selection criteria (Table 1). All bacterial genera are represented in this study. In genera with higher number of strains (*Pseudomonas* and *Flavobacterium*) different levels of tolerance, sensitive (S -EC_{50} < 10%PEG), medium tolerant (MT -EC_{50} ≥ 10% and < 15% PEG) and tolerant (T -EC_{50} ≥ 15%PEG) strains were used.

2.2. Experimental conditions

Strains were grown in flasks containing 50 mL of yeast broth mannitol (YMB) medium (Somasegaran and Hoben, 1994) supplemented at control (no PEG addition) and PEG (at EC_{50} PEG %s -Table 1, rounded to unit %). Inoculated tubes were incubated at 26 °C in an orbital shaker (150 rpm) until late exponential phase (48 h). Growth was determined by measuring optical density at 620 nm. The relationship between optical density and cell concentration was obtained by direct cell counting in a Neubauer chamber. Cell

concentration was expressed in million cells per milliliter (M cells mL^{-1}). Three independent experiments were carried out with 3 replicates per condition. Cells from each tube were collected separately after centrifugation at 10,000 × g for 10 min at 4°C, washed twice with deionized water, and frozen at -80°C for further use. The growth media and cell wash water from one replicate of each experiment were pooled and also frozen at -80°C for extracellular alginate quantification.

2.3. Cell damage

A replicate from each experiment was suspended in 20% (v/v) trichloroacetic acid (TCA) and lysed in an ultrasonic probe for 30 s, keeping tubes in an ice bath, and extracts centrifuged at 12,000 × g for 10 min at 4°C. Lipid peroxidation (LPO) was measured by quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust (Buege and Aust, 1978). TBARS were quantified spectrophotometrically at 532 nm and calculated using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}). Results were expressed in nmol of MDA equivalents per million cells (nmol MDA eq M $cells^{-1}$).

2.4. Osmotolerance

2.4.1. Alginate

Alginate determination was adapted from the method described by Johnson et al., (2011), using dimethyl methylene blue. A replicate from each experiment was suspended in sodium phosphate buffer (0.1M pH 7.25), lysed in an ultrasonic probe for 30 s and extracts centrifuged at 12,000 × g for 10 minutes at room temperature. Dimethyl blue was added both to the supernatant and to the growth media in order to quantify intra and extracellular

alginate, respectively. Absorbance was immediately measured at 525 nm and alginate standards (1.25 – 25 μg mL^{-1}) were used. Results were expressed in ng alginate per million cells (ng M $cells^{-1}$).

2.4.2. Betaine

Betaine was determined following the method described by Grieve and Grattan (1983). A replicate from each experiment was suspended in deionized water, lysed in an ultrasonic probe for 30 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, sulfuric acid (1M) and KI-I_2 were added and incubated overnight in ice. Tubes were centrifuged at 12,000 × g for 30 min at 4°C, the supernatant discarded, dichloroethane was added and tubes vortexed. After 2h the absorbance was measured at 365 nm and betaine standards (50 – 200 μg mL^{-1}) were used. Results were expressed in ng alginate per million cells (ng M $cells^{-1}$).

2.4.3. Proline

Proline was determined following the method described by Bates et al., (1973) with some modifications. A replicate from each experiment was suspended in 3% sulfosalicylic acid, lysed in an ultrasonic probe for 30 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, 2.5% acid ninhydrin and glacial acetic acid were added and incubated 1h at 100 °C and cooled in an ice bath. Toluene was added and vortexed vigorously for 15-20 s. The toluene phase was separated from the aqueous phase, and absorbance measured at 520 nm and proline standards (1 – 6.25 μg mL^{-1}) were used. Results were expressed in ng proline per million cells (ng M $cells^{-1}$).

2.4.4. Trehalose

Trehalose was determined following the method described by Dahlqvist (1968) with some modifications. A replicate from each experiment was suspended in citric acid buffer (0.135 M, pH 5.7), lysed in an ultrasonic probe for 30 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, trehalase solution (0.2 unit/ml) was added and incubated 15 min at 37 °C. The glucose formed was determined using an assay kit (NZYTech, Portugal). Absorbance was measured at 510 nm and trehalose (15-240 μg mL^{-1}) and glucose (15-240 μg mL^{-1}) standards were used. Results were expressed in ng trehalose per million cells (ng M $cells^{-1}$).

2.5. Antioxidant and biotransformation responses

Cells were suspended in sodium phosphate buffer (50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dihydrate; 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT), pH 7.0) and lysed in an ultrasonic probe for 30 s, keeping tubes in an ice bath, and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was immediately used or frozen (-80°C) for protein content, catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferases (GSTs).

Protein content was determined by the Biuret method (Robinson and Hogden, 1940). The amount of protein was determined spectrophotometrically at 540 nm, using bovine serum albumin (BSA) as standard (1.25 to 10 mg mL^{-1}). Results were expressed in μg protein per million cells (μg M $cells^{-1}$).

2.5.1. Catalase

Catalase (CAT) activity was determined by the reaction of CAT with methanol in the presence of H_2O_2 (Johansson, 1988). A standard curve was built using formaldehyde standards (2.5 – 30 μM). One unit (U) was defined as the amount of enzyme that caused the formation of 1.0 μmol formaldehyde, per min. Results were expressed in milliunits (mU) per million cells (mU M cells^{-1}).

2.5.2. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined by the reaction of nitro blue tetrazolium (NBT) with superoxide radicals to form NBT diformazan, based on the method described by (Beauchamp and Fridovich, 1971). Absorbance was measured at 560 nm and SOD standards (0.01 – 60 U) were used. One unit of enzyme activity (U) corresponds to a 50% reduction of NBT. Results were expressed in milliunits (mU) per million cells (mU M cells^{-1}).

2.5.3. Glutathione S-transferases

Glutathione-S-transferases (GSTs) activity was determined using the method described by (Habig et al., 1974) and modified by (Corticeiro et al., 2013). GSTs catalyze the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH), forming a thioester product. The reaction can be followed by the absorbance increase at 340 nm and GSTs activity determined using CDNB extinction coefficient (9.6 mM cm−1). Results were expressed in nanounits (mU) per million cells (nU M cells^{-1}).

2.6. Statistical analysis

All parameters tested were submitted to hypothesis testing. One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA). Parameters were analyzed following a one-way hierarchical design, with PEG conditions (0-control and EC_{50}) as fixed factor. The null hypothesis tested was: no significant differences exist between tested conditions. Significant differences were considered for $p \leq 0.05$, and were identified in figures with different lowercase (for control) and uppercase (PEG) letters.

A matrix gathering the descriptors (LPO, trehalose, alginate, betaine proline, Prot, SOD, CAT, GSTs) per strain and condition was used to calculate a Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the strains and conditions, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biomarkers (correlation > 0.60) were provided as supplementary variables and superimposed on the PCO graph, allowing to identify the descriptors that contributed more to differences among strains and between conditions.

3. Results

3.1. Cell density

Since osmotolerance of strains was already published (Cardoso et al., 2018) and in this study the concentrations of PEG used were the EC_{50} of each strain, only cell density in control condition was presented (Supplementary Fig. 1).

3.2. Cell damage

LPO levels of strains in the absence (control) and presence of PEG were represented in a coordinate plan (control in the abscissa and PEG in the ordinate) (Fig. 1A), in order to evidence the relationship of LPO levels at both conditions. The dotted line marks identical LPO values between the two conditions, symbols (circles) above the line represent higher values in PEG than in the control condition, circles below the line represent higher values in control than in the PEG condition. Variability in LPO levels among strains is observed. For most strains LPO increased (between 1.5 and 9.1 times) in PEG relatively to control. In five strains LPO did not vary or even decreased in PEG relatively to control condition.

LPO by strain's osmotolerance level of PEG and control conditions are shown in Fig. 1B. In control, no significant differences were observed among osmotolerance levels. PEG increased LPO relatively to control but significant differences were only noticed in T and MT strains. Significant lower LPO in S compared to T and MT strains was observed (Fig. 1B).

3.3. Osmotolerance

The osmolytes to which cells resorted to counteract the effects of exposure to increased osmolarity are presented in Fig. 2. This figure presents strains separately (Fig. 2A, 2C, 2E and 2G) and grouped by tolerance level (Fig. 2B, 2D, 2F and 2H).

Proline concentrations of most strains (80%) were lower than 0.5 ng M cells^{-1} in control, but four strains displayed higher proline concentrations (between 0.75 and 3 ng M cells^{-1}). PEG induced (above dashed line) proline in 60% of strains and reduced (below dashed line) in 15% of strains (Fig. 2A). Osmotolerance level influenced proline concentrations in control and PEG conditions (Fig. 2B). MT strains had significantly lower proline

concentrations than S and T strains at control. Proline levels were not changed by PEG in T strains but increased in the MT and S strains, although only significantly in MT ones (Fig. 2B).

In one strain betaine was not increased by PEG, in the other 19 PEG induced increases of this osmolyte between 3 and 37-fold relatively to control (Fig. 2C). Betaine concentration of strains grouped by osmotolerance level (Fig. 2D) showed significantly higher betaine levels in S strains compared to T and MT strains in control. PEG quashed this difference and betaine concentration was not significantly different among osmotolerance levels. Comparing conditions, the large betaine increase by PEG relatively to control was noticed for all levels of tolerance. These increases were more expressive for the T and MT strains (13 and 12-fold, respectively) than for S strains (8-fold) (Fig. 2D).

Variability in trehalose content (between 8.3 and 78.6 ng M cells^{-1}) were observed among strains. Most strains (65%) increased trehalose levels in presence of PEG. However, some strains (6) are over the line (no concentration change between control and PEG) and one strain is below the line (trehalose decrease by PEG) (Fig. 2E). The average response of strains per tolerance level evidenced an inverse (though not statistically significant) relationship between trehalose content and osmotolerance in both control and PEG conditions. However, trehalose concentrations were higher in PEG than in control for all tolerance levels, but significant increases were only observed for strains with higher osmotolerance (T and MT) (Fig. 2F).

Two strains were not able to synthesize alginate nor in control neither in PEG conditions. The amount of alginate present extracellularly was considered negligible (less than 5%) and therefore were not presented. Among the strains able to synthesize alginate, PEG increased

between 1.5 and 9.1-fold the amount of alginate in 70% of them (Fig. 2G). Results presented by osmotolerance level (Fig. 3H) showed the lower ability of S strains to synthesize alginate both in control and PEG conditions, evidencing the inability of S strains to induce alginate synthesis in presence of PEG. On the contrary, PEG increased by 1.7- and 2.4-fold alginate levels in MT and T strains, respectively (Fig. 2H).

3.4. Antioxidant and biotransformation response

The metabolic effort to adapt to changes induced by high osmolarity is presented by protein levels (Fig. 3A and 3B). The antioxidant (SOD, CAT) and biotransformation (GSTs) mechanisms strains resorted as a way to minimize oxidative damage and toxicity of endoxenobiotics by exposure to high osmolarity is also presented separately (Fig. 3C, 3E and 3G) or grouped by tolerance level (Fig. 3D, 3F and 3H).

Most strains do not show variation in the amount of protein between conditions (PEG and control), but three strains had 50% lower and two 100% more protein in the presence of PEG relatively to control (Fig. 3A). Protein content by osmotolerance level (Fig. 3B) showed that in control condition S strains presented 2-fold ($p< 0.05$) more protein than MT and T strains, but PEG narrowed this difference and at PEG condition no significant difference was observed among tolerance levels (Fig. 3B).

In most strains PEG duplicated CAT activity, but in five strains no increase was observed in CAT activity between conditions (Fig. 3C). Overall performance by osmotolerance level (Fig. 3D) showed that S strains exhibited significantly higher activity than MT strains both in presence and absence of PEG. However, PEG increased CAT activity relatively to the control at all tolerance levels (Fig. 3D).

SOD activity was not changed by PEG in 55% of strains, but increases (between 3 and 19-fold) and decreases (between 2 and 6-fold) were recorded in 3 and 6 strains, respectively (Fig. 3E). Overall performance by osmotolerance level (Fig. 4F) evidenced significant differences among osmotolerance levels both in presence and absence of PEG, but no differences were observed between conditions (Fig. 3F).

With the exception of four strains, PEG increased (1.5 to 2.7-fold) the activity of GSTs (Fig. 3G). Analysis by osmotolerance level (Fig. 4H) showed higher activity in S strains in both conditions and increased activity by PEG relative to the control at all osmotolerance levels (Fig. 3H).

3.5. Multivariate analysis

Principal Components Ordination (PCO) diagram evidenced that the two axes represented explained more than 90% of the total variation obtained among strains exposed to two conditions (Supplementary Fig. 2). Exposure to PEG was highly correlated with osmolytes (betaine -r=0.94, trehalose -r=0.66 and alginate –r=0.65) and enzymes activity (CAT -r=0.79, GSTs -r=0.74 and SOD –r=0.96), evidencing the effort of cells to regulate osmotically and to fight oxidative stress imposed by PEG exposure. Despite the activation of these mechanisms exposure to PEG was also highly correlated with LPO (r=0.70), evidencing the damage that PEG-induced oxidative stress caused in membranes.

4. Discussion

The present study aimed to investigate the mechanisms underlying the tolerance differences observed in soil bacteria isolated from wild legumes growing at different edaphoclimatic

environments (Cardoso et al., 2018) and the relation between tolerance level and bacteria diversity (genus level). Several studies addressed the effect of osmotic stress on bacteria (Benabdellah et al., 2011; Chang et al., 2007; Csonka, 1989; Fierer and Schimel, 2002; Freeman et al., 2013; Marulanda et al., 2009; Ngumbi and Kloepper, 2016; Patel et al., 2017; Schimel et al., 2007; Wood, 2015; Yaakop et al., 2016; Yan et al., 2015; Yancey et al., 1982) and some assessed the mechanisms of tolerance (Benabdellah et al., 2011; Chang et al., 2007; Csonka, 1989; Freeman et al., 2013; Yaakop et al., 2016; Yancey et al., 1982), yet these studies focus on a strain or a set of strains of the same species and results are hardly extrapolable to other bacterial groups or accepted as a general bacterial response. Thus, representatives of all genera isolated, and of strains with different osmotolerance levels from the same genera (*Pseudomonas* and *Flavobacterium*) were studied in order to test the hypothesis that strains displaying different osmotolerance levels have differences in the efficiency of osmoadaptation and antioxidant mechanisms.

Differences observed among strains of the same genus overlapped differences among genera. On the other hand, similar response patterns among strains with the same level of osmotolerance were noticed, regardless the strain genus and the edaphoclimatic provenance.

The osmotolerance level of strains was not limited by the level of oxidative stress, since when exposed to PEG sensitive strains presented lower LPO levels than more tolerant ones. Sensitive (S) strains already presented higher activity of antioxidant (CAT, SOD) and biotransformation (GSTs) enzymes in the control, which only increased significantly in the presence of PEG for CAT, but that were sufficient to control oxidative stress and limit

LPO. Indeed, the presence of PEG did not increase LPO significantly relatively to control in S strains.

Since strains sensitivity was not associated with oxidative stress, it must be related to changes in other cellular functions, such as osmotic adaptation. General osmotolerance mechanisms are similar across diverse bacteria and include the accumulation of compatible solutes by *de novo* synthesis and uptake (Yancey et al. 1982). Accumulation of compatible solutes such as proline, glycinebetaine and trehalose increase the thermotolerance of enzymes, inhibit proteins denaturation, and help maintain membrane integrity (Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007; Schobert and Tschesche, 1978; Welsh, 2000). Freeman and collaborators (2013) observed that for *Pseudomonas* trehalose was a much larger contributor to osmotolerance than other osmolytes. (Measures (1975) found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. Csonka (1989) reported that some species respond equally to both proline and glycinebetaine, other are stimulated more dramatically by glycinebetaine than by proline. In soil bacteria, such as *Rhizobium meliloti* glycinebetaine also accumulated in cells grown in media with high osmolarity (Smith et al., 1988). In our study, glycinebetaine increased much more in PEG-exposed cells relatively to control than did other osmolytes (trehalose, proline), constituting a preferential mechanism of osmotolerance in strains of all levels of tolerance and providing a particularly strong fitness benefit to osmotically stressed cells. Yet, there were no significant differences in the osmolytes commonly referred in the literature (trehalose, proline and glycinebetaine) among strains form different osmotolerance levels.

Therefore, the present study searched for alternative mechanisms that could explain the differences in the osmotolerance observed. The production of exopolymeric substances, such as alginate, was proposed by several authors (Chang et al., 2007; Freeman et al., 2013) to increase bacteria osmotolerance (Chang et al., 2007), by encapsulating bacterial cells and thus protecting them from desiccation (Chang et al., 2007). In our study, quantification of extracellular alginate evidenced very low concentrations, falling to evidence differences among strains with different levels of osmotolerance. However, S strains accumulated low intracellular concentrations of alginate at control and were unable to increase alginate synthesis in presence of PEG. In contrast, T (tolerant) and MT (medium tolerant) strains had constitutively (control) higher intracellular concentrations of alginate, and were able to significantly increase alginate concentration under PEG, especially T strains, where the average response was a 2.4-fold increase of alginate concentration and with one strain increasing 9-fold alginate concentration intracellularly. Freeman et al. (2013) also observed osmoinduction of the alginate biosynthesis genes and alginate production in a *Pseudomonas* osmotolerant strain but not in the sensitive one and hypothesized that the lack of osmoinduction of the alginate biosynthesis in the sensitive strain could be associated with its lower osmotolerance. The results of this study demonstrate the ability to synthesize alginate under high osmotolerance conditions as a competitive advantage for soil bacteria to tolerate and survive in environments with high osmolarity. Although it is assumed that alginate is produced by bacteria belonging to *Pseudomonas* genus (Chang et al., 2007; Fialho et al., 1990; Flores et al., 2013; Freeman et al., 2013), in our study of the five *Pseudomonas* strains used three presented high amounts of alginate (both at control and PEG conditions) and two not only produced low alginate levels at control condition, but in the presence of PEG the alginate biosynthesis was reduced. Moreover, other bacteria

genera (*Flavobacterium*, *Erwinia*, *Herbaspirillium*, *Stenotrophomonas*, *Achromobacter*, *Lysobacter*) produced higher alginate levels than *Pseudomonas* at both conditions, leading to question the nearly universal ability of *Pseudomonas* species to produce alginate (Chang et al., 2007) and the prevalence of alginate biosynthesis capabilities among pseudomonads (Fialho et al., 1990).

Contrary to most literature that describes alginate as an exopolimeric compound (Chang et al., 2007; Flores et al., 2013), less than 5% of the alginate produced was excreted outside cells, being accumulated intracellularly and contributing preponderantly to the level of osmotolerance observed in strains. But how does alginate accumulation inside cells increase bacteria osmotolerance?

Alginate was described as having antioxidant properties. Kelishomi et al. (2016) reported the antioxidant and radical scavenging properties of alginate, which they attributed to functional groups such as hydroxyl, carbonyl and carboxyl groups and double bonds between C-4 and C-5. Sellimi et al. (2015) also reported alginate to have high free-radicals scavenging activity, including hydroxyl radicals and high reducing power of Fe^{3+}. Moreover, alginate hygroscopic properties (Robyt, 1998; Sutherland, 2001) contribute to keep cells hydrated (Chang et al., 2007) and its interaction with various proteins (Imeson et al., 1977; Schwenke et al., 1977) increase the stability of enzymes to heat (Wingender and Winkler, 1984) and possibly to other stresses. Thus, alginate may reduce cell dehydration, stabilize enzymes and decrease oxidative stress originated by high osmolarity, increasing cell osmotolerance. In fact, S strains synthesized low amounts of alginate, whereas strains with higher levels of alginate presented higher osmotolerance with or without higher

concentrations of other osmolytes (trehalose, betaine, proline), and many with mild increases in the activity of antioxidant enzymes (CAT and SOD).

Thus, results evidence alginate as an efficient mechanism to increase osmotolerance in soil bacteria, but its protective effect is not extensive to membranes and tolerant strains, which were exposed to high PEG concentrations evidenced high damage, as LPO levels prove. The metabolism of lipid peroxides originates endoxenobiotics, such as aldheydes, some of them presenting high toxicity towards cells, which in strains with higher osmotolerance (T and MT) should be abundant, since lipid peroxidation is high. However, GSTs can convert the products resulting from the metabolism of lipid peroxides in less toxic compounds (El-Aal, 2012; Korpi et al., 2009; Schmidt et al., 2015), thus minimizing the interference with biomolecules with important functions, such as proteins and nucleic acids. Indeed, in our study GSTs activity of T and MT strains increased significantly in PEG exposed relatively to control cells, evidencing the cell effort to contain toxicity resulting from lipid peroxides metabolism and rendering cells more tolerant to osmotic effects.

5. Conclusions and applications

This study relates for the first time the level of osmotolerance with the ability of soil bacteria of different genera to accumulate alginate intracellularly, and therefore more likely to survive in environments subjected to frequent droughts, being excellent candidates for and implementation in natural and agricultural environments subjected to drought after validation in controlled conditions, such as mesocosm studies. Some of these strains have the capacity to promote plant growth, either by production of phytohormones (indole acetic acid), siderophores, or volatile organic compounds (Cardoso et al., 2018). Moreover, some of the osmoprotectants (such as proline, choline and trehalose) synthesized by bacteria in

drought stress can be excreted, and if absorbed by plants can directly increase drought stress, or induce osmoprotective mechanisms in plants. For example trehalose metabolism in plant growth promoting rhizobacteria (PGPR) is key for signaling plant growth, yield, and adaptation to abiotic stress, and its manipulation may have a major agronomical impact on plants (Rodríguez-Salazar et al., 2009). PGPR also improves the stability of plant cell membranes by activating the antioxidant defense system (SOD, CAT, APX, GR, POD), also enhancing drought tolerance in plants (Gusain et al., 2015).

Since the strains with greater capacity to produce alginate are those that present higher osmotolerance and also produce high amounts of proline, glycine betaine and trehalose, the ability to synthesize alginate should be an attribute present in bacteria to be used as inoculants at sites potentially subjected to conditions generating low water activities in soils both at natural and agronomic systems. Additionally, since alginate is able to form complexes with divalent cations (Grant et al., 1973) and (Davis et al., 2003), some of them being micronutrients (Cu, Mn, Mg, Fe, Ca) its application can thus further promote plant growth in soils affected or not by drought.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure and table legends

Table 1-Selected endophytic bacteria differing in osmotolerance. Percentage of PEG that inhibits 50% growth (PEG%). and osmotolerance (OsmT) level (Sensitive - PEG% < 10, Moderately tolerant - 10 ≤ PEG% < 15 and Tolerant- PEG% >15%). Strains were isolated from the root nodules of wild legumes (Legume host species) growing in four sites (Aljustrel-At, Alvito-Av, Murtosa-M and Vale de Cambra-V) in Continental Portugal (Site). Bacteria genera identified by 16S rRNA gene sequencing.

Fig. 1. Lipid peroxidation (LPO) in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. A) LPO levels for each strain in control versus PEG condition; dashed line represents equal concentrations in both conditions for each strain; values are means of at least 3 replicates. B) LPO levels for strains grouped by tolerance level; values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

Fig. 2. Intracellular concentration of osmolytes in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. Concentration of osmolytes for each strain in control versus PEG condition (A- proline, C-

betaine, E- trehalose, G- alginate); dashed line represents equal concentrations in both conditions for each strain; values are means of at least 3 replicates. Osmolytes concentrations for strains grouped by tolerance level (B- proline, D- betaine, F- trehalose, H- alginate); values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

Fig. 3. Soluble protein and activity of antioxidant (CAT, SOD) and biotransformation (GSTs) enzymes in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. Protein concentration and enzyme activity for each strain in control versus PEG condition (A- protein, C- CAT, E- SOD, G- GSTs); dashed line represents equal levels or activity in both conditions for each strain; values are means of at least 3 replicates. Protein concentration and enzyme activity for strains grouped by tolerance level (B- protein, D- CAT, F- SOD, H- GSTs); values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

Table 1

Strain	PEG%	Legume host species	Site	Bacteria genera
Sensitive				
Q1	7.69	*Medicago* sp.	AT	*Flavobacterium*
U6	7.41	*Ornithopus pinnatus* (Mill.) Druce	MT	*Agrobacterium/Rhizobium*
M1	7.25	*Vicia sativa* subsp. *sativa* L.	AV	*Flavobacterium*
D4	5.94	*Lotus corniculatus* L.	VC	*Flavobacterium*
Moderately Tolerant				
N1	14.06	*Scorpiurus vermiculatus* L.	AT	*Herbaspirillum*
K6	13.43	*Ornithopus compressus* L.	AV	*Achromobacter*
K1	13.29	*Vicia sativa* subsp. *sativa*	AV	*Erwinia*
U1	14.15	*Medicago lupulina* L.	MT	*Acinetobacter*
H5	13.17	*Medicago lupulina*	MT	*Pseudomonas*
I9	11.18	*Ornithopus sativus* subsp. *sativus* Brot.	MT	*Pseudomonas*
V4	14.80	*Medicago lupulina*	MT	*Stenotrophomonas*
E1	10.91	*Vicia sativa* subsp. *sativa*	VC	*Flavobacterium*
A7	13.86	*Ornithopus sativus* subsp. *sativus*	VC	*Pseudomonas*
Tolerant				
O4	18.71	*Medicago* sp.	AT	*Herbaspirillum*
Q4	15.56	*Medicago* sp.	AT	*Lysobacter*
N9	15.69	*Vicia benghalensis* L.	AT	*Variovorax*
O3	25.8	*Scorpiurus vermiculatus*	AV	*Herbaspirillum*
B3	20.50	*Ornithopus compressus*	VC	*Flavobacterium*
A10	18.19	*Ornithopus compressus*	VC	*Paenibacillus*
C11	17.82	*Ornithopus compressus*	VC	*Pseudomonas*

Highlights

Betaine has an important role in the tolerance of bacteria to drought

Lipid peroxidation was higher in drought simulated by polyethylene glycol

Betaine insufficient to explain differences in tolerance to drought among strains

Antioxidant mechanisms insufficient to explain observed differences

Intracellular alginate could be associated to differences in tolerance to drought

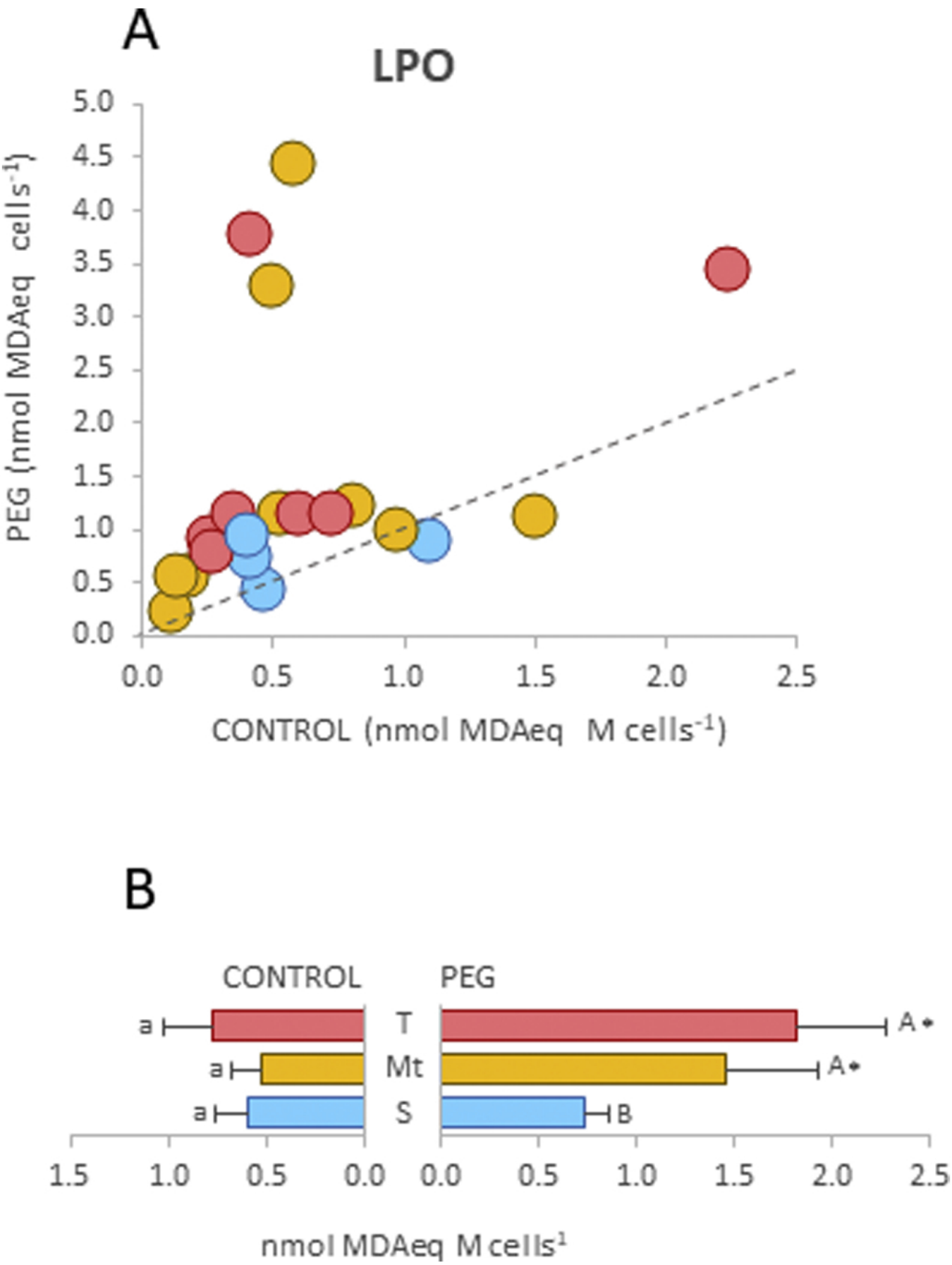
A
LPO
PEG (nmol MDAeq cells-1)
CONTROL (nmol MDAeq M cells-1)
B
CONTROL
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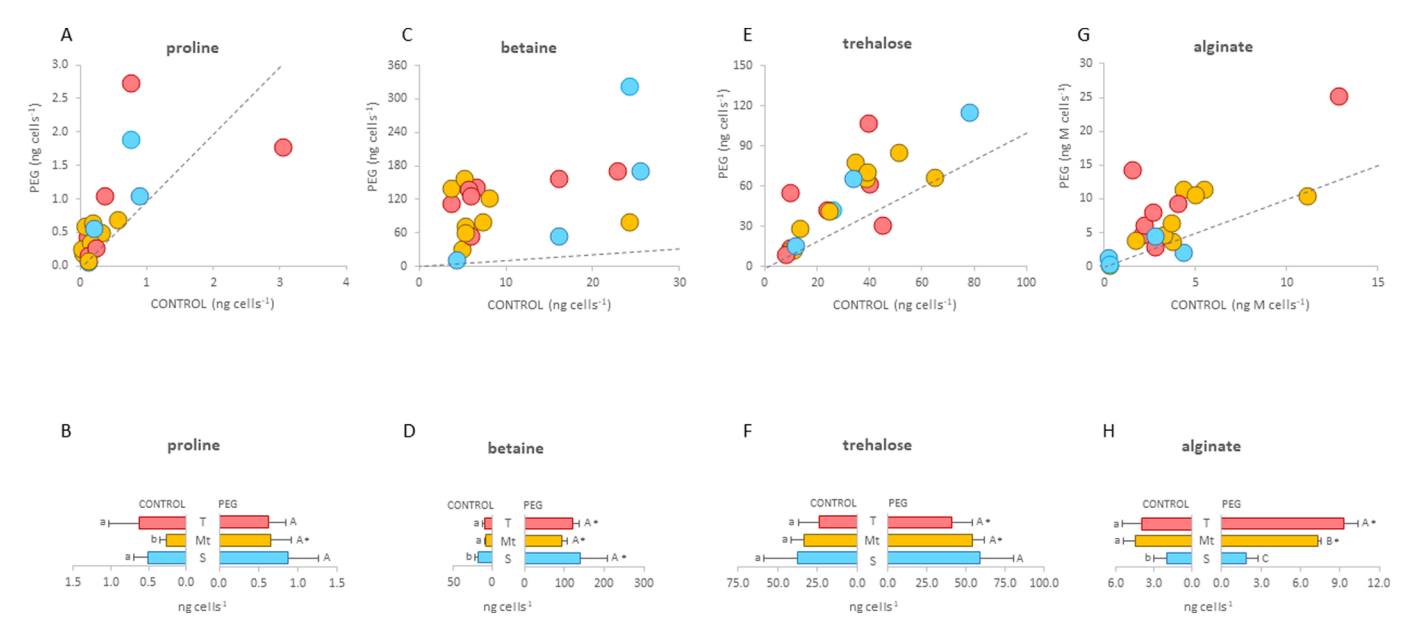
Figure 1


A
proline
PEG (ng cells-1)
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C
betaine
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CONTROL (ng cells-1)
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trehalose
PEG (ng cells-1)
CONTROL (ng cells-1)
G
alginate
PEG (ng M cells-1)
CONTROL (ng M cells-1)
B
proline
CONTROL
PEG
T
Mt
S
ng cells-1
D
betaine
CONTROL
PEG
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Mt
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Figure 2

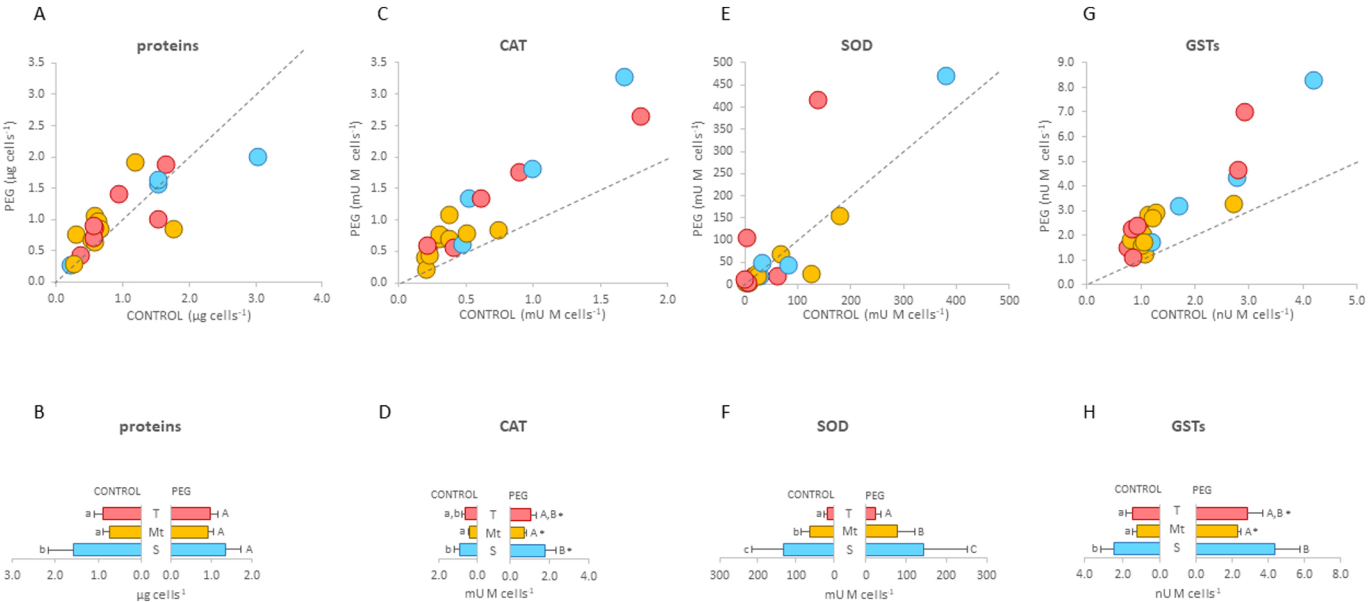
A
proteins
PEG (µg cells-1)
CONTROL (µg cells-1)
C
CAT
PEG (mU M cells-1)
CONTROL (mU M cells-1)
E
SOD
PEG (mU M cells-1)
CONTROL (mU M cells-1)
G
GSTs
PEG (nU M cells-1)
CONTROL (nU M cells-1)
B
proteins
CONTROL
PEG
T
Mt
S
µg cells-1
D
CAT
mU M cells-1
F
SOD
mU M cells-1
H
GSTs
nU M cells-1

Figure 3

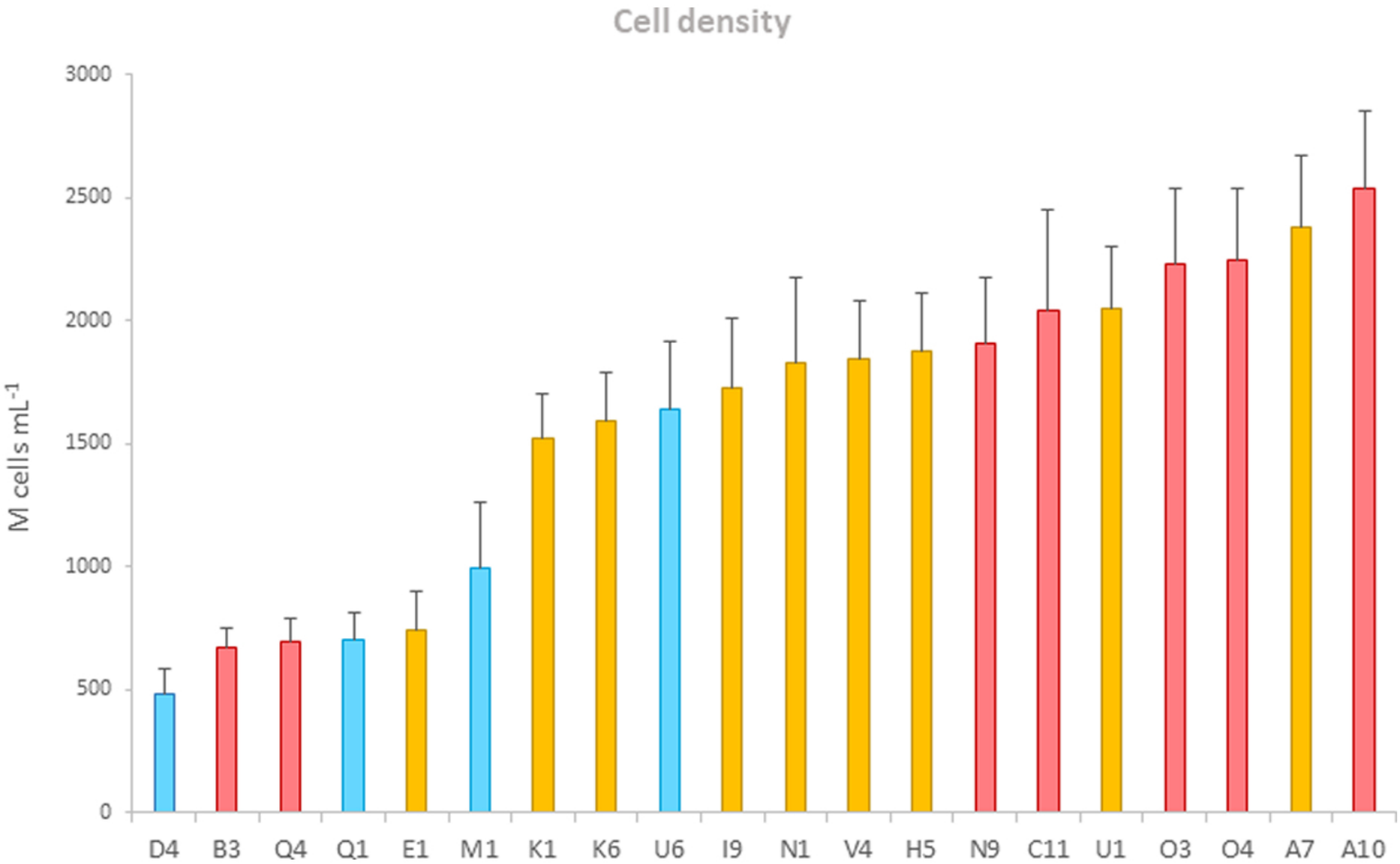
Cell density
M cells mL-1
3000
2500
2000
1500
1000
500
0
D4
B3
Q4
Q1
E1
M1
K1
K6
U6
I9
N1
V4
H5
N9
C11
U1
O3
O4
A7
A10

Figure 4

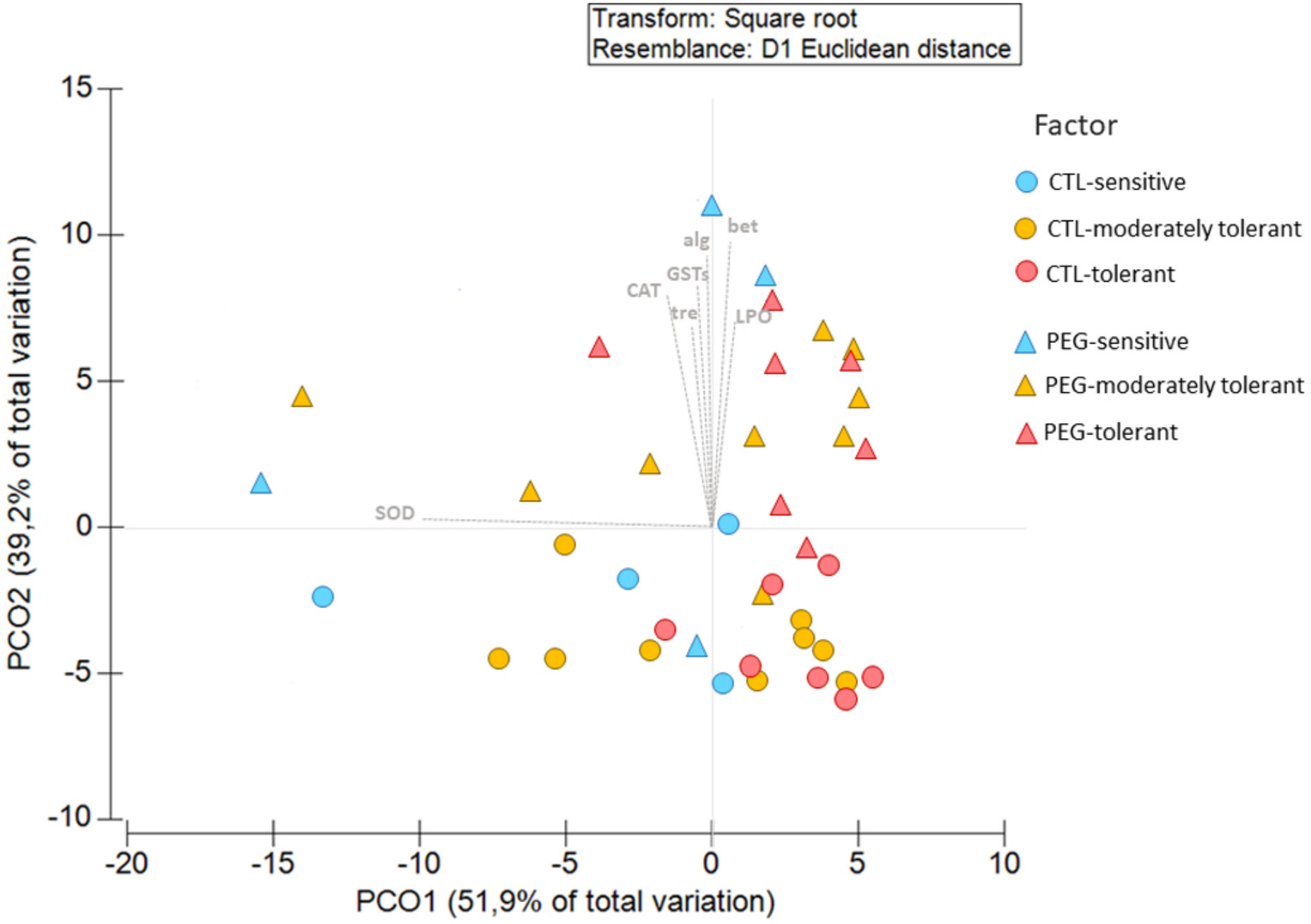
Transform: Square root
Resemblance: D1 Euclidean distance
Factor
CTL-sensitive
CTL-moderately tolerant
CTL-tolerant
PEG-sensitive
PEG-moderately tolerant
PEG-tolerant
bet
alg
GSTs
CAT
tre
LPO
SOD
PCO2 (39,2% of total variation)
PCO1 (51,9% of total variation)
15
10
5
0
-5
-10
-20
-15
-10
-5
0
5
10

Figure 5