Universidade de Aveiro Departamento de Biologia
Ano 2018

Bruno Miguel Ribeiro Soares

Effect of halophilic bacteria from Aveiro salt pans in the attenuation of saline stress in plants

Efeito de bactérias halófilas das salinas de Aveiro na atenuação do stresse salino em plantas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Professora Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Maria Helena Abreu Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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Aos meus pais que se abdicaram de todo o seu conforto em troca da minha felicidade.

o júri

presidente	Professora Doutora Ana Maria de Jesus Rodrigues Professora Auxiliar Departamento de Biologia da Universidade de Aveiro
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palavras-chave

agricultura salina, bactérias halófilas, bactérias promotoras do crescimento de plantas, salinização dos solos, stresse salino

resumo

A salinização dos solos é um problema crescente a nível global e têm sido várias as abordagens propostas para atenuar os seus efeitos na produtividade de plantas de interesse económico. O uso de bactérias halófilas ou halotolerantes como promotoras do crescimento de plantas, é uma das estratégias preconizadas para a mitigação do stresse salino. No entanto, são normalmente usadas como inóculo bactérias halotolerantes isoladas da rizosfera de plantas halófitas. O objetivo deste trabalho foi avaliar o potencial de bactérias halófilas, isoladas de uma marinha de sal, na atenuação do stresse salino em *Lactuca sativa,* usada como modelo de glicófita de interesse agrícola. Uma coleção de estirpes isoladas da marinha de Santiago da Fonte (Aveiro) representando os géneros *Bacillus, Halobacillus, Idiomarina e Marinobacter,* foi analisada quanto a algumas caraterísticas consideradas como vantajosas na colonização e promoção do crescimento de plantas. Testou-se a produção de enzimas extracelulares em salinidades 0, 20 e 100 de NaCl bem como a capacidade para solubilizar fosfato e produzir ácido 1-aminociclopropano-1-carboxilato desaminase. *H. locisalis* e *I. seosinesis,* considerados como mais interessantes face às características promotoras do crescimento, foram testados separadamente e em conjunto, como inóculo em sementes de alface. Foi aplicado um desenho experimental fatorial para testar o efeito da inoculação e da salinidade da água de irrigação sobre a eficiência de germinação das sementes e crescimento das plantas. A eficiência de germinação foi fortemente afetada pela salinidade não tendo sido observados efeitos significativos de nenhum dos inóculos testados. Na condição de salinidade 10, a eficiência de germinação foi mais baixa do que com salinidade 0 e o peso das plantas foi significativamente menor nas plantas inoculadas com o consórcio de isolados do que nas plantas não inoculadas.

As plantas inoculadas com o consórcio e cultivadas na salinidade 10 apresentaram menor teor de água. As plantas inoculadas separadamente com *H. locisalis* ou com *I. seosinesis* cultivadas em salinidade 0, revelaram um aumento do tamanho das folhas relativamente ao controle não inoculado. Embora não tenham sido encontradas evidências de atenuação do stresse salino, o inóculo *H. locisalis* apresentou um efeito positivo no crescimento das plantas em condições não-salinas, o que demonstra um potencial como bactéria promotora do crescimento de plantas de interesse agrícola.

keywords halophilic bacteria, plant growth-promoting bacteria (PGPB), saline agriculture, saline stress, soil salinization

abstract Soil salinization is a globally growing problem, and several approaches have been proposed to mitigate its effects on the productivity of plants of economic interest. The use of halophilic or halotolerant bacteria as plant growth promoters is one of the strategies recommended for the mitigation of salt stress. However, halotolerant bacteria isolated from the rhizosphere of halophyte plants are the most commonly used inoculum. The objective of this work was to evaluate the potential of halophilic bacteria, isolated from a salt pan, in the attenuation of saline stress *Lactuca sativa*, used as a model crop glycophyte. A collection of strains isolated from Santiago da Fonte saltpans (Aveiro) representing the genera *Bacillus, Halobacillus, Idiomarina and Marinobacter*, was analyzed for some characteristics considered as advantageous in the colonization and promotion of growth of host plants. The production of extracellular enzymes in presence of 0, 20 and 100 NaCl, as well as the ability to solubilize phosphate and produce 1-aminocyclopropane-1-carboxylate deaminase were tested. *H. locisalis* and *I. seosinesis*, considered as more interesting in terms of plant growth promoting traits, were tested separately and together as inoculum in lettuce seeds. A factorial experimental design was applied to test the effect of inoculation and salinity of the irrigation water on the efficiency of seed germination and plant growth. Germination efficiency was strongly affected by salinity and no significant effects of inoculation were observed. The germination efficiency was lower at 10 NaCl than at salinity 0 and the weight of the plants was significantly lower in the plants inoculated with the consortium of isolates than in the uninoculated plants. Plants inoculated with the consortium and grown at salinity 10 had lower water content. When used separately, *H. locisalis* or *I. seosinesis* caused an increase in leaf size in plants cultivated in salinity, in relation to the inoculated control. Inoculation did not cause a significant effect on chlorophyll fluorescence. Although no evidence of attenuation of saline stress by inoculation was detected, *H. locisalis* inoculum showed a positive effect on the growth of plants in non-saline conditions, indicating a potential as a growth promoting bacterium of plants of agricultural interest.

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List of abbreviations

ABA - Abscisic acid

ACC - 1-aminocyclopropane-1- carboxylate

CAM - Crassulacean acid metabolism

EC - Electrical conductivity

EC_e - Electrical conductivity of the saturation extract

Fs - Fluorescence (steady state)

GE - Germination efficiency

IAA - Indole acetic acid

NCC - Nitrogen-containing compounds

PAM - Pulse amplitude modulated

PGPB - Plant growth promoting bacteria

PGPR - Plant growth promoting rhizobacteria

PSI - Photosystem I

PSII - Photosystem II

ROS - Reactive oxygen species

rRNA - Ribosomal ribonucleic acid

SA - Saline agar

SB - Saline broth

SCW - Sterilized crystallizer water

1. Introduction

1.1. Soil salinization

A soil is considered as saline when the electrical conductivity (EC) of the saturation extract (EC_e) in soil surpasses 4 dS m^{-1} (approximately 40 mM NaCl) at 25 °C and has a content of exchangeable sodium of 15% (Munns, 2005). High salinity is currently considered as one of the major threats to agriculture by causing reductions of area of soils suitable for cultivation and also a decrease of the productivity and quality of crops (Yamaguchi and Blumwald, 2005). In fact, it is estimated that soils with high salinity correspond to a total of 20% of cultivated and 33% of irrigated lands worldwide (Epstein et al., 1980; Flowers et al., 1986).

According to the processes that cause it, soils salinization as considered as primary or secondary. Primary soils salinization is associated with natural processes like weathering of native rock constituents, high evapotranspiration, lack of rainfall, tidal flooding and wind in coastal area (Singh, 2015). Secondary salinization is associated has anthropogenic causes like over exploitation of coastal groundwater aquifers causing seawater intrusion, waterlogging without adequate drainage, and climate change impacts like sea level rise caused by melting of the polar caps and lack rainfall (Singh, 2015). The area of salinized soils is increasing at a rate of 10% annually and it is estimated that more than 50% of cultivable land would be salinized in 2050 (E. V. Maas, 2012).

1.2. Salt tolerant and salt sensitive plants

Salt tolerance corresponds to the capacity of plants to withstand high salt concentrations in the root zone or in the leaves without dramatic adverse effects, still being able to grow and complete the life cycle (Shannon and Grieve, 1998). According to tolerance, plants are classified as halophytes or glycophytes (Levitt, 1985). Halophytes can survive, reproduce and complete the life cycle under high concentrations of salt (Flowers et al., 1986; Parida and Das, 2005; Colmer and Flowers, 2008). Obligate halophytes can tolerate irrigation with up to 50% sea water whereas facultative halophytes grow under more moderate concentrations of salt (Parida and Das, 2005). Glycophytes are significantly

affected by salinity and all major crop species are included in this category (Munns and Tester, 2008).

Tolerance to salt may significantly vary between plants and along the different stages of development of each species (Vicente et al., 2004; Omami, 2005; Manchanda and Garg, 2008). In general, germination and seedling stages are more susceptible to saline stress effects (Maas and Poss, 1989; Vicente et al., 2004). However, the selection for salinity tolerance at germination, seedling stage or early vegetative growth may not ensure that plants will be equally tolerant in subsequent development stages (Kingsbury et al., 1984; El-Hendawy et al., 2005). Although the intensity of the responses to high salinity may differ, it is generally accepted that same general salt tolerance regulatory mechanisms, and differences between halophytic and glycophytic species are a quantitative rather than qualitative nature (Omami, 2005).

1.3. Saline stress effects

Soil salinity causes osmotic stress, nutrient deficiency, ion toxicity and oxidative stress (Bano and Fatima, 2009) which lead to changes in productivity in agriculture of crops negatively affecting the germination, vegetative growth, absorption of some nutrients and reproductive success (Blaylock, 1994; Hu and Schmidhalter, 2002; Ashraf, 2004).

The root is the first organ of plant to be affected by salinity (Waisel and Breckle, 1987). However, since the root is involved in ion accumulation roots are also involved in stress responses and mechanisms of salt tolerance (Munns, 2002).

High concentrations of NaCl in soil reduce water availability to the roots and the water potential of leaves limiting nutrient uptake (Sohan et al., 1999; Romero-Aranda et al., 2001). This decrease in water potential was demonstrated in some *Brassica* species although with significant differences between species. *B. campestris* and *B. carinata* were more tolerant maintaining higher leaf water potentials at 200 mM of NaCl (Ashraf, 2001). A significant decrease in water content loss of turgor due to elevated salinity was observed in sugar beet and the effect was associated to the accumulation of Na^+ and Cl^- in the tissues and a decrease in the transport of water from the roots to leaves (Katerji et al., 1997; Ghoulam at al., 2002) that will ultimately lead to senescence (Lutts et al., 1996).

In saline soils, Na+ and Cl- compete with macronutrients such as K+, N, P, and Ca2+ in terms of root uptake, which creates nutritional imbalance (Grattan and Grieve, 1998). Elevated NaCl concentrations in root zone causes accumulation of Na^+ and Cl^- in shoot tissues and a decrease in the concentration of Ca^{2+}, K^+ and Mg^{2+} (Pérez-Alfocea et al., 1996; Khan et al., 2000; Bayuelo-jiménez et al., 2003). The absorption of nitrogen is also affected causing a reduction in nitrogen accumulation in plants (Pardossi et al., 1999; Silveira et al., 2001). This was observed in eggplant, in which the accumulation of Cl^- in leaves was accompanied by a decrease of the concentration of NO_3^- (Savvas and Lenz, 2000). Depending on the plant species, growth stages, and level of salinity, the content of phosphorus also decreases in saline conditions. In most of the plants, P concentration in plant tissues is negatively correlated with soils (Sonneveld and De Kreij, 1999; Kaya et al., 2001).

In the leaves, the reduction of stomatal conductance (Brugnoli and Lauteri, 1991) and consequent reduction of CO_2 supply is one of the primary effects of saline stress, that affect CO_2 fixation and respiration rates (Marler and Zozor, 1996; Ashraf, 2001; Romero-Aranda et al., 2001). Under NaCl stress, net photosynthetic rate and chlorophyll content decrease whereas respiration rate increase (Khavari-Nejad and Chaparzadeh, 1998). There is evidence of a negative effect on the quantum efficiency of photosystem II (PSII) activity accompanied by a stimulation of photosystem I (PSI). (Lu and Vonshak, 1999a). Tests conducted on wheat demonstrated a two-step inhibition of photosynthesis with an initial phase of gradual reduction of photosynthetic efficiency and a later phase second characterized by a rapid decrease decline of the energy conversion efficiency in photosystem II (Muranaka et al., 2002). The may imply that plants can withstand a certain loss in photosynthetic activity without an immediate effect in growth (Alarcón et al., 1993). Some other environmental effects may enhance the effect of salinity. Tests in sorghum indicate that fluorescence parameters Fv/Fm describing the function of PSII are rather resilient to saline but respond dramatically to combined saline stress and elevated temperature (Lu and Zhang, 1998; Lu et al., 2003). Other studies report that chlorophyll fluorescence in spinach is not directly affected by salinity and that the inhibition of photosynthesis is due to the reduction of CO_2 diffusion associated with by stomatal

closure and damage on the mesophyll (Delfine et al., 1998; Delfine et al., 1999). Salinity also affects growth and induces structural, histological and cytological changes.

There is evidence of anatomical changes associated with salinity stress. The increase in the diameter of spongy cells and palisade cells, the increase of palisade cell length and reduction of the epidermis and mesophyll thickness as well as of the intercellular spaces has been reported (Longstreth and Nobel, 1979; Delfine et al., 1998; Parida et al., 2004). A reduction of stomatal density has also been observed (Romero-Aranda et al., 2001). A study on the cytological effects of salinity conducted with sweet potato reported vacuolization and partial swelling of endoplasmic reticulum, swelling and reduction of cristae in mitochondria, increase of vesicle release from stacks of the Golgi apparatus, mixture of cytoplasmic and vacuolar matrices (Mitsuya et al., 2000).

1.4. Mechanisms of adaptation to salt

Plants used a wide range of biochemical mechanisms to overcome saline stress and preserve the capacity to develop and grow. Those mechanisms involve the uptake or synthesis of solutes, the active exclusion or sequestration of ions, adaptations of the cellular membrane and expression of stress-mitigation enzymes and phytohormones and ultimately, complex changes in photosynthetic and respiratory pathways (Flowers et al., 1977; Ashraf and Harris, 2004; Yamaguchi and Blumwald, 2005). When exposed to high levels of salinity exclude salt or accumulate it selectively. At low to moderate salt concentrations, the exclusion mechanism can be effective but at high levels of salinity halophytes make use of compartmentalization mechanism (Cheeseman, 1988; Bohnert et al., 1995). Salt secretion involves unique cellular structures (salt glands) that secrete salt (especially NaCl) from leaves and maintain internal ion concentration at lower level (Marcum and Pessarakli, 2006). Salt exclusion through the roots contributes to regulate the concentration of salt in the leaves of many halophytes (Levitt, 1985). Halophytes can restrict the excess of salt in the vacuole or use a compartmentalization strategy that limits the transport of Na to tissues where damage can have more dramatic effects (Zhu, 2003; Manchanda and Garg, 2008).

Osmotic adjustment corresponds to the inclusion of ions, like Ca^{2+} in the intracellular compartments, reducing the toxic effects of NaCl (Halperin and Lynch, 2003). Low molecular mass compounds (compatible solutes) like proline, glycine betaine and other nitrogen-containing compounds (NCC), sugars and polyols, accumulating in the vacuoles contribute to ionic balance without affecting with normal biochemical reactions (Ashihara et al., 1997; Hasegawa and Bressan, 2000; Manchanda and Garg, 2008)

Increasing osmotic potential in cells causes an increase in reactive oxygen species (ROS) (Imlay, 2003) and like in other situations of stress, there is an imbalance between the production of ROS and the antioxidant defences (Spychalla and Desborough, 1990; Imlay, 2003; Mittova et al., 2004). The increase in the expression of antioxidants in response to high salinity has been reported in halophytes and in crop plants (Bandeoğlu et al., 2004; Amor et al., 2006).

Some phytohormones such as abscisic acid (ABA), jasmonates and cytokinins activate salt-stress-induced genes that mitigate the negative effects of NaCl on photosynthesis, growth and transport of nutrients by promote stomata closure and shifts in the C-fixation pathway (Aldesuquy et al., 1998; Pedranzani et al., 2003).

High salinity reduces photosynthesis and C-fixation rates. Some facultative halophytes growing in arid conditions operate a shift from C3-metabolism to crassulacean acid metabolism (CAM) in which CO_2 is captured during the night and stored as malate so that stomata remain closed during the day, as a strategy to reduce the loss of water (Cushman, 1989; Parida and Das, 2005).

1.5. Mitigation of saline stress by rhizosphere engineering

The rhizosphere is the zone of the soil or sediments that is directly influenced by plant roots. Considering that high NaCl concentrations in soil affect primarily root functions and trigger root-related responses, this compartment has a paramount relevance in salinity stress in plants. The bacterial communities of the rhizosphere (rhizobacteria) are involved in mutually beneficial relations with the plant and some are considered as plant growth promoting rhizobacteria (PGPR) because their activities are associated with a wide spectrum of positive effects on plant condition, development and productivity. PGPR can

mitigate stress effects like those associated with salinized soils and, consequently, enhance growth (Shrivastava and Kumar, 2015). This effect can be exerted directly in the plant expressing siderophores that contribute to iron acquisition, or phosphatases that solubilize phosphate facilitating nutrient uptake (Hayat, et al., 2010). By secreting phytohormones, PGPR attenuate ethylene-mediated stress responses and stimulate growth. The level of this hormone is influenced by biotic and abiotic stress (Hardoim, Overbeek and Elsas, 2008). Under stress conditions ethylene is biosynthesized, regulating plant homeostasis and leading to a reduction on root and shoot growth (Page and Malcolm, 1997). The presence of bacteria capable of synthetizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase allow bacterial cells to use ACC as supply of nitrogen source and energy and in this way the bacteria can attenuate the negative effect caused by ethylene and promote plant growth (Glick et al., 2007). Other hormones synthetized by PGPR, like indole acetic acid (IAA) and gibberellins, induce increases in root length, root surface and number of root tips, which will also lead to enhanced nutrient and a general improvement in plant performance and growth under salinity stress (Egamberdieva and Kucharova, 2009).

Indirectly, PGPR may control phytopathogens protecting the plant against soil-borne diseases (Lugtenberg and Kamilova, 2009), or degrade toxic compounds (Dimkpa, Weinand and Asch, 2009).

The use of PGPR inoculants to manipulate the rhizosphere microbiota, an approach referred as rhizosphere engineering, has been proposed as useful tool to reduce the impact caused by salinity stress on plants (Yao et al., 2010). Halotolerant bacteria inoculated in wheat and rice stimulated the growth under 320 mM NaCl in relation to non-inoculated controls, expressed by increases in root length and dry weight (Ramadoss et al. 2013). *P. mendocina* attenuated the effect of saline stress on nutrient uptake in *Lactuca sativa* with significantly increase on shoot biomass and root length when compared with non-inoculated controls (Kohler et al., 2006).

Most commonly, halotolerant bacterial strains isolated from salt affected soils or salt-marshes, expressing plant-growth promoting effects, are regarded as interesting as inoculants for the mitigation of saline stress by rhizosphere engineering approaches.

Klebsiella, *Pseudomonas*, *Agrobacterium*, and *Ochrobactrum* isolated from roots of the halophyte *Arthrocnemum indicum* showed ACC-deaminase activity, N_2 fixation and phosphate solubilization capacities and promoted the growth of peanut seedlings (Sharma, Kulkarni and Jha, 2016) Halophilic strains expressing IAA production, ACC-deaminase activity, phosphate solubilization and nitrogen fixation activities isolated from salt effected soils were successfully used for promote the growth of wheat grown at 200 mM NaCl, increasing the root and shoot length and total fresh weight (Orhan, 2016). Hypersaline environments are still underexplored in the perspective of the isolation of PGP strains. However, Archaea expressing plant-growth traits like phosphorus solubilization, nitrogen fixation, siderophore and IAA production were already isolated from extreme environments (Yadav et al., 2017). *Halomonas* strains capable expressing plant-growth promoting traits like IAA production and phosphate solubilization in with 5% NaCl were isolated from hypersaline ecosystems in Tunisia and successfully used to colonized *Salicornia* roots (Mapelli et al., 2013).

1.6. Objective

The objective of this work was to evaluate the potential of halophilic bacteria, isolated from an active salt pan of Ria de Aveiro, as plant-growth promoting bacteria suitable for the attenuation of saline stress of *Lactuca sativa*, used as a model glycophyte of agricultural interest.

2. Methods

2.1. Bacterial isolates

The collection of bacterial strains used in this study was obtained in a previous study conducted by Sofia Cruzeiro (Cruzeiro, 2018). Isolates were retrieved from water of one solar saltern (Santiago da Fonte, 40.628676 N, 8.660874 W) owned by the University of Aveiro and still operating according to the traditional method of sea salt production. Water samples were collected from the crystallizers (salinity ~30) in early autumn 2016.

The isolates were identified by 16S rRNA gene sequencing as belonging to the *Bacillus*, *Idiomarina*, *Halobacillus* and *Marinobacter* (Table 1).

Table 1. Identification of isolates and corresponding percentages of similarity (Cruzeiro, 2018).

Isolate	Identification	% Similarity	Blast
1	*Bacillus licheniformis*	99%	*Bacillus licheniformis* strain M63
2	*Idiomarina zobellii*	95%	*Idiomarina zobellii* strain NIOSSD020#90
3	*Idiomarina seosinensis*	100%	*Idiomarina seosinensis strain NIOSSK079#67*
4	*Halobacillus sp.*	99%	*Halobacillus sp. JC 137*
5	*Halobacillus locisalis*	100%	*Halobacillus locisalis* strain K-W48
6	*Idiomarina sp.*	99%	*Idiomarina sp TP368*
7	*Bacillus licheniformis*	99%	*Bacillus licheniformis* strain M63
8	*Marinobacter salsuginis*	99%	*Marinobacter salsuginis* strain NIOSSK56#5
9	*Marinobacter salsuginis*	99%	*Marinobacter salsuginis* strain NIOSSK56#5
10	*Idiomarina zobellii*	99%	*Idiomarina zobellii* strain NIOSSD020#90
11	*Marinobacter sp.*	100%	*Marinobacter sp. strain 7002-278*
12	*Idiomarina sp.*	100%	*Idiomarina sp. TP368*
13	*Idiomarina seosinensis*	99%	*Idiomarina seosinesis* culture-collection MCCC:1A02681
14	*Idiomarina zobellii*	100%	*Idiomarina zobellii* strain NIOSSD020#90

2.2. Cultivation conditions

Work cultures were routinely maintained in Saline Agar (SA) containing 5 g/L tryptone (Oxoid), 4g/L yeast extract (Liofilchem) and 15g/L agar (Liofilchem). A mixture of sterilized crystallizer water (SCW; filtered by Whatman GF/C Glass Microfiber filters and

autoclaved) and distilled water was used to achieve a salinity of 20 (SA20) and, whenever necessary, 100 (SA100). Work cultures were incubated at 37 °C until the development of isolated colonies, stored in the refrigerator (37 °C) and renewed every two weeks. For the revivification of work cultures, isolated colonies were inoculated in Saline Broth (SB20 or SB100) prepared as described for SA but excluding the agar.

2.3. Production of extracellular hydrolytic enzymes

The production of extracellular hydrolytic enzymes under different salt concentrations was tested by a culture-dependent approach using culture media containing specific substrates in which salinity was adjusted to 0, 20 or 100 with the convenient proportions of distilled water and SCW.

2.3.1. Amylase

The activity of extracellular amylase (α-amylase) was tested in solid media containing starch as substrate (Abel-Nabey and Farag, 2016). Tryptic Soy Agar (Liofilchem) was amended with 20 g/L rice starch (Liofilchem) and salinity was adjusted as previously described. Strains were streak-plated (3 replicate plates for each salinity condition) and *Bacillus cereus* ATCC 11778 was included as positive control. The cultures were incubated at 37 °C until growth was observed (~48 h). To detect the degradation of starch, lugol solution 1% was poured over the culture medium. A clear halo around the growth zone was interpreted as a positive result. The strains were considered as amylase-positive in each salinity condition if positive results were observed in the 3 corresponding replicate plates.

2.3.2. Lecithinases

The activity of extracellular lecithinases (phospholipases) was tested in solid media containing egg yolk as substrate (Kushner, 1957; Lakshmipathy and Kannabiran, 2009). Tryptic Soy Agar (Liofilchem) was amended with 20% egg yolk emulsion (Liofilchem) and salinity was adjusted as previously described. Strains were streak-plated (3 replicate plates for each salinity condition) and *Bacillus cereus* ATCC 11778 was included as positive

control. The cultures were incubated at 37 °C until growth was observed (~48h). An opaque halo around the growth zone, corresponding to the degradation of egg yolk lecithin to insoluble diglycerides, was interpreted as a positive result. The strains were considered as lecithinase-positive in each salinity condition if positive results were observed in the 3 corresponding replicate plates.

2.3.3. Lipases

The activity of extracellular lipases (triacylglycerol acylhydrolases) was tested in solid media containing olive oil as lipid substrate (Kouker and Jaeger, 1987). Tryptic Soy Agar (Liofilchem) was amended with 2.5% (wt/vol) commercial "extra virgin" olive oil (acidity < 0.8%) and 0.001% (wt/vol) rhodamine B (Sigma-Aldrich). Salinity was adjusted as previously described. Strains were streak-plated 3 replicate plates for each salinity condition) and *Pseudomonas aeruginosa* ATCC 27853 was included as positive control. The cultures were incubated at 37 °C until growth was observed (~48h). The appearance of orange fluorescent halos around bacterial colonies, visible under UV light, was interpreted as a positive result. The strains were considered as lipase-positive in each salinity condition if positive results were observed in the 3 corresponding replicate plates.

2.3.4. Protease

The activity of extracellular proteases (caseinase) was tested in solid media containing milk (Sokol *et al.*, 1979). Tryptic Soy Agar (Liofilchem) in which salinity was adjusted as previously described, was amended with 10% (vol/vol) skim milk (Liofilchem). Strains were streak-plated (3 replicate plates for each salinity condition) and *Pseudomonas aeruginosa ATCC 27853* was included as positive control. The cultures were incubated at 37 °C until growth was observed (~48h). The appearance of clear zone surrounding the growth zone was interpreted as a positive result. The strains were considered as protease-positive in each salinity condition if positive results were observed in the 3 corresponding replicate plates.

2.3.5. Chitinase

The activity of extracellular chitinase was tested in solid medium containing colloidal chitin as substrate (Dunne *et al.*, 1997). The basal medium was prepared with 0.001 g/L $ZnSO_4.7H_2O$, 0.2 g/L KH_2P0_4, 0.8 g/L K_2HPO_4, 0.5 g/L $(NH4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.2H_20$), 0.01 g/L $FeCl_3.6H_2O$, 2 g/L casamino acids and 15 g/L agar (Liofilchem). Salinity was adjusted with SCW as previously described. A colloidal chitin suspension (Sigma) was added to obtain a final concentration of 10 g/L, NaCl 20 g/L. Strains were streak-plated (3 replicate plates for each salinity condition) and *Pseudomonas aeruginosa ATCC 27853* was included as positive control. The appearance of a clearing zone (halo) surrounding the growth zone was interpreted as a positive result. The strains were considered chitinase-positive in each salinity condition if positive results were observed in the 3 corresponding replicate plates. All reagents were purchased from Merck, except when otherwise indicated.

2.4. Phosphate solubilization

Phosphate solubilization was evaluated in solid medium (Nautiyal, 1999) containing 10 g/L glucose, 0.5 g/L NH_4SO_4, 0.2 g/L KCl, 0.3 g/L $MgSO_4.7H_2O$, 0.004 g/L $MnSO_4.H_2O$, 0.002 g/L $FeSO_4.7H_2O$, 20 g/L NaCl, 0.5 g/L yeast extract, 0.1 g/L bromocresol purple and 15 g/L agar (pH 7.2). After autoclaving, a sterile suspension of insoluble $Ca_3(PO_4)_2$ was added to achieve a final concentration of 5 g/L. Strains were streak-plated in triplicate plates and *Pseudomonas aeruginosa ATCC 27853* was included as positive control. The development of yellow colour in the culture medium surrounding the growth zone was interpreted as a positive result. The strains were considered as positive for phosphate solubilization if positive results were observed in the 3 replicate plates.

2.5. ACC deaminase activity

The activity of ACC deaminase was demonstrated as the capacity of growing in liquid medium containing ACC (1-aminocyclopropane-1-carboxylate) as sole nitrogen source (Ali *et al.*, 2014). Aliquots from fresh SB20 cultures were inoculated in 3 different non-saline media. (i) DF minimal salt medium (Dworkin and Foster, 1958) containing 2.0 g/L glucose,

2.0 g/L gluconic acid, 2.0 g/L citric acid, 4.0 g/L KH_2PO_4, 6.0 g/L Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 10 mL/l trace-element solution (negative control); (ii) DF minimal salt medium supplemented with 3.0 mM ACC as sole nitrogen source (test); (iii) DF minimal salt medium supplemented with 2.0 g/L $(NH4)_2SO_4$ (positive control). Trace element solution was prepared by combining an iron solution (10 g/L $FeSO_4.7H_2O$) with a mineral solution (100 mg/L H_3BO_3, 111.9 mg/L $MnSO_4.H_2O$, 1.246 g/L $ZnSO_4.7H_2O$, 782.2 mg/L $CuSO_4.5H_2O$, 100 mg/L MoO_3). The solutions were sterilized and mixed in equal proportions to obtain the sterile trace-element solution. Cultures were incubated at 37 °C for 2 weeks and growth was assessed by measuring the OD600 of the cultures, against blanks of non-inoculated medium.

2.6. Inoculation experiment

To test the effect of inoculation with selected halophilic strains on germination efficiency and growth of a non-halophyte plant exposed to saline stress, lettuce (*Lactuca sativa* L.) was used as a model crop plant. Seeds (Alface Grand Rapids, lot 79, brand Flora Lusitana) were surface-sterilized by immersion in sodium hypochlorite (5 % active chlorine) for 15 min and rinsed 4-5 times with sterilized distilled water. Pure cultures of the chosen isolates (*Halobacillus locisalis* and *Idiomarina sp.*) were grown in SB20 at 37 °C and 100-fold diluted in sterile physiological saline solution. Sterilized seeds were immersed in mono-specific suspensions of either strains and in a 1:1 mixture of both and incubated at room temperature for 5 min. The suspensions were centrifuged at 14000g for 10 min to induce the settlement of cells over the seeds. Excess liquid was discarded, and seeds were at room temperature for 2 h, to allow bacterial adhesion to seed surface. Control seeds followed the same treatment, but sterile physiological saline solution was used instead of the bacterial suspensions.

A factorial experimental design was used to test the effect of inoculation, being “inoculation” and “salinity” the two factors tested. For the “inoculation” factor, 4 conditions were tested: (i) no inoculation (control), (ii) inoculation with *Halobacillus locisalis* strain KW48 (HL); (iii) inoculation with *Idiomarina* (I); (iv) inoculation with both strains (HL+I). For the “salinity” factor, 5 conditions were tested, corresponding to the

different salinities (0, 10, 20, 30, 40). Groups of 15 seeds were sown in plastic perforated pots (aprox. 11 cm height, 7 cm diameter, with a basal support improvised with a petri plate lid) containing washed sterilized sand and buried just below the sediment surface. Five replicate pots were prepared for each experimental condition.

The pots were tagged to identify the experimental conditions, randomly arranged in plastic trays and incubated indoor (room temperature ranging ~20-38 °C) in conditions of wide exposure to natural sunlight. Immediately after sowing and twice a week during the experiment, the pots were watered with 25 mL of ¼ Hoagland´s modified basal salt mixture solution (MP Biomedicals) in which salinity was adjusted to 0, 10, 20, 30, 40 by addition of NaCl (Merck).

2.6.1. Germination efficiency

Germination efficiency (GE) was determined 18 days after seeding when the number of germinated seeds stabilized. Plantlets in each pot were counted and the germination efficiency was determined as GE=Np/15*100, being Np the number of plants in each pot and 15 the initial number of seeds per pot. GE values for the 5 replicate pots corresponding to each experimental condition were averaged.

2.6.2. Plant growth and photosynthetic performance

Parameters describing plant growth and condition were assessed only for salinities 0 and 10. After 29 days of cultivation, the plants were analysed for indicators of photosynthetic performance and later, harvested. The harvested plants (5 specimens from each experimental condition) were used to determine growth parameters. Limbo length and width were measured with a ruler, as descriptors of leaf size, and the ratio length/width was used as elongation index (Radice and Arena, 2015). Fresh weight was determined after gentle rinsing in distilled water and drying with filter paper. Dry weight was determined after drying in an oven (70 °C) for 48 h. Water content was calculated as the ratio between the loss of weight and the initial fresh weight. The values determined in the 5 replicate specimens corresponding to each experimental condition were averaged.

The same parameters were determined in the cultivated plants at the end of the experiment (54 days).

Chlorophyll fluorescence, as descriptor of photosynthetic activity, was determined with a portable Pulse Amplitude Modulated (PAM) fluorometer (FluorPen FP 100, Photon Systems Instruments) in cultivated plants (5 replicate specimens for each experimental condition) before mid-harvest (29 days) and at the end of the experiment (54 days). Measurement representing steady-state conditions (Fs), were made directly on the leaves under light conditions (Maxwell and Johnson, 2000). Replicate values were averaged.

2.7. Data analysis

All statistical data were checked for normality (Shapiro-Wilk) and an Equal Variance Test (Brown-Forsythe). Then an ANOVA was performed with a p-value < 0.05 using SPSS Statistics 25 software.

3. Results

3.1. Extracellular enzyme activity

The profile of extracellular enzymatic activities expressed by the collection of 13 isolates that could be cultivated in solid media containing the corresponding substrate is summarized in Table 3.

None of the isolates expressed activity of amylases, lecithinases or chitinases. Lipolytic activity was detected in isolates #2, #5, #10 and #12 (*Idiomarina* sp., *Halobacillus locisalis*, *Idiomarina zobelli* and *Idiomarina seosinesis,* respectively) in all tested salinities. Extracellular proteolytic activity was detected in isolates #2, #3, #10, #13 and #14 (*Idiomarina* sp., *Idiomarina seosinensis*, *Idiomarina zobelli*, *Idiomarina seosinesis*, and *Idiomarina zobellii,* respectively), all belonging to *Idiomarina* genus. In isolate #3, *Idiomarina seosinensis*, extracellular degradation of casein was only observed in the medium corresponding to the highest salinity (100).

3.2. Phosphate solubilization and ACC-deaminase activity

Phosphate solubilization (Table 2) in solid medium with salinity 20 was only detected in *Halobacillus locisalis* (isolate #5). ACC-deaminase activity (Table 2) was detected in isolates #2 (*Idiomarina* sp.), #5 (*Halobacillus locisalis*), #10 (*Idiomarina zobelli*), #12 (*Idiomarina seosinesis*) and #14 (*Idiomarina zobellii*).

Table 2. Phosphate solubilization tested in solid media and ACC-deaminase tested in liquid media with salinity 20.

	Phosphate solubilization	ACC-deaminase activity
1. Bacillus licheniformis	-	-
2. Idiomarina sp.	-	+
3. Idiomarina seosinensis	-	-
4. Halobacillus sp.	(*)	(*)
5. Halobacillus locisalis	+	+
6. Idiomarina seosinesis	-	-
7. Bacillus licheniformis	-	-
8. Marinobacter salsuginis	-	-
9. Marinobacter salsuginis	-	-
10. Idiomarina zobelli	-	+
11. Marinobacter adhaerens	-	-
12. Idiomarina seosinesis	-	+
13. Idiomarina seosinesis	-	-
14. Idiomarina zobellii	-	+

(*) Not determined.

Table 3. Extracellular activity of amylases, lipases, lecithinases, proteases and chitinases expressed by halophilic isolates, tested in solid media with salinities 0, 20 and 100.

	Amylases			Lipases			Lecithinases			Proteases			Chitinases		
Salinity	0	20	100	0	20	100	0	20	100	0	20	100	0	20	100
1. Bacillus licheniformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. Idiomarina sp.	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-
3. Idiomarina seosinensis	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
4. Halobacillus sp.	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)
5. Halobacillus locisalis	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
6. Idiomarina seosinesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. Bacillus licheniformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8. Marinobacter salsuginis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. Marinobacter salsuginis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10. Idiomarina zobelli	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-
11. Marinobacter adhaerens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12. Idiomarina seosinesis	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
13. Idiomarina seosinesis	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
14. Idiomarina zobellii	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-

(*) No growth detected

3.3. Plant growth promotion effects

3.3.1. Germination efficiency

The effect of inoculation in the efficiency of germination of *L. sativa* seeds is represented in Figure 1. Maximum germination efficiency (91 %) was observed in non-inoculated controls irrigated with non-saline solution. The lowest germination efficiency (19 %) was observed in seeds inoculated with the bacterial consortium and exposed to salinity 20. Salinity had a significant negative effect in germination which was totally inhibited at 30 and 40.

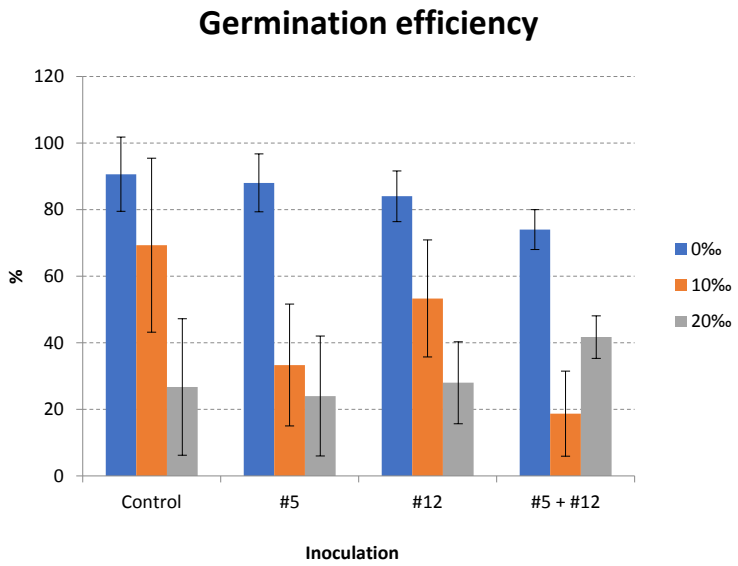


Figure 1. Efficiency of germination of *Lactuca sativa* seeds inoculated with solate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 18 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. With salinities of 30 and 40 germination was totally inhibited. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation.

Overall, inoculation did not cause a significant effect on the germination efficiency. However, in seeds inoculated with the consortium (*Halobacillus locisalis* and *Idiomarina sp.*), germination efficiency with salinity 10 was significantly higher (ANOVA, $p < 0.05$) than with 10, although still lower than non-saline conditions.

3.3.2. Fresh weight

The fresh weight of plants resulting from inoculated and non-inoculated after 29 days of cultivation in different salinity conditions is represented in Figure 2. In non-saline conditions, plants weighted 3.8-4.5 g without significant differences (ANOVA, $p < 0.05$) in relation to inoculation. Salinity had a very significant negative effect (ANOVA, $p < 0.05$) on plant weight. In saline conditions, inoculation with the consortium of *Halobacillus locisalis* and *Idiomarina* sp. had a further negative effect and the lowest fresh weight value (0.02) was observed in the plants of the experimental condition.

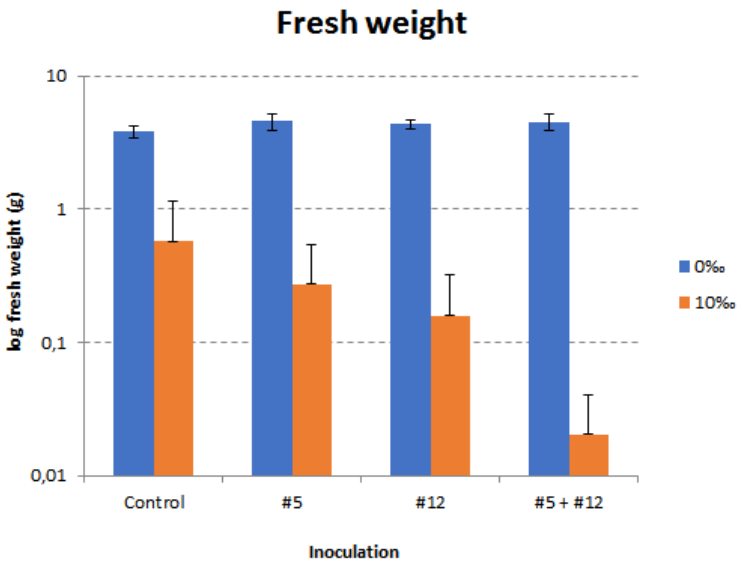


Figure 2. Fresh weight of *Lactuca sativa* plants grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation.

3.3.3. Dry weight

The dry weight of plants resulting from inoculated and non-inoculated after 29 days of cultivation in different salinity conditions is represented in Figure 3. In non-saline conditions, values ranged 0.16-0.23 g and with 10 salinities, the dry weight of the plants varied between 0.005 and 0.02 g. Differences associated to salinity are significant (ANOVA, $p < 0.05$).

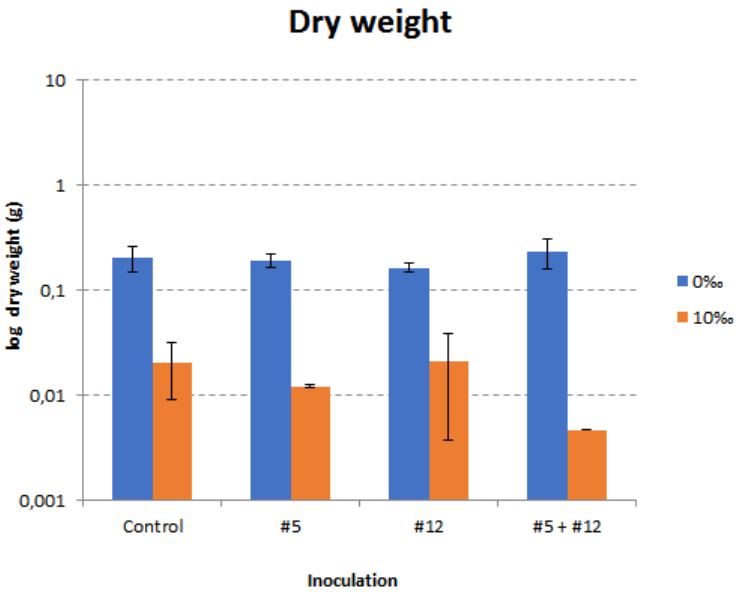


Figure 3. Dry weight of *Lactuca sativa* plants grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation

3.3.4. Water content

The water content, expressed in relation to fresh weight, of plants resulting from inoculated and non-inoculated after 29 days of cultivation in different salinity conditions is represented in Figure 4. In non-saline conditions, values ranged 95-95 % and were not different between inoculation conditions. In saline conditions, water content was significantly lower (ANOVA, $p < 0.05$) ranging 77-96 %. In saline conditions, the water content of plants inoculated with the consortium of *Halobacillus locisalis* and *Idiomarina* sp. was the lowest, and different from the other inoculation conditions.

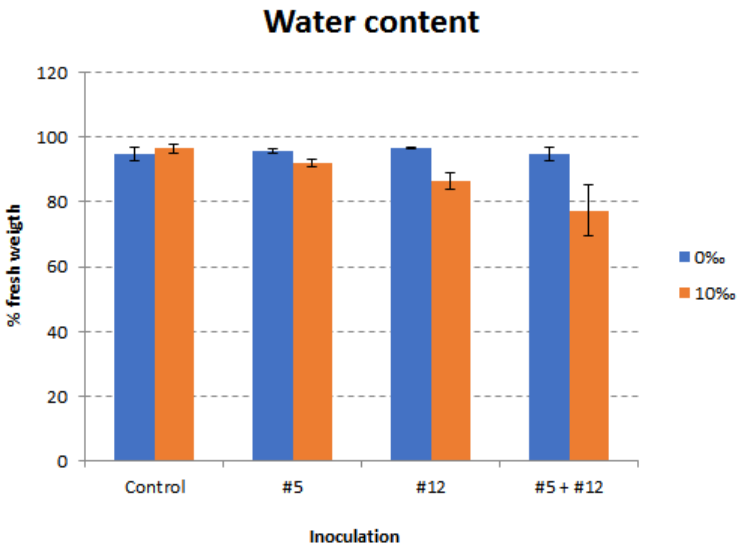
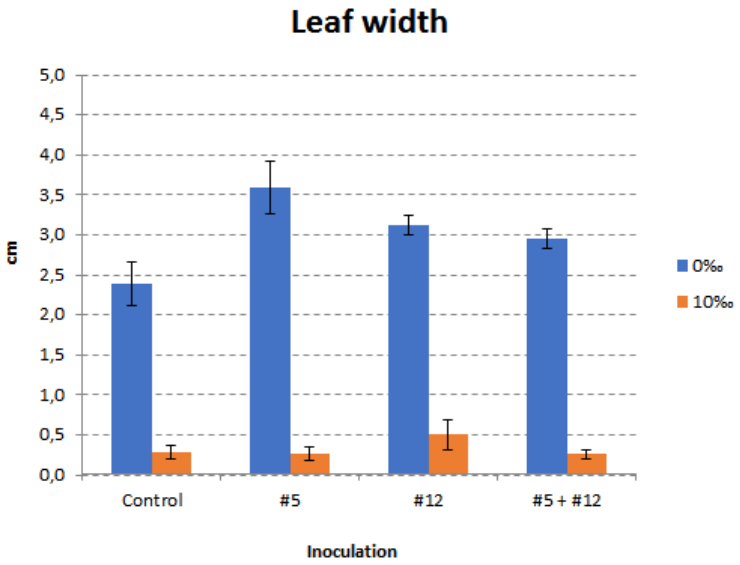


Figure 4. Water content in *Lactuca sativa* plants grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation

3.3.5. Leaf dimensions and elongation index

Leaf dimensions (width and length) of plants developing from inoculated and non-inoculated after 29 days of cultivation in different salinity conditions is represented in Figure 5. In non-saline conditions, leaf width and length varied within the ranges of 2.4-3.6 cm and 4.2-8.1 cm, respectively. In this condition (0), leaf dimensions of inoculated plants were significantly larger (length and width) (ANOVA, $p < 0.05$) than those of the non-inoculated control. Maximum values were observed in plants inoculated with Isolate #5 (*Halobacillus locisalis*). Salinity has a significant effect on leaf dimensions (ANOVA, $p < 0.05$). In saline conditions, the corresponding ranges for width and length 0.3-0.8 cm and 0.4-0.7 cm, respectively. In these condition, inoculated had no significant effect in leaf dimensions (ANOVA, $p < 0.05$).

A



B

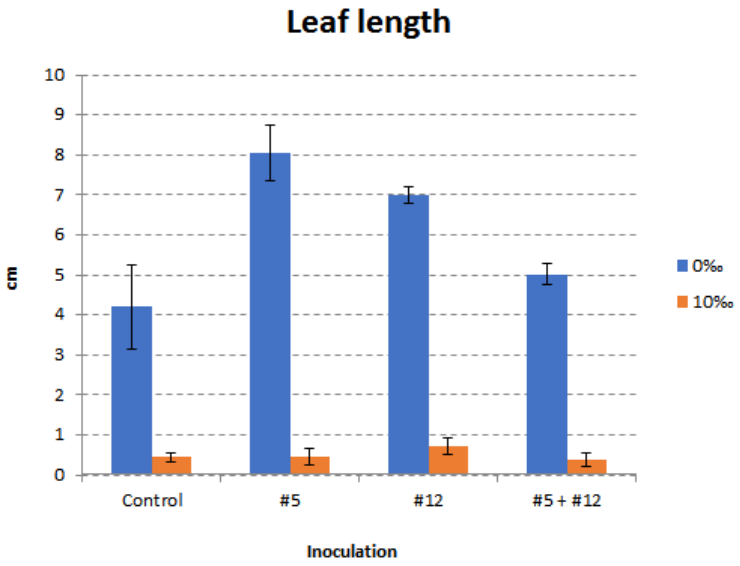


Figure 5. Lead dimensions (A-width, B-length) in *Lactuca sativa* plants grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina sp*.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation.

The elongation index calculated from average leaf dimensions for each experimental condition is represented in Figure 6. In non-saline conditions, the minimum elongation index (1.7) corresponded to plants inoculated with the consortium *Halobacillus locisalis* and *Idiomarina* sp. and the maximum (2.3) to plants inoculated with #5 (*Halobacillus locisalis*) and the values calculated for non-inoculated plants or inoculated with the bacterial consortium were significantly different (ANOVA, $p < 0.05$) than the values calculated for plants inoculated with either the bacterial strains. In saline conditions, the elongation index was significantly lower (1.4-1.7) with significant differences between inoculation conditions (ANOVA, $p < 0.05$).

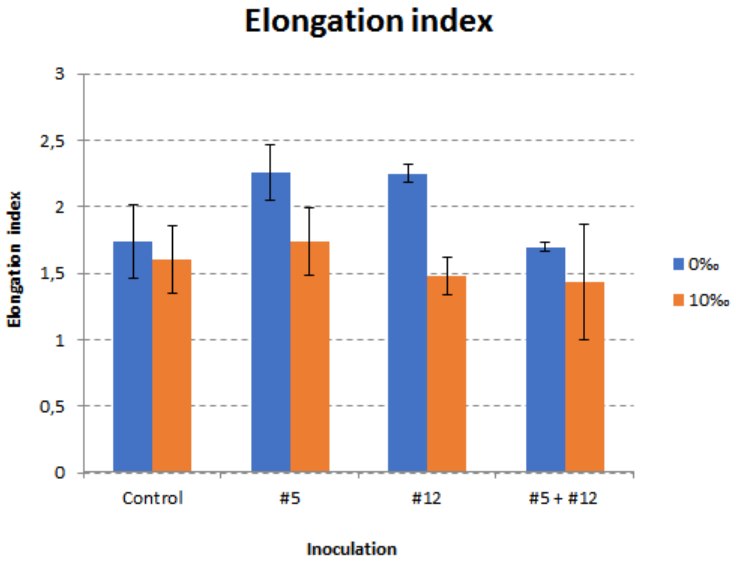


Figure 6. Elongation index calculated for the leaves of *Lactuca sativa* grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation.

3.3.6. Chlorophyll fluorescence

Chlorophyll fluorescence (steady state, F_s) measured under ambient lights in plants developing from inoculated and non-inoculated after 29 days of cultivation in different salinity conditions, is represented in Figure 7. F_s values of plants in non-saline conditions varied between 0.798 and 0.806 without significant differences (ANOVA, $p < 0.05$)

between inoculation conditions. In saline conditions (10), Fs values were lower (0.742-0.783) and in plants inoculated with # 5 (*Halobacillus locisalis*) Fs was significantly lower (ANOVA, $p < 0.05$) than in the other inoculation conditions.

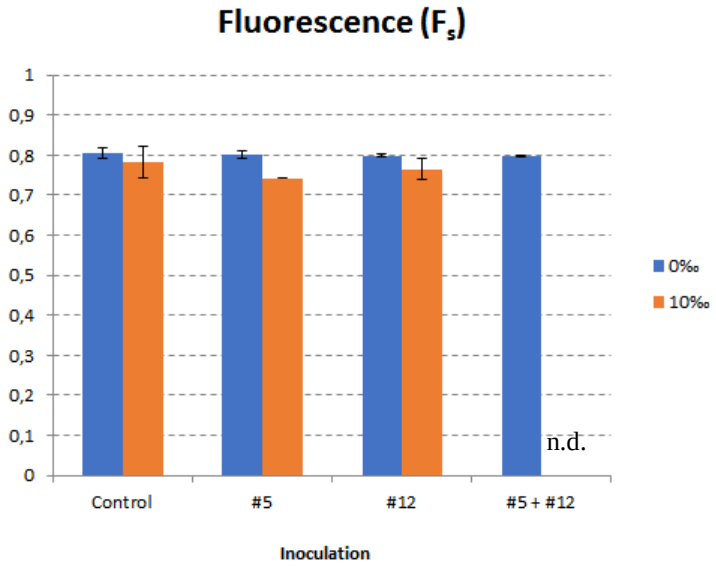


Figure 7. Steady state chlorophyll fluorescence (F_s) of *Lactuca sativa* grown from non-inoculated seeds, and seeds inoculated with isolate #5 (*Halobacillus locisalis*), isolate #12 (*Idiomarina* sp.) or a combination of both, after 29 days cultivation in a sterilized sand substrate and irrigation with Hoagland´s modified basal salt mixture solution in which salinity was adjusted by addition of NaCl. Control seeds were not inoculated. The values correspond to the average of 5 replicate pots and the error bars represent the standard deviation. n.d.=not determined.

4. Discussion

In general, when the inoculation has the objective of improving the performance of plants exposed to saline stress, halotolerant or halophilic PGPR are used (Siddikee et al., 2010) and bacteria expressing plant-growth promoting traits have been successfully isolated from the rhizospheres and saline habitats (Sgroy et al., 2009; Sadeghi et al., 2012).

This study had the objective of further characterizing potential plant-growth promoting traits in bacteria previously isolated from an active salt pan in Ria de Aveiro (Cruzeiro, 2018) and the assessment of the effect of inoculation with selected strains in the mitigation of saline stress in model glycophyte. During this study, one of the isolates of

the initial collection was unable to grow in solid media and could not be tested (#4 *Halobacillus* sp).

Potential extracellular enzymatic, had already been assessed by the degradation of dissolved model fluorescent substrates (Cruzeiro, 2018). In this study, a cultivation approach using solid media added of polymeric substrates was used to demonstrate the activity of amylase, lecithinase, protease and chitinase. None of the isolates expressed amylolytic, phospholipolytic (lecithinases) or chitinolytic effects. Lipolytic activity was detected in 5 isolates and proteolytic activity detected in 5 isolates. Except for *#2 Idiomarina*, that only expressed hydrolytic activity at the highest salinity (100), in all other isolated that tested positive for extracellular enzymatic activity in the 3 salinity conditions tested. Only isolates #2 *Idiomarina* sp. and # 10 *Idiomarina zobellii* tested positive for lipolytic and proteolytic activity, and even so it was a partial match since #2 only expressed lipolytic activity at the highest salt concentration. Lipolytic has been reported in *Idiomarina* (Babavalian et al., 2013; Li et al., 2014) although it is considered as a modest protease producer (Zhou et al., 2009). *Idiomarina* strains isolated from the endosphere of *Halimione portulacoides* also demonstrated proteolytic and lipolytic activity (Fidalgo, 2017). Extracellular enzymatic activity represents a plant growth promoting trait because it is involved in mechanisms of inhibition of phytopathogenic fungi (Van Loon, 2007). Previous tests had already shown that in this collection of isolates, strains of *Idiomarina* exhibited the strongest inhibitory effect on the phytopathogenic fungus *Alternaria* (Cruzeiro, 2017). The results indicate that lipolytic and proteolytic activity may underlie the biocontrol potential of *Idiomarina* species.

Phosphate solubilization capacity was only detected in isolate *#5 Halobacillus* sp. Potential phosphatase activity already been detected in this isolate by the degradation of MUF-phosphate and it has been reported in strains isolated from a solar saltern (Baati et al, 2010). The solubilization of phosphate by bacterial phosphatases contributes to the supply of inorganic P to the plants and it is, therefore a valuable plant growth promoting trait particularly in arid or saline soils, in which nutrient imbalance is a consequence of ionic stress (Xiang et al., 2011).

The production of ACC-deaminase is one of the most interesting traits in PGPB because it decreases the ethylene levels that rise in response to stress (Bharti and Barnawal, 2019) particularly in the situations of saline stress (Ali et al., 2014). ACC-deaminase activity was detected in 4 strains of *Idiomarina* (#2, #10, #12 and #14) and in *Halobacillus locisalis* (#5). However, this activity was not detected in *Idiomarina* strains isolated from the endosphere of *Halimione portulacoides* (Fidalgo, 2017) nor in *Halobacillus* isolated from saline habitats indicating that this can be a strain-specific trait (Orhan, 2016).

Considering the profile of plant-growth promoting traits, #12 (*Idiomarina seosinensis*) expressing proteolytic and ACC-deaminase activities and #5 (*Halobacillus locisalis*) expressing also the capacity to solubilize phosphate were selected for the inoculations experiments. *Idiomarina seosinensis* (#12) has also been characterized as being very motile producing siderophores and having quorum quenching and biocontrol effects whereas #5 (*Halobacillus locisalis*) was also motile but did not exhibit any other of the plant-growth promoting traits (Cruzeiro, 2018) and therefore, it was chosen because of the phosphate solubilization capacity.

The experiments of cultivation of *Lactuca sativa* under different salinities demonstrated a dramatic effect in the germination efficiency and at the highest salinities (30 and 40) germination was completely inhibited. These results confirm that germination and growth of the plant are negatively affected by salt (Zapata et al., 2003; Ünlükara et al., 2008). Growth of plantlets could only be assessed for salinities 0 and 10 because the number of germinated seeds with higher salinity was too small to ensure for statistical significance and furthermore, and most of the plantlets died during the experiment. Irrigation with 10 NaCl also a decrease in fresh and dry weight but water content was not significantly affected. In non-inoculated plants, salinity 10 did not significantly affect chlorophyll fluorescence indication that other processes, like root function and water and nutrient absorption might have been more affected than the photosynthetic apparatus (Martínez-Ballesta et al., 2003).

Inoculation with halotolerant bacteria as a strategy of alleviating the effects of saline stress in glycophyte plants has been extensively tested (Etesami and Beattie, 2018) and consortia of bacteria with different plant promoting can achieve a more efficient effect by

a combination of different biochemical mechanisms (Ibiene et al., 2012). Inoculation of rice with halotolerant ACC-deaminase producing *Brevibacterium linens* decreased ethylene levels under saline stress and has a positive effect on photosynthetic performance (Chatterjee et al., 2018). Halophylic endophytic bacteria from *Salicornia europaea*, increased the germination efficiency, accelerated growth and increased root length and dry weight in wheat (Piernik et al., 2017) Halotolerant isolates able to solubilize phosphate, and produce phytohormones, siderophores and ACC-deaminase improved the tolerance of tomato to salt (Tank and Saraf, 2010).

However, inoculation with isolates #5 and #12 failed to attenuate the negative effects of salinity on seed germination. Seed germination, as other development processes in plants, is regulated by phytohormones like IAA and gibberellins (Miransari and Smith, 2014) and the positive effect of PGPB in mitigating salt stress has been often associated with the production of phytohormone. The production of phytohormones was not assessed in this study and the results may indicate that the lack of effect may be related to the low release of phytohormones by the bacterial inoculants. The screening for IAA production will be included in further characterization of these isolates.

Inoculation with the consortium of *Halobacillus locisalis* and *Idiomarina* sp. caused a significant decrease in water content in relation to the non-inoculated controls but this effect was only observed in plants exposed to salinity 10. Experiments with lettuce exposed to saline irrigation demonstrated a reduction in dry matter that was related with poor quality of this vegetable (Al-Maskri et al., 2010). The effect of the combined inoculation with strains #5 and #12 seem to have induced the opposite effect by reducing the water content in saline stressed plants to levels that were even lower than in the plants cultivated in non-saline medium.

In non-stressing conditions, inoculation had a significant effect on leaf morphology with an increase in length, width and elongation index indicating an effect in terms of size and shape. Inoculation of maize with *Pseudomonas* and *Azospirillum* caused enhancement of growth and changes in plant height and leaf area (Gholami et al., 2009). In tomato plants, inoculation with a consortium of phosphate-solubilize *Nitrobacter* and *Nitrosomona*s and IAA-producing *Azotobacter* species caused significant increase in leaf area (Ibiene et al.,

2012). Therefore, inoculation had a positive effect on some morphological attributes in non-stressing conditions indicating that plant-growth promotion effects may not be dependent on plant stress responses.

5. Conclusion

The cultivation of *Lactuca sativa* seeds under saline stress had a dramatic effect on seed germination and plant growth. The inoculation with a consortium of *Halobacillus locisalis* and *Idiomarina* sp. isolated from salt pans expressing proteolytic and ACC-deaminase activities and the capacity to solubilize phosphate, failed to alleviate the effects of saline stress in terms of seed germination and plant growth. However, in non-saline conditions, inoculation caused an increase in leaf size and a change in shape.

The production of phytohormones by the bacteria, that may underlie morphological changes in the plant, needs to be addressed as a continuation of this work. Also, longer cultivation experiments are necessary to confirm that the changes detected during the initial stages of plant development are maintained during maturation.

The use of PGPR represents a promising approach for stress agriculture (Saleem et al., 2007) but it may also represent as a powerful agrobiotechnological tool to enhance crop productivity in non-stressing conditions (Bhattacharyya and Jha, 2012).

6. References

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