Università degli Studi di Padova
Dipartimento di Biologia
Corso di Laurea Magistrale in Biotecnologie Industriali


# Inoculation of rhizospheric microbiome from coastal soil is able to enhance salinity tolerance in plants

Relatore: Prof. Alessandro Alboresi
Dipartimento di Biologia

Correlatori: Dr. Sílvia Busoms González
Dipartimento di Plant Physiology

Controrelatore: Prof. Michela Zottini
Dipartimento di Biologia

Laureando: Lorenzo Bettella

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# ABSTRACT

In the past decades the importance of plant microbiome interactions has become clear, playing a vital role in plant health, growth promotion and tolerance to both abiotic and biotic stress. Soil salinity is an increasingly prevalent abiotic stress which by 2050 is expected to affect 50% of agricultural lands. In addition, Mediterranean soils are also affected by alkalinity since they are usually calcareous and carbonated soils. The research in this thesis is focused on: (1) indicate how the microbiome of the rhizospheric soil of *Brassica fruticulosa,* a Mediterranean endemic wild brassica, is able to modulate plant tolerance to salt and salt-alkaline stress; and (2) reveal the role of tolerant PGPBs, selected from the coastal microbiome, on the performance of soybean plants exposed to salinity. To achieve these goals, microbial inocula were extracted from four coastal and three inland soil samples of Catalonia, where natural populations of *B. fruticulosa* can be found. Coastal microbiomes were used to inoculate salt-sensitive *B. fruticulosa* plants collected from the inland (non-saline) locations exposed to salinity. The results demonstrated the beneficial relationship that exists between rhizospheric microbiome and salt stress tolerance of *Brassica* plants, that leads to significant changes in total biomass, pigment, proline, hydrogen peroxide and TBARs content in salt and salt-alkaline stress. The research continued with the isolation of the different bacterial populations present in the coastal-adapted microbiome. Subsequently, these microbes were screened for their plant growth promoting activities through different assays. Four different bacterial strain were selected for their potential in growth improvement of plants, other than *Brassica*, exposed to salt stress. These bacteria were inoculated separately in soybean (*Glycine max* L.) commercial plants, a valuable moderate salt-tolerant leguminous. The preliminary results of this experiment showed that a couple of these bacterial candidates might be modulating the oxidative response of soybean plants growing under salt stress, pointing out its potential as a future bioinoculant.

# 1. INTRODUCTION

## 1.1 Environmental stress in plants

Plants are influenced by a variety of environmental stresses, which are able to diminish and strongly limits growth of agricultural crops. In general, a stress can be defined as any external and internal factor that alters the photosynthetic process and reduces the energy conversion ability of a particular plant (Atafar et al., 2010). In particular, a stress can be classified in two different categories: abiotic and biotic stress. An abiotic stress is an environmental factor that acts on plants resulting on a variation of physical or chemical stress (Finch et al., 2016) whereas biotic stress is a stress that occurs as a result of damage done to an organism by another living organisms, such as bacteria, virus fungi, parasites, beneficial and harmful insects, among others (Gimenez, 2018). To cope with these stresses, a number of strategies have been developed by plants that are able to detect the particular environmental stress and generate an appropriate cellular response. Specifically, abiotic stresses have really strong effects on plant growth and productivity and are becoming increasingly important considering the direct or indirect effects of climate change. It is getting imperative to provide crops with multi-stress tolerance to reduce the pressure of environmental changes and to being able to meet the demand of population growth. Most common known abiotic stress are: temperature, drought, saline, UV, ozone, low nutrients availability, heavy metals and hypoxia.

## 1.2 Plant response and general defenses to abiotic stress

Due to their sessile nature, plants had to confront the stresses previously mentioned and develop potent tactics to avoid or tolerate their adverse effects so as to survive and to thrive. During evolution, plenty of cellular, physiological and morphological defenses have been established. One example can be represented by the cuticle, the most evident and the universal outmost shield (Shepherd et al., 2006). It is an exterior translucent lipid structure that seals the aerial surfaces of the organs. From a structural point of view, this thin hydrophobic layer is basically a cutin matrix filled in and coated by cuticular waxes. It plays a crucial role in restricting liquid and gas exchange with the environment defending both from biotic and abiotic stresses and represents an elegant innovation of land plant to deploy the outermost shield derived from simple molecules. Tremendous progress has been made toward understanding the biochemical and molecular mechanisms that control the defenses, owing to forward and reverse genetic approaches as well as genome wide association analyses conducted on various model species.

A lot of interest is arising nowadays on the beneficial relation that the plants develop with microorganisms that help to contrast these stresses. These microorganisms can influence the accumulation of Reactive Oxygen Species (ROS), the peroxidation of the membrane lipids, the fixation of the nitrogen, among others (Ren et al., 2019). This particular relation and the concept of Plant Growth Promoting Bacteria (PGPB) in response of different abiotic stresses will be treated later and separately on in this thesis.

### 1.2.1 Reactive Oxygen Species

In the normal metabolism of aerobic organisms there is an endless generation of Reactive Species (RS), particularly Reactive Oxygen Species (ROS) including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$), as well as reactive carbonyl species (RCS) like malondialdehyde [MDA; $CH_2(CHO)_2$] and methylglyoxal (MG; $CH_3COCHO$). The two types of RS are intertwined with each other. Indeed, RCS can arise from ROS-induced lipid peroxidation, while ROS can be raised by RCS activities. In particular, ROS molecules, were initially recognized as toxic products of aerobic metabolism. More recently, it has become clear that they play an important role in plants, controlling growth, development, and response to biotic and abiotic environmental stimuli (Das, 2014). The main locations of ROS biosynthesis in plants are chloroplast, mitochondria and peroxisomes. While in normal conditions ROS molecules act as secondary messages in different key physiological processes, in several environmental stress condition they can induce oxidative damages. It exists a delicate balance between ROS production and elimination that, if disturbed, can lead to critical damages to the plant.

The cellular damages present in form of degradation of biomolecules, like pigments, proteins lipids, carbohydrates and DNA (Das, 2014). All these cellular damages can lead to oxidative catastrophe including enhanced photoinhibition and membrane lesions which can be measured by the production of thiobarbituric acid reactive substances (TBARs), mainly in the MDA form derived from unsaturated fatty acid (UFA) peroxidation (Takahashi and Murata, 2008). In particular, $H_2O_2$, a moderately reactive ROS, is formed when $O_2^{\cdot-}$ undergoes both univalent reduction as well as protonation (Quan et al., 2008). $H_2O_2$ is produced by plant cells under normal condition, but overaccumulated by oxidative stress, caused by factors like drought, chilling, UV radiation, among others. Compared to other ROS molecules, $H_2O_2$ has a significantly longer half-life, of 1 ms approximately, for that reason it is able to traverse longer distances and cross plant cell membranes. Specifically, it can cross membrane *via* aquaporins and cover high lengths within the cell causing significant oxidative damages (Bienert et al., 2007). In any cases, $H_2O_2$ is moderately reactive and its damage is fully realized when is converted into more reactive species (Dat et al., 2000).

### 1.2.2 Unsaturated fatty acid

Saturated fatty acids, C16 and C18, are not only the main component of the cuticle but are also the fundamental blocks of the biological membranes, the fundamental biological barrier. Plants cellular membranes are made mainly by phospholipids and glycolipids that both contain a glycerol core liked with two FA-derived attached. These FAs have a profound impact on the membrane properties, in particular, their unsaturation degree is fundamental for the membrane fluidity (Hazel, 1995). Membrane fluidity is strongly linked to various abiotic stresses, extreme temperature in particular. Both cold-driven rigidification and heat-driven fluidization can cause biomembrane dysfunction. For that reason, membrane remodeling is of paramount importance in plants. Indeed, adjusting the unsaturation degree of the FA tails in bilayer interior is favored by plants in offsetting thermal

perturbations to maintain the optimal range of fluidity. Particularly, there is a close relationship between chilling tolerance and the unsaturation level of chloroplastic phosphatidylglycerol (PG) (Nishida and Murata, 1996). Desaturation of PG has been shown to protect photosystem II (PSII) cold-enhanced photoinhibition, which contributes to chilling tolerance (Moon et al., 1995). This can also be applicable to different kind of stresses that intensify photoinhibition, for example, upon NaCl treatment the content of UFAs including PG increased.

## 1.3 Salt and salt-alkaline stress

### 1.3.1 Salinity and Its effect on Plants

Salinity is considered one of the most devastating environmental stresses that drastically affects the productivity and the quality of crops across the world. More than 20% of the world's cultivable lands are dealing with the adversity of salt stress and these salt-sensitive areas are continuously increasing, due to both natural and anthropogenic activities. Like other abiotic stresses, it negatively affects plant growth and reproduction in many ways. It produces nutritional and hormonal imbalances, ion toxicity, oxidative and osmotic stress, and an increase in plant susceptibility to diseases. As a consequence, these primary stresses result in oxidative stress and can cause a series of secondary stresses like complications in taking $K^+$ into cells, decreased photosynthetic activity, generation of ROS and programmed cell death (Zhu et al., 2002). As a whole, these primary and secondary stresses lead to various physiological and molecular changes affecting plant growth by inhibiting photosynthesis (Van et al., 2020). Moreover, increased levels of ions, such as $Na^+$ and $Cl^-$, trigger ion toxicity due to the disruption of ion homeostasis and the unavailability of essential nutrients which are essential for a correct growth.

From a molecular point of view, salt stress affects light-harvesting complex formation (Chen et al., 2015), key enzymes in the photosynthesis process as ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), sugar signaling key molecules as sucrose, fructose and glycolysis process (Shumilina et al., 2019), among others. Finally, salt-induced water deficit conditions declined stomatal conductance, thus reducing photosynthetic activities of the plants and accelerating the accumulation of ROS. In general, different crops respond to salinity in different ways; glycophytes are salt-sensitive plants and mostly show growth and total yield reduction during salt stress. On the other hand, halophytes are salinity tolerant plants, which have adapted to salinized environments and even take advantage from high salt concentrations for optimal growth (Su et al., 2020). The majority of plants are glycophytes strongly affected by the high salinity in the soil.

### 1.3.2 How does the salt enter the plant?

Ion uptake can occur *via* the symplastic and the apoplastic pathway (Gao et al., 2007). In most conditions, the contribution of the apoplastic flux in the total transpirational volume flow is less than 1%, nevertheless, it can be increased when the transpirational demand is high (Pitman, 1982). In some species, as rice, this particular flux is especially pronounced and could be responsible for up to 50% of total $Na^+$ uptake (Malagoli et al., 2008) and, in addition, it was shown that up to

50% of $Cl^-$ translocation to rice shoots is also apoplastic (Shi et al., 2013). Regarding net uptake *via* the symplastic pathway of $Na^+$ into roots, it is assumed to be catalyzed by a specific complement of transporters. The evidence points towards a large number of different systems, but their relative contribution, and therefore physiological relevance, is often unclear. Nonselective cation channels (NSCCs) are encoded by two big gene families: glutamate receptor-like channels (GLRs) and cyclic nucleotide-gated channels (CNGCs) and blocked by $Ca^{2+}$(Leng et al., 2002). Even though, in plants like *Arabidopsis*, it appears that a large fraction of inward $Na^+$ flux is carried by NSCCs, either the genetic identity of the contributing channels is obscure or their putative role has not been quantified. From another point of view, in monocotyledonous plants, the situation is likely to be different. In contrast to *Arabidopsis*, which contains only the subclass 1, $Na^+$ selective, AtHKT1 isoform (particular kind of ion transporter), monocots have multiple HKT isoforms. *Arabidopsis* HKT1 functions in long-distance transport of $Na^+$ *via* xylem and phloem but in several cereals HTKs can mediate $Na^+$ uptake (Berthomieieu et al., 2003).

### 1.3.3 Salt stress sensing and response

The sensing of salt stress signals initiates a wide array of complex transduction pathways in plants. Early signals that trigger a salt stress response include $Na^+$, the alteration of intracellular $Ca^{2+}$ levels and the accumulation of ROS. Under salt stress, excess $Na^+$ is perceived and triggers downstream sodium stress responses (**Figure 1)** (Gong, 2021).

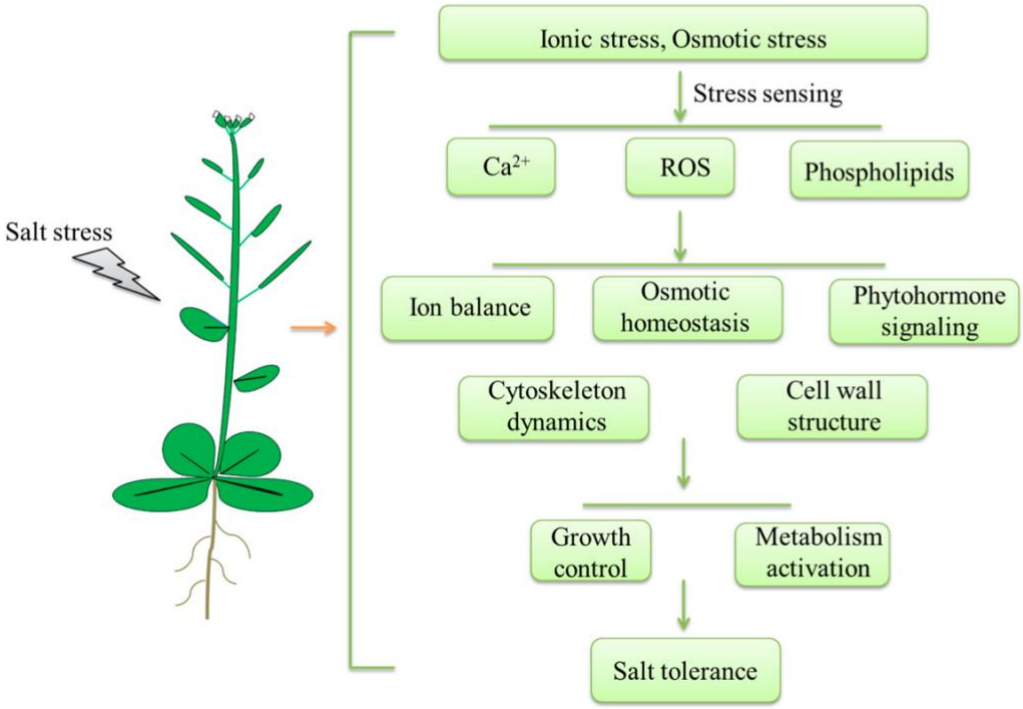


**Figure 1.** A simplified model of the plant salt stress sensing and response (Zhao et al., 2021)

Salt stress induces ion and osmotic stress, which leads to an increase in the $Ca^{2+}$ concentration in the cytosol. In fact, salt stress is always associated with the activation of $Ca^{2+}$ channels. This ion works as an important second messenger with

a specific calcium signaling cascade associated. The accumulation of $Ca^{2+}$ activates ROS signaling and alter their phospholipid composition. These signals are able to activate adaptive processes to alleviate salt stress, including maintaining an ion balance and osmotic homeostasis, inducing phytohormone signaling, regulating cytoskeleton dynamics, cell wall integrity and structure. Subsequently, through an array of signal transduction pathways, plant growth is temporarily slowed and metabolism is activated to increase salt tolerance in different ways depending on the plant species (Zhao et al., 2021).

### 1.2.4 Physiological and biochemical adaptation against salinity

In order to determine the responses of plant to salt stress, firstly the factors that cause the stress has to be addressed. It is a priority to know whether the toxic effect caused by excessive salt accumulation in the plant or the osmotic stress caused by soluble salts in the soil in which the plant is growing. In general, plants give rapid effective responses to external induced osmotic stress, they give slower responses to accumulation of $Na^+$ in the leaves. As a whole, it is possible to categorize the main different physiological and biochemical response during saline stress in:

- *Osmoprotectants:* These are high rated soluble compounds (McNeil et al., 1999). Compatible osmolytes protect the structure and maintain osmotic balance within the cell by continuous water influx. These compatible solutes or osmolytes consist of sugars, sugar alcohols, amino acid derivatives, and sulfonium compounds. Glycine betaine, sugars, Proline (Pro) and polyols are the most important osmoprotectants (Yokoi et al., 2002). As a response to salinity and drought stress, soluble carbohydrates accumulate in plant tissues and lead to osmoprotection, osmotic adjustment, carbon storage and radical scavenging. In particular, a large body of data suggests a positive correlation between Pro accumulation and plant stress. Pro, an amino acid, plays a highly beneficial role in plants exposed to various stress condition. It is able to act in three different ways during stress, i.e, as metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012). The phenomenon of Pro accumulation is known to occur under water deficit (Checin et al., 2006), salinity (Munns et al., 2005), low temperature, heavy metal exposure, UV radiations (Verbruggen et al., 2008), among others. For example, it has been demonstrated that Pro accumulation is responsible for scavenging ROS and other free radicals in oxidative stress condition (Chen et al., 2005). As demonstrated in *Arabidopsis* roots, when applied Pro exogenously reduced the levels of ROS, indicating the strong ROS scavaging potential of this osmoprotectant (Cuin et al., 2007). In response to salt stress, the concentration of Pro increases in plant cells while other amino acids as cysteine, arginine and methionine decrease (El-Shintinawy et al., 2001). In addition to that, intracellular Pro can also function as an organic nitrogen reserve during this stress recovery.

- *Antioxidants:* Antioxidants enzymes and non-enzymatic compounds play an essential role in detoxifying ROS induced by salinity and other stresses. In general, salt tolerance is strongly correlated with the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), among others. Non-enzymatic compounds are also able to enhance salt tolerance in plants like for example ascorbate, anthocyanin and glutathione. Other

non-enzymatic antioxidant such as vitamin E, carotenoids and lipoic acid have been reported to protect the plant subjected to oxidative stress

- *Polyamines:* Polyamines are small, polycationic, aliphatic molecules with low molecular weight. The most common examples are putrescine, spermidine and spermine. When plants are exposed to salinity stress, the endogenous level of polyamine increases (Takahashi et al., 2010). In addition to that, the application of exogenous polyamine increases the level of endogenous polyamines during stress reminding a sort of positive feedback amplification during a stress condition. The positive effects of these molecules include maintaining membrane integrity, reduction in ROS production, controlling the accumulation of $Na^+$ and $Cl^-$ ions in different organs, the regulation of gene expression for the synthesis of osmolytes (Roy et al., 2005).

- *Hormone Regulation:* Abscisic acid (ABA) is considered a stress phytohormone that mitigates the effect of the different stresses in plants. Indeed, it has been studied that the expression of ABA is upregulated under stress condition including osmotic stress. Salinity causes water deficit and osmotic stress and, as a consequence, the production of ABA increases in roots and shoots (Cabot et al., 2009). ABA is an important cellular signal that modulates the expression of several salt and osmotic stress-responsive genes (Fukada et al., 2006). In addition to that, Salicylic acid (SA) and brassinosteroids (BR) also are involved in abiotic stress response in plants. For example, the endogenous level of SA and the activity of SA biosynthetic enzymes increased in rice under salt stress (Sawada et al., 2006). Similarly, also the exogenous application of SA and BR leads to improved salt tolerance in plants. For instance, the application of BR enhanced the activity of different antioxidant enzymes such as SOD, POX and APX (El-Mashad et al., 2012).

### 1.2.5 Salt-alkaline combined stress

The effect of saline-alkaline soil on plants include the effect of both salt and alkaline stress. Depending on the salt and content and pH value the degree of salt alkaline condition are classified as mild (salt content less that 3%, pH 7.1-8.5), moderate (salt content 3-6%, pH 8.5-9.5) or severe (salt content more than 6%, pH exceeds 9.5) (Oster et al., 1999). Both of these aspects are able to cause metabolic disorders. In particular, alkaline stress is induced by the presence of $NaHCO_3$ and $Na_2CO_3$ that cause a rise in pH value with further damages to the plant metabolism. Therefore, in addition to ion toxicity and osmotic stress, high pH can disturb pH stability, destroy cell membrane integrity, decrease root vitality and photosynthetic function (Kaiwen et al., 2020; Zhang et al., 2017). In addition to that, many studies have elucidated the causative role of these stress in more serious trophic ion imbalances, reduced osmotic adjustment capacity, inhibition of the antioxidant system and an overall plant growth reduction (Amirinejad et al., 2017; Chen et al., 2017; Wang et al., 2020).

## 1.3 Plant Growth Promoting Bacteria (PGPB)

### 1.3.1 PGPB definition and main characteristics

Soil is filled with a high variety of microscopic life forms including, bacteria, fungi, protozoa and algae. Among them, bacteria are by far the most common with a concentration often from $10^8$ to $10^9$ cells *per* gram soil, from which, only 1% is cultivable (Schoenborn et al., 2004). Both the number and the types of bacteria that are found in the soils are influenced by soil condition and characteristics, as well as the number and the type of plants present in those soils (Glick and Bernard, 2012). In addition to that, bacteria are not generally equally distributed in the soil, the highest concentration of microorganisms is found around the roots of the plant (i.e rhizosphere). This unequally distribution is due to the presence of different nutrients as sugars, amino acids, organic acids and other organic molecules that derives from the plant metabolism (Badri et al., 2009). Independently from the different number of bacteria present in a soil, they can interact with the plant in three different ways. The interaction can be: beneficial, neutral or harmful (from the perspective of the plant) (Brimeconde, 2000). In any cases, the particular effect that a bacterium has on a plant could change depending on the different conditions. For example, a particular bacterium that helps plant growth by providing either nitrogen or phosphorus, is beneficial only in the case that these elements are limited in a particular soil and would be neutral when a significant amount of chemical fertilizer is provided.

The bacteria that are able to promote plant growth are indicated as a whole as plant growth-promoting bacteria (PGPB) and include those that are free-living, those that form specific interactions with plants, bacterial endophytes that colonize internal plant tissues and cyanobacteria, also called blue green algae (Glick and Bernard, 2012). All these different categories of bacteria act with two main mechanisms on the plant growth: direct (improving resource acquisition or modulating plant hormones levels) and indirect mechanisms (decreasing the inhibitory effects of biotic and abiotic stresses) (Glick et al., 2012). In particular, as direct mechanisms they can act in processes as: Nitrogen fixation, Phosphate solubilization, sequestering iron, modulating Phytohormones levels, among others. On the other hand, as indirect mechanisms there are: Antibiotics and lytic enzymes, Siderophore production, Ethylene production and Induced Systemic resistance. In addition, it has been studied that the mixture of PGPB is more efficient in its positive action due to their synergistic action in enhancing plant growth and protection. In the context of crop health management, the use of PGPB has been studied as an alternative or integrated approach to reduce the use of chemical fertilizers or toxic pesticides. In general, the characteristics of these bacteria are quite conspicuous, they are naturally occurring non-pathogenic bacteria that enhance plant growth through their excellent root-colonizing ability (Van Loon., 1998). These bacteria have also been used for wastewater treatment (Bashan et al., 2003), to reduce soil erosion and to restore marine mangroves. The most widely studied group of PGPB is rhizobacteria, that is associated with plant growth proportion and disease control (Kloepper 2003).

Some example of PGPB are: *Agrobacterium radiobacter*, *Acinetobacter* spp., *Arthrobacter* spp., *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus fimus*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus mucilaginous*, *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Delfitia acidovorans*, *Paenobacillus macerans*, *Pantoea agglomerans*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas solanacearum*, *Pseudomonas syringae*, *Serratia entomophilia*, *Streptomyces griseoviridis*, *Streptomyces lydicus*, and *Rhizobia* spp. (Bashan et al., 2003).

## 1.3.2 Mechanisms of action

### 1.3.2.1 Biological nitrogen fixation

Diazotrohpic bacteria have the ability to fix atmospheric nitrogen ($N_2$) to make it available for absorption by the plants. The most studied example of nitrogen fixation in PGPB is represented by the group of mycrosimbionts collectively known as rhizobia. Rhizobia were studied extensively form physiological, biochemical and molecular point of view for what regards its role in legumes plant growth promotion (Andrews et al., 2017). In addition to that, there is a wide variety of free-living bacteria, for example *Azospirillum* spp., that are able to contribute to the nitrogen fixation process in non-leguminous plants. For example, it has been studied that Nitrogen-fixing diazotrophic bacteria, such as *Gluconacetobacter diazotrophicus* PAL5, *Herbaspirillum rubrisubalbicans* M4 and *Azospirillum brasilense* SP7 improve the total nitrogen uptake in sugarcane plants (Somasekhar et al., 2003). In detail, nitrogenase (*nif*) genes are required to allow biological nitrogen fixation (BFN) in bacteria. They include structural genes, genes involved in the activation of a Fe protein, iron molybdenum cofactor biosynthesis, electron donation and regulatory genes. Structurally, are found in a cluster of 20-24 kb with seven operon encoding a total of 20 proteins (Dixon, 2004). Because of the high complexity of this system, genetic strategies to improve BFN have been complicated. The nitrogen fixation process occurs only in a clade of angiosperms known as nitrogen-fixing clade (NFC) which include, for example, the Leguminosae family of the Fabales order. In response to nitrogen starvation and the presence of these particular nitrogen-fixing bacteria, these particular plants form symbiotic organ on their roots known as nodules (Fred et al., 2002). Nodules are infected by a large population of these bacteria, which are able to convert atmospheric nitrogen into ammonia in a particular protected environment.

### 1.3.2.2 Phosphorus Solubilization

Phosphorus (P) is a key nutrient in the plant nutrient and its deficiency could cause multiple problems during plant growth and development. The amount of phosphorous in the soil is generally high, nevertheless most of it is insoluble and not available to support plant growth (Peix et al., 2001). Indeed, the insoluble P is present in the soil in form of apatite or one of the several organic forms including inositol phosphate and phosphotriester, among others. Some PGPB called phosphate-solubilizing bacteria are able to solubilize and mineralize phosphorus in order to increase the accessibility of this nutrient (Richardson et al., 2001). Specifically, the solubilization of P is subordinate to the action of low molecular

weight organic acids as gluconic and citric acid synthesized by different soil bacteria (Rodriquez et al., 1999). On the other hand, the process of P mineralization occurs through enzymes called phosphatases that induce the hydrolysis of phosphoric esters. It is important to underline that solubilization and mineralization can occur in the same bacterial strain (Guang-Can, 2008). There are different examples of the activity of PGPBs in the P solubilization process, for instance, it has been studied that the introduction of a particular bacterium called *Baciullus megaterium* into the rhizosphere of rice increase the availability of P from insoluble sources of soil-bound phosphate (Lucero et al., 2021).

#### 1.3.2.3 Sequestering iron

Iron is the fourth most abundant element on earth mainly in the form of ferric ion or $Fe^{3+}$ that cannot be assimilated rapidly by either bacteria or plants. Iron, in this form, is only not soluble and for that reason the total amount of iron available is extremely low (Guang-Can et al., 2008). To being able to survive, bacteria synthesize low-molecular mass siderophores, molecules with a really strong affinity for $Fe^{3+}$, and membrane receptors that can bind the Fe-siderophore complex. It significantly helps the iron uptake by these microorganisms (Hider et al., 2010). There are more than 500 known siderophores and 270 of these have the chemical structure defined (Hider et al., 2010). The benefits of the bacterial siderophores on plant development has been demonstrated in a multitude of experiment, furthermore, the provision of iron to plants is fundamental when the plants are subjected to an environmental stress such as heavy metal stress. In this particular case, siderophores help to reduce the stress on plants by high concentration of metal in the soil (Belimov et al., 2005).

#### 1.3.2.4 Phytohormones

Plant hormones play several fundamental roles in plant growth and in the response of plant to various stresses (Davies et al., 2004). In addition, during the life of a plant, this can be exposed to a wide number of non-lethal stresses that can limit its growth until either the stress is eliminated or the metabolism is adjusted to overcome this particular condition (Glick et al., 2007). When subjected to a particular stress, plants tend to adjust the level of endogenous phytohormones in order to decrease the negative effect of the environmental stress but is not always enough to overcome the adverse condition (Salamone, 2005). In this context, PGPBs are known to produce Indole-3-acetic acid (IAA) cytokinin, giberellins and ethylene stress mediating enzymes like 1-aminocyclopropane-1-carboxylic acid (ACC)deaminase. Inoculation with IAA-producing PGPR has stimulated seed germination, accelerated root growth and modified the architecture of the root system with a general increase of the root biomass (Zhang et al., 2015). Vessey (2003) has also specifically reviewed the production of hormone by PGPR and its implication in biofertilization for plant growth promotion. Concerning ACC deaminase, this particular enzyme produced by PGPB acts on ACC that is an immediate ethylene precursor in plants (Saravanakumar et al., 2007). Decreased ethylene levels allow the plant to be more tolerant to a wide variety of environmental stresses. Additionally, the use of PGPR possessing ACC deaminase in mitigating flooding, salinity, drought and pathogenic stresses has been demonstrated in several studies (Glick, 2014).

### 1.3.3 Role in salt stress tolerance

#### 1.3.3.1 Osmolyte accumulation

Under high salinity, the prevention of water loss is necessary to overcome the osmotic stress and maintaining osmotic balance in the cell (Hussain et al., 2013). During an osmotic stress, low molecular weight, electrically neutral and highly soluble solutes termed as osmolytes are accumulated into the cell. In addition to the ones produce by the plant, the osmolytes produced by PGPB can further improve water potential and hydraulic conductivity that positively affects stomatal opening and transpiration rate in the plant (Ilangumaran and Smith, 2017). Many reports show that PGPB inoculation significantly increases the level of osmoprotectants in several plants under salt stress condition (Saberi-Riseh et al., 2020). For example, it has been studied that a rice cultivar inoculated with the PGPB *Bacillus amyloliquefaciens* was found to increase the level of Pro and total soluble sugars compared to uninoculated seedlings (Tiwari et al., 2017).

#### 1.3.3.2 Aquaporins

Plants aquaporins (AQPs) are proteins member of the highly-conserved membrane protein family known as major intrinsic protein (MIP) reported in many organisms (Abascal et al., 2014). In particular, in plants AQPs enable water uptake from the soil helping root hydraulic conductivity maintenance. PGPBs under salt stress can regulate the expression of these proteins that in turn improve plant water relationship. For example, in a study, the bacterium *Azospirillum brasilense* was found to enhance the expression of the *HvPIP2;1* transcript in the roots of barley which helps in establishing better plant-water relationship along with increased salt tolerance (Zawoznik et al., 2011). Some phytohormones such as ABA produced by PGPBs are also linked to the upregulation of a specific family of AQPs, the plasma membrane intrinsic protein (PIP) under stress conditions. For instance, several genera of PGPBs such as *Bacilluls, Pseuudomonas, Azospirillum* have reported to produce ABA and to be involved in the regulation of PIPs under salt and drought condition (Salomon et al., 2014; Cohen et at., 2015). Currently, a large number of aquaporin intrinsic membrane proteins are known, however, amongst them only PIPs are reported to be expressed by certain PGPB under salt stress. More detailed studies are needed to find out connection of PGPB in modulating activities of other AQPs that are involved in the protection of plants from osmotic stress as stress responses.

#### 1.3.3.3 Ion homeostasis

Apart from osmotic stress, high intracellular concentration of $Na^+$ and $Cl^-$ may create ion toxicity affecting many cellular functions (Serrano et al., 1999). As mentioned before, the different ions enter the root cells mainly through simplastic (mainly *via* non-selective cation channels) or through apoplastic movement (*via* cell wall). To maintain the ion homeostasis and reduce the $Na^+$ influx, plants use salt overly sensitive (SOS) signaling pathway. Different cascade of reactions in the SOS signaling pathways (SOS1, SOS2, SOS3) are known to mediate cellular signaling under saline conditions to maintain ion homeostasis (Ji et al., 2013). Inoculation of PGPB could alleviate effectively the salt stress in plants by modulating the related

gene expressions. In addition, PGPB are able to upregulate the SOS genes pathways in saline stress condition. In particular, it has been studied that they are able to influence the activity of the $Na^+/H^+$ antiporter, the $H^+$ pumping pyrophosphatase ($H^+$-ppase), HKT1 and RNCS and rbcL (encoding RuBisCo subunits) to decrease ion toxicity in plants (Ji et al., 2013; Chen et al., 2016; El-Esawi et al., 2018; Isayenkov and Maathuis, 2019). Furthermore, PGPB can help ion homeostasis increasing $K^+$ level and maintain $K^+/Na^+$ ratio by removing excess $Na^+$ out of the cell in plant stressed with salinity. In a specific study, PGPB *Azospirillum lipoferum* and *Azotobacter chroococcum* were reported to enhance the level of $K^+$, balancing the $K^+/Na^+$ ratio and consequently reducing the $Na^+$ level in maize plants affected with salinity (Abdel Latef et al., 2020). It has been clearly proved that the maintenance of ion homeostasis is necessary to improve the survival of plants under salinity. It would be interesting to further elucidate the mechanisms of gene regulation and cross talks between plants and PGPB for maintaining the ionic balance under multiple stresses caused by soil salinity.

## 1.4 Plants in the research

During the different experimentations two different plants were used. The first one, a novel model plant namely *Brassica fruticulosa* is an endemic plant of the Mediterranean and is relatively new. The second one is Soybean (*Glicyne max* L.), already strongly characterized and studied. In the next section these two plants will be described.

### 1.4.1 *Brassica fruticulosa* and the importance of *Brassica* crops

**Figure 2.** *B. fruticulosa plant.*

The genus *Brassica* belong to the Brassicaceae or mustard family, that includes approximately 435 genera and over 3500 species distributed across the globe. The genera *Brassica* contains nearly 100 species, many of which are grown globally as a vegetable in form of cabbage, broccoli, kale and raddish; as spice in various colors of mustard; as an oil crop placing third after palm and soil. This genus is relatively salt tolerant, in particular, the polyploid species (as *B. juncea* and *B. napus*) have a higher salt tolerance compared to their diploid counterparts. In addition to that, there are difference in salinity resistance between inter and intra specific members of the *Brassica* genus. *Brassica fruticulosa* is an endemic plant of the Mediterranean, is a wild diploid considered to be salt tolerant with a genotypic variance within the species. *B. fruticulosa* has proven to be a valuable source of resistance against various insect pests and pathogens (Agrawal et al., 2011; Arti et al., 2012;). Additionally, there have been different successful attempts in crossing *B. fruticulosa* with cultivated *Brassicas* developing different introgression lines, for instance, with *B. juncea* to introduce resistance to mustard aphids (Agrawal et al., 2011).

### 1.4.2 Soybean (*Glicyne max* L.)

**Figure 3.** *Glycine max L. plant.*

The soybean (*Glycine max* (L.); 2n=40) is an economically important leguminous crop with high nutritional quality due to high protein and low carbohydrates content. Soybean contain significant amounts of phytic acid, dietary minerals and B Vitamins. It is native to East Asia and produced mainly by Argentina, United states, Brazil and China. It is ranked number one in world production in the international trade markets among the major oil crops, such as cottonseed, groundnut (peanut), sunflower seed, etc. In addition, it is the most important protein source for feed farm animals. Soybean plants are able to assimilate nitrogen (N) in form of nitrate ($NO_3^-$) and ammonia ($NH_4^+$). But the major source of N is obtained by the BNF. As explained before, one of the most efficient nitrogen fixation system is constituted by the symbiotic interaction established between soybean and members of the rhizobia family (Bianucci et al., 2018) that take place in a new organ formed in the root called nodule. An example of nitrogen fixation bacteria in Soybean plants is represented by *Bradyrhizobium diazoefficiens* USDA110 (Bianucci et al., 2018) that has been proved to cause the development of nodules in this particular leguminous.

It has been studied that environmental stresses have an important impact on the BNF process reducing or completely inhibiting the process (Zaharan, 1999). Concerning to salt resistance, Soybean is classified as a moderately salt-sensitive crop and the salt stress is able to negatively affect its yield (Phang, 2008). High salt levels impose negative impacts on growth, nodulation, agronomy traits, seed characteristics. With increasing salinity levels, soybean production can be reduced by as much as 40% (Papiernik et al., 2005).

# 2. OBJECTIVES

The objectives in this research can be divided into two parts corresponding the two different experiments performed. The first one, regarding *B. fruticulosa* plants, is of explorative nature to prove the causative role of microbiome inoculation in the saline stress resistance. The second one, regarding Soybean (*Glicyne max* L.) plants, is of more specific nature, representing the natural follow-up of the first experiment to investigate the effects of some candidate plant growth promoting bacteria derived from *B. fruticulosa* microbiome in another plant species. In particular:

*Brassica fruticulosa* experiment objectives:

- Evaluate the impact of the *B. fruticulosa* rhizosphere microbiome on plant growth exposed to salt/salt-alkaline stress.
- Decipher the oxidative response of *Brassica* plants exposed to saline or combined stress and evaluate the role of the microbiome in this response;
- Isolate plant growth promoting bacteria that are able to improve plant growth and abiotic stress resistance from the microbiome of *Brassica fruticulosa* rhizosphere.

Soybean (*Glicyne max* L.) experiment objectives:

- Analyze the effect of selected bacterial strain candidates (BS), derived from *B. fruticulosa* rhizospheric microbiome, when inoculated in *Glicyne max* L. plants during salt stress;
- Decipher the oxidative response of Soybean plants to salt stress condition when inoculated with different BSs.
- Select the most effective bacterial-legume interaction in terms of salt-stress inducing resistance for future studies.

# 3. MATERIAL AND METHODS

## 3.1 Seeds and soil collection

During March 2022, soil samples and seeds from different *B. fruticulosa* populations were collected from four coastal (saline) locations: Tossa del Mar (TOS), Garraf (GAR), Escala (ESC), Roses (ROS); and from three inland (non-saline) locations: Palafrugell (PALA), Pau (PAU), and Tordera (TOR). GAR and ESC refer to locations with salt-alkaline soils while TOS and ROS refer to salt-siliceous soils. **Figure 2** shows the different locations from where soil samples and *B. fruticulosa* plant seeds were retrieved. Coastal locations are indicated in blue while inland locations are indicated in yellow. The samples were retrieved from various locations to maximize the number of possible plant growth promoting bacteria identification effective against salt and salt alkaline stresses.


**Figure 4.** Location of the different *B. fruticulosa* plants and soil samples

In particular, soil sample were taken from approximately 5 cm underground, from *B. fruticulosa* rhizosphere, and stored in polyethylene bags at 4°C until use. The microbiome and plant populations derived from non-saline locations are referred as M_SS (salt sensitive microbiome) and P_SS (salt sensitive plant population) while the ones derived from the coastal locations are referred as M_ST (salt tolerant microbiome) and P_ST (salt tolerant plant population) or M_SAT (salt-alkaline tolerant microbiome) and P_SAT (salt-alkaline tolerant plant population) depending on the precise location of the sample. The microbiome and the plants derived from salt-siliceous soil and from the salt-alkaline soil will be treated separately because they were subjected to a different kind of stress and they adapted in a different way to tolerate these specific conditions. Therefore, M_ST were inoculated exclusively in P_SS and P_ST plant populations and studied in control and salt stress conditions, while M_SAT were inoculated only in P_SS and P_SAT plant populations in control and salt-alkaline stress conditions.

## 3.3 Disinfection of the seeds

Seeds were surface sterilized using 30% bleach (mixed with dd$H_2O$) for 15 minutes before being washed with dd$H_2O$ a total of 5 times, then submerged in $KNO_3$ and placed in a 4°C fridge for 3 days priming. Seeds were consequently sown in plates with MS medium (0.8% agar, pH 6) (Murashige and Skoog, 1962).

## 3.4 Microbiome extraction

The microbial communities were extracted from the soil samples using the method described by Salas-Gonzalez et al., (2020). Briefly, 500 ml of soil and 500 ml of d$H_2O$ were vigorously shaken and then settled for 20 minutes. After that, the mixtures were poured through a mesh-lined funnel into beakers, allowing soil to settle again before pipetting the supernatant into a 500 ml centrifuge tube. The samples were spun at 4000*g* until the supernatant was no longer turbid. The pellet was subsequently resuspended in MES (0.5 g/L, pH 6.0), and the centrifugation was repeated. The resulting pellet was resuspended again in 100 ml of MES, and this final step was repeated before a final resuspension with 50 ml of MES. One ml of each soil sample was taken and mixed with 80 glycerol at a 1:1 ratio and stored at -80°C for future sequencing. All material was autoclaved prior to use.

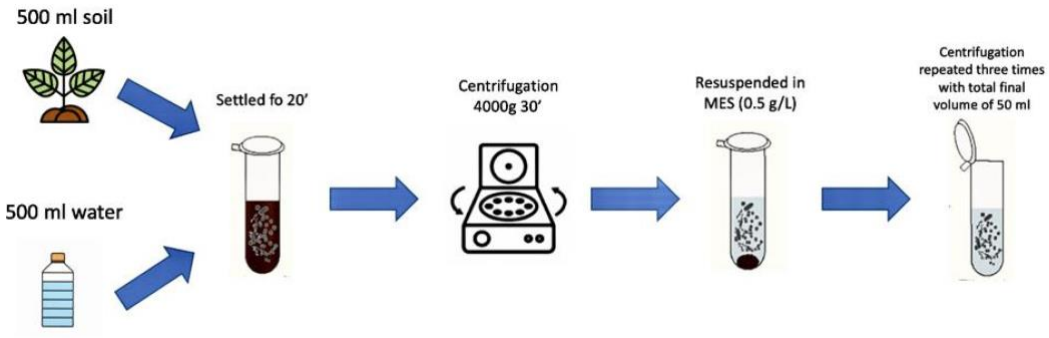


**Figure 5.** Representative scheme of the microbiome extraction technique.

## 3.5 Bacterial microbiome salinity tolerance

The number of viable cells in each microbiome was determined as colony forming units (CFU/ml) by the drop–plate method described by Somsegeran and Hoben (1994). Briefly, from the 1 ml aliquots of the extracted microbiome, serial dilutions were cultured in 10% TY (tryptone-yeast) agar solid media supplemented with 200 g/ml of cycloheximide, an antifungal. To determine the salinity tolerance of the microbiome, the dilutions were also drop plated into TY agar plates that were supplemented with [0mM], [100mM] and [150mM] NaCl. Afterwards, cultures were incubated for 72 h at 28 °C and after 24 h CFUs/ ml were counted.

## 3.6 Plant cultivation and microbiome interaction assay

### *Brassica fructiculosa* plants

Seeds were surface sterilized as previously detailed. The seeds were sown in 120 x 120 mm square Petri dishes containing MS medium (Murashige and Skoog, 1962) with 0.8% agar pH 6. After 7 days, seedlings of population were transferred to a semi-hydroponic system with a sterilized mixture of sand:perlite (ratio 2:1) irrigated with ½ Hoagland nutrient solution (HS) (Hoagland and Arnon, 1950) adjusted to a pH 6. After a week, plants per population were inoculated close to the root system with 1 ml of each microbiome suspension, non-inoculated plants remained without bacterial addition. Plants were exposed to two different abiotic stress, salt (S) and the combined salt-alkaline (SA) treatment. For that, two days post-inoculation, for the treatment groups, the NaCl concentration was increased every 3 days following this specific order: [50mM], [100mM], [150 mM]. Once reached the maximum concentration, plants under the Salt treatment (S) were irrigated with ½ HS + 150 mM NaCl, pH 6, for 10 days and harvested. Plants submitted to Salt-Alkaline treatment (SA) were irrigated with ½ HS + 135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3, for 10 days and harvested. Plants were germinated and grown in a controlled growth chamber (22°C, 10 h light/dark photoperiod, irradiance 80 $mmolm^{-2}s^{-1}$).

## 3.7 Photosynthetic pigments quantification

For chlorophyll content determination, the method adapted from Vernon (1960) was followed. 0.1 g of fresh leaf was homogenized in 15 ml of ethanol 80 % (v/v) and was placed in 100°C water bath for 15 minutes. Afterwards, the resulting solution was measured with the spectrophotometer at 650, 665 and 450 nm corresponding to the maximum absorbance for chlorophyll *a*, chlorophyll *b* and carotenoids respectively. Data were expressed as mg of chlorophyll ($mg^{-1}$) dry weight. For the calculation, Vernon and Mac Kinney formula was applied (modified by Mac Kinney, 1941):

Chlorophyll *a*: **11.63** x ($OD_{665nm}$) - **2.39** x ($OD_{650nm}$)

Chlorophyll *b*: **20.11** x ($OD_{650nm}$) - **5,18** x ($OD_{665nm}$)

Total Chlorophyll: Chlorophyll *a* + Chlorophyll *b*

Carotenoids: **0.02** x ($OD_{450nm}$)

## 3.8 Hydrogen peroxide content determination

The hydrogen peroxide content was estimated spectrophotometrically by its reaction with KI (Alexieva et al., 2001). Leaves were homogenized with 0.1% trichloroacetic acid (TCA) in liquid nitrogen. The homogenate was centrifuged at 10,000*g* for 20 min and the supernatant (0.16 ml) was mixed with 0.16 mL of 100

mM K-phosphate buffer and 0.68 mL reagent (1M KI w/v in water). The reaction was kept in the dark and developed for 1 h after which the absorbance at 390 nm was recorded. The $H_2O_2$ content was estimated from a standard curve prepared with aliquots of 1 mM $H_2O_2$.

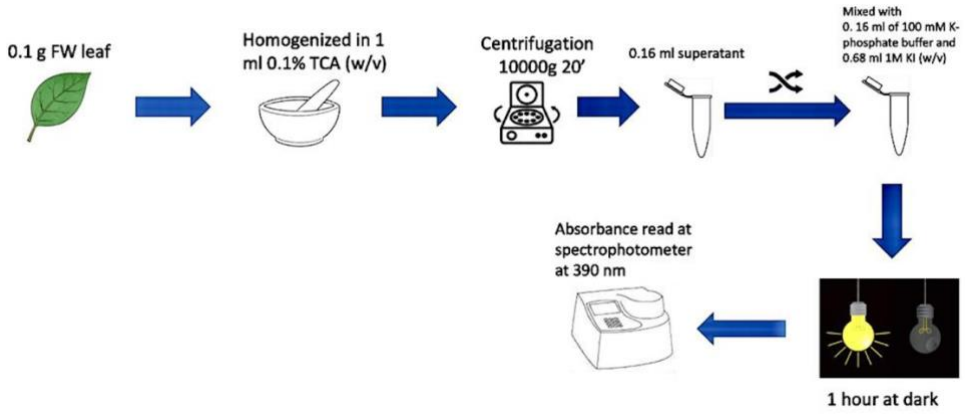


**Figure 6.** Representative scheme of the hydrogen peroxide content determination technique.

## 3.9 Lipid Peroxidation measurement

To determine the level of lipid peroxidation in the plant the method from Heath and Packer (1968) was performed. In brief, thiobarbituric acid (TBA) reacts with the aldehyde group of MDA (final and major product of lipoperoxidation) and other aldehyde reactive substances (TBARs) to give a pink compound with maximum absorbance at 532 nm. Leaves (0.3 g) were homogenized in 3 mL of 0.1% (w/v) TCA solution. The homogenate was centrifuged at 10,000*g* for 5 min and the supernatant (0.75 mL) was mixed with equal volume of 20% TCA containing 0.5% (w/v) TBA. The mixture was heated at 95 °C (30 min), cooled on ice and centrifuged at 10,000*g* (15 min). TBARs were determined at 532 nm, corrected by the non-especific absorption at 600 nm, and its concentration was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

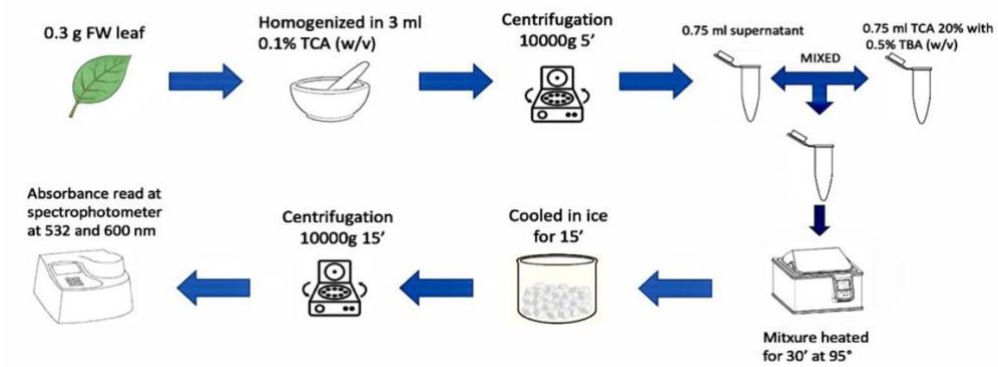


**Figure 7.** Representative scheme of the TBARs content determination technique.

## 3.10 Proline quantification

Proline content was quantified by following the method of Bates et al. (1973). Leaves (100 mg) were homogenized in 3% (w/v) sulphosalicylic acid, and

centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was supplemented with ninhydrin (47 mM), phosphoric acid (0.8 M) and glacial acetic acid (0.25 M) in a test tube. The mixture was heated for 60 min at 98 °C in a water bath and then allowed to cool at room temperature. The mixture was extracted with 0.3 vol of toluene and the absorbance was read at 520 nm. The amount of proline was calculated using a standard curve prepared with known concentrations of the amino acid**.**

## 3.11 Bacterial isolation and DNA extraction from microbiome

Individual bacterial colonies were obtained with the drop-plate method. Briefly, each colony was streak plated onto fresh plates contained 200 g/ml cycloheximide, this was repeated until uncontaminated growth was observed (Kuklinsky-Sobral et al. 2004). The long-term preservation of the microorganisms was carried out by taking aliquots of cultures in late logarithmic phase supplemented with sterile glycerol until reaching a final concentration of 40%. The prepared suspensions were stored at -20°C.
Total genomic extraction was performed using a kit by Nzytech called NZY Microbial gDNA Isolation kit. In addition to that some selected bacteria stain were sequenced for what regards the RNA 16S to be able to recognize the specific bacteria thanks to the informatics tool BLAST.

## 3.12 Plant cultivation and Plant-Microorganisms interactions assay

**Soybean** (*Glicyne max* L.)

Soybean seeds cv. Don Mario 4200 RR (IVC group) were surface sterilized with ethanol and $H_2O_2$ (6%) and germinated at 28 ºC in Petri dishes on a layer of Whatman N°1 filter paper and moistened cotton, until the radicle reached 2-3 cm (around 72 hours) (Vincent, 1970). Pre-germinated seeds were aseptically transferred into pots as explained before. The plants were grown in a controlled environment chamber (light intensity of 200 µmol $m^{-2}s^{-1}$, 16 h day/8h night cycle, a constant temperature of 28ºC and a relative humidity of 50%) and irrigated with Hoagland nutrient solution (Hoagland and Arnon, 1950). 10-day post-emergence plants were inoculated with a mixture of *Bradyrhizobium diazoefficiens* USDA110 and the isolated strains obtained as explained before (1:1). From this moment increasing concentration of NaCl was added every 4 days in the following order: [50mM], [100mM], [150 mM]. The plants were harvested 10 days after the maximum salinity had been reached (35 days old) in order to assure nodule formation.

## 3.13 Plant harvest

*Brassica* and Soybean plants were harvested by washing their roots with tap water, roots were separated from shoot and growth measurements were performed. Root length (cm), fresh weigh (g) of the aerial and root zones were measured independently. One leaf was weight and dried for two days at 60 °C to measure water content and estimate the total dry weight (Biomass). The rest of roots and leaves were flash frozen in liquid nitrogen and stored at -80°C for further analysis.

In soybean leaves, $H_2O_2$, Lipid peroxidation and proline level was determined following the procedures explained before.

## 3.14 Statistical analysis

One-way or multivariate ANOVA was used to test for significant differences between means of all variables determined for each microbiome type, treatment and population. To test for correlations between two variables, a bivariate fit was applied. To perform multiple comparisons of group means we used the Tukey's HSD test. All statistical analyses were performed using SAS Software JMP v.16.0 (https:// www. jmp. com/ es_ es/ home. html).

# 4. RESULTS AND DISCUSSION

## 4.1 *Brassica fruticulosa*

In this study, the rhizospheric microbiome of *Brassica fruticulosa* populations was explored in order to evaluate the contribution of the core microbiome to the salinity tolerance of this species and subsequently select keystone microorganisms. To identify the effects of the microbiome during salt and salt alkaline stress, the microbiome was inoculated into various *B. fruticulosa* plant populations as described previously.

### 4.1.1 Impact of salinity on the microbiome viability

Prior of investigating the effect of the microbiome on plants, the bacterial viability and tolerance to salinity of the *B. fruticulosa* rhizosperic microbiome was analyzed (**Table 1**). As conducted in similar studies (e.g. Kearl et al., 2019), it was mandatory to confirm that the bacteria in the rhizospheric soil were adapted to a saline environment in order to provide beneficial effects to the *B. fruticulosa* plants.

Table 1. Effect of salt on bacteria derived from different *Brassica fruticulosa* plant populations

| | Bacteria viability (UFC/ml) | | |
|---|---|---|---|
| | 0 mM NaCl | 100 mM NaCl | 150 mM NaCl |
| PAU (M_SS) | $1{,}2 \pm 0{,}12 \times 10^{7\ A}$ | $3{,}3 \pm 0{,}3 \times 10^{6\ B}$ | $2{,}5 \pm 0{,}19 \times 10^{6\ C}$ |
| PALA (M_SS) | $1 \pm 0{,}19 \times 10^{7\ A}$ | $3{,}4 \pm 0{,}4 \times 10^{6\ B}$ | $2{,}7 \pm 0{,}4 \times 10^{6\ B}$ |
| ROS (M_ST) | $3{,}5 \pm 0{,}37 \times 10^{6\ A}$ | $3{,}3 \pm 0{,}33 \times 10^{6\ A}$ | $3{,}5 \pm 0{,}28 \times 10^{6\ A}$ |
| TOS (M_ST) | $2{,}7 \pm 0{,}11 \times 10^{6\ B}$ | $4{,}2 \pm 0{,}62 \times 10^{6\ A}$ | $4{,}8 \pm 0{,}58 \times 10^{6\ A}$ |
| GAR (M_SAT) | $1{,}3 \pm 0{,}41 \times 10^{5\ A}$ | $7{,}3 \pm 2{,}95 \times 10^{4\ A}$ | $9 \pm 1{,}4 \times 10^{4\ A}$ |
| ESC (M_SAT) | $2{,}7 \pm 0{,}31 \times 10^{4\ A}$ | $2{,}3 \pm 0{,}14 \times 10^{4\ A}$ | $3 \pm 0{,}17 \times 10^{4\ A}$ |

Values represent the mean ± SE (n = 5). Different letters indicate significant differences among treatments according to the Duncan`s test ($P < 0.05$).

The viability test results showed that all the tolerant *B. fruticulosa* populations (ROS, TOR, ESC and GAR) did not modify this variable under any salt concentration tested. However, PALA and PAU populations, the sensitive ones, reduced the viability under 100 and 150 mM of salt in a significant way compared to control treatment. The reduction of the bacteria viability observed under increasing [NaCl] concentration in the M_SS derived bacterial culture shows the limited tolerance that these bacteria have to the applied stress. It is important to underline that the M_SAT derived bacteria showed a lower microorganism density compared to M_ST and M_SS. It is probably due to the fact that these bacteria were extracted from a soil where the combined stress (salt-alkaline) is present. Indeed,

these bacteria are adapted to survive in adverse conditions and their viability can be lower but their performance in providing positive effect on the plant growth and resistance may be higher. Indeed, it has been studied that different abiotic stresses are able to alter the rhizospheric composition and their abundance and it has a beneficial effect on counteracting the salinity stress on plants (Santos et al., 2021). In addition, the correlation between between stress tolerant bacteria and plant growth promotion was confirmed in multiple previous studies (Aisha et al., 2011; Naser et al., 2022)

### 4.1.3 Total biomass of *B. fruticulosa* plants exposed to stress

The comparison between control and salt treatment (**Figure 8a**) showed that, regardless of the inoculation condition, the total aerial biomass was significantly reduced in all populations exposed to salt treatment compared to control, with the exception of the sensitive populations (P_SS) inoculated with the tolerant microbiome (M_ST). The comparison among inoculation conditions in a same treatment indicates that in control treatment no differences were observed, however in the salt treatment, P_SS inoculated with the M_ST exhibited higher biomass in a significant way compared to non-inoculated plants. Regarding tolerant populations, no differences were observed between inoculated and no inoculated plants in the same treatment group. Similarly, the analysis of aerial biomass on plants exposed to the combined treatment (salt/alkaline) (**Figure 8b**) showed similar results as in the salt treatment just with some exceptions. Not only the sensitive population increased the biomass level when inoculated with the tolerant microbiome (M_SAT) but also the tolerant population (P_SAT).

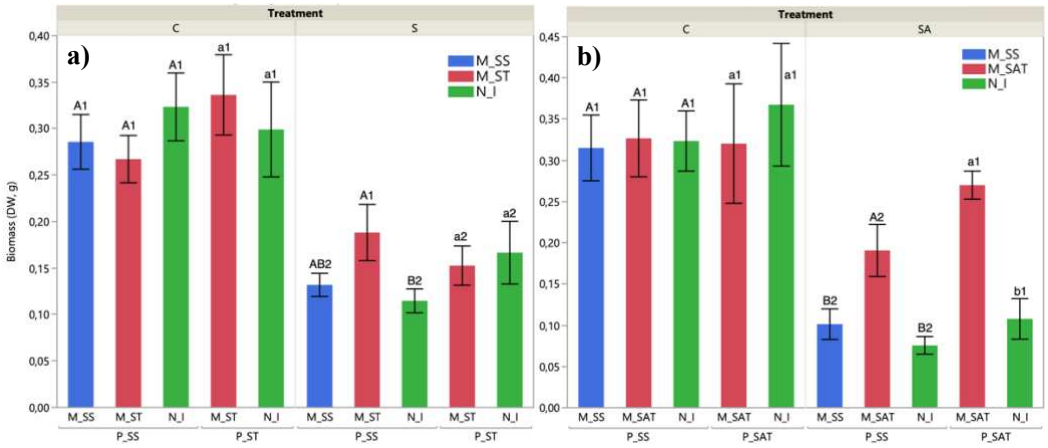


**Figure 8**. Total aerial biomass (g) of: **a)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt treatment for 10 days; and **b)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline conditions for 10 days. DW = dry weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between inoculation for the same treatment (c or s) ($p < 0.05$) according to ANOVA test for P_ST and P_SAT and to multiple mean comparison ($p < 0.05$) according to Tukey's Test for P_SS. Different numbers

indicate significant differences between treatments for each growth condition ($p < 0.05$) according to ANOVA test.

These results confirm that sensitive *B. fruticulosa* plants are experiencing symptoms of stress due to the salt and salt-alkaline treatments, impacting their growth and in consequence a total biomass reduction. These results are in agreement with previous studies performed with other plant species such as cotton (Guo et al., 2019), rice (Tian et al., 2016) and wheat (Li et al., 2020). Regarding the different microbiome inoculation, our results suggest that inoculation of sensitive populations with a tolerant microbiome, independently of the treatment applied (Salt or Salt/alkaline), promoted the growth of the plant by increasing the total biomass. These results are supported by Santos et al. (2021) and Yuan et al. (2019) studies, where rice and *Hibiscus* plants inoculated with tolerant microbiomes also showed higher biomass under salt stress conditions.

When focusing on the tolerant populations, only in the combined treatment a significant increase of the biomass was observed compared to non-inoculated plant, suggesting a more specific plant microbiome interaction. It seems that P_ST are less dependent of the microbiome than P_SAT in order to cope with salinity stress, therefore the genetic component of the host is more relevant when we only apply a single stress. On the other hand, in a salt-alkaline environment the role of the microbiome is more critical. Many studies have shown that when an abiotic stress intensifies, plants ask for help and specific plant-microorganism interactions increase proportionally (Sandrini et al., 2022), justifying why non-inoculated P_SAT and P_SS plants are the most impacted by the salt/alkaline stress.

### 4.1.4 Evaluation of leaf pigments content

Photosynthetic efficiency in plants depends on photosynthetic pigments, such as chlorophyll *a* and *b* and carotenoids. These pigments play important roles in the photochemical reactions during photosynthesis (Olaiya and Poloamina 2013) and are essential to a correct growth of a plant. The comparison among inoculation conditions in a same treatment showed that in control no differences in pigments content were observed, however in the salt treatment, sensitive populations showed that the inoculation with the tolerant microbiome enhanced significantly the total chlorophyll and carotenoids compared to non-inoculated plants. In P_ST, no differences were observed between inoculated and no inoculated plants (**Figure 9a**/**b**).

In plants exposed to the combined treatment (salt/alkaline), inoculation of tolerant microbiome in control condition showed a significant increase of the variable when compared to non-inoculated plants, or inoculated with the sensitive microbiome (**Figure 9c/d)**. Curiously, the inoculation of the M_SS in P_SS plants submitted to salt-alkaline stress enhanced the carotenoids content in a similar way to the inoculation with the M_SAT (**Figure 9d**), indicating that their own microbiome also has an effect in pigments synthesis despite the stress.

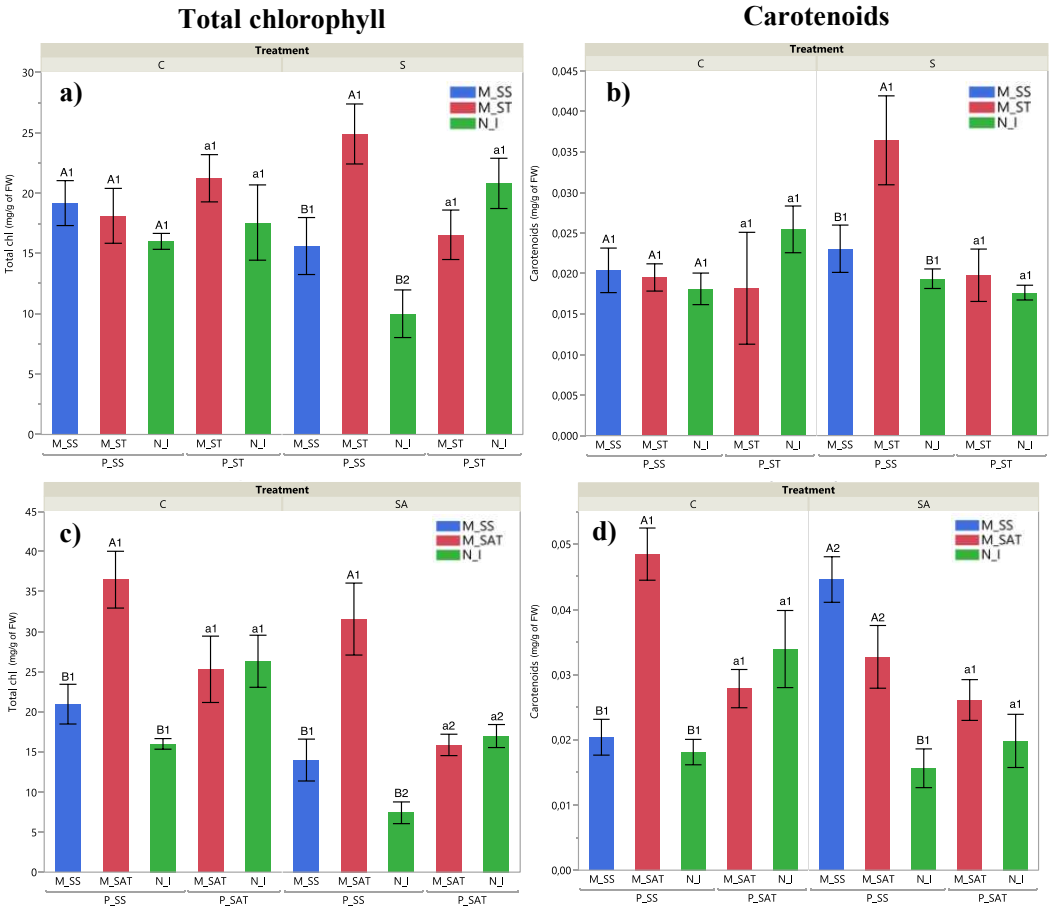


**Figure 9.** Total chlorophyll content (mg/g FW) of **a)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt treatments for 10 days; and **c)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* plants no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline treatments for 10 days. Total carotenoids content (mg/g FW) of **b)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* plants no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt conditions for 10 days; and **d)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* plants no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline conditions for 10 days. FW = fresh weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between inoculation for the same treatment (c or s) ($p < 0.05$) according to ANOVA test for P_ST and P_SAT and to multiple mean comparison ($p < 0.05$) according to Tukey's Test for P_SS. Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to ANOVA test.

Chlorophyll content decreasing has been linked with salinity stress previously (Sayyad-Amin et al., 2016) and it is an indicator of the health status of the plant that is directly correlated with the plant growth (Adams et al., 2005). However, research regarding pigment content in *Brassica fruticulosa* plant or other, subjected to the stress applied in this thesis and inoculated with a tolerant or sensitive microbiome is scarce or inexistent. The results obtained suggest that the inoculation of sensitive

population with tolerant microbiome, independently of the treatment applied, promote the synthesis of chlorophyll by the plant.

Carotenoids are tetraterpene pigments fundamental in the photosynthesis process acting along with chlorophylls. In addition, these molecules act as photo-protectors, antioxidants and precursors of plant hormones in non-photosythetic organs of the plant (Maoka et al., 2020). Furthermore, they act as scavangers of ROS molecules formed during photooxidative stress in order to reduce the damage caused by these molecules (Strazalka et al., 2003). In this sense, the increase of carotenoids content consequent to the inoculation of the tolerant microbiome could be linked to the particular ability of these molecules in reducing the stress status of the plant. For all these reasons, a microbial consortium able to increase the carotenoid content under stressful conditions is of high interest. The results in this thesis show a strong correlation between carotenoids content and tolerant microbiome inoculation in both salt and salt-alkaline stress. Similar result was obtained in *Raphanus sativus* and maize during salt stress after inoculation of a selected bacterial strain, isolated from a natural microbiome (Mohamed et al., 2012; Rojas et al., 2012).

### 4.1.5 Ionome analysis of plants exposed to salt

Plants able to adapt to alkaline-saline conditions must efficiently manage multiple stress factors: high $Na^+$, low osmotic potential, low availability of micronutrients, especially Fe and Zn, and imbalance of carbon metabolism due to dark fixation of inorganic carbon (Poschenrieder et al., 2018). Maintenance of a high $K^+/Na^+$ ratio is critical for salinity tolerance (Rubio et al., 2020) and alkaline salinity has an especially severe inhibitory effect on this parameter (Jixiang., 2012), especially in Brassica species (Pérez-Martín et al., 2021). To study the nutritional status of our plants, the leaf ionome (10 elements) was quantified when plants were harvested.

Under control condition, in *B. fruticulosa* sensitive plants each microbiome is modulating differently the nutrition profile of each plant, enhancing in general the absorption of essential nutrients such as P, Ca, Mg, Mn and Fe (**Figure 10a/b**). Contrastingly, we did not detect a clear effect in salt-alkaline tolerant population, inoculated with their own microbiome in any condition (**Figure 1c**), with the exception of a significant K reduction in all cases (**Figure 1c/f/h**). This suggest that salt alkaline tolerant plants have not a strong interaction with their microbiome and their salinity tolerance might be more intrinsic of the plant ecotype. Despite this, when the salt-alkaline tolerant microbiome was inoculated to sensible plants submitted to neutral salt stress, plants were able to accumulate more K and less Na in their tissues (**Figure 1d**), preventing that Na reached toxic levels. Under salt-alkaline stress we could not observe this pattern (**Figure 1g**) probably because the stress is too severe and, even with the inoculation of a tolerant microbiome, sensible plants are not able to cope with the effects.

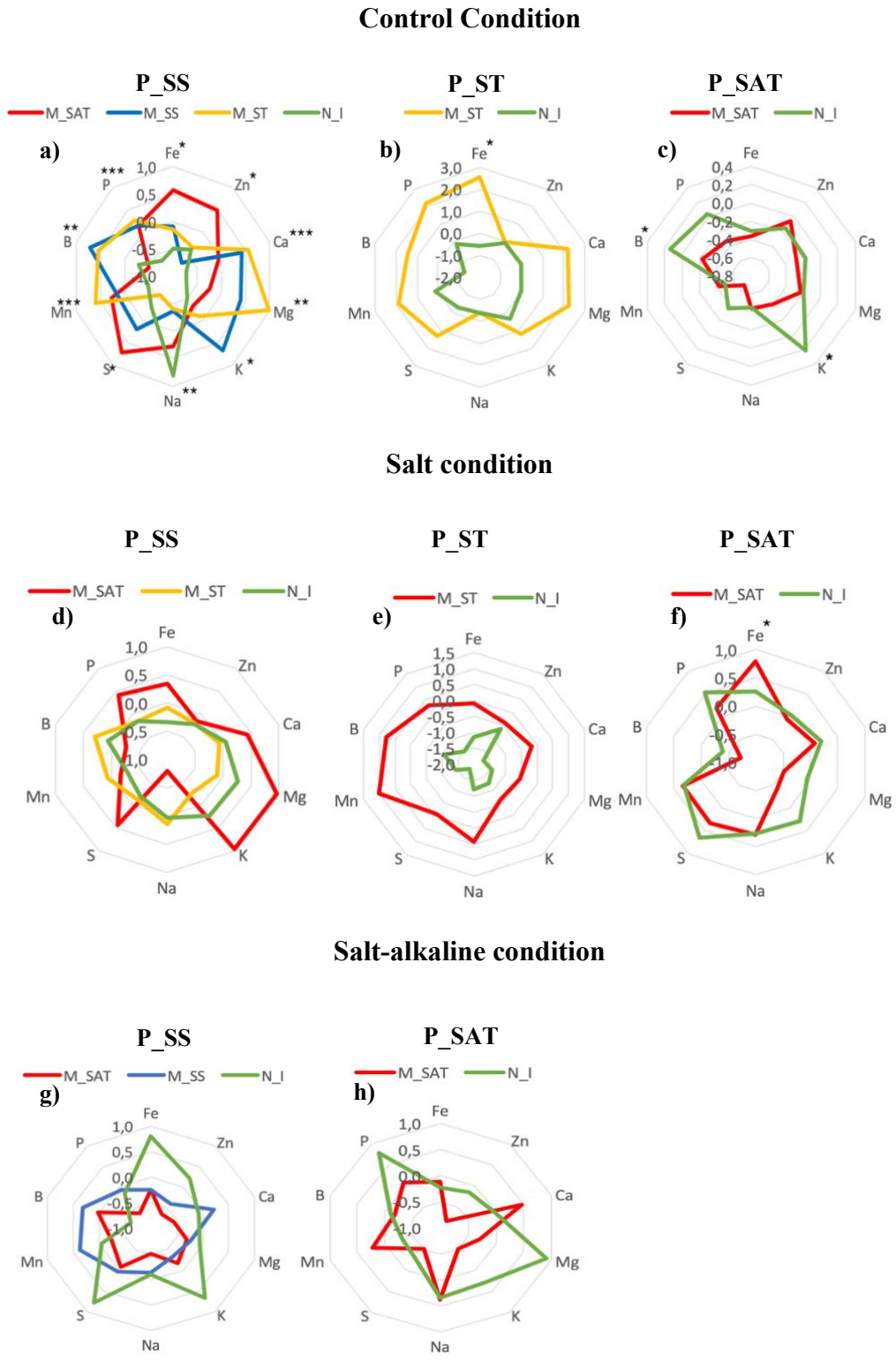


**Figure 10.** Normalized difference of 10 elements in control (**a)**, **b)**, **c)**), salt (**d)**, **e)**, **f)**) and salt-alkaline (**g)**, **f)**) condition of salt sensitive (P_SS), salt tolerant (P_ST) and salt-alkaline tolerant (P_SAT) *B. fruticulosa* plants inoculated with M_SS (blue line), M_ST (yellow line), M_SAT (red line) or without inoculation (N_I, green line) for 10 days (n=10). C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Elements exhibiting significant differences between M_ST or M_SAT inoculation and N_I (according to a ANOVA test) are marked with an asterisk (*, $P < 0.05$).

We also observed that salt tolerant plants inoculated with their own microbiome exhibited better nutrition status than non-inoculated plants under control and salt stress (**Figure 1b/e**). However, when the salt tolerant microbiome was inoculated to the sensitive population submitted to salinity stress, the ionomic profile of these plants did not substantially changed (**Figures 1d/g**), indicating that the inoculation with this particular microbiome is not preventing the Na uptake under salinity but might be enhancing other mechanisms of salinity tolerance. It has been studied that It is possible that these bacteria are able to interact with plants via various routes that can and enhance resistance against stresses in different ways as upregulating of lipid metabolism, modifying plant hormones metabolism and enhancing different abiotic and biotic stress tolerance mechanisms (Glick et al., 2012)

### 4.1.6 Evaluation of the oxidative status under salinity

#### 4.1.6.1 Hydrogen peroxide content

The hydrogen peroxide content is an indicator of the oxidative status of plants. As anticipated before this molecule is a ROS widely generated in aerobic biological system and over accumulated in response to a biotic and abiotic stress (Shu-Hsien et al., 2005). The comparison between treatments showed that salt addition increased the content of $H_2O_2$ in N_I sensitive plants compared to control (**Figure 11a)**. On the other hand, the comparison among inoculation conditions in a same treatment showed that under control conditions no differences were observed, independently of the tolerance of each population (**Figure 11a)**. However, inoculation of plants (both P_SS and P_ST) with the tolerant microbiome significantly reduced the $H_2O_2$ content compared to non-inoculated plants (**Figure 11a**). On the other hand, in the SA treatment, the comparison between control and treated conditions showed that sensitive and tolerant populations inoculated with the tolerant microbiome, enhanced the $H_2O_2$ content when compared to each control (**Figure 11b**). Moreover, in the SA treatment, no significant differences were found regardless of which microbiome was inoculated (**Figure 11b**).

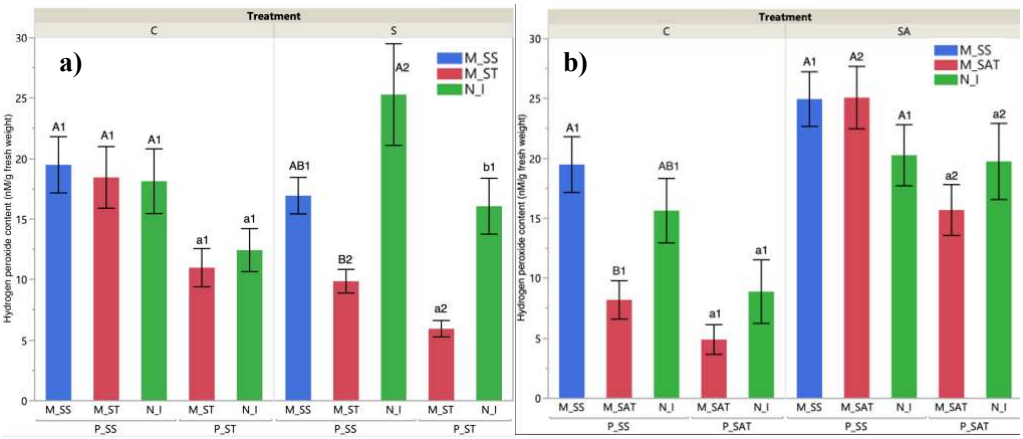


**Figure 11.** Total Hydrogen peroxide content (nM/g FW) of **a)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt treatments for 10 days; and **b)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* populaations no inoculated (N_I, green bar) or inoculated with M_SS

(blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline treatments for 10 days. FW = fresh weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between inoculations for the same treatment (c or s) ($p < 0.05$) according to ANOVA test for P_ST and P_SAT and to multiple mean comparison ($p < 0.05$) according to Tukey's Test for P_SS. Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to ANOVA test.

The bacteria present in the microbiome of salt tolerant plant seems to modify the total level of $H_2O_2$ in the plant by reducing it content under S treatment. This positive effect is extended to both sensitive and tolerant populations indicating the fundamental role that the microbiome is playing in helping plant to cope with saline stress environment. In contrast, when analyzing the SA stress, an enhance of the radical level was observed when inoculating tolerant microbiome into plants, indicating that the plant oxidative response is treatment specific. Although no references could be found regarding this variable on plants inoculated with a tolerant microbiome this results could be extrapolated with those referring to PGPB inoculation. In this sense, these results are supported by previous discoveries that confirmed the link between PGPB inoculation and $H_2O_2$ reduction during salt stress (Li et al., 2017; Santos et al., 2018). Regarding the combined stress condition, the results showed that there is no correlation between $H_2O_2$ content and the tolerant microbiome inoculation.

#### 4.1.6.2 TBARs content

TBARs are formed as a bioproduct of the lipid peroxidation. Assay of TBARs mainly measures the MDA content present in the sample. Here, the comparison between control and salt treatment (**Figure 12a**) showed that regardless the population the TBARs content was significantly enhanced in non-inoculated plants exposed to salt treatment compared to control. The comparison among inoculation conditions in a same treatment showed that in control treatment no differences were observed however, in the salt treatment, sensitive populations showed that the inoculation with the sensitive and tolerant microbiome reduced the TBARs content in a significant way compared to non-inoculated plants (regardless of the population analyzed). In a similar way, the analysis of the TBARs content levels on plants exposed to the combined treatment (salt/alkaline) (**Figure 12b**) showed similar results as in the salt treatment with a strong TBARs content reduction in inoculated plant during salt-alkaline stress.

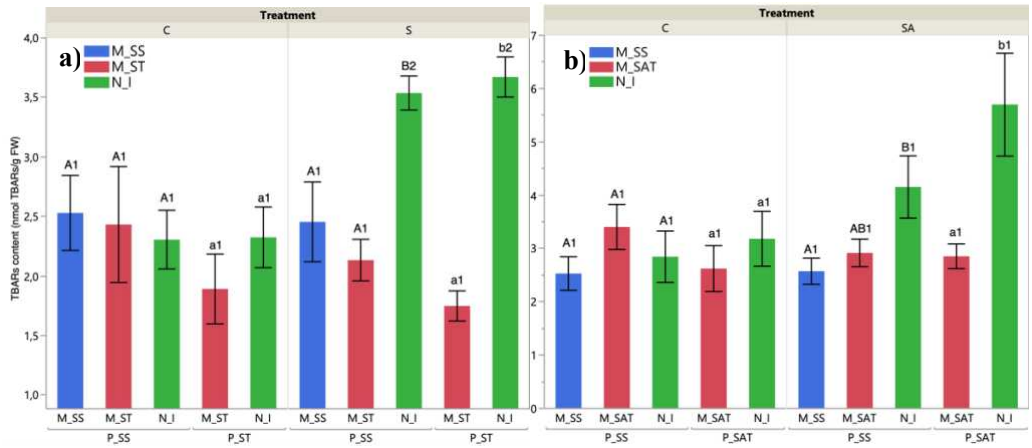


**Figure 12.** Total TBARs content (µg/g FW) of **a)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt conditions for 10 days; and **b)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline conditions for 10 days. Data represent the mean ± SE (*n=10*). FW = fresh weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Different letters indicate significant differences between inoculations for the same treatment (c or s) ($p < 0.05$) according to ANOVA test for P_ST and P_SAT and to multiple mean comparison ($p < 0.05$) according to Tukey's Test for P_SS. Different numbers indicate significant differences between inoculation for a same growth condition ($p < 0.05$) according to ANOVA test.

In general, lipid peroxidation is a physiological index of both biotic and abiotic stress responses, hence is often used as a biomarker to assess stress-induced cell damage or death. Concerning the results obtained in this study, it can be suggested that, although a high level of $H_2O_2$ was detected, the inoculation of tolerant microbiome in salt and salt alkaline stress condition leads to a significantly decrease in TBARs contents indicating that these microorganisms are able to modulate the oxidative response by increasing the level of ROS, acting as a signal molecule, to activate antioxidant mechanisms and avoid severe oxidative damage. The peroxidation of unsaturated lipids in membrane is the most apparent symptom of oxidative stress. It is a highly deleterious process in plants, which affects membrane properties, ultimately triggering the cell death process (Yamamoto et al., 2001). In particular, MDA content, was found to enhanced in rice (Ma et al., 2012), Pea (Yamamoto et al., 2001) and Soybean (Cakmak and Horst, 1991) when exposed to salt. In addition, several researches showed that inoculating a single PGPB into plants subjected to different environmental stresses, reduced TBARs or carbonylation of proteins, indicating the protective role of these bacteria under an unfavorable growth condition (Bianucci et al., 2018; Furlan et al., 2013).

### 4.1.6.3 Proline Content

The comparison between control and salt treatment (**Figure 13a**) showed that, regardless of the inoculation conditions, the level of total proline content was significantly enhanced in all populations exposed to salt treatment compared to control.
The comparison among inoculation conditions in a same treatment showed that in control treatment no differences were observed, however in the salt treatment, both populations showed that the inoculation with the tolerant microbiome enhanced the amino acid content in a significant way compared to non-inoculated plants and to plant inoculated with the sensitive microbiome.
In the same way, the analysis of the proline content on plants exposed to the combined treatment (salt/alkaline) (**Figure 13b**) showed similar behavior as in the salt treatment with a significant higher proline content when inoculating plants with the tolerant microbiome inoculation in the salt-alkaline condition.

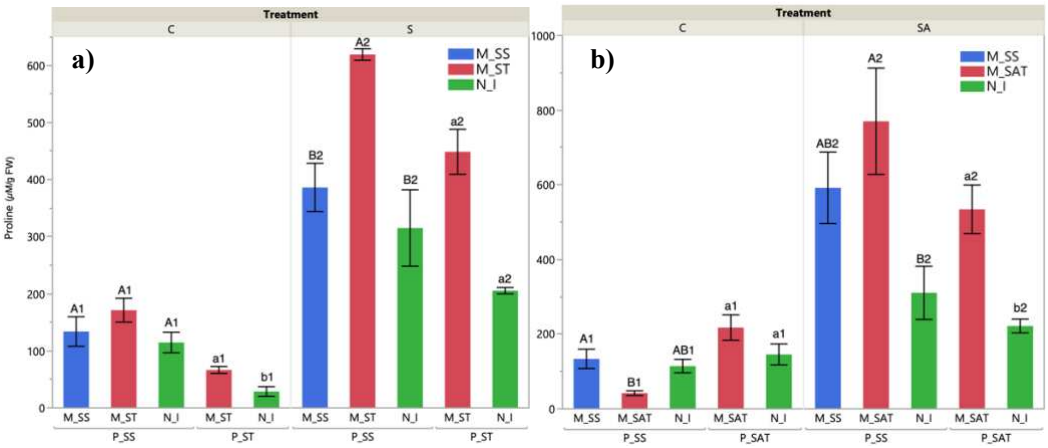


**Figure 13** Total proline content (µg/g FW) of **a)** salt-sensitive (P_SS) and salt-tolerant (P_ST) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_ST (red bar) microbiomes cultivated under control and salt treatments for 10 days; and **b)** salt-sensitive (P_SS) and salt-alkaline-tolerant (P_SAT) *B. fruticulosa* populations no inoculated (N_I, green bar) or inoculated with M_SS (blue bar) or M_SAT (red bar) microbiomes cultivated under control and salt-alkaline treatments for 10 days. FW = fresh weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); SA= Salt Alkaline (135 mM NaCl + 15 mM $NaHCO_3$, pH 8.3); M_SS = Salt sensitive microbiome; M_ST = salt tolerant microbiome; M_SAT = Salt alkaline tolerant microbiome. Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between inoculations for the same treatment (c or s) ($p < 0.05$) according to ANOVA test for P_ST and P_SAT and to multiple mean comparison ($p < 0.05$) according to Tukey's Test for P_SS. Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to ANOVA test.

As anticipated before, besides acting as an excellent osmolyte, proline plays three major roles during stress as metal chelator, anti-oxidative defense and as signal molecule. In particular, it has been found a positive correlation between proline accumulation and plant stress. The measure of proline content is a strong indicator of how the plant is responding to the stress, indeed, the synthesis of proline is an effective tolerant mechanism against the salt stress fundamental to overcome this particular stress. It has been studied that proline is responsible for scavenging ROS and other free radicals (Rejeb et al., 2014), in particular, when proline was applied exogenously to roots of *arabidopsis*, resulted in a reduced level of ROS, elucidating

the scavenging potential of proline (Cuin et al., 2007). Regarding its specific role against saline stress, in a study performed by Gadallah (1999), exogenous proline application increased leaf chlorophyll content, leaf relative water content and overall plant growth. Furthermore, in a study with *Mesambryanthemum crystallinum* L. exogenous addiction of proline drastically decreased the oxidative damage caused by salinity resulting in reduced lipid peroxidation rate and increased the chlorophyll content in the leaves of salt stressed plants (Shevyakova et al., 2009). The results regarding the proline content in this study it was shown the strong positive correlation that exists between microbiome inoculation and the total proline content, in both salt and salt-alkaline stress condition.

During a stress condition, also the non-inoculated plants were experiencing a rise in total proline content, confirming previous studies that addressed proline as a stress coping mechanism intrinsically activated by plant metabolism (Hayat et al., 2012).

## 4.2 Soybean (*Glicyne max* L.) results

To verify if the candidate bacterial strains are beneficial when inoculate a non-brassica crop species such as Soybean we studied the effects of inoculation on these BS during salt stress analyzing the morpho-physiological characteristics as total shoot biomass, nodulation activity, $H_2O_2$ content, lipid peroxidation and proline content.

### 4.2.1 Shoot Biomass

The comparison of soybean growth between control and salt treatment showed that, regardless of the different bacterial strain inoculated, the level of total biomass was significantly reduced in plants exposed to salt treatment compared to control (**Figure 14**). The comparison among inoculation conditions in a same treatment did not show significance difference among inoculated strain under salt stress, however in control plants, non-inoculated plants were significantly smaller compared to the ones inoculated with the BS1 (**Figure 14**).

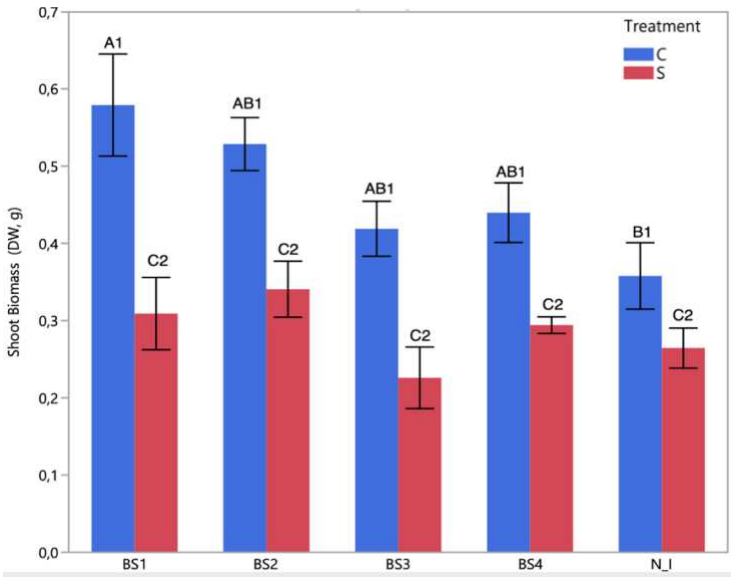


**Figure 14** Total shoot biomass content (DW, g)) of Soybean (*Glicyne max* L.) plants no inoculated (N_I) or inoculated with different bacterial strain (BS) cultivated under control (blue bar) and salt (red bar) conditions for 10 days. DW = dry weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between growth conditions for the same treatment (c or s) ($p < 0.05$) according to ANOVA test ($p < 0.05$). Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to ANOVA test.

These results were supported by previous studies that describes soybean plants as a relatively salt sensitive species (Phang et al., 2008; Lu et al., 2009; Le et al., 2021). These results confirmed that the plants are experiencing a stress, and once this condition is achieved, it is possible to analyze the different effect of the microbiome inoculation during the different treatment conditions. Interestingly, the inoculation of the different bacteria strain did not cause significant difference in shoot biomass during salt stress. This can be due to an inefficient volume of microbiome inoculated or to an excessive degree of salt stress that did not allow a significant variation in shoot biomass among different inoculation treatment.

### 4.2.2 Nodulation variable

Nodulation and subsequent nitrogen fixation are important factor to determine the productivity of soybean. Indeed, the formation of nodules in leguminous plants is an indicator of the process of nitrogen fixation by the nitrogen-fixing bacteria that fulfill the N demand of leguminous plants. The comparison of the number of nodules between control and salt treatment indicates that salt addition decreased the nodule number per plant in non-inoculated (with PGPB) plants and, in plants inoculated with BS1 and BS2 compared to control (**Figure 15**). The comparison among inoculation conditions in a same treatment showed that only in the salt treatment, the inoculation with the PGPB bacterial strains enhanced the developing of nodules in a significant way compared to non-inoculated plants. In particular, the most effective strains were BS3 and BS4 followed by BS2 and finally BS1 (**Figure 15**).

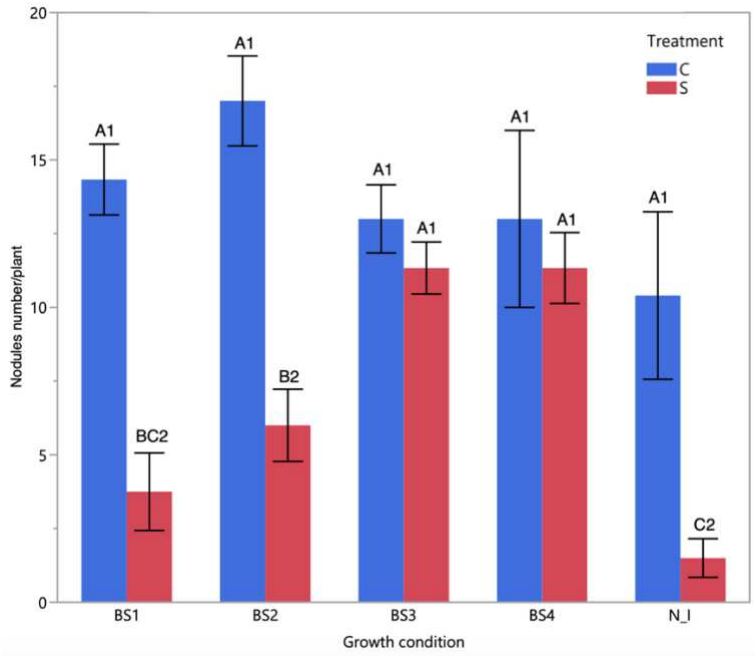


**Figure 15.** Nodules number/plant of Soybean (*Glicyne max* L.) no inoculated (N_I) or inoculated with different bacterial strain (BS) cultivated under control and salt treatments for 10 days. DW = dry weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between growth conditions for the same treatment (c or s) ($p < 0.05$) according to ANOVA test ($p < 0.05$. Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to Tukey's test.

Regarding the different bacterial strain inoculation, the results obtained suggest that inoculation of soybean plants with the isolated bacterial strains promoted the formation of nodules in the salt stress condition. Nodulation and subsequent nitrogen fixation are important factor to determine the productivity of soybean. Indeed, the formation of nodules in leguminous plants is an indicator of the process of nitrogen fixation by the nitrogen-fixing bacteria that fulfill the N demand of leguminous plants. Nodule initiation in the legume-bacteria symbiosis involves a complex interaction between host root, rhizobial strain and environment (Suzaki et al., 2015). In particular, the process of nodule development in soybean was reported

to be extremely sensitive to NaCl (Phang et al., 2006). Furthermore, past studies suggested that the beneficial effects of nodulation can be enhanced when rhizobial inoculation is combined with PGPB (Mishra et al., 2009).

### 4.2.3 Hydrogen peroxide content and lipid peroxidation determination

The comparison among inoculation conditions showed that addition of salt decreased the $H_2O_2$ content significantly when plants were inoculated with microbiome isolated bacteria strains compared to non-inoculated plants (**Figure 16a).** Similarly, the comparison among inoculation condition regarding the TBARs content showed that the inoculation with BS candidtes reduced significantly this variable compared to non-inoculated plants (**Figure 16b**). In particular, bacterial strain 3 and 4 seem to be the most effective in reducing lipid peroxidation in soybean plants during salt stress.

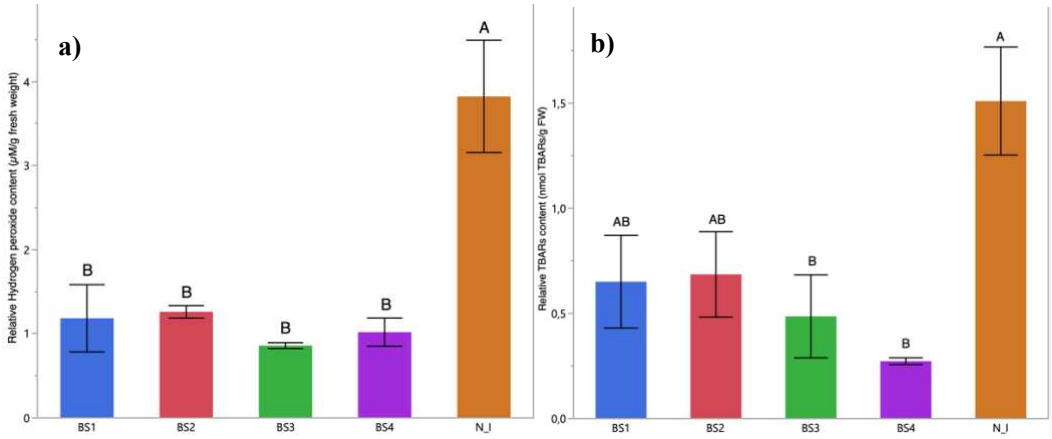


**Figure 16. a)** Relative Hydrogen peroxide content (S/C) of Soybean (*Glicyne max* L.) plants no inoculated (N_I, orange bar) or inoculated with different bacterial strain (BS) cultivated under control and salt treatments for 10 days. **b)** Relative TBARs content (S/C) of Soybean (*Glicyne max* L.) plants no inoculated (N_I) or inoculated with different bacterial strain (BS) cultivated under control and salt conditions for 10 days. DW = dry weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between inoculations for the same treatment (c or s) ($p < 0.05$) according to Tukey's test ($p < 0.05$).

The results obtained suggest that inoculation of soybean plants with these PGPB strains reduced the formation of $H_2O_2$ and consequently the level of TBARs in salt stress conditions. As explained before, TBARs is an important index useful for measure the degree of oxidative stress that the plant is experiencing. As confirmed by other previous studies, during salt stress the degree of both $H_2O_2$ and TBARs increased in soybean plants (Weisany et al., 2019). In addition, this detrimental effect observed in soybean plants can be attenuated by the inoculation of PGPB that are effective against different abiotic and biotic stress conditions (Bùttros et al., 2022; Moretti et al., 2021; Nigam et al., 2022).

### 4.2.4 Proline Content

Proline is an important metabolite accumulated during different stress condition with consequent beneficial effects to the plant (Hayat et al., 2012). The comparison of proline content among inoculation conditions showed that addition of salt significantly enhanced proline content when plants were inoculated with the isolated bacterial strains compared to non-inoculated plants with the exception of BS2 and BS3 that did not show any significant difference compared to non-inoculated plant. In particular, BS1 showed to be the most effective in terms of proline content accumulation (**Figure 17**)

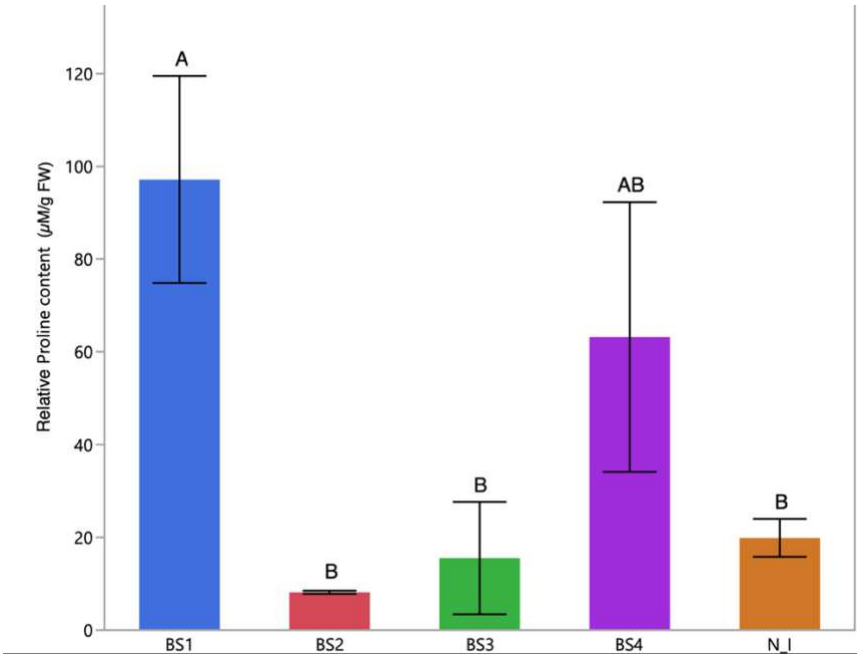


**Figure 17.** Relative Proline content (S/C) of Soybean (*Glicyne max* L.) plants no inoculated (N_I, orange bar) or inoculated with different bacterial strain (BS) cultivated under control and salt treatments for 10 days. DW = dry weight; C = Control (0mM NaCl, pH 6); S = Salt (150 mM NaCl, pH 6); Data represent the mean ± SE (*n=10*). Different letters indicate significant differences between growth conditions for the same treatment (c or s) ($p < 0.05$) according to ANOVA test ($p < 0.05$. Different numbers indicate significant differences between treatments for a same growth condition ($p < 0.05$) according to Tukey's test.

The results obtained in this section suggest that only bacterial strain 1 and 4 are able to promote the accumulation of proline in plant leaves. In particular, BS1 was the most effective compared to other inoculated strains. These results are supported by previous studies in soybean that correlated the accumulation of proline with the inoculation of beneficial rhizospheric bacteria (Moretti et al., 2022; Nigam et al., 2022). Proline accumulation is a fundamental response against salt stress since this particular amino acid is able to act as an efficient osmolyte limiting the osmotic stress and as an important second messenger stimulating a wide variety of stress response mechanisms. (Hayat et al., 2012)

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Inoculation of salt sensitive *Brassica fruticulosa* plants exposed to salt and salt-alkaline stress with a salt-tolerant microbiome is able to improve plant salinity tolerance and growth compared to non-inoculated plants. In detail, the total biomass, pigments content and proline increased in inoculated plants. In addition, microbiome inoculation could reduce the oxidative burst and damage caused by the applied treatment. These results confirm that the inoculation of a specific rhizospheric microbiome modulates the salt and alkaline stress response conferring tolerance to the plant.

Regarding the four selected bacteria strain inoculated into soybean plants, they have exhibited beneficial effect during salt stress, improving nodules formation, reducing $H_2O_2$ and TBARs concentration, and promoting proline synthesis, causing an overall enhanced of salinity tolerance. In particular, the selected strain 3 and 4 have shown to be the most effective against this particular stress. It is important to mention that these were the first analysis on the effectiveness of these particular bacterial strains derived from the *Brassica fruticulosa* microbiome and more replicates are needed to support our observations. Further studies are required to confirm these results and to identify the most effective strain, or the most effective PGPBs consortium, that could be used as a commercially bio-stimulant for salt stress tolerance.

The following up steps of this project will include (i) a further investigation of the *B. fruticulosa* tolerant microbiome in order to select a synthetic community of bacterial strains that help plants to cope with salt and salt alkaline stress; (ii) additional studies on the effect of the four bacterial strain inoculation with a larger number of replicates of soybean plants during salt stress including a co-inoculation of the different bacteria in the same plant in order to verify if the beneficial effects are cumulative; (iii) further experiments to validate the beneficial effects of the selected PGPB in other crop species.

In summary, this research represents a starting point on the study of the *B. fruticulosa* rhizospheric microbiome and could lead to the discovery of beneficial PGPBs for salt and salt-alkaline stress potentially useful for attenuating the damage of these abiotic stresses on a large scale.

# 6. AKNOWLEDGMENTS

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# 7. BIBLIOGRAFY


Abascal, Federico, Iker Irisarri, and Rafael Zardoya. "Diversity and evolution of membrane intrinsic proteins." *Biochimica et Biophysica Acta (BBA)-General Subjects* 1840, no. 5 (2014): 1468-1481.

Abdel Latef, Arafat Abdel Hamed, Mona Fawzy Abu Alhmad, Mojtaba Kordrostami, Abo-Baker Abd-Elmoniem Abo–Baker, and Ali Zakir. "Inoculation with Azospirillum lipoferum or Azotobacter chroococcum reinforces maize growth by improving physiological activities under saline conditions." *Journal of Plant Growth Regulation* 39, no. 3 (2020): 1293-1306.

Adams, S. R., and F. A. Langton. "Photoperiod and plant growth: a review." *The Journal of Horticultural Science and Biotechnology* 80, no. 1 (2005): 2-10.

Agrawal, Neha, Mehak Gupta, Chhaya Atri, Javed Akhatar, Sarwan Kumar, Pat JS Heslop-Harrison, and Surinder S. Banga. "Anchoring alien chromosome segment substitutions bearing gene (s) for resistance to mustard aphid in Brassica juncea-B. fruticulosa introgression lines and their possible disruption through gamma irradiation." *Theoretical and Applied Genetics* 134, no. 10 (2021): 3209-3224.

Aisha, Waheed Qurashi, and Nasim Sabri Anjum. "Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress." *African Journal of Microbiology Research* 5, no. 21 (2011): 3546-3554.

Alexieva, V., I. Sergiev, S. Mapelli, and E. Karanov. "The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat." *Plant, Cell & Environment* 24, no. 12 (2001): 1337-1344.

Amirinejad, A-A., M. Sayyari, F. Ghanbari, and S. Kordi. "Salicylic acid improves salinity-alkalinity tolerance in pepper (Capsicum annuum L.)." *Advances in Horticultural Science* 31, no. 3 (2017): 157-164.

Andrews, Mitchell, and Morag E. Andrews. "Specificity in legume-rhizobia symbioses." *International journal of molecular sciences* 18.4 (2017): 705.

Atafar, Zahra, Alireza Mesdaghinia, Jafar Nouri, Mehdi Homaee, Masoud Yunesian, Mehdi Ahmadimoghaddam, and Amir Hossein Mahvi. "Effect of fertilizer application on soil heavy metal concentration." *Environmental monitoring and assessment* 160, no. 1 (2010): 83-89.

Atri, Chhaya, Bharti Kumar, Hitesh Kumar, Sarwan Kumar, Sanjula Sharma, and Surinder S. Banga. "Development and characterization of Brassica juncea–fruticulosa introgression lines exhibiting resistance to mustard aphid (Lipaphis erysimi Kalt)." *BMC genetics* 13, no. 1 (2012): 1-9.

Badri, Dayakar V., et al. "Rhizosphere chemical dialogues: plant–microbe interactions." *Current opinion in biotechnology* 20.6 (2009): 642-650.

Bashan, de-Bashan, L. E., and Y. Bashan. "Microalgae growth-promoting bacteria: a novel approach in wastewater treatment." *Revista Colombiana de Biotecnologia*5, no. 2 (2003): 85-90.

Bates, L. S, R. P Waldren, and I. D. Teare. "Rapid determination of free proline for water-stress studies." *Plant and soil* 39, no. 1 (1973): 205-207.

Belimov, A. A., N. Hontzeas, V. I. Safronova, S. V. Demchinskaya, G. Piluzza, S. Bullitta, and B. R. Glick. "Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.)." *Soil Biology and Biochemistry* 37, no. 2 (2005): 241-250.

Berthomieu, Pierre, Geneviève Conéjéro, Aurélie Nublat, William J. Brackenbury, Cécile Lambert, Cristina Savio, Nobuyuki Uozumi et al. "Functional analysis of AtHKT1 in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance." *The EMBO journal* 22, no. 9 (2003): 2004-2014.

Bianucci, Eliana, Ana Furlan, Jesica Rivadeneira, Juan Sobrino-Plata, Ramón O. Carpena-Ruiz, María del Carmen Tordable, Adriana Fabra, Luis E. Hernández, and Stella Castro. "Influence of cadmium on the symbiotic interaction established between peanut (Arachis hypogaea L.) and sensitive or tolerant bradyrhizobial strains." *Journal of environmental management* 130 (2013): 126-134.

Bianucci, Eliana, Andrea Godoy, Ana Furlan, Juan Manuel Peralta, Luis E. Hernández, Ramón O. Carpena-Ruiz, and Stella Castro. "Arsenic toxicity in soybean alleviated by a symbiotic species of Bradyrhizobium." *Symbiosis* 74, no. 3 (2018): 167-176.

Bienert, Gerd P., Anders LB Møller, Kim A. Kristiansen, Alexander Schulz, Ian M. Møller, Jan K. Schjoerring, and Thomas P. Jahn. "Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes." *Journal of Biological Chemistry* 282, no. 2 (2007): 1183-1192.

Brimecombe, Melissa J., Frans A. De Leij, and James M. Lynch. "The effect of root exudates on rhizosphere microbial populations." *The rhizosphere*. CRC Press, (2000): 111-156.

Buttrós, Victor Hugo, Neílton Antônio Fiusa Araújo, Vinícius de Abreu D'Ávila, Maysa Mathias Alves Pereira, Dirceu de Sousa Melo, Moacir Pasqual, and Joyce Dória. "A Little Helper: Beneficial Bacteria with Growth-Promoting Mechanisms Can Reduce Asian Soybean Rust Severity in a Cell-Free Formulation." *Agronomy* 12, no. 11 (2022): 2635.

Cakmak, Ismail, and Walter J. Horst. "Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max)." *Physiologia plantarum* 83, no. 3 (1991): 463-468.

Cechin, Inês, S. C. Rossi, V. C. Oliveira, and Terezinha de Fátima Fumis. "Photosynthetic responses and proline content of mature and young leaves of sunflower plants under water deficit." *Photosynthetica* 44, no. 1 (2006): 143-146.
Chen, Changbin, and Martin B. Dickman. "Proline suppresses apoptosis in the fungal pathogen Colletotrichum trifolii." *Proceedings of the National Academy of Sciences* 102, no. 9 (2005): 3459-3464.

Chen, Yanmei, and Wolfgang Hoehenwarter. "Changes in the phosphoproteome and metabolome link early signaling events to rearrangement of photosynthesis and central metabolism in salinity and oxidative stress response in Arabidopsis." *Plant Physiology* 169, no. 4 (2015): 3021-3033.

Chen, Lin, Yunpeng Liu, Gengwei Wu, Kimani Veronican Njeri, Qirong Shen, Nan Zhang, and Ruifu Zhang. "Induced maize salt tolerance by rhizosphere inoculation of Bacillus amyloliquefaciens SQR9." *Physiologia plantarum* 158, no. 1 (2016): 34-44.

Chen, Youyuan, Yangyang Li, Ping Sun, Guanglin Chen, and Jia Xin. "Interactive effects of salt and alkali stresses on growth, physiological responses and nutrient (N, P) removal performance of Ruppia maritima." *Ecological Engineering* 104 (2017): 177-183.

Cuin, Tracey Ann, and Sergey Shabala. "Compatible solutes reduce ROS-induced potassium efflux in Arabidopsis roots." *Plant, cell & environment* 30, no. 7 (2007): 875-885.

Das, Kaushik, and Aryadeep Roychoudhury. "Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants." *Frontiers in environmental science* 2 (2014): 53.

Dat, J., Steven Vandenabeele, E. V. M. M. Vranova, Marc Van Montagu, and F. Van Breusegem. "Dual action of the active oxygen species during plant stress responses." *Cellular and Molecular Life Sciences CMLS* 57, no. 5 (2000): 779-795.

Davies, Peter J., ed. *Plant hormones: biosynthesis, signal transduction, action!*. Springer Science & Business Media, 2004.
Dixon, Ray, and Daniel Kahn. "Genetic regulation of biological nitrogen fixation." *Nature Reviews Microbiology* 2.8 (2004): 621-631.

El-Esawi, Mohamed A., Ibrahim A. Alaraidh, Abdulaziz A. Alsahli, Saud M. Alzahrani, Hayssam M. Ali, Aisha A. Alayafi, and Margaret Ahmad. "Serratia liquefaciens KM4 improves salt stress tolerance in maize by regulating redox potential, ion homeostasis, leaf gas exchange and stress-related gene expression." *International journal of molecular sciences* 19, no. 11 (2018): 3310.

El-Mashad, Ali Abdel Aziz, and Heba Ibrahim Mohamed. "Brassinolide alleviates salt stress and increases antioxidant activity of cowpea plants (Vigna sinensis)." *Protoplasma* 249, no. 3 (2012): 625-635.

El-Shintinawy, F., and M. N. El-Shourbagy. "Alleviation of changes in protein metabolism in NaCl-stressed wheat seedlings by thiamine." *Biologia plantarum*44, no. 4 (2001): 541-545.
Farmer, Edward E., and Celine Davoine. "Reactive electrophile species." *Current opinion in plant biology* 10, no. 4 (2007): 380-386.

Fich, Eric A., Nicholas A. Segerson, and Jocelyn KC Rose. "The plant polyester cutin: biosynthesis, structure, and biological roles." *Annual review of plant biology* (2016): 207-233.
Fred, Edwin Broun, Ira Lawrence Baldwin, and Elizabeth McCoy. *Root nodule bacteria and leguminous plants*. No. 5. UW-Madison Libraries Parallel Press, (2002).

Fukuda, Atsunori, and Yoshiyuki Tanaka. "Effects of ABA, auxin, and gibberellin on the expression of genes for vacuolar H+-inorganic pyrophosphatase, H+-ATPase subunit A, and Na+/H+ antiporter in barley." *Plant Physiology and Biochemistry* 44, no. 5-6 (2006): 351-358.

Furlan, Ana, Eliana Bianucci, Micaela Sequeira, Lucía Álvarez, Juan Manuel Peralta, Carina Valente, Valmiro Guarnieri, and Stella Castro. "Combined application of microbial and non-microbial biostimulants to improve growth of peanut plants exposed to abiotic stresses." In *Microbial Probiotics for Agricultural Systems*, pp. 239-256. Springer, Cham, 2019.

Gadallah, M. A. A. "Effects of proline and glycinebetaine on Vicia faba responses to salt stress." *Biologia plantarum* 42, no. 2 (1999): 249-257.

Gao, Ji-Ping, Dai-Yin Chao, and Hong-Xuan Lin. "Understanding abiotic stress tolerance mechanisms: recent studies on stress response in rice." *Journal of Integrative Plant Biology* 49, no. 6 (2007): 742-750.

Garcia Salamone, Ines E. de, Russell K. Hynes, and Louise M. Nelson. "Role of cytokinins in plant growth promotion by rhizosphere bacteria." *PGPR: biocontrol and biofertilization* (2005): 173-195.

Gimenez, Estela, Maria Salinas, and Francisco Manzano-Agugliaro. "Worldwide research on plant defense against biotic stresses as improvement for sustainable agriculture." *Sustainability* 10, no. 2 (2018): 391.

Glick, Bernard R., Zhenyu Cheng, Jennifer Czarny, and Jin Duan. "Promotion of plant growth by ACC deaminase-producing soil bacteria." *New perspectives and approaches in plant growth-promoting Rhizobacteria research* (2007): 329-339.

Glick, Bernard R. "Plant growth-promoting bacteria: mechanisms and applications." *Scientifica* 2012 (2012).

Glick, Bernard R. "Bacteria with ACC deaminase can promote plant growth and help to feed the world." *Microbiological research* 169, no. 1 (2014): 30-39.

Gong, Zhizhong. "Plant abiotic stress: New insights into the factors that activate and modulate plant responses." *Journal of Integrative Plant Biology* 63, no. 3 (2021): 429.

Guang-Can, T. A. O., T. I. A. N. Shu-Jun, C. A. I. Miao-Ying, and X. I. E. Guang-Hui. "Phosphate-solubilizing and-mineralizing abilities of bacteria isolated from soils." *Pedosphere* 18, no. 4 (2008): 515-523.

Guo, H., Hu, Z., Zhang, H., Min, W., & Hou, Z. (2019). Comparative effects of salt and alkali stress on antioxidant system in cotton (Gossypium hirsutum L.) leaves. *Open Chemistry*, *17*(1), (2019): 1352-1360.

Hare, P. D, W. A Cress, and J. Van Staden. "Dissecting the roles of osmolyte accumulation during stress." *Plant, cell & environment* 21, no. 6 (1998): 535-553.

Hashem, Abeer, Elsayed Fathi Abd_Allah, Abdulaziz A. Alqarawi, Stephan Wirth, and Dilfuza Egamberdieva. "Comparing symbiotic performance and physiological responses of two soybean cultivars to arbuscular mycorrhizal fungi under salt stress." *Saudi journal of biological sciences*26, no. 1 (2019): 38-48.

Hayat, Shamsul, Qaiser Hayat, Mohammed Nasser Alyemeni, Arif Shafi Wani, John Pichtel, and Aqil Ahmad. "Role of proline under changing environments: a review." *Plant signaling & behavior* 7, no. 11 (2012): 1456-1466.

Hazel, Jeffrey R. "Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?." *Annual review of physiology* 57, no. 1 (1995): 19-42.

Heath, Robert L., and Lester Packer. "Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation." *Archives of biochemistry and biophysics* 125, no. 1 (1968): 189-198.

Hider, Robert C., and Xiaole Kong. "Chemistry and biology of siderophores." *Natural product reports* 27.5 (2010): 637-657.

Hoagland, Dennis Robert, and Daniel Israel Arnon. "The water-culture method for growing plants without soil." *Circular. California agricultural experiment station* 347, no. 2nd edit (1950).

Hussain Wani, Shabir, Naorem Brajendra Singh, Athokpam Haribhushan, and Javed Iqbal Mir.
"Compatible solute engineering in plants for abiotic stress tolerance-role of glycine betaine." *Current genomics* 14, no. 3 (2013): 157-165.

Ilangumaran, Gayathri, and Donald L. Smith. "Plant growth promoting rhizobacteria in amelioration of salinity stress: a systems biology perspective." *Frontiers in Plant Science* 8 (2017): 1768.

Isayenkov, Stanislav V., and Frans JM Maathuis. "Plant salinity stress: many unanswered questions remain." *Frontiers in plant science* 10 (2019): 80.

Jha, Yachana, and R. B. Subramanian. "Regulation of plant physiology and antioxidant enzymes for alleviating salinity stress by potassium-mobilizing bacteria." In *Potassium solubilizing microorganisms for sustainable agriculture*, pp. 149-162. Springer, New Delhi, (2016).

Ji, Hongtao, José M. Pardo, Giorgia Batelli, Michael J. Van Oosten, Ray A. Bressan, and Xia Li. "The salt overly sensitive (SOS) pathway: established and emerging roles." *Molecular plant* 6, no. 2 (2013): 275-286.

Jixiang, Lin, Li Xiaoyu, Zhang Zhaojun, Yu Xingyang, Gao Zhanwu, Wang Ying, Wang Junfeng, Li Zhuolin, and Mu Chunsheng. "Salinity-alkalinity tolerance in wheat: Seed germination, early seedling growth, ion relations and solute accumulation." *African Journal of Agricultural Research* 7, no. 3 (2012): 467-474.

Kaiwen, Guo, Xu Zisong, Huo Yuze, Sun Qi, Wang Yue, Che Yanhui, Wang Jiechen, Li Wei, and Zhang Huihui. "Effects of salt concentration, pH, and their interaction on plant growth, nutrient uptake, and photochemistry of alfalfa (Medicago sativa) leaves." *Plant Signaling & Behavior* 15, no. 12 (2020): 1832373.

Kearl, Jennifer, et al. "Salt-tolerant halophyte rhizosphere bacteria stimulate growth of alfalfa in salty soil." *Frontiers in microbiology* 10 (2019): 1849.

Kuklinsky-Sobral, Júlia, Welington Luiz Araújo, Rodrigo Mendes, Isaias Olívio Geraldi, Aline Aparecida Pizzirani-Kleiner, and João Lúcio Azevedo. "Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion." *Environmental microbiology* 6, no. 12 (2004): 1244-1251.

Kumar, R.M.; Murthy, A.G.K.; Subbiah, S.V.; Mishra, B. Effect of phosphorus solubility bacteria (PSB) in different rice based cropping systems. In Proceedings of the Sixth International PGPR Workshop on Plant Growth Promoting Rhizobacteria, Calicut, (2003): 157–158.

Le, Ly Thi Thanh, Lukasz Kotula, Kadambot HM Siddique, and Timothy D. Colmer. "Na+ and/or Cl− toxicities determine salt sensitivity in soybean (Glycine max (L.) Merr.), mungbean (Vigna radiata (L.) R. Wilczek), cowpea (Vigna unguiculata (L.) Walp.), and common Bean (Phaseolus vulgaris L.)." *International journal of molecular sciences* 22, no. 4 (2021): 1909.

Leng, Qiang, Richard W. Mercier, Bao-Guang Hua, Hillel Fromm, and Gerald A. Berkowitz. "Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels." *Plant Physiology* 128, no. 2 (2002): 400-410.

Li, H. Q., and X. W. Jiang. "Inoculation with plant growth-promoting bacteria (PGPB) improves salt tolerance of maize seedling." *Russian Journal of Plant Physiology* 64, no. 2 (2017): 235-241.

Li, Xiaoyu, Shuxin Li, Jinghong Wang, and Jixiang Lin. "Exogenous abscisic acid alleviates harmful effect of salt and alkali stresses on wheat seedlings." *International Journal of Environmental Research and Public Health* 17, no. 11 (2020): 3770.

Lu, K.X., Cao, B.H., Feng, X.P., He, Y. and Jiang, D.A., 2009. Photosynthetic response of salt-tolerant and sensitive soybean varieties. *Photosynthetica*, *47*(3), pp.381-387.

Lucero, Cinthia Tamara, Graciela Susana Lorda, María Soledad Anzuay, Liliana Mercedes Ludueña, and Tania Taurian. "Peanut endophytic phosphate solubilizing bacteria increase growth and P content of soybean and maize plants." *Current Microbiology* 78, no. 5 (2021): 1961-1972.

Ma, Baohui, Lu Gao, Hongxiao Zhang, Jin Cui, and Zhenguo Shen. "Aluminum-induced oxidative stress and changes in antioxidant defenses in the roots of rice varieties differing in Al tolerance." *Plant cell reports* 31, no. 4 (2012): 687-696.

Mackinney, G. "Absorption of light by chlorophyll solution." *J. biol. Chem* 140 (1941): 315-322.

Malagoli, Philippe, Dev T. Britto, Lasse M. Schulze, and Herbert J. Kronzucker. "Futile Na+ cycling at the root plasma membrane in rice (Oryza sativa L.): kinetics, energetics, and relationship to salinity tolerance." *Journal of Experimental Botany* 59, no. 15 (2008): 4109-4117.

Maoka, Takashi. "Carotenoids as natural functional pigments." *Journal of natural medicines* 74, no. 1 (2020): 1-16.

McNeil, Scott D., Michael L. Nuccio, and Andrew D. Hanson. "Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance." *Plant Physiology* 120, no. 4 (1999): 945-949.

Mishra, Pankaj K., Smita Mishra, G. Selvakumar, Samresh Kundu, and Hari Shankar Gupta. "Enhanced soybean (Glycine max L.) plant growth and nodulation by Bradyrhizobium japonicum-SB1 in presence of Bacillus thuringiensis-KR1." *Acta Agriculturae Scandinavica Section B–Soil and Plant Science* 59, no. 2 (2009): 189-196.

Mohamed, H. I., and E. Z. Gomaa. "Effect of plant growth promoting Bacillus subtilis and Pseudomonas fluorescens on growth and pigment composition of radish plants (Raphanus sativus) under NaCl stress." *Photosynthetica* 50, no. 2 (2012): 263-272.

Moon, Byoung Yong, S. Higashi, Zoltan Gombos, and Norio Murata. "Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants." *Proceedings of the National Academy of Sciences* 92, no. 14 (1995): 6219-6223.

Moretti, Luiz Gustavo, Carlos Alexandre Costa Crusciol, João William Bossolani, Juliano Carlos Calonego, Adônis Moreira, Ariani Garcia, Letusa Momesso, Eiko Eurya Kuramae, and Mariangela Hungria. "Beneficial microbial species and metabolites alleviate soybean oxidative damage and increase grain yield during short dry spells." *European Journal of Agronomy* 127 (2021): 126293.

Munns, Rana. "Genes and salt tolerance: bringing them together." *New phytologist* 167, no. 3 (2005): 645-663.

Munns, Rana. "Plant adaptations to salt and water stress: differences and commonalities." *Advances in botanical research* 57 (2011): 1-32.

Murashige, Toshio, and Folke Skoog. "A revised medium for rapid growth and bio assays with tobacco tissue cultures." *Physiologia plantarum* 15, no. 3 (1962): 473-497.

Naser, Iftekhar Bin, Nur Uddin Mahmud, Aniruddha Sarker, M. Nazmul Hoque, and Tofazzal Islam. "A Highly Salt-Tolerant Bacterium Brevibacterium sediminis Promotes the Growth of Rice (Oryza sativa L.) Seedlings." *Stresses* 2, no. 3 (2022): 275-289.

Nigam, Bhavna, Rama Shanker Dubey, and Dheeraj Rathore. "Protective role of exogenously supplied salicylic acid and PGPB (Stenotrophomonas sp.) on spinach and soybean cultivars grown under salt stress." *Scientia Horticulturae* 293 (2022): 110654.

Oster, J. D., I. Shainberg, and I. P. Abrol. "Reclamation of salt-affected soils." *Agricultural drainage* 38 (1999): 659-691.

Peix, A., A. A. Rivas-Boyero, P. F. Mateos, C. Rodriguez-Barrueco, E. Martınez-Molina, and E. Velazquez. "Growth promotion of chickpea and barley by a phosphate solubilizing strain of Mesorhizobium mediterraneum under growth chamber conditions." *Soil Biology and Biochemistry* 33, no. 1 (2001): 103-110.

Pérez-Martín, Laura, Silvia Busoms, Maria Jose Almira, Nicole Azagury, Joana Terés, Roser Tolrà, Charlotte Poschenrieder, and Juan Barcelo. "Evolution of salt tolerance in Arabidopsis thaliana on siliceous soils does not confer tolerance to saline calcareous soils." *Plant and Soil* (2022): 1-21.

Phang, Tsui-Hung, Guihua Shao, and Hon-Ming Lam. "Salt tolerance in soybean." *Journal of Integrative Plant Biology* 50, no. 10 (2008): 1196-1212.

Pitman, M. G. "Transport across plant roots." *Quarterly reviews of biophysics* 15, no. 3 (1982): 481-554.

Poschenrieder, Charlotte, José Antonio Fernández, Lourdes Rubio, Laura Pérez, Joana Terés, and Juan Barceló. "Transport and use of bicarbonate in plants: current knowledge and challenges ahead." *International Journal of Molecular Sciences* 19, no. 5 (2018): 1352.

Quan, Li-Juan, et al. "Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network." *Journal of Integrative Plant Biology* 50.1 (2008): 2-18

Rejeb, Kilani Ben, Chedly Abdelly, and Arnould Savouré. "How reactive oxygen species and proline face stress together." *Plant Physiology and Biochemistry* 80 (2014): 278-284.

Ren, Xue-Min, Shi-Jun Guo, Wei Tian, Yan Chen, Hui Han, E. Chen, Bai-Lian Li, Yu-Ying Li, and Zhao-Jin Chen. "Effects of plant growth-promoting bacteria (PGPB) inoculation on the growth, antioxidant activity, Cu uptake, and bacterial community structure of rape (Brassica napus L.) grown in Cu-contaminated agricultural soil." *Frontiers in microbiology* 10 (2019): 1455.

Richardson, Alan E. "Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants." *Functional Plant Biology* 28.9 (2001): 897-906.

Rodríguez, Hilda, and Reynaldo Fraga. "Phosphate solubilizing bacteria and their role in plant growth promotion." *Biotechnology advances* 17.4-5 (1999): 319-339.

Rojas-Tapias, Daniel, Andrés Moreno-Galván, Sergio Pardo-Díaz, Melissa Obando, Diego Rivera, and Ruth Bonilla. "Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (Zea mays)." *Applied Soil Ecology* 61 (2012): 264-272.

Roy, Papiya, Kamala Niyogi, Dibyendu N. Sengupta, and Bharati Ghosh. "Spermidine treatment to rice seedlings recovers salinity stress-induced damage of plasma membrane and PM-bound H+-ATPase in salt-tolerant and salt-sensitive rice cultivars." *Plant science* 168, no. 3 (2005): 583-591.

Rubio, Francisco, Manuel Nieves-Cordones, Tomoaki Horie, and Sergey Shabala. "Doing 'business as usual'comes with a cost: evaluating energy cost of maintaining plant intracellular K+ homeostasis under saline conditions." *New Phytologist*225, no. 3 (2020): 1097-1104.

Saberi-Riseh, Roohallah, Fariba Fathi, and Mojtaba Moradzadeh-Eskandari. "Effect of some Pseudomonas fluorescens and Bacillus subtilis strains on osmolytes and antioxidants of cucumber under salinity stress." *Journal of Crop Protection* 9, no. 1 (2020): 1-16.

Salas-González, Isai, Guilhem Reyt, Paulina Flis, Valéria Custódio, David Gopaulchan, Niokhor Bakhoum, Tristan P. Dew et al. "Coordination between microbiota and root endodermis supports plant mineral nutrient homeostasis." *Science* 371, no. 6525 (2020): eabd0695.

Sandrini, M., Nerva, L., Sillo, F., Balestrini, R., Chitarra, W. and Zampieri, E., 2022. Abiotic stress and belowground microbiome: The potential of omics approaches. *International Journal of Molecular Sciences*, *23*(3), p.1091.

Santos, Alexandra de Andrade, Joaquim Albenísio Gomes da Silveira, Aurenivia Bonifacio, Artenisa Cerqueira Rodrigues, and Márcia do Vale Barreto Figueiredo. "Antioxidant response of cowpea co-inoculated with plant growth-promoting bacteria under salt stress." *brazilian journal of microbiology* 49 (2018): 513-521.

Santos, Susana Silva, Klara Andrés Rask, Mette Vestergård, Jesper Liengaard Johansen, Anders Priemé, Tobias Guldberg Frøslev, Ana M. Martín González, Huan He, and Flemming Ekelund. "Specialized microbiomes facilitate natural rhizosphere microbiome interactions counteracting high salinity stress in plants." *Environmental and Experimental Botany* 186 (2021): 104430.

Saravanakumar, D., and R. Samiyappan. "ACC deaminase from Pseudomonas fluorescens mediated saline resistance in groundnut (Arachis hypogea) plants." *Journal of Applied Microbiology* 102, no. 5 (2007): 1283-1292.

Sawada, Hiroko, Ie-Sung Shim, and Kenji Usui. "Induction of benzoic acid 2-hydroxylase and salicylic acid biosynthesis—modulation by salt stress in rice seedlings." *Plant Science* 171, no. 2 (2006): 263-270.

Sayyad-Amin, Parvaneh, Mohammad-Reza Jahansooz, Azam Borzouei, and Fatemeh Ajili. "Changes in photosynthetic pigments and chlorophyll-a fluorescence attributes of sweet-forage and grain sorghum cultivars under salt stress." *Journal of biological physics* 42, no. 4 (2016): 601-620.

Schoenborn, Liesbeth, Penelope S. Yates, Bronwyn E. Grinton, Philip Hugenholtz, and Peter H. Janssen. "Liquid serial dilution is inferior to solid media for isolation of cultures representative of the phylum-level diversity of soil bacteria." *Applied and Environmental Microbiology* 70, no. 7 (2004): 4363-4366.

Serrano, Ramón, Jose M. Mulet, Gabino Rios, Jose A. Marquez, Iñigo F. De Larrinoa, Martin P. Leube, Iratxe Mendizabal et al. "A glimpse of the mechanisms of ion homeostasis during salt stress." *Journal of experimental botany* (1999): 1023-1036.

Shepherd, Tom, and D. Wynne Griffiths. "The effects of stress on plant cuticular waxes." *New Phytologist* 171, no. 3 (2006): 469-499.

Shevyakova, N. I., E. A. Bakulina, and Vl V. Kuznetsov. "Proline antioxidant role in the common ice plant subjected to salinity and paraquat treatment inducing oxidative stress." *Russian Journal of Plant Physiology* 56, no. 5 (2009): 663-669.

Shi, Yu, Yichao Wang, Timothy J. Flowers, and Haijun Gong. "Silicon decreases chloride transport in rice (Oryza sativa L.) in saline conditions." *Journal of plant physiology* 170, no. 9 (2013): 847-853.

Shu-Hsien, Hung, Y. U. Chih-Wen, and Chin Ho Lin. "Hydrogen peroxide functions as a stress signal in plants." *Botanical Bulletin of Academia Sinica* 46 (2005).

Shumilina, Julia, Alena Kusnetsova, Alexander Tsarev, Henry C. Janse van Rensburg, Sergei Medvedev, Vadim Demidchik, Wim Van den Ende, and Andrej Frolov. "Glycation of plant proteins: regulatory roles and interplay with sugar signalling?." *International journal of molecular sciences* 20, no. 9 (2019): 2366.

Somasegaran, Padma, and Heinz J. Hoben. "Quantifying the growth of rhizobia." In *Handbook for rhizobia*, pp. 47-57. Springer, New York, NY, (1994).

Somasekhar, N.; Hari, K.; Sankaranarayanan, C. Comparative performance of three diazotrophic rhizobacteria in growth promotion and suppression of root-knot nematode in sugarcane. In Proceedings of the Sixth International PGPR Workshop on Plant Growth Promoting Rhizobacteria, Calicut, India, (2003): 194–195.

Strzałka, K., A. Kostecka-Gugała, and D. Latowski. "Carotenoids and environmental stress in plants: significance of carotenoid-mediated modulation of membrane physical properties." *Russian Journal of Plant Physiology* 50, no. 2 (2003): 168-173.

Su, Tong, Xuezhi Li, Mingyue Yang, Qun Shao, Yanxiu Zhao, Changle Ma, and Pingping Wang. "Autophagy: an intracellular degradation pathway regulating plant survival and stress response." *Frontiers in plant science* 11 (2020): 164.

Suzaki, Takuya, Emiko Yoro, and Masayoshi Kawaguchi. "Leguminous plants: inventors of root nodules to accommodate symbiotic bacteria." *International review of cell and molecular biology* 316 (2015): 111-158.

Takahashi, Shunichi, and Norio Murata. "How do environmental stresses accelerate photoinhibition?." *Trends in plant science* 13, no. 4 (2008): 178-182.
Takahashi, Taku, and Jun-Ichi Kakehi. "Polyamines: ubiquitous polycations with unique roles in growth and stress responses." *Annals of botany* 105, no. 1 (2010): 1-6.

Tian, Zhijie, Jingpeng Li, Xueying Jia, Fu Yang, and Zhichun Wang. "Assimilation and translocation of dry matter and phosphorus in rice genotypes affected by salt-alkaline stress." *Sustainability* 8, no. 6 (2016): 568.

Tiwari, Shalini, Vivek Prasad, Puneet S. Chauhan, and Charu Lata. "Bacillus amyloliquefaciens confers tolerance to various abiotic stresses and modulates plant

response to phytohormones through osmoprotection and gene expression regulation in rice." *Frontiers in Plant Science* 8 (2017): 1510.

Van Loon, L. C., P. A. H. M. Bakker, and C. M. J. Pieterse. "Systemic resistance induced by rhizosphere bacteria." *Annual review of phytopathology* 36 (1998): 453-483.

Van Zelm, Eva, Yanxia Zhang, and Christa Testerink. "Salt tolerance mechanisms of plants." *Annual review of plant biology* 71 (2020): 403-433.

Verbruggen, Nathalie, and Christian Hermans. "Proline accumulation in plants: a review." *Amino acids* 35, no. 4 (2008): 753-759.

Vernon, Leo P. "Spectrophotometric determination of chlorophylls and pheophytins in plant extracts." *Analytical Chemistry* 32, no. 9 (1960): 1144-1150.

Vessey, J. Kevin. "Plant growth promoting rhizobacteria as biofertilizers." *Plant and soil* 255, no. 2 (2003): 571-586.

Wang, Juan, Yunxiang Zhang, Xingrong Yan, and Jinping Guo. "Physiological and transcriptomic analyses of yellow horn (Xanthoceras sorbifolia) provide important insights into salt and saline-alkali stress tolerance." *PLoS One* 15, no. 12 (2020): e0244365.

Weisany, Weria, Yousef Sohrabi, Gholamreza Heidari, Adel Siosemardeh, and Kazem Ghassemi-Golezani. "Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean ('Glycine max'L.)." *Plant Omics* 5, no. 2 (2012): 60-67.

Yamamoto, Yoko, Yukiko Kobayashi, and Hideaki Matsumoto. "Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots." *Plant physiology* 125, no. 1 (2001): 199-208.

Yokoi, Shuji, Francisco J. Quintero, Beatriz Cubero, Maria T. Ruiz, Ray A. Bressan, Paul M. Hasegawa, and Jose M. Pardo. "Differential expression and function of Arabidopsis thaliana NHX Na+/H+ antiporters in the salt stress response." *The Plant Journal* 30, no. 5 (2002): 529-539.

Yuan, Yongge, Caroline Brunel, Mark van Kleunen, Junmin Li, and Zexin Jin. "Salinity-induced changes in the rhizosphere microbiome improve salt tolerance of Hibiscus hamabo." *Plant and Soil* 443, no. 1 (2019): 525-537.

Zahran, Hamdi Hussein. "Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate." *Microbiology and molecular biology reviews* 63, no. 4 (1999): 968-989.

Zawoznik, Myriam S., Mayra Ameneiros, María P. Benavides, Susana Vázquez, and María D. Groppa. "Response to saline stress and aquaporin expression in

Azospirillum-inoculated barley seedlings." *Applied microbiology and biotechnology* 90, no. 4 (2011): 1389-1397.

Zhang, L., S. E. Khabbaz, A. Wang, H. Li, and P. A. Abbasi. "Detection and characterization of broad-spectrum antipathogen activity of novel rhizobacterial isolates and suppression of F usarium crown and root rot disease of tomato." *Journal of applied microbiology* 118, no. 3 (2015): 685-703.

Zhang, Hui, Xiao-Long Liu, Rui-Xue Zhang, Hai-Yan Yuan, Ming-Ming Wang, Hao-Yu Yang, Hong-Yuan Ma, Duo Liu, Chang-Jie Jiang, and Zheng-Wei Liang. "Root damage under alkaline stress is associated with reactive oxygen species accumulation in rice (Oryza sativa L.)." *Frontiers in plant science* 8 (2017): 1580.

Zhao, Shuangshuang, Qikun Zhang, Mingyue Liu, Huapeng Zhou, Changle Ma, and Pingping Wang. "Regulation of plant responses to salt stress." *International Journal of Molecular Sciences* 22, no. 9 (2021): 4609.

Zhu, Jian-Kang. "Salt and drought stress signal transduction in plants." *Annual review of plant biology* 53 (2002): 247.