Universidade de Aveiro Departamento de Química
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Carolina Lobão de Jesus Figueira

Isolamento e caracterização de rizobactérias associadas à halófita *Salicornia ramosissima* com efeito de biocontrolo

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo alimentar, 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 científica da Professora Doutora Maria Helena Abreu Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

“Knowledge is a big subject. Ignorance is bigger. And it is more interesting.”
Stuart Firestein, *Ignorance: How it drives science*

o júri

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palavras-chave

Halófitas; *Salicornia ramosissima*; rizosfera; bactérias promotoras do crescimento; efeito de biocontrolo; *Bacillus aryabhattai*

resumo

O cultivo da halófita *Salicornia ramosissima* representa uma atividade económica emergente em zonas costeiras e estuarinas. No entanto, o crescimento da planta é negativamente afetado pela salinidade elevada e por agentes fitopatogénicos. As bactérias promotoras do crescimento de plantas (*plant-growth promoting bacteria*, PGPB) com efeito de biocontrolo são consideradas uma alternativa promissora aos fungicidas comerciais, bem como um fator de melhoramento da produtividade de culturas melhorando a sua resistência a diversos fatores de stresse. O objetivo deste trabalho foi o isolamento e caracterização de rizobactérias associadas à halófita *Salicornia ramosissima* que demonstrassem efeito de biocontrolo e potencial de melhoria do cultivo desta planta.

De uma fase inicial de isolamento a partir de plantas colhidas em sapais da Ria de Aveiro, obteve-se um total de 54 isolados. Destes, 23 foram estudados quanto a diversas características de biocontrolo como a inibição do crescimento do fungo fitopatogénico *Alternaria* sp., a produção de cianeto de hidrogénio (HCN), a produção de hidrolases extracelulares (proteases e lipases) e outras características (mobilidade e tolerância ao sal) potencialmente vantajosas na interação com a planta.

Todos os isolados apresentaram pelo menos uma característica de biocontrolo, embora apenas 2 isolados tenham produzido resultado positivo para todas as características testadas. O isolado SP1016-20, identificado como *Bacillus aryabhattai*, foi selecionado para ser testado como inóculo por apresentar também mobilidade. Os testes de germinação de sementes de *S. ramosissima* mostraram que as sementes inoculadas com *B. aryabhattai* SP1016-20 apresentaram melhor eficiência de germinação sob stresse salino (30 ‰ de NaCl). *B. aryabhattai* SP1016-20 pode assim ser perspetivada como uma estirpe promissora para aplicação como PGPB no cultivo de *S. ramosissima* em sedimentos salinos como os da Ria de Aveiro.

keywords

Halophytes; *Salicornia ramosissima*; rhizosphere; plant-growth promoting bacteria; biocontrol effect; *Bacillus aryabhattai*

abstract

The crop cultivation of the halophyte *Salicornia ramosissima* is an emergent activity in coastal and marine regions. However, plant growth is negatively affected by high salinity and by phytopathogenic agents . Plant-growth promoting bacteria (PGPB) with biocontrol effect are regarded as a promising alternative to commercial fungicides, as well as a factor of improvement of crop productivity, enhancing plant resistance to stress factors. The objective of this work was the isolation and characterization of rhizobacteria associated to the halophyte *Salicornia ramosissima* that demonstrated biocontrol effect and potential to improve the crop productivity.

From an initial phase of isolation from plants harvested in salt marshes of Ria de Aveiro, a total of 54 isolates were obtained. Of these, 23 were studied for various biocontrol characteristics such as inhibition of the phytopathogenic fungus *Alternaria* sp., production of hydrogen cyanide (HCN), production of extracellular hydrolases (proteases and lipases) and other characteristics (motility and salt tolerance) potentially associated with mutually beneficial interactions with the plant.

All isolates showed at least one biocontrol characteristic, although only 2 isolates produced a positive result for all the characteristics tested. Isolate SP1016-20, identified as *Bacillus aryabhattai*, was selected to be tested as inoculum because it also showed motility. Seed germination tests showed that the seeds of *S. ramosissima* inoculated with *B. aryabhattai* SP1016-20 presented better germination efficiency under salt stress (30 ‰ NaCl). *B. aryabhattai* SP1016-20 can thus be considered as a promising strain for application as PGPB in the cultivation of *S. ramosissima* in saline sediments such as those in the Ria de Aveiro.

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ACC – 1-aminocyclopropane-1-carboxylate

BCA – Biocontrol Agent

BLAST – Basic Local Alignment Research Tool

CTAB – Cethyltrimethylammonium Bromide

DAPG – 4-Diacetyl Phloroglucinol

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediamine Tetraacetic Acid

FG – Final Germination

HCN – Hydrogen Cyanide

IAA – Indole-3-acetic Acid

ISR – Induced Systemic Resistance

LSD – Least significant difference

MDG – Mean Daily Germination

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

PERMANOVA – Permutational Multivariate Analysis of Variance

PGPB – Plant-Growth-Promoting Bacteria

PGPR – Plant-Growth-Promoting Rhizobacteria

ROS – Reactive Oxygen Species

SD – Standard Deviation

TAE – Tris-Acetate-Ethylenediamine tetraacetic acid

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

1. Introduction

1.1. Soil salinity, a global issue

By the year 2050, the world population is expected to stabilize at around 9.5 billion people and global production will need to increase by up to 70 % (relative to 2009 levels) in order to feed this population appropriately (FAO, 2011). Therefore, there is a strong economic and demographic pressure to raise crop productivity in a sustainable manner to fulfil global food demand. The severity of this problem is enhanced by factors related to global climate change, such as soil salinization, that threatens soil quality and negatively impacts crop production (Howden *et al.,* 2007). Soil salinization is the process of increasing the concentration of total dissolved salts in soil due to natural (primary salinization) or anthropogenic (secondary salinization) processes (Ghassemi *et al.*, 1995). Primary salinization affects about a billion hectares in areas that are largely coastal salt marshes or inland deserts, but it is the secondary salinization that poses the major threat to crop production since it affects about 20 % of all irrigated land in the most active agricultural areas of the world (FAO, n.d.; Royal Society, 2009). Recent data from the FAO report on soil resources indicate that the increase of soil salinity problems is taking an estimated 0.3 to 1.5 million hectares of farmland out of production each year. In Europe, significant parts of Spain and areas in Italy, Hungary, Greece, Portugal, France and Slovakia are affected by soil salinization (FAO, 2015).

Most crop species employed in modern agriculture are salt sensitive (glycophytes) and can only tolerate a very limited concentration of salt in their growth media, which is not compatible with the salt levels observed on salt-affected soils (Shannon *et al.*, 1994). Once salinity in the soil surpasses a critical level, the productivity is reduced making the crops commercially unviable (Ventura *et al.,* 2015). Maas (1990) reviewed several studies that show that salinity higher than 1.3 g/L resulted in yield reduction of vegetable crops such as beans (yield decline of 19 %), peppers (yield decline of 14 %), corn (yield decline of 12 %) and potatoes (yield decline of 12 %). Note that 1 g/L (weight of total dissolved solids to volume of water) is approximately equal to 1.6 dS m^{-1} of electrical conductivity. The limits of electrical conductivity of irrigation water may reach 3.0 dS m^{-1} (equivalent to approximately 1.9 g/L), a significantly higher value than those observed in drinkable water (maximum value of 0.7 dS m^{-1}) (Fipps, n.d; Rhoades *et al.*, 1992).

1.2. Biosaline agriculture

To efficiently manage irrigation water and farm soil, the use of brackish water and salinized soils, in a strategy designated as biosaline agriculture, is a promising strategy. Biosaline agriculture is defined as 'agriculture under a range of salinity levels in groundwater, soils, or a combination of both' (Masters *et al.*, 2007). Biosaline agriculture is not a novel strategy since the first studies using seawater for crop production date back to 1959, when two Israeli scientists

attempted to develop high salinity agriculture and demonstrated that some crops could be grown in highly saline water on sand-dunes or similar soils with high permeability (Boyko & Boyko, 1959). The aim of biosaline agriculture is to increase the number of crop species that could produce viable economic yields while growing under saline conditions. To attain this, two main research directions are being followed: (1) improving the salt tolerance of salt-sensitive crops through conventional breeding methods or genetic engineering (Epstein *et al.*, 1980; Flowers & Yeo, 1995); and (2) domestication of naturally salt-tolerant plants (halophytes) as alternative crops (Gallagher, 1985).

Attempts to enhance the salt tolerance of salt-sensitive crops through conventional breeding have been largely unsuccessful (Flowers, 2004; Rozema & Schat, 2013). In 1980, Epstein *et al.* proposed a new strategy to genetically modify plants to tolerate saline conditions involving the use of salt-tolerant germplasm, however this approach was also unsuccessful since only a few salt-tolerant lines emerged (Flowers & Yeo, 1995). Furthermore, although many studies have been published regarding production of transgenic plants for salt tolerance enhancement, few of these products were tested in the field (Yu *et al.*, 2012; Panta *et al.*, 2014). The difficulty of improving salt tolerance of crop species by conventional breeding or genetic engineering is probably related to the high complexity of salinity tolerance as a trait, both physiologically and genetically, since it is a multigenic trait (Flowers *et al.*, 2010; Ventura *et al.,* 2015). Due to lack of success of this approach, researchers have considered other options such as finding alternative crops like halophytes for farming in these saline conditions. The use of crop halophytes opens perspective of regaining, for farming activities, soils suffering from secondary salinization as well as exploring natural salty soils (such as salt marshes) that are currently unexploited.

1.3. Halophytes

1.3.1. Definition of halophytes

In terms of plant-salt relationships, higher plants are divided into two main groups: glycophytes (salt-sensitive plants) and halophytes (salt-tolerant plants). In reality, the two groups merge with each other, since there is a continuum in the relative salt tolerance of plants from very sensitive species like chickpea (*Cicer arietinum*) and rice (*Oryza sativa*), to the most tolerant halophytes, as demonstrated by Yadav *et al.* (2011). Throughout the years, several definitions of halophytes have been proposed (Flowers & Colmer, 2008; Aslam *et al.,* 2011). Nevertheless, the definition proposed by Flowers *et al.* (1986) is one of the most widely accepted by the scientific community and states that a halophyte has the ability to 'complete the life cycle in a salt concentration of at least 200 mM of sodium chloride under conditions similar to those that might be encountered in the natural environment'. Halophytes are thus naturally-evolved salt-tolerant plants, in opposition to plants that tolerate salt but do not normally live in saline environments. Halophytes represent only about 2 % of terrestrial plant species (Glenn *et al.*, 1999) although they are present

in a wide diversity of plant forms. The largest group of halophytes is the Chenopodiaceae family with over 380 halophytic species. This family provides some of the most used halophyte models through which the physiology and genetics of salt tolerance can be studied and understood (Flowers & Colmer, 2008; Hamed *et al.*, 2015).

1.3.2. Salinity tolerance in halophytes

Salinity tolerance in halophytes has been extensively studied (Flowers *et al.*, 1977; Glenn *et al.*, 1999; Shabala, 2013), and it appears to be an integration of many adaptive physiological mechanisms, allowing the plant to cope with salinity levels that are damaging or lethal to non-halophytes (Shabala, 2011; Flowers *et al.*, 2014). The common characteristic of all halophytes is their ability to sequester inorganic ions such as sodium (Na^+) and chloride (Cl^-), the predominant ions in saline environments, in their cell vacuoles for osmotic adjustment (Glenn *et al.*, 1999). Although in high concentrations in vacuoles, the concentration of Na^+ in the cytoplasm must be maintained within tolerable limits, since ions associated with high salinity may damage metabolic processes directly (Wyn & Gorham, 2002; Flowers *et al.*, 2008). For instance, high Na^+ concentrations in relation to other salts can disrupt root permeability to ions, by displacing calcium in the plasma membrane (Shannon *et al.*, 1994). Thus, vacuoles have a set of transport channels that prevent efflux of sodium back to the cytoplasm, where salt-sensitive metabolic processes take place (Shabala, 2013). In the cytoplasm, low molecular weight organic solutes are accumulated to adjust the osmotic potential and prevent adverse effects on metabolism, by stabilizing membrane and enzyme structures and scavenging free radicals (Bohnert *et al.*, 1995; Rhodes *et al.,* 2002). Among these, there are a variety of molecules such as sugar alcohols (e.g. sorbitol), free amino acids (e.g. proline) and betaines (e.g. glycine betaine) (Flowers & Colmer, 2008).

In addition to the ability to sequester ions in vacuoles and produce compatible osmolytes in the cytoplasm, some halophytes have developed a range of secondary mechanisms to handle the excess of salt. These mechanisms are mainly associated with transpiration inhibition and involve reduced or inexistent leaves (Ungar, 1991), salt bladders (Shabala *et al.*, 2014) and salt glands, modified cells in the leaf epidermis that secrete excess salt from the leaves (Thomson *et al.*, 1988). Other typical morphological response to salinity is succulence, defined as 'water content per unit area of leaf' (Flowers *et al.,* 1986). Succulence is a typical characteristic of dicotyledonous species but is seldom observed in monocotyledonous (Shannon *et al.*, 1994). This characteristic allows plants to have large cells with greater water content and larger vacuoles, thus reducing salt concentration in the cytoplasm (Aslam *et al.,* 2011). Increased succulence may also be beneficial to CO_2 exchange and therefore photosynthetic activity, by increasing the internal surface area where CO_2 diffusion can occur (Longstreth & Nobel, 1979).

1.3.3. Applications of halophytes

Mainly because of their preadaptation to salt through the mechanisms described above and because of their diversity (Glenn *et al.*, 1999), halophytes are not only valuable as scientific models, but also have potential for diverse industrial applications, with acknowledged economic importance. The study of halophytes in an economic perspective goes back to 1989, when Aronson developed a database of halophytes and their economic uses, motivated by the results of research on saline agriculture in Israel during the 1960s. More recently, an interactive version, the eHALOPH database, was compiled and can be found at http://www.sussex.ac.uk/affiliates/halophytes. So far, halophytes have been tested for potential use as ornamentals (Cassaniti *et al.,* 2013), for revegetation and remediation of industrially polluted or salty soils (Cambrolle *et al.,* 2008) or even as biofilters in aquaculture effluents (Buhmann & Papenbrock, 2013). Although there is a wide range of opportunities for the use of halophytes, the most promising applications are their direct use as crops for forage (El Shaer, 2010), oilseed crops (Glenn *et al.*, 1991) and as food for human consumption (Ventura *et al.*, 2011; Rozema & Schat, 2013).

While halophytes have long had a place in the diet of people across the world, most of the research has been focused on the value of halophytes as forage in animal feeding systems (Rozema & Schat, 2013). These studies demonstrate that despite the high protein content (ranging from 10 to 20 % of dry matter) of these plants, their high mineral content (15 to 50 % salts of dry matter) and the fact that they are a poor energy source lowers its value as forage, since increased amount of salt in the animal diet results in increased water consumption which might affect animal weight or have implications in the long term, as noted by Panta *et al.* (2014). Nevertheless, it is possible to equilibrate the amount of salt present in animal food, by using halophytes as a replacement to a percentage of the conventional feed in animal diets (ICBA, 2007). Unlike leaves that accumulate a large quantity of salt, the seeds of halophytes have a very low salt content, even when growing under saline conditions, a great advantage for the use of halophytes as seed crops (Glenn *et al.,* 1999). In the context of the food industry, at least 50 species of seed-bearing halophytic plants are potential sources of edible oils. The halophytes *Salicornia bigelovii* and *Suaeda fruticosa* are commonly used for seed oil extraction due to the high amount of oil present in their seeds, that varies between 25 and 30 % (Glenn *et al.,* 1991; Weber *et al.*, 2007). Halophytes are also a source of bioactive compounds such as antioxidants that are produced within the powerful antioxidant system of halophytes, promoted by the unfavourable conditions in the environment where halophytes grow, such as salt constraints. Ksouri *et al.* (2012) reviewed the most common bioactive compounds present in halophytes and their potential. Furthermore, Boestfleisch *et al.* (2014) demonstrated that by altering the conditions of the growing environment of the halophytes from

different families it is possible to alter the concentration of various bioactive compounds such as phenols and flavonoids in seedlings and plants. Being possible, the manipulation of the type and number of bioactive compounds present in halophytes, will allow the commercialization of halophytes with improved characteristics for food industries. Cultivation of certain halophytes for human food can contribute to mitigate the problem of food loss due to increasing salinization. For example, halophytes such as *Salicornia* spp. (glasswort), *Aster tripolium* (sea aster) and *Atriplex triangularis* (salt bush) have been consumed by humans for centuries, mainly because of their salty taste. The market for products of biosaline agriculture is only starting and is still limited compared with 'conventional agriculture', but it is growing exponentially (Ventura *et al.*, 2015). For instance, the halophyte quinoa (*Chenopodium quinoa*) has been consumed for years but only recently it began being sold as a premium product, especially in Europe. According to FAO (2013), the global export value of quinoa has been growing since 2000 (Panta *et al.*, 2014).

1.3.4. Halophytes as crops

Several studies were made regarding cultivation of various halophytes with the objective of studying their growth behaviour, properties and yield obtained under different growing conditions, such as temperature, amount of water, soil type and above all, soil salt levels since this is one of the main limiting factors (O'Leary *et al.,* 1985). However, despite many laboratory studies carried out, there have been few field trials that replicate agronomic conditions (Glenn *et al.*, 1999). One of the first field trials was made by O'Leary *et al.* in Mexico (O'Leary *et al.*, 1985). The researchers found that, under irrigation with seawater, the most productive halophytes yielded the equivalent of 8 to 17 tons of dry matter per hectare (t DM ha^{-1}) per year, comparable with the yield obtained annually with a conventional forage crop, such as alfalfa, grown on fresh water (5 to 20 t DM ha^{-1}) (O'Leary *et al.*, 1985). In the same year, Pasternak *et al.* (1985) evaluated the behaviour of the halophyte *Atriplex nummularia*, which was irrigated with 100 %, 75 % and 15 % seawater. The corresponding annual yields of dry matter were 15.3, 21.2 and 28.9 t DM ha^{-1}, making them one of the most promising halophytes regarding yield of dry matter. These reports demonstrate that halophytes can be cultivated and irrigated even under full seawater and have productivities within the range of conventional crops (Glenn *et al.*, 1999; Ventura *et al.*, 2011). However, many studies note that the optimal salinity for growth of halophytes vary between 200 to 340 mM NaCl (approximately 11.4 to 19.4 g/L) (Glenn & O'Leary, 1985; Flowers & Colmer, 2008). Thus, under high-salinity irrigation, halophytes are beyond their growth optimum.

Although a wide range of halophytes can be cultivated in salt affected areas, to succeed as crops, according to Glenn *et al.* (1999), four conditions must be met: 1) they must have high yield potential; (2) the irrigation of halophytes should be similar to that of conventional crops, and must not damage the soil; (3) halophyte products must be able to substitute conventional crop products;

and (4) high-salinity agriculture must be able to be integrated in the existing agricultural infrastructure. Whether halophytes can meet these conditions depends on their performance as agronomic crops, which is associated to their physiology and biochemistry regarding different conditions such as salt stress (Glenn *et al.*, 1999). As a way of choosing the halophytes with the greatest potentials to be domesticated as vegetable crops, it is necessary to compile existing knowledge about traditional uses and applications, which frequently can be found at ethnobotanic literature (Tardío *et al.,* 2006). Furthermore, it is important to note that the successful incorporation of halophytes into future farming systems will also depend on the farmer's acceptance of new practices, crop nutritional quality, overall cost-benefit of production, market development and price and government policies (Panta *et al.*, 2014). Ventura & Sagi (2013) concluded that *Salicornia* probably is one of the most successful examples of halophyte cultivation (Ventura & Sagi, 2013).

1.3.5. Salicornia

Salicornia (Chenopodiaceae) is a genus of annual, apparently leafless halophytic plants that is widely distributed worldwide, in temperate boreal and subtropical habitats of the northern hemisphere and in South Africa, although absent from South America and Australia (Kadereit *et al.*, 2007). *Salicornia* grows in periodically wet saline coastal and inland habitats such as salt marshes, mud flats and salt pans, usually occupying the zone of highest salinity (Chapman, 1960; Ungar, 1979). In their habitat, they protect against erosion caused by wave force on the coast, and are involved in the biofiltration of pollutants from the sediment (Silva *et al.*, 2007; Han *et al.,* 2010). Furthermore, two species of *Salicornia* (*S. europaea* and *S. bigelovii*) are able to inhibit the growth of *Skeletonema costatum*, a marine diatom responsible for water eutrophication (Jiang *et al.,* 2010; Jiang *et al.,* 2012). The involvement in these important processes makes *Salicornia* species crucial for the tidal ecology.

1.3.6. Taxonomy of Salicornia

The genus *Salicornia* currently includes about 80 species being *S. bigelovii, S. europaea, S. prostata* and *S. ramosissima* those of wider occurrence (GBIF, 2016). This number is just an estimate, since albeit numerous species have been described over the last 250 years (Davy *et al.*, 2001), there is still unsatisfactory taxonomic classification and it is frequently impossible to assign published information to specific taxa within *Salicornia* (Kadereit *et al.*, 2007). For example, the same specimen (P. Teege chen 968, MJG) collected in Normandie (France) was identified as *S. europaea* (Stace, 1997), *S. brachystachya* (Lahondère, 2004) and *S. ramosissima* (Ball & Akeroyd, 1993). The species considered in this investigation is the annual halophyte *Salicornia ramosissima* J. Woods. Analysis of morphological variation in the field failed to support a distinction between the species *S. europaea* and *S. ramosissima* (Ingrouille & Pearson, 1987), although Jefferies &

Gottlieb (1982) had found consistent differences at loci coding for six enzymes. Commonly, they are classified as species included in the species aggregate *S. europaea* agg. since it is extremely difficult to differentiate them due to their morphological similarity, phenotypic plasticity and local differentiation of populations at different sites (Kadereit *et al.,* 2012). The two species occur in the same habitat, however there are preferential distributions. The upper-marsh plants most closely resembled the description of *S. ramosissima*, whereas plants from the lower marsh appeared to be *S. europaea* (*sensu stricto*), although this restriction to different habitats is not exclusive and at most sites both species are present (Jefferies & Gottlieb, 1982).

1.3.7. Salicornia ramosissima

S. ramosissima, included in the species aggregate *S. europaea agg.* (Stace, 1997), is widely distributed in northwest Europe and can be found in many salt marshes of the Iberian Peninsula (Castroviejo *et al.,* 1990). The distribution of *S. ramosissima* in the Iberian Peninsula is represented in Figure 1. In Portugal, it is frequent in the salt marshes of 'Ria de Aveiro' and less frequent in the Minho estuary, in Ria Formosa (Algarve), in Mondego estuary and in Sado estuary (Castroviejo *et al.,* 1990; AMBIECO, 2011). It preferentially occupies small areas not invaded by other halophytes such as *Halimione portulacoides* (Silva *et al.,* 2007).

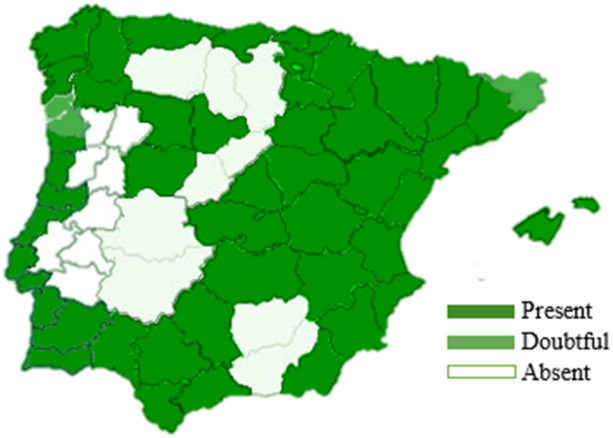

Figure 1 - Distribution map of *Salicornia ramosissima* from the Iberian Peninsula. Adapted from Woods (1987) and Castroviejo *et al.* (1990).

As other coastal systems in the world, agriculture fields surrounding the Aveiro coastal region have been experiencing severe saltwater intrusion because of the interaction of the Atlantic Ocean, the estuarine system 'Ria de Aveiro' and local freshwater aquifers (Martinho *et al.*, 2004). As *S. ramosissima* occurs naturally in 'Ria de Aveiro' and is frequently the first higher plant to colonize the tidal zones, it is important to study its behaviour since *S. ramosissima* can potentially be used to mitigate the losses associated with arable land salinization or to aid in preservation and conservation of these type of ecosystems (Davy *et al.,* 2001; Silva *et al.,* 2007).

Morphologically, *S. ramosissima* is a small herb, usually less than 40 cm high (Figure 2). The root system of this halophyte tends to be superficial, often penetrating less than 10–20 cm into the sediment. *S. ramosissima* has articulated and succulent stems (Figure 2b**)** composed of cylindrical photosynthetic internodes with two opposite three-flowered cymules each (Figure 2c). Each cymule holds one large central flower, responsible to produce a large seed, and two smaller lateral flowers, responsible for the production of two smaller seeds (Castroviejo *et al.,* 1990; Davy *et al.*, 2001). The larger seed (Figure 2d) has a length of about 1.0 to 1.4 mm, while smaller seeds have a medium length of about 0.8 to 1.3 mm (Ungar, 1979). Many species of the genus *Salicornia* have green stems that usually become reddish in the fall. This fact is particularly noticeable in *S. ramosissima*, sometimes becoming purple-red. This distinctive purple-red colouration of many forms of *Salicornia* is mainly due to the presence of a betacyanin pigment (Chiji, 1976). The life cycle of *Salicornia* is typically annual, although in subtropical environments plants can persist for more than a year (Davy *et al.*, 2001). Especially for annuals, such as *S. ramosissima*, which have only one opportunity in their life to reproduce, the success of reproduction is highly dependent on the germination responses of their seeds (Ungar, 1991).

Seeds reach maturity from late-September onwards, and fall from the dead or dying parent plant. Although seeds can be dispersed by salt water, water birds or the wind (Wilson, 1980; Ungar, 1987), most of the seeds produced remain in the proximity of parent plant (Jefferies, 1981) and almost all germinate after few months. Some studies report that the seeds of *S. ramosissima* exhibit different germination success, with small seeds (lateral flowers) more dormant and less salt-tolerant than large seeds (central flowers) (Ungar, 1979; Philipupillai & Ungar, 1984). For successful germination, a combination of environmental conditions that may include abiotic factors, such as soil characteristics, and biotic factors, such as the absence of competition or predation, is required (Ungar, 1991). As previously mentioned, *S. ramosissima* grows mostly in the upper-marsh region. Characteristically, lower marsh populations, such as *S. dolichostachya* and *S. europaea,* tend to germinate earlier than upper marsh ones, e.g. *S. ramosissima* and *S. pusilla* (Smith, 1985). The upper marsh, unlike the lower marsh, is not tidal during most of the summer, and, because of evaporation, hypersaline conditions develop in the summer in most years. Thus, *S. ramosissima* shows delayed growth, a genetically determined response to adverse conditions (Jefferies, 1981). In spring, with greater rainfall and lower salinity, there is an interruption of dormancy and germination takes place (Vleeshouwers *et al.,* 1995). These observations agree with those of Ungar (1991) which proposed that, even within halophytes, high concentrations of salt may delay growth (Ungar, 1991).

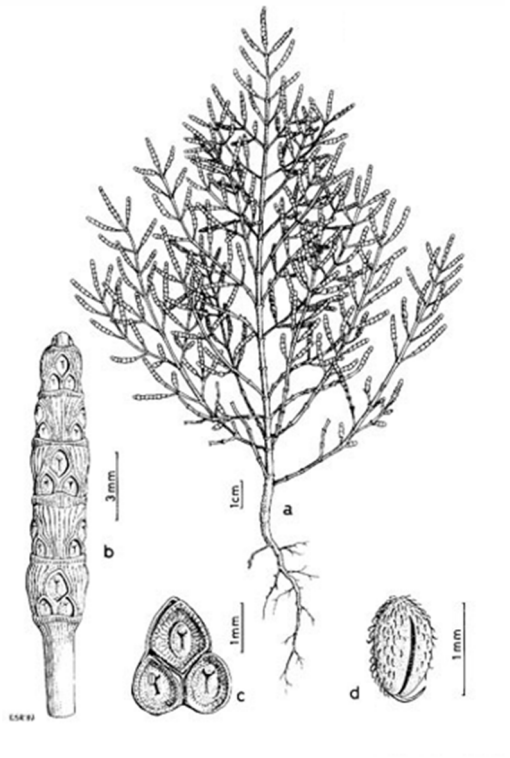

Figure 2 - *Salicornia ramosissima*: a) plant; b) fertile stem; c) cymule; d) seed. Adapted from Castroviejo *et al.* (1990).

1.3.8. Potential applications of Salicornia

Salicornia species have a long history of applications namely as food for human, forage for animals and in traditional medicine, mainly due to their medicinal properties and high salt contents (Davy *et al.*, 2001). The use of *Salicornia* by ancient communities is well documented in a review by Chevalier (Chevalier, 1922). Ancient Native Americans (Indian tribes) boiled *S. rubra* in water and let the solution evaporate to obtain salt. The Native American tribe Gosiute grinded the seeds to powder and used it to bake salty bread. In the present, *Salicornia* species are used as a seasonal vegetable and fermented food in Korea. In Nova Scotia, Canada, the annual glasswort *S. europaea* has been used freshly in salad or boiled for jarring as pickles (Isca *et al.,* 2014).

Scientists have reported several uses of *Salicornia* as food for animals and demonstrated that despite its high salt content, animals fed with moderate amounts of *Salicornia* gained as much weight as those whose diet included other terrestrial weeds (Swingle *et al*., 1996). *Salicornia* is acceptable as a forage component of the diet fed to goats and fish (Glenn *et al.*, 1992; Belal & Al-Dosari, 1999). *Salicornia* species have also been used as oilseed crop, with *S. bigelovii* being one of the most frequently used halophytes for seed oil extraction due to the high amount of oil present in the seeds (about 30 %) and its good oil properties (Glenn *et al.,* 1991; Ho & Cummins, 2009; Glenn *et al.*, 2012). Also, *S. europaea* seems to be a suitable candidate for oilseed crop, since under seawater irrigation the seeds of *S. europaea* have an oil content of 28 %, with a large percentage of unsaturated fatty acids (Ó Leary *et al*., 1985; Zhao & Feng, 2001). However, to use *Salicornia* as an oilseed crop can be a waste of the nutritional proprieties of the stems and other constituents of

the plant, since the chemical profile in halophytic plants can be unique due to the environmental stress of their habitat (Isca *et al.*, 2014).

In plants growing in habitats that impose significant environmental stress such as saline environments, the increased production and accumulation of reactive oxygen species (ROS) leads to cellular damage, metabolic disorders, and senescence processes (Menezes-Benavente *et al.*, 2004). Halophytes are known for their ability to withstand these toxic ROS, since they are equipped with powerful antioxidant systems. The production of antioxidants can delay the oxidation of lipids or other molecules by inhibiting the initiation or propagation of the oxidative chain reaction (Ksouri *et al.*, 2012). In addition to antioxidant activity, *Salicornia* has omega-3 fatty acids, which are a major constituent of plant lipids located in the chloroplast membrane. These lipids are known for their beneficial properties for human health (Simopoulos, 2004). O'Leary *et al.* (1985) made a nutritional analysis of three halophyte species and concluded that, under seawater irrigation, *S. europaea* has a high content of fibre (17 % of dry weight) and high nutritional value in terms of minerals (Lu *et al.*, 2001). Halophytes are also known for their ability to synthesize secondary metabolites that perform a variety of functions inside the plant. Detailed reviews on the bioactive compounds of *Salicornia* and similar species were stated by Ksouri *et al*. (2012) and Isca *et al.* (2014).

Although the primary reason to produce secondary metabolites is the mitigation of salt stress, those changes are also responsible for the enhancement of their nutritional value and sensorial properties (Maggio *et al*., 2011). Interest in 'functional foods' (foods that scientifically proved health benefits beyond basic nutrition) is increasing and halophytes present a level of nutritionally valuable metabolites, meeting these special nutritional demands (IFIC, 2013; Ksouri *et al.*, 2012). Because of their salty taste and nutritional value, *Salicornia* species have been commercialized as a singular vegetable for United States and European markets at comparatively high prices (Ventura *et al.*, 2011). Despite the lack of reports publicly available (most consumer studies are market surveys made by commercializing companies), it is known that *Salicornia* species are well accepted by the consumer, who is mostly interested in the young green plant stems that are sold in the markets as "samphire", "sea asparagus", "glasswort" or in Portugal, "erva-salada" (Ventura & Sagi, 2013; Isca *et al.*, 2014). In opposition to the use of *Salicornia* as forage crop or as oilseed crop, if used for human consumption as a gourmet product, the high NaCl content in the shoot of *Salicornia* has only a minor impact on the nutritional value of the plant and provides different sensorial experiences since as a gourmet product it is consumed in small quantities (Ventura *et al.*, 2015).

The market for gourmet vegetables requires products of the highest quality. In addition to be visually appealing in terms of freshness, colour and packaging, the product should also reach the

market at the same maturity level, have a particular taste recognized by its consumers, and be of sufficient nutritional value to certificate its gourmet status (Van der Voort *et al*., 2007). Since non-cultivated plant material presents low uniformity and unpredictable quality, it is necessary to select superior genotypes of the plant and define growing conditions (Ventura *et al.*, 2015). Thus, Ventura & Sagi (2013) reviewed several agrotechnical practices for *Salicornia* cultivation, reaching the conclusion that practices such as daylight manipulation and multiple harvesting enhance the characteristics of *Salicornia* ensuring a higher market value. The same authors reported that currently, the easiest and most straightforward way to produce *Salicornia* is to cultivate it in native soils watered with drip irrigation. More recently, Gunning (2016) extensively reviewed several techniques for the successful *S. europaea* cultivation, as well as some of the companies that are commercializing this halophyte worldwide.

1.3.9. Salicornia as a crop – perspectives and limitations

Despite most scientific records of *Salicornia* biomass production and yields refer to laboratory experiments, which fail to fully represent plant densities under field conditions, there are some field studies that report a good yield of biomass for several species of *Salicornia*. Glenn *et al.* (1998) reported a yield of 17 t DM ha^{-1} per year for *S. bigelovii* when grown with seawater. Generally, coastal halophyte communities exhibit relatively high annual biomass productivity comparable of that of glycophytes in contrast with that presented by plant communities from dry or cold environments (Flowers & Colmer, 2008). As a salt marsh pioneer plant, *Salicornia* possesses extreme salt tolerance and can be grown with saline irrigation water with salinities as high as that of seawater (Ventura *et al.,* 2010). However, Ungar (1991) reported that the initial establishment of halophyte seed is delayed under conditions of high salt stress. Aghaleh *et al.* (2009) concluded that the shoot growth of *S. europaea* increased under low NaCl concentration (100 mM) and then decreased with increasing NaCl concentrations. Ventura *et al.* (2011) also reported that the best germination conditions for *Salicornia* were either freshwater or low-salinity water. Rubio-Casal *et al.* (2003) studied the germination responses of *S. ramosissima* and demonstrated that *Salicornia* seeds germinate better under low salinities, and with a salinity of 3 % the germination was significantly delayed. The inhibition of growth of halophytes under high salinity could be attributed to imbalances in phytohormone levels and the toxicity of Na^+ that cause decrease in cell metabolism (Shannon *et al.*, 1994; Khan & Ungar, 1997). On the other hand, excess Na^+ and Cl^- ions may interfere with the absorption of potassium and calcium and this can result in deficiencies of nutritional elements, affecting plant growth and development (Borsani *et al*., 2001). Consequently, although *Salicornia* can be grown with saline irrigation water with salinities as high as that of seawater, for this halophyte to become a commercial halophyte crop it must also be capable of high-yield production under saline conditions (O'Leary, 1998), which includes

achieving an adequate rate of seed germination and a sufficient quantity of plant biomass in high salt concentrations.

Furthermore, several studies conducted in distinct locations worldwide report the high prevalence of different genus of fungi associated with this halophyte, especially the genus *Alternaria*, that accounts for 9 species found associated with *Salicornia europaea* (Davy *et al.*, 2001), probably attributed to the succulent nature of this plant (Muhsin, 1996). The occurrence of fungi can be a stress factor for *Salicornia* growth and development, and can significantly decrease their market value, since foliar pathogens such as *Alternaria* create necrotic lesions in the stems of the plant that severely reduces their photosynthetic ability, accelerating plant senescence. The fungus is in the centre of the lesion, which is surrounded by an un-invaded chlorotic halo created by the diffusion of fungal metabolites like toxins (Tewari, 1983). *Alternaria* frequently causes quiescent infections in which the fungus enters the tissue where it remains dormant until conditions favour the progression of the infection. The fungus can survive for a considerable time as mycelium or spores on decaying plant debris, or as a latent infection in seeds (Rotem, 1994). If seed-borne, the fungus can attack the seedling once the seed has germinated. In other cases, once the spores are produced, they are mainly spread by wind on to plant surfaces where infection can occur (Thomma, 2003). In Portugal, a rust-like fungus in stems of *S. ramosissima* was observed, probably *Uromyces salicorniae* de Bary that was previously observed in Britain (Davy *et al.*, 2001). Chemical pesticides have been used since many decades in agriculture to successfully control fungi diseases and thus increasing the crop production. However, this strategy has led to increased concerns over environmental contamination and has resulted in pathogens developing resistance to individual chemicals over time, needing a constant development of new pesticides (Tariq *et al.*, 2017). Furthermore, the growing cost of pesticides, and consumer demand for pesticide-free food has led to a search for substitutes for these products (Compant *et al.*, 2005). Biological control is thus being considered as a promising alternative or a supplement to reduce the use of synthetic chemicals in agriculture, since biopesticides are safe to use as compared to synthetic pesticides and have targeted activity against specific pathogens, being most easily decomposed than conventional pesticides (Tariq *et al.*, 2017). In 2015, biofungicides compounded only 5% of the worldwide crop protection market (approx. \$3 billion year^{-1}), but this segment of the industry is growing, and it is projected to increase by 8.84 % annually, reaching more than 7 % of the total crop protection market by 2025 (more than \$4.5 billion year^{-1}) (Olson, 2015).

One of the most promising solutions to improve crop yields in adverse conditions is the inoculation of crop plants with plant-growth-promoting bacteria (PGPB), bacteria associated with plants that stimulate their growth and aid in the control of pathogenic fungi affecting seeds, soil or

stems of the plant, through their biocontrol traits (Gunning, 2016). When these bacteria are associated with the rhizosphere of plants, are called plant-growth-promoting rhizobacteria (PGPR).

1.4. Plant-growth-promoting rhizobacteria (PGPR)

1.4.1. The rhizosphere as a microbial microniche

The rhizosphere is defined as a region or volume of soil that is influenced by the plant root system activity and generally extends out 1-2 mm from the surface of roots (Gregory, 2006). Due to the microenvironment that the plant root creates, the rhizosphere has a range of different physical, biological and chemical characteristics different to those of the bulk soil such as larger microbial biomass (Gregory, 2006). Bacteria are generally not evenly distributed in soil, being present at high densities on plant root surfaces (Campbell & Greaves, 1990). This is due to the greater availability of oxygen as well as nutrients including sugars, amino acids, organic acids and other small molecules released from plant root (Glick, 2012).

1.4.2. PGPR definition and biochemical traits

All plants have local bacterial communities (rhizobacteria) associated with their rhizospheres. These microorganisms can establish relations with their host plants (Schmid *et al.,* 2009; Martínez-Viveros *et al*., 2010). Interaction of rhizobacteria and growing plants can be neutral, negative or positive. Neutral interactions are related to commensals bacteria exhibiting no visible effect on growth or physiology of the host. Negative interactions are related to phytopathogenic rhizobacteria and their metabolic products, while positive interactions enhance plant growth (Beattie, 2006). Rhizobacteria that are beneficial to plant growth have been widely studied and have been termed by Kloepper and Schroth (1978) plant growth-promoting rhizobacteria (PGPR). PGPR may promote plant growth and development through direct mechanisms, usually by either promoting nutrient acquisition or balancing plant hormone levels, or through indirect mechanisms by decreasing or preventing the inhibitory effects of pathogenic agents on plant development, thus acting as biocontrol bacteria (Glick, 1995; Compant *et al.,* 2005). Direct mechanisms may be especially beneficial when attempting to cultivate plants under nutrient-limiting conditions and include (1) nitrogen fixation to improve nutrient availability, (2) phosphate solubilisation, (3) production of siderophores and (4) production of phytohormones such as auxins (especially indole-3-acetic acid (IAA)) and gibberellins (Hong *et al.*, 1991; Glick, 1995; Patten & Glick, 1996). In addition to promote plant development, these mechanisms may improve the plant tolerance to abiotic stresses such as high salinity, drought, metal toxicity and pesticide load without causing disease (Bashan *et al.*, 2008). Indirect mechanisms are associated with the effect of biocontrol and indirectly promote plant growth by preventing negative effects of viruses, phytopathogenic bacteria, fungi, and invertebrates (Compant *et al.*, 2005). The same strain of

PGPR may cause both growth promotion and biological control (Kloeppe *et al.*, 1999). In this study, some of the biological control mechanisms associated with PGPR isolated from *S. ramosissima* are evaluated.

1.4.3. Biological control mechanisms

Several species of bacteria are known to increase plant growth and productivity by preventing or controlling the harmful effects of phytopathogens (Lam & Gaffney, 1993; Whipps, 2001; Tariq *et al.*, 2017). Pathogen prevention can involve competition for niches within the rhizosphere, production of siderophores, induced resistance, production of specific biocontrol molecules such as hydrogen cyanide (HCN) and antibiotics, or hydrolytic enzymes (Fridlender *et al.,* 1993; Lo, 1998; Tariq *et al.*, 2017). These mechanisms are not mutually exclusive (Lo, 1998) and competition may result from several different mechanisms that confer competitive advantages to the bacteria, for example, production of siderophores or motility for better colonisation of roots (Martínez-Viveros *et al*., 2010).

Production of bioactive metabolites (antibiotics and hydrogen cyanide)

The production of antibiotics is considered the most effective antagonistic mechanism, showing positive results against phytopathogenic agents under laboratory conditions (Martínez-Viveros *et al*., 2010; Tariq *et al.*, 2017). Antibiotics are organic compounds of low molecular weight such as 4-diacyetyl phloroglucinol (DAPG), phenazine-1-carboxylic acid and viscosinamide that are involved in the inhibition of growth and metabolic activities of various microbes (Tariq *et al.*, 2017). However, the increased use of antibiotics may develop resistance by some phytopathogens to specific antibiotics. To prevent this from happening, some researchers have utilized biocontrol strains that synthesize hydrogen cyanide (HCN) as well as one or more antibiotics. While HCN may not have much biocontrol activity by itself, it appears to act synergistically with bacterially encoded antibiotics (Glick, 2012). HCN is a volatile, secondary metabolite that prevents the development of microorganisms, by inhibition of several metalloenzymes such as catalase, peroxidase and superoxide dismutase and especially copper containing cytochrome C oxidases, the final component of the aerobic respiratory chain in many organisms (Solomonson, 1981). Furthermore, in the soil, HCN can regulate micronutrient availability by producing stable complexes with transient metals like Fe, Zn and Cu (Rennert & Mansfeldt, 2002). The HCN cluster is formed by three contiguous structural genes, *hcnABC*, which encode a membrane-bound HCN synthase complex. This synthase complex is responsible for catalysis of glycine, the immediate metabolic precursor of cyanide in bacteria, into HCN and carbon dioxide (Castric, 1997; Martínez-Viveros *et al*., 2010). Many different bacterial genera have shown to produce HCN such as *Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Martínez-Viveros *et al*., 2010).

Production of extracellular hydrolytic enzymes

The degradation of fungal cell walls and membranes caused by extracellular hydrolytic enzymes (such as chitinases, β-glucanases, lipases and proteases) is one of the most important mechanisms for biocontrol of phytopathogenic fungi (Weller, 2007; Elshafie *et al.*, 2012; Bouizgarne, 2013). One of the most important microbial hydrolytic enzymes are proteases [E.C. 3.4.24], that play a significant role in cell wall lysis of phytopathogenic fungi, since chitin and the fibrils of β-glucan (the major constituents of fungal cell walls) are embedded into a protein matrix. Cell walls contain proteins involved in iron transfer and those promoting survival under stress. The protein composition of the fungal cell wall varies depending on the environmental conditions and the development stage and these proteins are covalently bound to polysaccharides (Feofilova, 2010). Thus, proteolytic activity is a prerequisite to lyse whole fungal cells, and thereby destroy their capacity to act on plant cells. In addition to fungal cell walls, hydrolytic enzymes affect hyphal tips and germ tubes leading to hyphal curling or hyphal tip bursting (Jadhav *et al.*, 2017).

Production of siderophores

Siderophores are low molecular weight molecules involved in the solubilization and sequestration of iron (III) from the soil which are synthesized by some species of bacteria such as *Bradyrhizobium* (Neilands, 1995; Antoun *et al*., 1998). Siderophore producing PGPR can prevent the proliferation of pathogenic microorganisms by sequestering Fe^{3+} in the area around the root, essential for fungal growth (Siddiqui, 2006). Iron deficiency in fungus causes growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation, and causes changes in cell morphology (Mathiyazhagan *et al*., 2004).

Induction of systemic resistance (ISR)

Some rhizobacteria can trigger induced systemic resistance (ISR) (Van Peer *et al.,* 1991; Pieterse *et al.*, 2014), a mechanism that does not target specific pathogens and is responsible to control diseases caused by a range of different pathogens by activating chemical or physical defence mechanisms in the host plant. ISR-positive plants are said to be 'primed' so that they react faster and more strongly to attack from several pathogens (Glick, 2012). ISR does not require direct interaction between the resistance-inducing PGPR and the pathogen. Thus, this mechanism enables that the control of soilborne pathogens can also be used to target, for example, seed-borne and foliar pathogens (Glick, 2012). Several bacterial traits (i.e., flagellation, production of siderophores and volatile organic compounds) have been proposed to trigger ISR, but there is no compelling evidence for an overall ISR signal produced by bacteria (Compant *et al.*, 2005).

1.4.4. Halotolerant bacteria as PGPR

In relation to salt requirements, bacteria can be either extreme (halophilic) or facultative (halotolerant). Halophilic microbes can be defined as microorganisms which require at least 1.5 M

(9 % w/v) NaCl for growth and optimum growth occurs at 3.0 M (18 % w/v) NaCl or higher concentrations. Halotolerant bacteria can grow under a wide range of salt concentrations, with optimum growth in the absence of salt. Therefore, halotolerance describes the ability that bacteria have to grow in sub-optimal conditions (Ollivier *et al.*, 1994; Bowers *et al*., 2009). Most bacteria found in saline ecosystems are Gram-negative, such as *Halomonas*, *Pseudomonas* and *Vibrio* (Proteobacteria) (Quesada *et al*., 1982; Trüper *et al.,* 1991). Gram-positive rods and cocci, namely *Bacillus* and *Salinicoccus* (Firmicutes) as well as *Micrococcus* also occur (Trüper *et al.* 1991). Although bacteria are present, the dominant microorganisms in hypersaline environments worldwide are halophilic archaea (Oren, 2006). In general, halotolerant or halophilic prokaryotes have two different strategies to grow under high concentrations of NaCl (Lowe *et al.*, 1993). One is the maintenance of the internal salt concentration at a level comparable to that of the environment and the exclusion of NaCl or the production of organic osmoregulants such as betaine (Burg & Ferraris, 2008). When microorganisms are exposed to high-osmolarity environments, there is a tendency for water to flow out of the cell, thus causing a reduction in turgor. Tolerant microbes have developed several adaptations to counteract this efflux of water. For maintaining the cytoplasmic salt concentration like that of the surrounding medium, the uptake of potassium (K^+) occurs and cells start to accumulate compatible solutes. K^+ serves as a messenger activating subsequent osmotic responses (Miller & Wood, 1996; Shabala, 2009). Compatible solutes, such as sugars and derivatives, amino acids and their derivatives and betaines work as osmoregulants and assure protein stability, folding and function *in vitro* and *in vivo*. These compatible solutes could be synthesised *de novo* or, if present in the medium, can be taken up by the microorganisms (Burg & Ferraris, 2008; Street *et al.,* 2006). Another mechanism to improve salt tolerance is by modification of the composition of the bacterial cell membrane and cell wall resulting in changes in proteins and exo and lipopolysaccharides. The production of exopolysaccharides is the main mechanism used by *Pseudomonas* to survive under high salinities, since these compounds protects them from hydric stress and fluctuations in water potential by enhancing water retention (Sandhya *et al.* 2009). Salt-tolerant rhizobacteria associated with halophytic plants and their halophilic or halotolerant proprieties were reviewed by Egamberdiyeva and Islam (2008). Mainly because their ability to adapt to different environmental pressures, rhizobacteria from halophytes are interesting for inoculation in agricultural crops, for the enhancement of crop survival and productivity, especially under saline stress (Cunha *et al.*, 2005; Egamberdiyeva & Islam, 2008; Nabti *et al.,* 2015). For example, bacteria associated with roots of halophytes are also known by producing exopolysaccharides as a root protective mechanism against high salt concentrations. They produce these exopolysaccharides not only during biofilm formation but also during the establishment of symbiotic interactions (Danese *et al.*, 2000; Jones *et al.,* 2007; Ruppel *et al.*, 2013).

The importance of plant growth promoting bacteria in improving salt tolerance in crop vegetables, such as tomatoes (Mayak *et al*., 2004) and wheat (Ramadoss *et al.,* 2013) has been highlighted in several studies. The application of halotolerant bacteria isolated from halophytes to cultivated glycophytes to enhance growth and improve productivity was reviewed by Paul & Lade (2014). Application of halotolerant PGPB to crops may also benefit farming practices by reducing the need for fertilizers as many of these bacteria are known for their abilities to supply plants with critical nutrients such as phosphorous and nitrogen (Nabti *et al.*, 2015). There are some reports of the use of PGPR in *Salicornia* either with bacteria isolated from the plant or with bacteria isolated from other halophytes. A recent study by Szymanska *et al.* (2016) evaluated the density and diversity of the rhizosphere communities associated with *S. europaea* and a study by Jha *et al.* (2012) suggests that *S. brachiata* may be a useful source of new halotolerant bacteria with plant growth-promoting potential. Rueda-Puente *et al.* (2003) evaluated the effects of a strain of *Klebsiella pneumoniae* on the germination and early seedling growth of *Salicornia bigelovii* and concluded that this bacterium has nitrogen-fixing abilities, advantageous to the halophyte. A study by Bashan *et al.* (2000) found that inoculation of *S. bigelovii* with pure cultures or cocktails of PGPB significantly increased plant height and dry weight. More recently, Mapelli *et al.* (2013) studied the rhizosphere of *Salicornia* plants in hypersaline ecosystems and the bacteria were characterized for the resistance of temperature, salt and plant-growth promoting features, in *vitro*. Results show that some species of *Halomonas* are capable of successfully colonising *Salicornia* roots in laboratory conditions and have plant-growth promoting characteristics. However, to the best of my knowledge, there are no reports on biocontrol effects of PGPR inoculated on *Salicornia*.

1.4.5. Application of PGPR as biocontrol agents

Implementation of PGPR as biological control agents (BCAs) has been prevented by the lack of consistency and variation in responses that are obtained in field trials from site to site, for different crops and poor rhizosphere competence (Benizri *et al*., 2001; Martínez-Viveros *et al*., 2010). Rhizosphere competence of BCAs comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable period, in the presence of the indigenous microflora (Compant *et al.*, 2005). Thus, it is advantageous to study each case and use microorganisms from wild-type plants in the native location (Requena *et al.,* 2001), since the introduction of a large quantity of 'exotic' microorganisms may disrupt a local ecosystem and produce ecological impacts on the rhizosphere microbiota (Jackman *et al.,* 1992). In addition to rhizosphere competence, difficulties in production, formulation and delivery of BCAs can also affect biocontrol efficacy in the field. Suggested solutions to overcome these constraints include combination of BCAs with chemical fungicides and modification of the bioformulation of BCA mixtures.

Methods for inoculation with either gram negative or gram positive PGPR bacteria require an easy to use carrier to deliver the inoculum into the soil, that maintain cell viability under adverse environmental conditions and ensure the maintenance of available bacteria population sufficient to exert the effects on the plant. This can involve low cost carriers such as calcined clay that is mixed with the bacterial suspensions and dried. Powder formulations (microorganisms concentrated into dry powders) are also frequently used by direct application to the soil, or dusting onto seeds. Alginate microbeads can also be used and provide many advantages by incorporating the cells into a protected matrix that undergoes decomposition in the soil and slowly releases the bacteria. Bacteria can also be introduced into the irrigation water via fermentation equipment that cultures the bacteria and pumps them into the irrigation water at desired intervals (Martínez-Viveros *et al*., 2010; Gupta *et al*., 2015). So far, the most commercially successful inoculants have been Gram positive spore forming bacteria, which can persist in storage from months to years, and can withstand temperature, moisture and other environmental stresses better than non-spore forming bacteria (Martínez-Viveros *et al*., 2010).

1.5. Scope and objectives

The general aim of this work is to obtain autochtonous PGPR strains to support a high-productivity strategy of crop cultivation of the halophyte *Salicornia ramosissima* in Ria de Aveiro. For that, PGPB from the rhizosphere of wild *S. ramosissima* plants collected in salt marshes of Ria de Aveiro, were isolated, tested for salt tolerance, characterized as to plant-growth promoting traits especially biocontrol activity and tested as inoculants on germination of *S. ramosissima* seeds. The results are expected to contribute for the strengthening of scientific basis of biosaline agriculture and for the development of PGPR-assisted crop cultivation protocols of halophytes in saline soils or estuarine sediments.

2. Methods

2.1. Study sites and sampling

Seeds and *Salicornia ramosissima* specimens were collected at salt marshes (Figure 3) in the Ria de Aveiro estuarine system (Aveiro, Portugal). The Ria de Aveiro is a shallow coastal lagoon on the north-west coast of Portugal (40.7°N, 8.7°W) formed by a complex network of channels and extensive intertidal zones (Dias *et al*., 1999). The sampling sites were chosen to represent different conditions in terms of salinity (Table 1). Sediments from sampling site no. 1 (Boco river, Vagos) represent the lowest salinity range, since this site suffers little influence from Atlantic Ocean, and it is a permanent source of freshwater input to the Ílhavo Channel. Salt marshes of Santiago da Fonte (sampling site no. 2) and Pontinha (sampling site no. 3) are closer to the city of Aveiro and are exposed to higher salinity (Dias *et al*., 1999).

S. ramosissima healthy specimens (20 cm average height) were harvested in October 2016 with a shovel, stored in individual sterile plastic bags and transported to the laboratory at the University of Aveiro. Seeds were collected in December 2016 from stems of dried plants and stored in the dark at room temperature until used. Bulk sediment was collected in January and June 2017 at the same tidal phase (one hour before lowest tide) from Pontinha (Table 1) at three monospecific banks of *S. ramosissima*. To avoid potential sampling bias caused by soil heterogeneity, five subsampling points were selected (around 3–5 m apart) in each site. Samples were transported to the laboratory for processing within one hour after collection in separate labelled bags to avoid contamination.

Table 1 - Location of the sampling sites and material collected in each site.

Sampling site	GPS information	Material collected
Boco (1)	40°33'35.9"N, 8°40'19.3"W	Seeds, plants (*S. ramosissima*)
Santiago da Fonte (2)	40°37'43.5"N, 8°39'38.8"W	Seeds, plants (*S. ramosissima*)
Pontinha (3)	40º38'57"N, 8º38'59.9"W	Seeds, plants (*S. ramosissima*), sediment

Figure 3 – Sampling sites represented with a circle and the correspondent number. Map created with Google tool 'My Maps'.

2.2. Sediment temperature and salinity

The salinity and temperature of the sediment were determined in situ in the interstitial water from the sampling soil with a handheld multi-parameter system (TetraCon® 325, WTW GmbH).

2.3. Isolation of rhizobacteria

Bacterial isolation from the rhizosphere of *S. ramosissima* was performed according to Domingues *et al.* (2011). Loose soil was carefully separated from the root of plants by manual shaking. Roots (10 g fresh weight) were washed thoroughly, cut in 2-3 cm fragments and transferred to sterile glass flasks with 200 mL Ringer solution (Merck, pH = 7.0 ± 0.2) and 20 g of glass beads (4 mm diameter) and shaken at 100 rpm for 5 min in an orbital incubator to detach bacteria and soil from root material. The suspension was serially diluted with Ringer solution and aliquots (100 µL) of each dilution were spread-plated on Tryptic Soy Agar (TSA, Liofilchem, pH = 7.2 ± 0.2). The cultures were incubated for 72 h at 37 ºC.

After visible growth, 54 representative bacterial strains were selected based on the morphological features of the colonies. Purification was conducted by standard streaking technique on TSA. The resulting cultures on solid medium were incubated for 24 h at 37 °C. Twenty-three isolates were then selected for further testing based on their efficiency of growth (colonies forming in 24 h) and the purity of the culture was checked by visual inspection of the colonies and by observation under optical microscope after Gram staining. For long-term storage, isolates were grown in Tryptic Soy Broth (TSB, Liofilchem, pH = 7.3 ± 0.2), and the resultant suspensions were

supplemented with 20 % glycerol and deep-frozen (-80 ºC) until further use. Whenever necessary, the isolates were reactivated by cultivation in 30 mL sterile TSB for 24 h at 37 ºC. Plate streaking was performed in TSA between every liquid reinoculation to confirm the purity of the cultures. Working cultures for routine tests were conserved in solid medium (TSA), stored in the refrigerator (4 °C) and renewed weekly.

2.4. Biochemical, physiological and biocontrol traits of rhizobacterial isolates

2.4.1. Gram staining and cell motility

Gram staining was performed according to the standard protocol. Cell motility was tested by using the wet-mount slide technique. Aliquots of 15 µL of fresh bacterial culture diluted in physiological saline solution were placed on a clean microscope slide and covered with a cover glass. The preparation was observed with an optical microscope (Leitz Laborlux K) with a total magnification of 1500 x.

2.4.2. Salt tolerance

Salt tolerance of the isolates was tested by inoculation of fresh bacterial cultures (50 µL) in 5 mL TSB medium supplemented with 0, 10, 20 and 30 ‰ NaCl, using non-inoculated medium as control. The cultures were incubated at 37 °C for 24 h and bacterial growth was determined by visual inspection of turbidity. Three independent tests were conducted.

2.4.3. Biocontrol effect against *Alternaria* sp.

Bacterial isolates were screened for *in vitro* antagonistic activity against the phytopathogenic fungus *Alternaria* sp. adapting the method described by Sgroy *et al.* (2009). One disc (5 mm) of mycelium of a young fungal culture in solid medium was placed on the centre of a plate of Potato Dextrose Agar (PDA, Liofilchem, pH = 5.6 ± 0.2). Two blank antibiogram disks were placed equidistantly on the edge of the plate, one soaked with 25 µL of fresh bacterial culture (*T*) and the other (control) soaked with 25 µL of sterilized *d*H_2O (*C*). Fluconazole disks were used as positive controls (Kowalsky & Dixon, 1991). The plates were incubated for 10 days at room temperature (approx. 25 ºC). Mycelium growth inhibition was calculated according to the equation (1), where *I* = mycelium growth inhibition in percentage, *C* = radius of mycelium growing towards the control, and *T* = radius of mycelium growing towards the test bacterium. The experiment was repeated three times and the results were averaged.

$$I = [(C - T)/C] \times 100 \quad (1)$$

2.4.4. Production of hydrogen cyanide

Hydrogen cyanide (HCN) production was examined by the method proposed by Lorck (1948). Bacterial isolates were inoculated in TSB medium supplemented with 4.4 g/L of glycine and incubated at 37 ºC for 24 h. After growth, bacteria were streaked on solid medium with the same concentration of glycine and a sterile filter paper (Whatman no. 1) soaked in a 2 % sodium carbonate in 0.5 % picric acid solution was placed on top of the medium. The test plates were sealed with Parafilm® and incubated at 37 ºC for 24 h. HCN production was detected by a change of colour of the filter paper (light-yellow to dark-brown). *Pseudomonas aeruginosa*, an isolate which was previously characterized as HCN-producing (Castric, 1977) was applied as positive control, while uninoculated medium was used as negative control. The assay was repeated 3 times for each isolate.

2.4.5. Proteolytic and lipolytic activity

The production of extracellular proteases was accessed by streaking fresh cultures of each bacterial isolate in skim milk agar 3 % (Sgroy *et al.*, 2009). Plates were sealed with Parafilm®, incubated at 37 °C for 24 h and observed for development of a clear zone around bacterial growth. Lipase production was tested by streaking fresh cultures of each bacterial isolate on 1/20 TSA medium amended with 2 % of Tween 80 (Merck), according to Howe and Ward (1976). Test plates were sealed with Parafilm® and incubated at 37 ºC for 24 h. Positive results show a calcium complex, visible as insoluble crystals around the inoculation site. *P. aeruginosa* was applied as positive control for assessment of both proteolytic (Oldak & Trafny, 2005) and lipolytic activity (Stuer *et al.*, 1986). In both tests, three independent assays were conducted for each isolate.

2.5. Molecular identification of a selected rhizobacterial isolate

Based on biocontrol activities exhibited by the isolates, one isolate designated as SP1016-20 which showed positive results in all tests, was selected for identification and further studies.

2.5.1. DNA extraction

A volume of 2 mL of liquid culture (TSB) was centrifuged at 16 000 *g* for 5 min. The pellet was resuspended in 800 µL of ethanol and transferred to a tube containing 500 mg of glass beads. The tubes with the samples and glass beads were stirred for 5 min in the vortex and centrifuged at 16 000 *g* for 5 min. The supernatants were discarded and 1 mL of extraction buffer (1 % CTAB, 2 % SDS, 1.5 M NaCl, 100 mM sodium phosphate buffer [pH 7.0], 10 mM Tris-HCl [pH 7.0], 1 mM EDTA [pH 8.0]) was added to the pellet. The mixture was gently homogenised and incubated at 65 ºC for 15 min. After centrifugation at 16 000 *g* for 5 min, the supernatants were transferred to a new tube and 1 mL 21:1 chloroform-isoamyl alcohol solution was added. The tubes were gently mixed and centrifuged at 16 000 *g* for 5 min. The aqueous phase was then transferred to a tube

containing 0.6 % isopropanol (v/v) and incubated at room temperature (approx. 25 ºC) for 30 min. After another centrifugation at 16 000 *g* for 20 min, the supernatant was discarded and the pellet was incubated at 55 ºC for 10 min until completely dry. The pellet was then resuspended in 80 µL TE buffer (10 M Tris-HCl [pH 7.4] containing 1 mM EDTA.Na_2 [pH 8.0]) and stored at -20 ºC.

2.5.2. Molecular typing by BOX-PCR

The BOX-PCR protocol was based in the method described by Martin *et al.* (1992). The PCR reaction mixture used in the procedure was composed of 8.75 µL *d*H_2O, 1.25 µL DMSO, 1.50 µL primer BOX_A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and 12.50 µL of Mastermix (Thermo Fischer Scientific) for 25.00 µL of reaction. The amplification protocol included a denaturation step of 7 min at 94 °C, followed by 30 thermal cycles of 1 min at 94 °C, 2 min at 53 °C, and 8 min at 65 °C, and an extension step at 65 °C for 16 min. PCR products were stored at -20 °C and run in agarose gel (1.5 %) electrophoresis, with 5.3x10-6 % (v/v) RedSafe™, at 100 V for 2 h in TAE buffer 1x (5 Prime). The profiles were visualized in a UV transilluminator (Benchtop UV) and were photographed using a Canon Powershot G10.

2.5.3. PCR-amplification of 16S rRNA gene fragments

PCR amplification of 16S rRNA gene fragments was made using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1378R (5' – CGG TGT GTA CAA GGC CCG GGA ACG – 3'). The composition of the reaction mixture (25 µL) was 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix, 0.25 µL of each primer, 1 µL BSA (2 mg/mL) and 10 µL *d*H_2O. The PCR cycle was composed by 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94 °C, 45 s at 56 °C, and 1.5 min at 72 °C, and a final extension step at 72 °C for 10 min. The success of the amplification of the 16S rRNA gene fragments was verified by agarose gel (1 %) electrophoresis, with 5.3x10-6 % (v/v) RedSafe™ as DNA staining agent, at 100 V for 30 min in TAE buffer 1x. The presence of bands was visualized in a UV transilluminator (Benchtop UV). The amplicons were sequenced by GATC Biotech (Germany). The obtained sequences were matched to the sequences available in the GenBank database using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov) to determine their closest relative.

2.6. Growth curves of the selected isolate under different salinities

Salt tolerance and growth behaviour of the chosen isolate (SP1016-20) was observed by inoculation of fresh bacterial culture (50 µL) in 5 mL TSB medium supplemented with 0, 10, 20 and 30 ‰ NaCl, using non-inoculated medium as control. The cultures were incubated at 37 °C and bacterial growth was determined by measuring the optical density at 590 nm every hour for 28 h, when a growth plateau was observed. Bacterial growth behaviour under different salinities was

investigated by growth curve analysis. Each culture was replicated three times within the experiment.

2.7. Seed germination tests

The effects of seed dimorphism, storage time and salinity on the germination efficiency were tested by an approach adapted from Ameixa *et al.* (2016). To assess the effect of seed dimorphism, small and large seeds were separated based on their size (≤1.4 and ≥1.5 mm, respectively), as described by Ungar (1979). For the evaluation of the effect of storage time, seeds collected in 2014, 2015 and 2016 in Pontinha salt-marsh stored in the laboratory at room temperature, were used. To test the germination efficiency under different salinities, all types of seeds (small and large seeds collected at Pontinha salt-marsh) were allowed to germinate under salinities of 0, 10, 20 and 30 ‰ NaCl. The preparation of seeds was similar for all tests. *S. ramosissima* seeds were surface-sterilized with sodium hypochlorite (5 % active chlorine) for 15 min, rinsed 4-5 times with sterilized dH$_2$O and placed on a sterile petri dish containing two layers of sterile Whatman no.1 filter paper soaked with 5 mL of NaCl solutions with different salinities (0, 10, 20 and 30 ‰). Three replicates for each treatment were performed in all germination treatments. For each replicate, twenty-five seeds per petri dish were arranged in five rows. The growth conditions were set to 24 °C and 16/8 h light/dark cycle in an incubator (Sanyo MLR 350 H Versatile Environmental Test Chamber). Emergence of radicle was daily monitored for 2 weeks. The data corresponding to the number of germinated seeds in each day and days to the first and last germination were registered, for further calculation of final germination (FG) percentage and mean daily germination (MDG: quotient of germination percentage by number of days to last germination, expressed as % day $^{-1}$).

2.8. Effect of the inoculation with selected bacterial isolate on seed germination under saline stress

A pure culture of the chosen isolate (SP1016-20) was grown in TSB at 37 ºC and 100-fold diluted in sterile physiological saline solution. *S. ramosissima* large and small seeds collected at 2016 from Pontinha salt marsh were used. Seeds were surface-sterilized as previously described, immersed in the bacterial suspension, immediately centrifuged at 16000 *g* for 10 min and incubated at room temperature for 2 h, to promote the adsorption of bacteria to the seeds. Control seeds were treated with dH$_2$O without bacterial culture. Germination of inoculated seeds and non-inoculated controls under different salinities was evaluated as described for the initial germination tests.

2.9. Statistical analyses

All statistical analyses were performed with Past® software, using two-way permutational multivariate analysis of variance (PERMANOVA) and a least significant difference (LSD) analysis at the 5 % probability level (Hammer *et al.*, 2001).

3. Results and discussion

3.1. Sediment temperature and salinity

The temperature and salinity values of the sediment are presented in Table 2. The temperature of the sediment ranged from 16.7 ºC in January to 26.3 ºC in June and did not vary significantly between sites. The highest salinity was observed in June (64.7 ± 3.04 ‰) that corresponded to the highest temperature values. There is a correlation between sediment temperature and sediment salinity, since at higher temperatures there are an increase in soil salinity, mostly due to evaporation phenomena. Falling of *S. ramosissima* seeds from the parent plant occurs in late-September or October (depending mostly on the climacteric conditions) and germination takes place between January and April, where salinity levels are lower.

Table 2 – Sediment properties in Pontinha salt-marsh on three sample sites (mean ± SD, $n = 5$)

Sample site	Temperature (ºC)		Salinity (‰)	
	January 2016	June 2016	January 2016	June 2016
1	17.0 ± 1.60	26.2 ± 0.25	28.3 ± 1.10	45.2 ± 1.44
2	16.7 ± 0.32	26.1 ± 0.07	28.5 ± 0.98	56.3 ± 0.93
3	17.2 ± 0.55	26.3 ± 0.09	28.7 ± 0.99	64.7 ± 3.04

3.2. Biochemical, physiological and biocontrol traits of rhizobacterial isolates

Initially, 54 representative bacterial strains were selected based on the morphological features of the colonies, but only bacteria that presented visible growth in 24 h at 37 ºC after purification in solid medium, were selected for further testing (23 isolates).

3.2.1. Gram staining

Gram-negative bacteria (78.3 %) were almost four times more represented in rhizosphere isolates than Gram-positive ones (21.7 %), independently of sampling site (Table 3). These results are in line with previous studies by Barin *et al.* (2015) and Szymanska *et al.* (2016) that reported the dominance of Gram-negative bacteria among rhizobacteria isolated from *S. europaea*. This can be due to the presence of cyclopropane fatty acids and outer lipopolysaccharide layer in the membranes of Gram-negative bacteria, which can counteract stressful conditions, such as high salt concentrations observed in saline environments (Trüper *et al.,* 1991; Kaur *et al*., 2005). Previous studies also report that *Salicornia* species selected similar bacterial communities in the rhizosphere, independently from the site of sampling (Mapelli *et al.,* 2013; Szymanska *et al.,* 2016), suggesting that rhizosphere acts as a selection factor that tends to uniform bacterial diversity independently of the soil type.

3.2.2. Motility

The motility of the isolates from *Salicornia* rhizosphere was examined as a plant-growth promoting trait and the results are shown in Table 3. Overall, 52 % of the tested isolates exhibited motility (Table 3). Bacteria can move by different mechanisms, including flagellar swimming, swarming, twitching and gliding motility (Jarrell & McBride, 2008), which were not discriminated in the present study.

Previous studies report that more motile bacteria are present in the proximity of roots than in the bulk soil (Czaban *et al.*, 2007; Barret *et al.*, 2011). This is consistent with the idea that motility can enhance rhizosphere competence, defined as the ability of survival and colonisation of new habitats and hosts (De Weger *et al.*, 1987; Bulgarelli *et al.*, 2013). Motility could enhance colonisation ability by enabling cells to find attachment sites more quickly, turning motile microbes more competitive that non-motile ones (Turnbull *et al*., 2001; Yao & Allen, 2006). Additionality, motility facilitates bacterial response to fluctuations experienced in stressful environments such as salt-marshes (Turnbull *et al*., 2001b). In cultivation experiments with seeds inoculated with bacteria, motility can aid the movement of microbes from seeds into protective microniches, which may enhance the survival of the introduced population in the presence of the indigenous microflora (Turnbull *et al*., 2001b). Furthermore, Choi *et al.* (2006) demonstrated that swarming motility may contribute to stable maintenance of antifungal compounds on plant surface, showing that motility is particularly relevant to achieve satisfactory biocontrol results (Choi *et al.,* 2006).

3.2.3. Salt tolerance

After 24 h, the isolates were characterized qualitatively as having strong, weak or no growth at all at different salinities, based on the turbidity of the TSB medium (Figure 4). Most of the isolates (60.9 %) grew well at lower (0 ‰ and 10 ‰) salinities, presenting weak growth or no growth at higher (20 ‰ and 30 ‰) salinities, while 26.1 % of the collection corresponded to halotolerant bacteria, able to grow well in all the tested salinities. Halophilic bacteria (unable to grow in the absence of NaCl in the medium) were not found (Figure 4). Naturally saline sediments such as in salt-marshes are mutable environments due to fluctuation of climatic conditions and precipitation throughout the year, which impact sediment salinity (Zahran, 1997). This type of habitat is inhabited by well adapted halophilic and halotolerant bacteria (Ollivier *et al.*, 1994). Several studies have reported higher abundance of halotolerant and halophilic bacteria in the rhizosphere, as compared to bulk soil, which may be related to the "rhizosphere effect" and the presence of root exudates that can contribute to salt tolerance in bacteria (Mapelli *et al.*, 2013). It is worth mentioning that in the soil, although NaCl is the most prevalent salt, there are more salts as well as other factors that can modify the overall salt tolerance of bacteria (Tavakkoli *et al.,* 2010; Wood, 2015). Since the final goal of this work is to inoculate seeds with the bacteria and to conduct

the cultivation in field conditions, the PGPB used must be halotolerant and survive under varying salinity especially during the initial stages of germination (Ungar, 1979). Furthermore, halotolerant bacteria are more promising as PGPB by enhancing crop survival and productivity under saline stress (Nabti *et al.*, 2015). Bacteria must also be easily cultivated (visible growth in 24 h) to be considered an efficient inoculum.

Figure 4 – Salinity tolerance of bacterial isolates under different salinities (0, 10, 20 and 30 ‰ NaCl). Isolates were characterized qualitatively as having strong (dark green), weak (light green) or no growth at all (yellow) by inspection of TSB turbidity.

3.2.4. Biocontrol effect against *Alternaria* sp.

The antagonistic potential of the isolates against the phytopathogenic mold *Alternaria* sp. was examined by the dual culture assay, which is a simple and efficient approach to evaluate growth inhibition, since both fungi and bacteria grew well on PDA at 25 °C, and 10 days was usually sufficient for full growth of all microbes. The results, shown in Table 3, are presented as the percentage of inhibition of growth of *Alternaria* sp. mycelium in the direction of the inoculated disk (Figure 5). Among the 23 isolates, the most effective fungal antagonists were the isolates

coded as SP1016-20 (27.54 ± 7.33 %) and SB1016-78 (23.80 ± 11.22 %), whereas about 48 % of the isolates exhibited little or no inhibitory activity (lower than 10 % inhibition).

Alternaria species were chosen for screening the antagonistic potential of the isolates due to the high prevalence of these fungi on *S. europaea* (Muhsin, 1996; Okane & Nakagiri, 2015). Despite their adaptability to saline environments, evidenced by their occurrence in halophytes, it has been shown that the presence of NaCl in the medium inhibits fungi in *S. europaea* (Szymanska *et al.*, 2016). This makes seed germination a critical stage for *Alternaria* infection, since seed germination occurs at low salinity, where fungus has a better chance of surviving. Plant pathogens that affect seeds reduce the quantity and quality of the seed harvested and can be preserved in seed lots, making seeds an efficient means of plant pathogen dissemination (Mancini & Romanazzi, 2013). Thus, the treatment of seeds with biocontrol agents can be very important for eradicating or reducing seedborne pathogens.

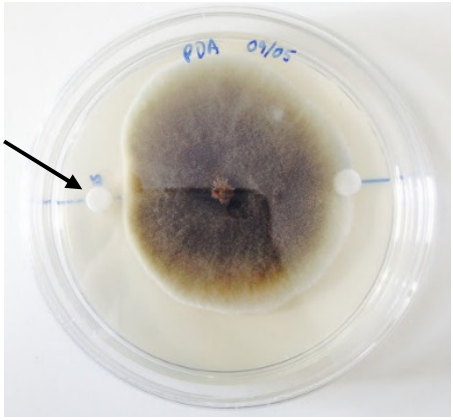

Figure 5 – Biocontrol effect of the isolate SF1016-12 against *Alternaria* sp. Inoculated disk is represented in the figure with an arrow.

3.2.5. Production of hydrogen cyanide

The ability of the rhizobacteria to control pathogens by producing hydrogen cyanide (HCN) was tested by assessing the colour change of the filter paper from light yellow to orange and brown (Figure 6). As shown in Table 3, 13 of the 23 tested isolates could synthesize HCN, a volatile organic compound (VOC) produced by rhizobacteria that is involved in biological control in seedlings (Voisard *et al.*, 1989; Flaishman *et al.,* 1996; Kang *et al.*, 2010). Production of HCN is highly dependent on glycine availability in the medium. Therefore, the level of HCN production in the rhizosphere may be lower compared to the tested *in vitro* conditions (where glycine is in excess), despite glycine being one of the predominant amino acids of root exudates (Lesuffleur *et al.,* 2007). HCN exerts its toxic effects through inhibiting cytochrome c oxidase, the final component of the aerobic respiratory chain in many organisms, as well as other essential

metalloenzymes (Solomonson, 1981). The host plant is generally not harmed by inoculation with HCN producing bacteria (Saharan & Nehra, 2011) and due to its high volatility, HCN does not accumulate to higher concentrations in the immediate vicinity of the producer, causing no harm to the environment (Kai *et al.*, 2010). It is important to note that, while production of HCN is a good strategy, it may not be an efficient inhibitor on its own (Voisard *et al.*, 1989; Glick, 2012).

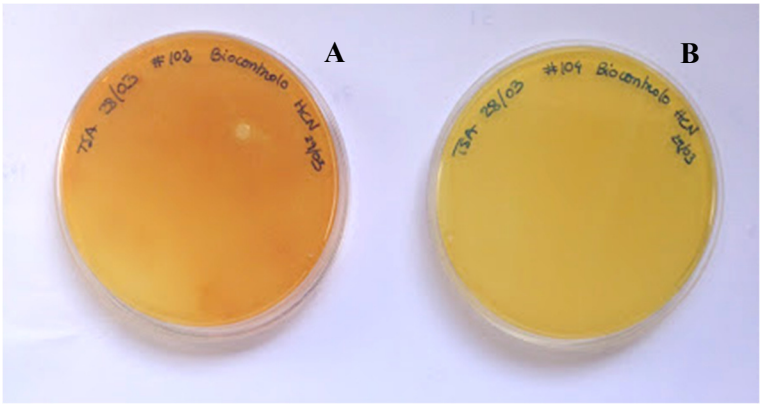

Figure 6 – Production of hydrogen cyanide by isolate SB1016-102 (A), demonstrating a positive result. Isolate SF1016-104 (B) did not produce hydrogen cyanide, since there are no changes in filter paper colour.

3.2.6. Proteolytic and lipolytic activity

Among the 23 evaluated bacteria, most isolates were positive for protease or lipase production (56.5 %), while seven isolates present both proteolytic and lipolytic activity (Table 3). Most soil microorganisms express proteolytic activity (Vranova *et al.*, 2013), assessed *in vitro* by the development of a clear zone around bacterial growth (Figure 7). Lipases are enzymes that hydrolyse triglycerides into fatty acids and glycerol (Jensen, 1983). *In vitro* lipase production by the microorganisms was evaluated using Tween 80 as substrate in agar media. The use of tweens (fatty acid esters of polyoxyethylene sorbitan) is one of the most widely used methods. When bacteria produce lipases, tweens are hydrolysed, and the liberated fatty acids bind with the calcium incorporated in the medium, originating a calcium complex, visible as insoluble crystals around the inoculation site (Kumar *et al.*, 2012).

Fungal cell walls are constituted predominantly by glucose (68 %) but also contain proteins and lipids (up to 3 %). Proteins in fungal cell walls are involved in critical processes in its operation such as iron transfer and promotion of survival under stress, while fungal lipids have a significant role on the structure and function of fungal cell walls (Feofilova, 2010). Therefore, hydrolytic enzymes that act on cell wall components such as proteins and lipids can have a serious

impact on fungi health and may cause its deterioration allowing, for example, penetration of small-molecular inhibitors such as antibiotics (Susi *et al.*, 2011).

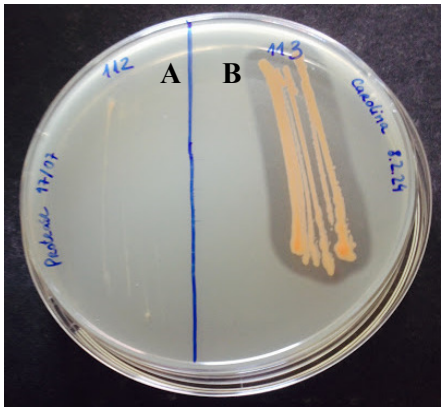

Figure 7 – Development of a clear zone around bacterial growth, demonstrating proteolytic activity of the isolate SB1016-113 (B), as opposed as SB1016-112 (A) that does not produce proteases.

3.3. Overall perspective on biocontrol and plant-growth promotion traits

Among 12 isolates antagonistic to *Alternaria* sp. (higher than 10 % of inhibition), those exhibiting lipolytic activity accounted for the highest percentage (75 %), followed by those showing proteolytic activity (58.3 %), while those that produce hydrogen cyanide had the lowest percentage (41.7 %) (Table 3).

This result indicates that HCN production is not the main factor affecting *in vitro* inhibition of *Alternaria* growth, which is in line with the conclusion of Voisard *et al.* (1989). Furthermore, isolates coded as SF1016-68 and SF1016-69 expressed two hydrolytic enzymes but not HCN and did not greatly inhibit *Alternaria* growth (lower than 10 % of inhibition); two isolates (SB1016-15 and SP1016-86**)** did not inhibit *Alternaria* growth (0 % of inhibition) and showed only one of the biocontrol traits studied. Since many of the isolates showed different traits, it is difficult to ascertain which mechanism is playing a key role in the *in vitro* inhibition of *Alternaria* growth and there are other biocontrol traits that were not evaluated in this study. Only the isolates SP1016-20 and SB1016-102 presented all the biocontrol traits studied and a good growth inhibition of *Alternaria* (27.54 ± 7.33 % and 16.96 ± 4.71 %, respectively). The higher inhibition observed with SP1016-20 (27.54 ± 7.33 %) may be attributable to a combination of traits, since this isolate displayed multiple traits related to biocontrol. Although both isolates showed all the biocontrol traits, only SP1016-20 is motile, a crucial physiological trait. Thus, it was chosen for identification and for subsequent tests. This isolate was collected from the rhizosphere of *S. ramosissima* from Pontinha salt marsh.

Table 3 – Motility, Gram staining and biocontrol traits of isolates from the rhizosphere of *S. ramosissima*. Quantitative traits are shown as mean of three replicate observations ± SD. Qualitative results are shown as + (positive) or – (negative) for each tested trait.

Bacterial isolate	*Alternaria* sp. growth inhibition[a]	HCN production[b]	Proteolytic activity	Lipolytic activity	Motility	Gram[c]
SB1016-4	0.93 ± 1.61	+	+	-	+	-
SB1016-8	2.67 ± 4.62	+	+	-	-	-
SF1016-12	16.60 ± 4.40	-	+	+	-	-
SB1016-15	0.00 ± 0.00	-	+	-	-	-
SP1016-20	27.54 ± 7.33	+	+	+	+	+
SF1016-23	16.06 ± 2.97	+	-	-	+	-
SF1016-27	8.45 ± 8.34	-	+	-	+	-
SP1016-32	1.28 ± 2.22	+	-	+	-	+
SF1016-59	1.01 ± 1.75	+	+	-	+	-
SF1016-68	2.78 ± 4.81	-	+	+	+	-
SF1016-69	1.33 ± 2.31	-	+	+	-	+
SF1016-77	11.89 ± 3.78	-	-	+	-	-
SB1016-78	23.80 ± 11.22	-	+	+	-	+
SP1016-86	0.00 ± 0.00	+	-	-	+	-
SF1016-91	9.29 ± 5.67	-	-	+	+	-
SF1016-92	1.23 ± 2.14	+	-	-	-	-
SF1016-100	2.86 ± 4.95	+	-	+	-	-
SB1016-102	16.96 ± 4.71	+	+	+	-	-
SP1016-103	18.10 ± 3.52	+	-	+	-	-
SF1016-104	9.05 ± 2.18	-	+	+	-	+
SB1016-112	2.15 ± 3.72	+	-	-	+	-
SB1016-113	19.02 ± 6.71	+	+	-	+	-
SB1016-115	22.55 ± 6.97	-	-	+	+	-

[a] % Mycelial inhibition was calculated as described in section 2.4.3.
[b] HCN, hydrogen cyanide production
[c] + (gram-positive); - (gram-negative)

3.4. Molecular identification of isolate SP1016-20

PCR amplified products were sequenced and compared with the 16s rDNA sequences in NCBI database. Based on the partial sequences comparison by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), isolate SP1016-20 was identified as *Bacillus aryabhattai* (Table 4). It is a motile, gram-positive rod with colonies that, on TSA plate, appeared creamy white, opaque and were approximately 2–3 mm in diameter.

Table 4 – Identification of bacterial strain with biocontrol potential isolated from *S. ramosissima* rhizosphere, based on 16S rDNA sequence

Isolate	Identified as	% Similarity	Accession number
SP1016-20	*Bacillus aryabhattai*	100 %	MG583717

Previous studies report the ability of *B. aryabhattai* isolated from rhizospheres of halophytes to promote the growth of *Xanthium italicum* (Lee *et al*., 2012), soybean (Park *et al*., 2017) and canola under salt stress (Siddikee *et al*., 2010). This growth promotion potential could be linked to production of 1 aminocyclopropane-1-carboxylate (ACC) deaminase and phytohormones (Lee *et al*., 2012; Park *et al*., 2017) as well as the ability of *B. aryabhattai* to form biofilms, solubilise phosphates, produce proteases, and efficiently utilize the root exudates as an energy source (Selim, 2015; Bhattacharyya *et al.*, 2017). *Bacillus* sp. has been reported as one of the most dominant genus among Gram-positive bacteria in the rhizosphere of several plants, including halophytes (Irshad *et al.*, 2014; Yaish *et al.*, 2015). This may be a result of the endospore-forming capability among this group of bacteria, in addition to their greater resilience and tolerance to environmental changes (Lalloo *et al*., 2009).

3.5. Growth curves of SP1016 under different salinities

Analysis of salinity tolerance is fundamental to predict the survival of the inoculum in the plant under saline conditions. The ability of SP1016-20 to grow in TSB supplemented with NaCl is shown in Figure 8. As expected after the preliminary salt tolerance test (section 3.2.3.), SP1016-20 could grow in all tested salinities although with a different kinetic profile. Without salt, the *lag* phase was about five hours, a relatively brief time for adaptation to a different medium. However, as the salinity increased, lag phase extended (11, 12 and 13 hours, respectively for 10, 20 and 30 ‰ NaCl) (Figure 8). After reaching the exponential phase, the isolate grew the fastest with 30 ‰ NaCl (growth rate of 0.28 h^{-1}) and more slowly with decreasing salinity (0.22 h^{-1}, 0.19 h^{-1} and 0.15 h^{-1}

respectively for 20, 10 and 0 ‰ NaCl), however the growth rates are not very different, so these results may only indicate that the isolate is halotolerant and grow well in all the salinities tested. The value of optical density reached at the end of the exponential phase was higher in salt-free medium, followed by 30 ‰ NaCl, 20 ‰ NaCl and 10 ‰ NaCl. After 20 hours, the strain had already reached the stationary phase at all salinities (Figure 8). Thus, in general, the strain showed halotolerance, which can be important to tolerate high and changing salinities in *S. ramosissima* habitat and indicates that, in field conditions, the isolate may have a selective advantage over slower growing competitors. This is particularly important for bacteria possessing biocontrol traits, since it has been shown that bacteria generally protect the plant by rapid colonization and thereby exhausting the limited available substrates so that none are available for pathogens to grow (Heydari & Pessarakli, 2010).

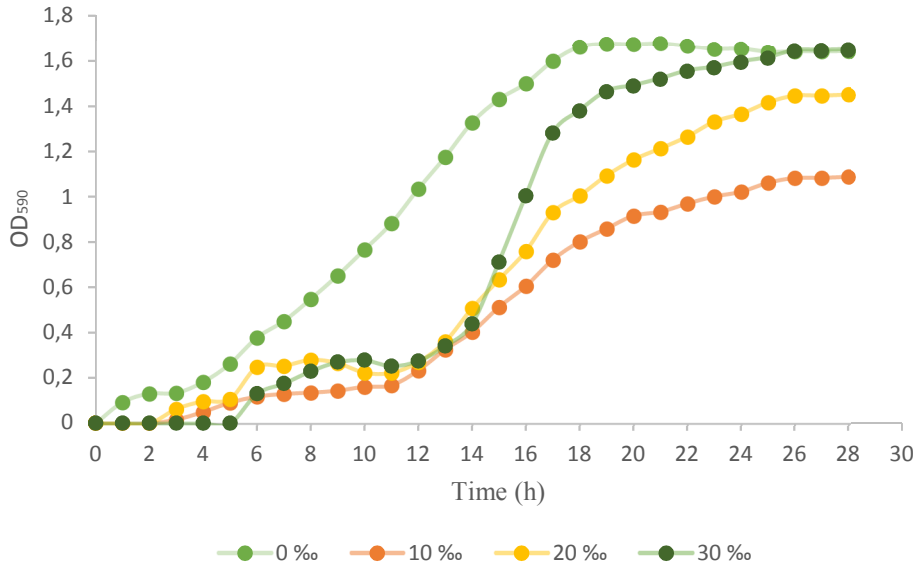

Figure 8 – Growth curve of SP1016-20 under a range of salinities (0, 10, 20 and 30 ‰ NaCl) in TSB medium at 37 ºC, determined by measuring optical density at 590 nm.

3.6. Seed germination tests

3.6.1. Effect of seed dimorphism and salinity on the germination efficiency

Seed dimorphism, the production by a single plant of two or more seed morphs with distinct characteristics, is common in halophytes and confers a selective advantage to plants that grow in fluctuating environments, by promoting the formation of a seed bank, which enables the long-term survival of the species (Philipupillai & Ungar, 1984). The aim of this experiment was the evaluation of seed dimorphism on the germination efficiency of *S. ramosissima* seeds under saline

stress. The experimental results showed that the seeds final percentage of germinated seeds (FG) was always higher in distilled water (up to 45 %), and a significant inhibition of germination with increasing salinity was observed, meaning that this species does not have a physiological requirement for salt to germinate. This is coincident with previous results on *Salicornia* (Ungar, 1979; Silva *et al.*, 2007; Ventura *et al.*, 2011; Ameixa *et al.*, 2016). The number of days until first germination was the same in all treatments (6 days after the beginning of the experiment), excluding small seeds at 30 ‰ NaCl, in which germination did not occur within the course of the experiment (14 days). The FG of large seeds was higher than that of the small seeds in all treatments (Figure 9), as previously described by Ungar (1979). At lower salinities (0 ‰ and 10 ‰), both seed types showed similar FG. However, there is a relevant decrease in FG at salinities over 10 ‰ (61 % for large seeds and 70 % for small seeds). These results are in line with the ones reported by Rubio-Casal *et al.* (2003) that stated a decrease of 81 % in germination of *S. ramosissima* seeds at salinity over 10 ‰. For large seeds, the minimum and maximum FG (mean ± SD) were 9.3 ± 9.2 % and 45.3 ± 8.3 %, at the highest and lowest salinity, respectively. For small seeds, the minimum FG was 0 ± 0 % at 30 ‰ and the maximum FG was 44.0 ± 6.9 % at 0 ‰ (Table 1, Appendix A).

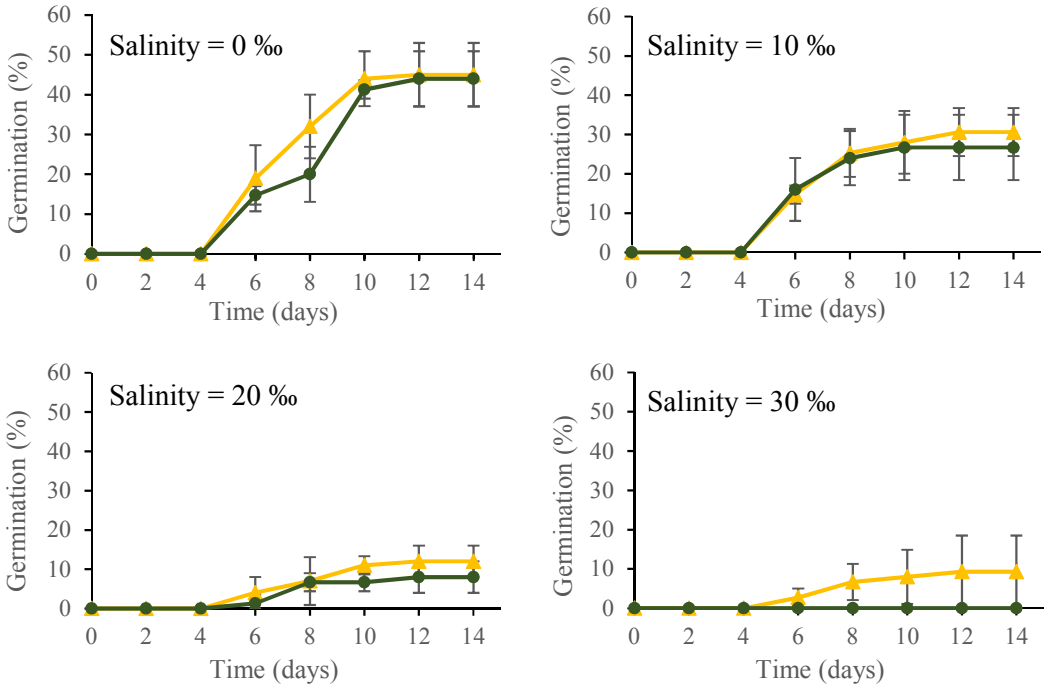

Figure 9 - Germination frequency (mean ± SD) of large (yellow triangles) and small (green circles) seeds exposed to four treatments of salinity (0, 10, 20 and 30 ‰ NaCl, respectively) over 14 days.

The PERMANOVA analysis (Table 1, Appendix B) on FG revealed that there is not a significant interaction (P = 0.5983) between salinity and seed type. However, FG decreases significantly with both salinity (P = 0.0001) and seed type (P = 0.0107). The highest average FG (45.3 ± 8.3 %) corresponded to large seeds germinated at a salinity of 0 ‰, while the lowest value (0 ± 0 %) was observed for small seeds germinated at a salinity of 30 ‰. The PERMANOVA analysis of the daily germination (MDG) (Table 2, Appendix B) revealed a significant effect of both salinity (P = 0.0001) and seed type (P = 0.0388). The highest MDG (3.78 ± 0.69 % day $^{-1}$) corresponded to large seeds at a salinity of 0 ‰ and the lowest value (0 ± 0 % day $^{-1}$) to small seeds germinated at a salinity of 30 ‰.

The higher germination efficiency of large seeds may be related to seed morphology. Both germination and salt tolerance require energy and large seeds have larger reserves, a greater ability to maintain ion homeostasis, and a thinner seed layer than small ones (Osmond *et al.*, 1980; Flowers & Colmer, 2008), making them more prone to germination. However, despite the higher variability in germination responses with increasing salinity, a similar trend could be observed for both seed types (Figure 9), thus emphasizing that seed type is not the main factor when evaluating the germination behaviour of *Salicornia*. Moreover, as noted by Singh *et al.* (2004), the time-consuming and tedious work of separating seeds is without any importance for the grower, since only negligible differences in germination are observed.

The effects of NaCl on *Salicornia* germination can be due to a reduction in water potential of the growth media, or to the toxicity of Na^+ and Cl^- ions (Munns, 2002; Wang *et al.*, 2002). However, salinity is not necessarily toxic as seeds will often recover and germinate when they are transferred to less-saline water (Ungar, 1978). The influence of salinity on seed germination are in line with what occurs in *Salicornia* natural environment since generally, seed germination in *Salicornia* occurs in early spring, when salinity is reduced by high soil moisture content, and temperatures are relatively low (Khan & Weber, 1989). Considering that although being halophytes, the germination of seeds of *Salicornia* is negatively affected by high soil salinity, the use of salt-tolerant PGPB may also contribute to mitigate salt stress and improve germination efficiency in field conditions.

3.6.2. Effect of seed storage time on the germination efficiency

Results demonstrated that seed germination is negatively correlated with storage time (Figure 10). PERMANOVA analysis showed that final germination (FG) of seeds significantly dropped as the storage time and salinity increased (P = 0.0001) (Table 3, Appendix B). For example, seeds from 2014 (2 years of storage), presented FG notably lower than seeds collected in 2016 (from 73.33 ± 9.24 % to 9.33 ± 4.62 % for 0 ‰), and for 20 ‰ and 30 ‰ treatments no germination was observed (Table 2, Appendix A). Storage time also delayed the start of

germination. For seeds collected in 2014, the first germinated seed was observed after 10 days, while for the 2015 seeds the mean time for the first germination was 7.6 days and for the 2016 seeds the corresponding period was 2 days (Table 2, Appendix A). Mean daily germination (MDG) also decreased significantly with both storage time (P = 0.0001) and salinity (P = 0.0005) (Table 4, Appendix B). Lower MDG values were observed in seeds stored for 2 years (2014) for both 20 and 30 ‰ treatments (0.00 ± 0.00 % day $^{-1}$) whereas seeds from 2016 germinating in distilled water presented the highest MDG (11.43 ± 5.16 % day $^{-1}$) (Table 2, Appendix A). The interaction between storage and salinity was significant for MDG (P = 0.0041), which indicate that storage time enhances the effect of salinity.

Loss of seed viability during storage might be due to occurrence of physical and chemical changes that alter tensile strength of seed coats and increased their permeability to water and gases, becoming more sensitive to stress, like salinity, during germination (Qaderi *et al.,* 2003). This loss of viability with time always occurs, but the rate depends strongly on storage conditions (Walters *et al.,* 2005). Thus, it is important to consider that this effect may have been aggravated due to inefficient storage, since is advisable to dry the seeds before storage and to maintain temperature below 20 ºC and relative humidity below 60 % (Mbofung *et al.,* 2013), conditions that have not been met in this experiment. The results emphasize the need for information on the factors that determine the efficiency of germination of *Salicornia*, since it is highly affected by storage conditions. This knowledge is crucial not only as basic knowledge on the biology of the species but also in the perspective of its crop cultivation, namely in the sowing seed storage time recommended.

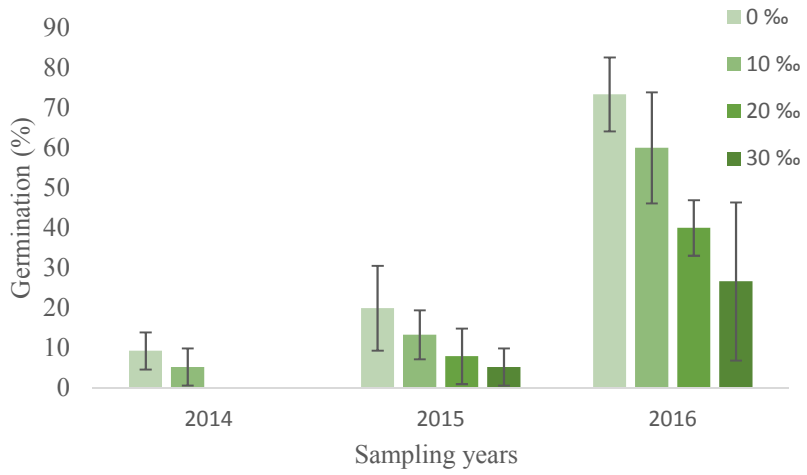

Figure 10 – Final percentage of germination (mean ± SD) of seeds collected in 2014, 2015 and 2016 exposed to four salinity conditions (0, 10, 20 and 30 ‰ NaCl, respectively) over 14 days.

3.6.3. Effect of inoculation with *Bacillus aryabhattai* SP1016-20 on seed germination under saline stress

Some bacteria, in addition to protecting the plant with their biocontrol traits, have the potential to promote seed germination and plant growth (Compant *et al.*, 2005). To test this hypothesis, seeds of *S. ramosissima* were inoculated with *B. aryabhattai* SP1016-20 and final germination and mean daily germination, of uninoculated (control) and inoculated seeds was determined.

PERMANOVA analysis revealed that in general, in both groups, seed final germination and mean daily germination were significantly reduced with increasing concentrations of NaCl ($P = 0.0016$ and $P = 0.0102$, respectively). Inoculation with *B. aryabhattai* did not have a significant effect on both final germination and mean daily germination ($P = 0.618$ and $P = 0.906$, respectively) (Tables 5 and 6, Appendix B). In lower salinities, final germination percentage was similar in inoculated and non-inoculated seeds (Table 3, Appendix A). This is in line with other studies that even report plant-growth inhibition by biocontrol agents, which demonstrates that biocontrol effect is usually not sufficient to promote plant growth (Whipps, 2001). However, at the salinity of 30 ‰, final germination values doubled in inoculated seeds (from 21.3 ± 24.4 % in control to 46.7 ± 26.6 % in inoculated seeds) (Table 3, Appendix A). This effect may be due to the production of ACC deaminase, not determined in this study but reported among *Bacillus* sp. (Barnawal *et al.*, 2013). Under stress conditions, like those generated by salinity, the level of ethylene is significantly increased, with overall negative effects for the plant. ACC deaminase acts by decreasing ethylene levels which, in turn, improves plant salt tolerance (Bhattacharyya & Jha, 2012). These results suggest that this strain of *B. aryabhattai* could enhance germination of *S. ramosissima* under salt stress, by increasing salt tolerance of seeds. However, a further characterization of the isolate in terms of other plant growth promoting traits and more germination tests are needed to prove this hypothesis, since the results are still not statistically significant.

4. Conclusion and future perspectives

Understanding microbial relations in soils and plants can lead to the discovery of microorganisms with agricultural potential. The aim of this study was to isolate and characterize bacteria with biocontrol traits, from *S. ramosissima* rhizosphere. The selected isolates were screened for biocontrol traits like the antagonist effect against *Alternaria* sp., production of hydrogen cyanide (HCN), production of extracellular hydrolytic enzymes (protease and lipase), and other related traits (salt tolerance and motility). All 23 bacterial isolates present one or more biocontrol traits, while only 2 isolates were positive for all tested traits. The isolate chosen for further studies (SP1016-20) displayed motility in addition to all biocontrol traits and was identified as *B. aryabhattai*. The growth behaviour of this strain under different salinities was evaluated, as well as its effect on *S. ramosissima* germination under salt stress. The results showed that *B. aryabhattai* SP1016-20 grew well at all the tested salinities and may improve seeds germination by enhancing salt tolerance. This effect is particularly important because this study demonstrated that both salinity and seed storage time significantly decrease germination of *S. ramosissima* seeds. *Bacillus* spp. are among the most commonly used biocontrol rhizobacteria. They are safe microorganisms able to synthesize several beneficial substances and, as all gram-positive bacteria, produce heat-resistant endospores. This is especially important for strain incorporation into biofungicides, since endospores make bacteria more able to survive harsh conditions when established in the field and enables its formulation into stable products such as dry powder.

Although *B. aryabhattai* SP1016-20 seems to be a promising strain for application in the cultivation of *Salicornia*, further studies are still necessary to harness their potential as bio-inoculant in agriculture. For example, since the ability of biocontrol agents to minimize the risk of infections in plants depends on their ability to colonize plant tissue, it is important to know if the strain forms biofilms and assess if the colonisation was successful, using for example immunofluorescence or molecular techniques. Furthermore, it is necessary to repeat the same experiments with different salinities, since all the biocontrol traits need to be maintained even in high salinities to ensure the beneficial attributes in saline environments. Further investigation is also needed on plant growth promoting traits of the strain to detect the possible reasons to the amelioration of salt stress on germination of seeds. Possible traits to evaluate are the production of phytohormones and siderophores and the ability of the strain to solubilize phosphate and fix nitrogen. The inconsistency of beneficial results when single microbes are used in the field trials has brought an emphasis on co-inoculation of microbes. This is a relevant research line, since co-inoculating this strain with other PGPR that have different biocontrol traits, can sinergicaly improve their effect. Finally, despite the importance of *in vitro* assays, it is crucial to evaluate the

biocontrol potential of *B. aryabhattai* SP1016-20 in field conditions where plants are actually exposed to the pathogens, and to assess if inoculation has any negative effects on the overall microbiological quality in the perspective of the use of *Salicornia* as food.

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6. Appendix A

Table 1 – Results (mean ± SD) on final germination (FG) (%), mean daily germination (MDG) (% day^{-1}) and days to first germination for different salinities and seed types.

	Salinity			
	0 ‰	**10** ‰	**20** ‰	**30** ‰
Central seeds				
FG (%)	45.30 ± 8.30	30.70 ± 6.10	12.00 ± 4.00	9.30 ± 9.20
MDG (% day $^{-1}$)	3.78 ± 0.69	2.56 ± 0.51	1.00 ± 0.33	0.78 ± 0.77
Days to first germination	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Lateral seeds				
FG (%)	44.00 ± 6.90	26.70 ± 8.30	8.00 ± 4.00	0.0 ± 0.0
MDG (% day $^{-1}$)	2.78 ± 0.19	2.67 ± 0.83	0.67 ± 0.33	0.0 ± 0.0
Days to first germination	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	n.a.

n.a. – non applicable

Table 2 – Results (mean ± SD) on final germination (FG) (%), mean daily germination (MDG) (% day^{-1}) and days to first germination for different salinities and sampling years.

	Salinity			
	0 ‰	**10** ‰	**20** ‰	**30** ‰
2014				
FG (%)	9.33 ± 4.62	5.33 ± 4.62	0.0 ± 0.0	0.0 ± 0.0
MDG (% day $^{-1}$)	0.82 ± 0.35	0.46 ± 0.41	0.0 ± 0.0	0.0 ± 0.0
Days to first germination	6.00 ± 0.00	14.0 ± 4.00	n.a.	n.a.
2015				
FG (%)	20.0 ± 10.58	13.33 ± 6.11	8.0 ± 6.93	5.33 ± 4.62
MDG (% day $^{-1}$)	1.58 ± 1.18	1.20 ± 0.26	0.51 ± 0.45	0.53 ± 0.46
Days to first germination	8.00 ± 6.9	5.30 ± 2.3	9.00 ± 7.7	8.00 ± 0.00
2016				
FG (%)	73.33 ± 9.24	60.00 ± 13.86	40.00 ± 6.93	26.67 ± 19.73
MDG (% day $^{-1}$)	11.43 ± 5.16	4.83 ± 1.63	3.54 ± 1.60	1.61 ± 1.21
Days to first germination	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00

n.a. – non applicable

Table 3 – Results (mean ± SD) on final germination (FG) (%), mean daily germination (MDG) (% day^{-1}) and days to first germination for different salinities with or without inoculation.

	Salinity			
	0 ‰	**10** ‰	**20** ‰	**30** ‰
Control				
FG (%)	97.3 ± 2.3	80.0 ± 8.0	61.3 ± 26.6	21.3 ± 24.4
MDG (% day^{-1})	9.60 ± 2.4	16.0 ± 8.7	7.4 ± 1.8	1.6 ± 1.7
Days to first germination	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Inoculated (*B. aryabhattai)*				
FG (%)	92.0 ± 6.9	60.0 ± 28.0	45.3 ± 14.0	46.7 ± 26.6
MDG (% day^{-1})	18.3 ± 9.8	7.9 ± 4.6	4.8 ± 1.1	4.5 ± 2.2
Days to first germination	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.0 ± 0.00

7. Appendix B

Table 1 – Results of two-way PERMANOVA analysis on final germination (FG), carried out on factors of salinity, seed type and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	4693.3	3	1564.4	41.905	0.0001
Seed type	322.67	1	322.67	8.6429	0.0107
Interaction	72	3	24	0.64286	0.5983
Residual	597.33	16	37.333		
Total	5685.3	23			

Table 2 – Results of two-way PERMANOVA analysis on mean daily germination (MDG), carried out on factors of salinity, seed type and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	34.586	3	11.529	40.371	0.0001
Seed type	1.498	1	1.498	5.2457	0.0388
Interaction	1.0903	3	0.36343	1.2727	0.3169
Residual	4.569	16	0.28557		
Total	41.743	23			

Table 3 – Results of two-way PERMANOVA analysis on final germination (FG), carried out on factors of salinity, seed sampling year and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	2983.1	3	994.37	12.226	0.0001
Sampling year	14721	2	7360.4	90.497	0.0001
Interaction	1446.2	6	241.04	2.9636	0.0283
Residual	1952	24	81.333		
Total	21102	35			

Table 4 – Results of two-way PERMANOVA analysis on mean daily germination (MDG), carried out on factors of salinity, seed sampling year and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	78.692	3	26.231	8.7792	0.0005
Sampling year	180.29	2	90.147	30.171	0.0001
Interaction	88.926	6	14.821	4.9604	0.0041
Residual	71.708	24	2.9878		
Total	419.62	35			

Table 5 – Results of two-way PERMANOVA analysis on final germination (FG), carried out on factors of salinity, bacteria and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	11917	3	3972.4	10.203	0.0016
Bacteria	96	1	96	0.24658	0.618
Interaction	1893.3	3	631.11	1.621	0.2306
Residual	6229.3	16	389.33		
Total	20136	23			

Table 6 – Results of two-way PERMANOVA analysis on mean daily germination (MDG), carried out on factors of salinity, bacteria and their interaction. *df*, degrees of freedom. *P*, P-value.

Permutation *N*: 9999

Source	Sum of sqrs	df	Mean square	*F*	*P*
Salinity	463.04	3	154.35	5.8439	0.0102
Bacteria	0.35042	1	0.35042	0.013267	0.906
Interaction	236.1	3	78.7	2.9798	0.0646
Residual	422.59	16	26.412		
Total	1122.1	23			