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PLANT-BASED BIOSTIMULANTS

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ASST. PROF. BAHAR YILDIZ KUTMAN

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

YENİ BİTKİ BAZLI BİYOSTİMÜLANTLAR OLARAK SÖĞÜT ÖZÜTLERİ

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SUMMARY

Salinity is one of the abiotic stresses threatening crop production and thus global food security. Biostimulants can be used as innovative and promising agents to address current challenges to sustainable agriculture. Plant-based biostimulants, also called botanicals, are attractive options due to their safety, renewability and low cost however, the mechanisms of their action are not fully explored.

Willow tree (*Salix* spp.) extracts are rich in many bioactive compounds including salicylates. Extracts of willow bark contain substantial amounts of salicin, which can be converted into the phytohormone salicylic acid, and have been used in traditional medicine for their anti-inflammatory and analgesic activities.

In this thesis study, the potential of willow bark and leaf extracts were evaluated as plant-based biostimulants to improve maize (*Zea mays* cv. Caramelo) growth in the absence and presence of salinity stress. Experiments were conducted on perlite, soil and solution culture under growth chamber conditions.

Perlite and soil experiments results suggest that willow tree extracts as a seed treatment agent could improve the maize seedlings performance and enhance various growth parameters including shoot and root lengths as well as biomass production under both control and saline conditions. Moreover, these extracts enhanced mineral content and protein concentration and reduced the negative effects of salinity in the early period.

In hydroponics experiments, especially, willow bark extracts enhanced root growth and development. The observed positive effects of willow extracts is thought to be due to its rich bioactive compounds. As a natural compound, willow extracts have a huge potential to be used as SA sources instead of chemical SA to increase the growth and development of plants.

Results suggest that aqueous extracts of willow tissues may be used as biostimulants to improve crop performance although effects may not be salinity specific. Further studies are needed to determine the compositions of extracts and their effects on other crops under different stress conditions.

Key words: biostimulant, salinity stress, seed treatment, willow leaf extract, willow bark extract, salicylic acid, hydroponic culture

ÖZET

Tuzluluk, bitki üretimini ve dolayısıyla küresel gıda güvenliğini tehdit eden abiyotik streslerden biridir. Biyostimülantlar, sürdürülebilir tarıma yönelik mevcut zorlukları gidermek için yenilikçi ve ümit verici ajanlar olarak kullanılabilir. Bitkisel olarak da adlandırılan bitki bazlı biyostimulantlar, güvenilir, yenilenebilir ve düşük maliyetleri nedeniyle cazip seçeneklerdir, ancak etki mekanizmaları tam olarak araştırılmamıştır.

Söğüt ağacı (*Salix* spp.) özütleri salisilatlar da dahil birçok biyoaktif bileşik bakımından zengindir. Söğüt kabuğunun özleri, fitohormon olan salisilik aside dönüştürülebilen ve geleneksel tıpta sıkça kullanılan önemli miktarlarda salisin içerir.

Bu tez çalışmasında söğüt kabuğu ve yaprak özütlerinin biyostimulant potansiyeli mısırın (*Zea mays* cv. Caramelo) tuz stresine karşı büyümesi açısından ele alınmıştır. Deneyler iklimlendirme odası koşullarında perlit, toprak ve su çözeltisi ortamlarında yürütülmüştür.

Perlit ve toprak deneyleri sonuçları, bir tohum uygulama maddesi olarak söğüt ağacı özütlerinin hem kontrol hem de tuzlu koşullarında mısırın biyokütle üretiminin yanı sıra sürgün ve kök uzunlukları dahil olmak üzere çeşitli büyüme parametrelerini arttırabileceğini göstermektedir. Bu uygulamalar mineral içeriği ve protein konsantrasyonunu arttırıp ve erken dönemde tuzluluğun olumsuz etkilerini azaltmıştır.

Su kültürü deneylerinde, özellikle söğüt kabuğu özütünün, kök büyümesini ve gelişimini arttırdığı görülmüştür. Söğüt özütünün gözlemlenen olumlu etkilerinin, içerdikleri zengin biyoaktif bileşiklerden kaynaklandığı düşünülmektedir. Söğüt özütleri, bitkilerin büyüme ve gelişimini arttırmak için kimyasal SA yerine doğal bir SA kaynağı olarak kullanılabilir.

Sonuçlar, söğüt doku özütlerinin tuz stresi olan ve olmayan koşullarda mısır performansını arttırmak için biyolojik uyarıcılar olarak kullanılabileceğini göstermektedir. Özütlerin kompozisyonlarının ve farklı stres koşulları altında başka bitkiler üzerindeki etkilerinin belirlemek için başka araştırmalara ihtiyaç vardır.

Anahtar Kelimeler: biyostimulant, tuzluluk stresi, tohum uygulamaları, söğüt yaprak ekstraktı, söğüt kabuk ekstraktı, salisilik asit, su kültürü

ACKNOWLEDGEMENTS

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Abbreviations and Acronyms		**Explanations**
EC	:	Electrical Conductivity
EC_e	:	Saturated Paste Extract
CAGR	:	Compound Annual Growth Rate
GB	:	Glycine Betaine
PHs	:	Protein Hydrolysates
ESP	:	Exchangeable Sodium Percentage
CK	:	Cytokinin
Al	:	Aluminum
Co	:	Cobalt
Na	:	Sodium
Se	:	Selenium
Si	:	Silicon
PS II	:	Photosystem II
ROS	:	Reactive Oxygen Species
PAL	:	Phenylalanine ammonia-lyase
SWE	:	Seaweed extract
$^1O_2$	:	Singlet oxygen
H_2O_2	:	Hydrogen peroxide
O_2^{-}	:	Superoxide radical
OH^-	:	Hydroxyl radical
K^+	:	Potassium
Ca^{+2}	:	Calcium
N	:	Nitrogen
JA	:	Jasmonic acid
GA	:	Gibberellins
ABA	:	Abscisic acid
IAA	:	Indole-3-Acetic Acid

SA	:	Salicylic acid
CAT	:	Catalase
APX	:	Ascorbate peroxidase
SOD	:	Superoxide dismutase
GR	:	Glutathione reductase
POD	:	Peroxidase
HCl	:	Hydrochloric acid
NaOCl	:	Sodium hypochlorite
ASC	:	Ascorbate
GPX	:	Guaiacol peroxidase
GSH	:	Reduced glutathione
ANOVA	:	Analysis of variance
n.s.	:	not significant
Mo	:	Molybdenum
ASA	:	Acetylsalicylic acid
IC	:	Isochorismate
PAs	:	Proanthocyanidins
Saligenin	:	Salicylic alcohol
Conc.	:	Concentration
Cont.	:	Content
cv.	:	cultivar
DAS	:	Days After Sowing
LSD	:	Fisher's least significant difference
HSD	:	Tukey's honestly significant difference
$Ca(NO_3)_2$	:	Calcium nitrate
WB	:	Willow Bark
WL	:	Willow Leaf
WS	:	Water Soaking

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each containing 30 seeds. The lower-case letters were given according to the Fisher's protected LSD test results in the control condition and salt stressed condition.

and the lower-case letters were given for to the salt stressed condition.

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1. INTRODUCTION

With a growth rate of at least 25%, human population is estimated to reach approximately 10 billion by 2050 [Schroeder et al., 2013], [Rouphael and Colla, 2018a]. However, the arable land in use is expected to increase by only 5% until 2050 [FAO, 2009]. Trend of shifting to non-agricultural activities like urbanization and industrialization, problems coming with the climate change, degradation of arable land due to wrong irrigation practices, pollution and desertification cause a decrease in readily used arable land [Motha and Baier, 2005].

Until 2050, to feed the increasing population, food production has to be approximately doubled in developing countries and increased by 70% globally to meet the demand [FAO, 2009]. Therefore, it will become even more important to achieve maximum crop yield from the unit area in the future [Tan et al., 2006], [Rouphael et al., 2018b].

On the other hand, the usage of chemical fertilizers in agriculture can be quite inefficient because over-fertilization or other wrong fertilization applications can cause soil degradation or salinization [Keeney and Olson, 1986], [Halpern et al., 2015]. The excess fertilizers can run away to nature for example via leaching from the agricultural area to underground waters and lead to environmental problems which negatively affect all living being and deteriorated soil properties.

The decreasing arable land, usage of chemical fertilizer and climatic changes threaten global food safety and security. So, sustainable, eco-friendly and also efficient agriculture practices are required for feeding the growing population [Duhamel and Vandenkoornhuyse, 2013].

1.1. Biostimulants

In recent years, natural plant biostimulants, which can be a promising solution as an alternative, safe and innovative and productive approach for sustainable agricultural production, are gradually replacing synthetic chemicals [Rouphael et al., 2017b], [Van Oosten et al., 2017]. As a result, the negative effects of chemical fertilizers and environmental pollution as well as farmer's production cost are expected to decrease [Ertani et al., 2016].

Biostimulants are neither fertilizers nor pesticides, but when applied in small quantities they can enhance the yield, growth and health of the plant [Calvo et al., 2014], [Brown and Saa, 2015], [Lovatt, 2015].

Although the exact mechanisms are not known or very much depend on the substance that is used as a biostimulant, biostimulants can increase nutrient uptake, nutrient use efficiency, photosynthesis capacity and biotic or abiotic stress tolerance of plants [du Jardin, 2015], [Brown and Saa, 2015], [Yakhin et al., 2017]. Moreover, biostimulants are reported to provide better crop yield, quality, and vigour as well as better germination, leaf and fruit number and root growth and development [Roupheal et al., 2018]. In biostimulant treated plants, increased amounts of primary metabolites like amino acid, protein, sugar and also secondary metabolites such as phenolic compounds are measured [Ertani et al., 2011]. Biostimulants can be applied to:

1) seeds by different seed treatment techniques,

2) directly to plants by foliar applications, or

3) plant roots by mixing with irrigation water or growing medium like perlite or soil.

Biostimulants may contain many compounds that are important for plant metabolism [du Jardin, 2015]. These compounds can be organic substances like proteins, carbohydrates, lipids, phenolic compounds, hormones, vitamins and nucleotides or inorganic substances like beneficial elements and play a critical role in primary and secondary plant metabolism [Yakhin et al., 2017]. Moreover, some biostimulants involve signaling molecules act as secondary messengers which stimulate signaling pathways and cellular responses [Mochida and Shinozaki, 2011], [Wang and Irving, 2011].

Biostimulants help various plants withstand to different stress condition by inducing of the signaling pathways and antioxidant system, reducing reactive oxygen species and enhancing secondary metabolites [Ertani et al., 2013]. The effectiveness of biostimulants may vary depending on the type of plant, developmental phase, period, amount and process of practice [Colla et al., 2015].

The global biostimulants market is rapidly expanding and by 2022 this market value is expected to reach from $ 1 billion to $ 3 billion with at a compound annual growth rate (CAGR) of approximately 11%. Europe (34%) has the largest share of the world biostimulant market, followed by North America (23%) and Asia-Pacific (22%) [Rouphael et al., 2018a].

As a result, the use of biostimulants in agriculture application has enormous potential that can be important contributions to early-stage plant growth and development through the seed soaking especially can help plants overcome stress situations, including salinity [de Vasconcelos et al., 2009].

1.1.1. Main Categories of Plant Biostimulants

Biostimulants can be classified in many different categories and found in alternative formulations. But they are generally classified into the following major groups: inorganic compounds, chitosan, humic and fulvic acids, seaweed extracts, botanicals, protein hydrolysates, beneficial fungi and bacteria [du Jardin, 2015].

1.1.1.1. Inorganic Compounds

Elements which can be used to optimize the plant growth and development but not essential for the life cycle of plants or essential for only particular plants are known as beneficial elements and they can be classified as a group of biostimulants (Kleiber and Markiewicz, 2013; Radkowski and Radkowska, 2013). Some examples are aluminum (Al), cobalt (Co), sodium (Na), selenium (Se), and silicon (Si) are called beneficial elements [Broadley et al., 2012].

The effects of these beneficial elements may vary depending on the type and dose [Pilon-Smits et al., 2009]. Beneficial elements can play a role in primary or secondary metabolism and nodulation of plants and can improve plant growth and tolerance to biotic and abiotic stresses, induce hormone synthesis and signaling and provide cell wall stability and osmoregulation [Vatansever et al., 2017], [Pilon-Smits et al., 2009].

Inorganic salts of such elements, like chloride, phosphate, phosphite silicate and carbonate are shown to enhance resistance against to biotic stress including fungal disease and thus can be used, at least conditionally, as antifungal agents instead of synthetic fungicides [Deliopoulos et al., 2010].

1.1.1.2. Chitosan

Chitosan is the second most common polysaccharide in nature after cellulose [Hejazi and Amiji, 2003] and can be found in the cell walls of crustacean shells insects

and fungi [Sandford, 2003]. This biopolymer has a wide application area including food, medicine, cosmetic due to its natural, non-toxic, biodegradable and antimicrobial properties [Pichyangkura and Chadchawan, 2015]. In addition, chitosan can also be used as biostimulant in agricultural area [Pichyangkura and Chadchawan, 2015].

Chitosan can be applied as a soil conditioner, antimicrobial or seed treatment agent, and these applications are reported to have a positive effect on plant metabolism [Lee et al., 2005]. Chitosan has beneficial effects on plant metabolism, including enhanced plant growth and development, better germination and crop quality [Kim et al., 2005].

Moreover, this biopolymer can act as an elicitor and induce the defense system, improve accumulation of secondary metabolites, and thus provide resistance to different biotic (fungi, viruses, and bacteria) and abiotic stresses (drought, salinity) [Katiyar et al., 2015].

1.1.1.3. Beneficial Fungi and Bacteria

Microorganisms-based biostimulants like beneficial bacteria (Rhizobium, Azotobacter, Bacillus etc.) and fungi (Arbuscular mycorrhizal, Trichoderma spp. etc.) are also classified as biofertilizers or biopesticides. The widespread usage of these microorganisms in agriculture is critical for sustainable agriculture as they have a potential to reduce the need of synthetic products which can be harmful to the environment [Vessey, 2003], [Selvakumar et al., 2009].

Beneficial bacteria and fungi are found in the rhizosphere of plants and can produce stimulant compounds like phytohormones which have critical roles in plant metabolism and thus may help the plants to tolerate various stress conditions [Bhattacharyya et al., 2012].

Moreover, these beneficial microorganisms can form symbiotic relationship with plants and cause changes in root morphology by enhancing root area, weight and length and, therefore can improve water-use-efficiency, crop yield and nutrient uptake particularly for phosphorus and nitrogen [Kloepper et al., 2007], [Ravensberg, 2015].

1.1.1.4. Humic and Fulvic acids

Humic and fulvic acids constitute more than half of the active organic compounds in the soil [Stevenson, 1994]. These substances are formed by biodegradation of dead

organic matters in soils in a quite long time and when mixed with soils, they can increase the organic matter content and increase agricultural production [Bulgari et al., 2015], [Canellas et al., 2015].

When humic substances are applied to the soil with the purpose of improving soil properties and fertility [Rouphael and Colla, 2018], nutrient availability and solubility [Zandonadi et al., 2007]. They can improve carbon and nitrogen metabolism of plants [Canellas et al., 2015] and enhance many growth and quality related parameters [Halpern et al., 2015].

These compounds can show biostimulant activity by affecting root morphology, growth, and development [Calvo et al., 2014], secondary metabolite production [de Pascale et al., 2017], reactive oxygen secies (ROS) scavenging, phenylalanine ammonia-lyase (PAL) activity [Olivares et al., 2015] as well as regulation of related genes. In this way, plants are reported to withstands stress condition such as drought and salinity [Battacharyya et al., 2015], [Calvo et al., 2014].

1.1.1.5. Seaweed Extract

Seaweed extract (SWE) or macroalgae are another subclass of biostimulants, which are often used for foliar or soil applications as powder or liquid extracts [Craigie, 2011], [Battacharyya et al., 2015]. These compounds can also be used in organic farming as organic fertilizers due to their marine sources and organic origin. The biostimulant effect of SWEs can be attributed the wide range of bioactive compounds such as osmolytes, nutrients, secondary metabolites, polysaccharides, and plant hormones [Khan et al., 2009].

SWE has been reported to enhance plant growth, germination capacity, flowering, nutrient uptake and remobilization, fruit setting, rhizosphere microorganism activity, nucleic acid and chlorophyll synthesis and root structure development [Birceno-Domínguez et al., 2014], [Hernández-Herrera et al., 2014], [Arioli et al., 2015]. Furthermore, these biostimulant substances can affect the regulation of genes, primary metabolism of plants such as photosynthesis, respiration [Sharma et al., 2014] and resistance against biotic [Allen et al., 2001] and abiotic stresses [Elansary et al., 2016].

1.1.1.6. Protein Hydrolysates (PHs)

Protein hydrolysates (PHs) include peptides and amino acids which are produced from either plant (legume seeds, alfalfa hay) or animal sources (leather, fish) by chemical and/or enzymatic hydrolysis [Kumar et al., 2019], [Schaafsma, 2009]. They can be applied in low amounts as leaf, seed or soil applications in various forms such as liquid, granule or powder [Colla et al., 2015].

When compared to animal-derived PHs, plant-derived PHs are preferred more in agricultural production due to food safety issues and vegetarian food nutrition or religious related customer preferences [Colla et al., 2014], [Cerdán et al., 2009]. According to the literature, the application of various plant-based protein hydrolysates on different plant species has improved germination capability, plant growth and development, crop yield and quality, root structure and nutrient uptake [Ertani et al., 2009], [Paul et al., 2019].

Moreover, these biostimulant substances play a critical role in the plant defense system and provide resistance to abiotic stresses by activation of signaling molecules and antioxidant enzymes, enhancing carbon and nitrogen metabolic activities and proline accumulation [Rouphael et al., 2017a], [Sestili et al., 2018].

1.1.1.7. Botanicals

Plant-based biostimulants, which are also called botanicals, are plant extracts or substances obtained from plants which can also be used as food additives or other products that are manufactured by pharmaceutical and cosmetic industries [du Jardin, 2015], [Seiber et al., 2014]. Botanical extracts include several important natural bioactive molecules like natural phenolics [Khattak et al., 2015] and these bioactive substances can increase yield and fruit quality, enhance photosynthesis, carbohydrate levels [Ziosi et al., 2012] and nodule development, improve secondary metabolite production [Yakhin et al., 2017] and [Bibi et al., 2016]. These plant-based biostimulants can be applied to agronomic valuable plants by seed treatment or foliar spraying both in the presence or absence of a stress condition.

A good example for plant-based biostimulants is Moringa leaves extract which have attracted the attention of agronomists due to its attractive properties such as being a natural, renewable, cheap, good source of cytokinin (CK), zeatin, antioxidants, vitamin, amino acids, protein and nutrients [Phiri and Mbewe, 2010], [Siddhuraju and Becker,

2003], [Bibi et al., 2016]. Application of moringa leaf extracts to different plants like maize [Bibi et al., 2016], [Afzal et al., 2012], [Iftikhar et al., 2009], [Rehman et al., 2015], [Basra et al., 2011], sunflower [Basra et al., 2009], rangeland grasses [Nouman et al., 2012], wheat [Yasmeen et al., 2013], lentil [Imran et al., 2014], pea [Merwad, 2018], radish [Ashraf et al., 2018] and tomato [Yasmeen et al., 2014] demonstrated stimulatory action and enhanced plant abiotic stress tolerance by increasing phenolics accumulation, root and shoot lengths, chlorophyll contents, fresh and dry weights of plants and provided better seed germination [Bibi et al., 2016], [Basra et al., 2011].

Another example of botanical biostimulants are garlic extracts. Improved plant growth and development, higher biomass, better quality and activation of key enzymes are observed when plants are treated with this compound [Hayat et al., 2018].

Finally, sugar beet extract are documented to contain many bioactive substances as well as glycine betaine (GB) which is one of the major organic osmolytes. Abbas et al. (2010) showed that many metabolic activities, GB level, growth, tolerance to salinity stress and yield of eggplant increased with leaf application of sugar beet extracts.

1.2. Abiotic Stress

Abiotic stress has arisen from excess or deficit of non-biological factors and can adversely affect food safety, crop production, yield and quality and result environmental degradation [Forni et al., 2017]. Plants are frequently exposed to these stress conditions including salinity, drought, UV, temperature (heat, cold, freezing), heavy metal toxicities, flooding, and inadequate oxygen [Hirayama and Shinozaki, 2010]. These stresses act as a serious threat to agricultural activities and can cause more than half of the world's major crop yield to be lost [Agarwal et al., 2018].

Development of cereals which are adapted to undesirable environmental conditions is very important for sustainable food production [Gong et al., 2014]. Today, various strategies are used to produce plants that can withstand these stresses but most of these methods are not easily applicable to farms or it takes a long time for the growers to adapt them [Ashkani et al., 2015].

1.2.1. Salinity Stress

Salinity stress is one of the most commonly observed abiotic stresses that seriously damage the agricultural sector and endanger the sustainable food supply of the growing global human population [Agarwal et al., 2018], [Botella et al., 2005]. More than one-third of the world's fertile lands are affected by soil salinity and especially in arid and semi-arid regions productivity of approximately 3.000 hectares of arable land is reduced significantly [Shabala, 2013], [Qadir et al., 2014]. Unfortunately, it is estimated that the impact of soil salinity will increase in the coming years and almost half of the fertile land will turn into barren lands before the 22nd century [Wang et al., 2003]. If not totally impossible, the transformation of the areas affected by salinity into a productive land is a difficult, time consuming and expensive [Ondrasek et al., 2011]. In Turkey, approximately 1.5 million hectares of land, which is about one-third of the fertile land, are affected by soil salinity in various degrees [Ekmekci et al., 2005].

Soil salinity consists of electrolytes of anions and cations resulting from the dissolution of various salts such as $NaCl$, KCl, Na_2SO_4, Na_2CO_3, $MgCl_2$, $MgSO_4$ in soil solution or water [Munns and Tester, 2008]. Soil salinization may be observed due to natural causes (primary salinity) or human activities (secondary salinity) [Parihar et al., 2015]. The primary salinity is caused by long term salt accumulation due to natural causes including global warming, insufficient rainfall, increase in evapotranspiration, weathering of native rocks, salty underground water and tides [Arzani, 2008], [Tahjib-Ul-Arif et al., 2018], [Rouphael et al., 2018c].

On the other hand, secondary salinity arises from anthropogenic activities such as intensive farming, improper irrigation practices and drainage systems (excessive, salt-rich and poor quality irrigation water, insufficient drainage), wrong fertilizer management (over-fertilization, Cl-containing fertilizers) and land clearing which alter the hydrological offset between soil and water [Manchanda and Garg, 2008], [Ilangumaran and Smith, 2017], [Rouphael et al., 2018c].

Electrical conductivity (EC) and exchangeable sodium percentage (ESP) can be used as parameters to determine the soil salinity level. With increasing electrical conductibility, the osmotic potential decreases and the soil solute concentration increases [Manchanda and Garg, 2008]. If the measured saturated paste extract (EC_e) value is 4 or

more and the ESP value is 15% or less, the soils are considered as saline soils and this corresponds to approximately to 40 mM NaCl [Munns and Tester, 2008]. The high ESP ratio indicates that Na is much more abundant than the other exchangeable cation. Soils with an ESP value greater than 15 are not only classified as saline soils but they are considered saline-sodic soils [Chhabra, 2017].

According to the EC mesaurements some threshold levels are reported for different plant species. The threshold value means the highest soil salinity which does not result in a yield reduction for a specific plant species. Since the salinity threshold value of each plant may be different, the impact level of this threshold may vary depending on plant species [Maas, 1986], [Munns, 2002]. If the EC of saturation extract of the soil is lower than this threshold value, that plant species is not expected to show a biomass or yield reduction due to salinity stress and above the threshold salinity stress is expected to effect that crop species. In addition, the slope level gives information about the expected percent decrease in yield or biomass per one unit increase in measured soil EC values above that salinity threshold [Tanji and Kielen, 2002]. Table 1 shows the specific threshold values of some plants species. For example according to this table maize has a threshold of 1.7 dS/m and the maize yield is expected to be reduced by about 13% for each 1ds/m rise in ECe of soil [Ma et al., 2008].

Table 1.1 Salt tolerance of some crop species

Crop species	EC of saturated soil extract	
	Threshold (EC_e) (ds/m)	Slope (% per ds/m)
Barley (*H. Vulgare*)	8.0	5.0
Sugar Beet (*B. vulgaris*)	7.0	5.9
Wheat (*T. aestivum*)	6.0	7.1
Soybean (*G. max*)	5.0	20.0
Sunflower (*H. annuus*)	4.8	5.0
Tomato (*L. esculentum*)	2.5	9.9
Maize (*Z. mays*)	1.7	12.9
Common Bean (*P. vulgaris*)	1.0	19.0

Halophytes, which are also known as salinity tolerant plants and constitute only a small part of the world flora, can accumulate Na^+ ions in their vacuoles and use it as

osmoticum [Wungrampha et al., 2018]. Low Na concentrations can exert a beneficial effect on plant growth, especially in natrophilic (salt-loving) species. By using osmotic adjustment, these plants can take up water from the soil and thus they can easily grow and develop in saline regions [Flowers and Colmer, 2015], [Shabala and Mackay, 2011].

1.2.1.1. Effects of Salinity on Plants

The adverse effects of salt stress on plants are related to the osmotic stress in short term and ionic toxicity in long term exposure [Carillo et al., 2011]. Firstly, the accumulation of soluble salts is detected by the root system immediate induces osmotic stress. As the amount of salt in the soil increases, the osmotic potential as well as total water potential of the soil decreases and the plant cannot take up water [Kader and Lindberg, 2010], [Acosta-Motos et al., 2017]. Secondly, high concentration of ions especially Na^+ and Cl^- that accumulate in the plant over time causes ionic stress. These ions enter the plant in the transpiration stream and start to accumulate on the old leaves of the plant and may cause progressive damage or death [Wungrampha et al., 2018]. The accumulation of excess Na^+ and Cl^- in the cytoplasm impair cell structure and function and inhibit growth and development by disrupting many metabolic processes [Pirasteh-Anosheh et al., 2017], [Parihar et al., 2015].

In general, when the plants are exposed to salt stress, their physiological and metabolic balances deteriorate and many critical steps and processes including germination, seedling establishment, reproduction, cellular structure, enzymatic activities, nutrient homeostasis, yield, respiration, water balance, hormonal status and photosynthesis are negatively affected due to direct or indirect effects of osmotic and ionic stress [Parihar et al., 2015], [Fardus et al., 2018], [Bulgari et al., 2018].

The seed germination, which is the first stage of plant development and very critical to determine the plant yield, is severely affected due to impaired water absorption of seeds as a result of osmotic stress [Fernández-Torquemada et al., 2013]. Under saline conditions germination may be delayed or totally blocked.

The growth-related parameters including plant height, fresh and dry weight, leaf number, size and area, number of flowers, seed setting, grain yield, and total biomass are decreased by the presence of excess salt ions [Guan et al., 2011], [Dolatabadian et al.,

2011]. The negative effect of salinity on shoot growth is greater when compared to root growth, so root to shoot ratio increase [Jimenez et al., 2003].

Photosynthesis is one of the most critical processes for plants and is negatively affected by salinity stress since photosystem II (PS II) activity, chlorophyll content, stomatal conductivity is reduced, thylakoid membrane stability is impaired and photosynthesis-related genes are downregulated [López-Climent et al., 2008].

The nutrient homeostasis is also impaired as the toxic ions reduce or interfere the uptake, translocation and remobilization mechanisms of essential minerals including potassium (K^+), calcium (Ca^{+2}) as well as nitrogen (N). In salt affected plants lower K^+/Na^+ and Ca^{+2}/Na^+ ratio are observed [Rady et al., 2017], [Zhu, 2001]. The excess of salts inhibits N uptake and disrupt nitrogen metabolism because of the relationship between Na^+ and NH_4^+ as well as between Cl^- and NO_3^- [Rozeff, 1995]. The Na^+ ions compete with K^+ ions because of their chemical similarities and causes K deficiency by inhibiting K uptake [Chinnusamy et al., 2006]. K^+ is one of the most critical and abundant essential element in plant cells and play a critical role in plant metabolism including stomatal control, photosynthetic efficiency, enzymatic activities, maintaining membrane stability and homeostasis and also protein synthesis. It is also an important determinant of plant tolerance to abiotic stress [Rodriguez-Navarro et al., 2006], [Isayenkov et a., 2019]. Ca is also another essential element and critical for cell wall stability [Hu et al., 2007].

Under the salinity stress, the production of ROS, which are highly reactive species containing oxygen such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH^-), etc., increases and oxidative stress is stimulated [Borsani et al., 2001]. Under normal conditions ROS is synthesized at low levels and act as signal transduction molecules in many important processes such as growth, development, signalling pathways, stress tolerance, cell cycle and apoptosis [Miller et al., 2008], [Miller et al., 2010]. However, under stress conditions, the increased ROS level causes oxidative stress as toxic compounds and significantly damages the plant cell [Chinnusamy et al., 2005]. High production of ROS can result in mutations in DNA level, deterioration of membrane function and chlorophyll pigments and degradation of proteins [Muchate et al., 2016]. Moreover, ROS disrupts the lipid metabolism and thus reduces the

membrane stability by decreasing membrane fluidity and deteriorating its integrity [Birben et al., 2012], [Ayala et al., 2014].

Salinity stress causes significant changes in the levels of phytohormones including abscisic acid (ABA), Indole-3-Acetic Acid (IAA) and salicylic acid (SA) [Zholkevich and Pustovaytova, 1993]. Under salt stress, ABA is mainly responsible for inhibition of stomatal opening, therefore it prevents the stomatal transpiration and water loss [Parida and Das, 2005], [Kawasaki et al., 2001]. The reduced levels of IAA and SA have negative effects on seed germination and growth [Zholkevich and Pustovaytova, 1993], [Verma et al., 2016]. Therefore, treating the plants with these hormones can mitigate the harmful impacts of salt on plants [Javid et al., 2011].

Salinity also impairs the soil structure and thus can cause adverse effects on root morphology of plants which are grown under salt-affected soils. The accumulation of salts in the soil change soil structure and properties, cause soil compaction, reduce aeration and water permeability capacity of soils. These soil related issues limiting root growth due to restricting air and water movement [Machanda and Garg, 2008].

The above-mentioned adverse effects of salt stress may vary depending on the species, genotype, and development stage of the plant subject to stress, salt type as well as the level and duration of stress [Dajic, 2006].

1.2.1.2. Plant Salinity Tolerance

Plants have developed many adaptation mechanisms to cope with saline conditions including osmotic adjustment, salts exclusion/ inclusion and compartmentalization, stimulation of antioxidative, hormonal, and secondary mechanisms, upregulated stress-related genes and expression of defense proteins for avoiding to salt stress [Hamed et al., 2018], [Pirasteh-Anosheh et al., 2017], [Sorahinobar et al., 2016], [Liu et al., 2016].

Osmotic adjustment is a mechanism where accumulation of compatible inorganic ions and organic solutes such as proline, glycine betaine, sugar alcohol (sorbitol, mannitol, pinitol), sugars reduce the water potential and thus plants can continue water uptake from the environment where water availability is limited [Parihar et al., 2015], [Fahad et al., 2015]. Accumulation of these compatible solutes do not cause any toxicity problems in the cytosol, and these compounds also exhibit antioxidant activity by eliminating ROS,

protection membrane stability and three-dimensional structure of proteins [Manchanda and Garg, 2008].

The levels of intracellular Na^+ depends on the activity of two Na^+/H^+ antiporters which are secondary active transports and use the proton gradient [Xu et al., 2010], [Apse and Blumwald, 2002]. One of these is Na^+/H^+ antiporters, which are located in the plasma membrane, exclude Na and thus inside the cytoplasm keep Na^+ concentration at a low level [Shi et al., 2003]. The other one, which is called is vacuolar Na^+/H^+ antiporter, is located on tonoplast and transport Na into the vacuole [Apse and Blumwald, 2007]. By using these antiporters plants can utilize these ions as osmolytes and continue taking up water under saline conditions due to low osmotic potential [Chinnusamy et al., 2005].

High ROS levels observed in saline conditions can be reduced by plants by inducing various antioxidative enzymes, such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD) and non enzymatic antioxidants like secondary metabolites (flavonoids, carotenoids, proline, other phenolics), ascorbate (ASC), reduced glutathione (GSH), etc. [Ertani et al., 2013], [Gill and Tuteja, 2010].

Another adaptation mechanism of plants under stress conditions is the stimulation of phytohormone including auxins, SA, CK, jasmonic acid (JA), ABA and gibberellins (GA) synthesis. These plant hormones are produced by various biochemical pathways and play a critical role in enhancing tolerance to salt stress conditions [Waśkiewicz et al., 2016], [Fahad et al., 2015].

1.3. Maize (*Zea mays L.*)

Cereals, which are the major part of the basic diet of people in most countries, compose a huge portion of agricultural production [Dudziak et al., 2019]. Among other cereals, maize is one of the fundamental food and feed crops [Campos et al., 2004]. Although area designated for maize production is lower when compared to wheat and rice, the yield obtained from the unit hectares is higher among all cereals [Ozcan, 2009]. It is estimated that maize production, which is approximately 800 million tons, will increase further in the future to feed the growing global human population [Alexandratos and Bruinsma, 2012], [Chulze, 2010].

The origin of the maize plant is Central America, especially Mexico. After the discovery of the American continent, maize seeds first spread to Spain and then to Africa and Asia [Ramirez-Cabral et al., 2017], [Matsuoka et al., 2002]. Introduction of maize to Turkey is thought to be through Egypt and Syria in 16th century. The Turkish name "mısır" is also related to its import from "Egypt" [Ozcan, 2009], [Comertpay, 2008]. Maize can be grown almost everywhere in the world especially in tropical, subtropical and temperate zones due to its wide adaptability [Gecit, 2009], [Kogbe and Adediran, 2003].

All over the world, the three leading countries in maize production are United States, China and Brazil [Ranum et al., 2014]. The United States, which accounts for about one-third of world maize production, is also the largest maize vendor and about one-fifth of the maize produced in US is exported [Kusvuran and Nazlı, 2014]. In Turkey, maize has the third highest cultivation area after wheat and barley and it is grown almost everywhere, especially in Çukurova region [Karlı et al., 2018].

Maize is a monocotyledonous plant and belongs to the Poaceae family [Schnable et al., 2009], [Zhang et al., 2009]. The consumable part of maize contains a high level of carbohydrates, water, protein, lipid and fiber as well as important minerals, vitamins and carotenes [White and Johnsan, 2003]. Besides its critical place in nutrition, maize, which is a product with high economic value, also has a wide usage area in other sectors including food, energy, textile, biofuel and cosmetic [Ozcan, 2009], [Yavuz et al., 2016], [Vaughan et al., 2018].

Maize is classified as a moderately sensitive plant to salinity stress [Goldsworthy, 1994]. Under saline conditions particularly early growth stages of maize like germination and seedling establishment are affected. Germination is delayed and due to osmotic stress related problems non-uniform germination can be observed [Farsiani and Ghobadi, 2009]. Salt stress may cause reduction in almost all growth parameters of maize such as shoot and root length, dry weight and leaf growth [Goldsworthy, 1994], [Devi et al., 2019]. Accumulation of the high concentrations of Cl^- and Na^+ ions as a result of salinity in roots and leaves of maize can cause nutrient imbalance by inhibiting uptake and transport of essential elements (K^+, Ca^{+2}, N, Mg^{+2}, Cu, Mo, Zn) [Hasegawa et al., 2000], [Turan et al., 2010], [Karimi et al., 2005]. The accumulation of these ions in maize leaves, may cause

dwarfing, inhibition of leaf expansion and eventually leaf abscission [(Qu et al., 2012], [Fortmeier and Schubert, 1995].

Another component of salt stress that negatively effects maize plant is oxidative stress and it results in increased production of ROS such as $O_2^{\bullet-}$ and H_2O_2 [de Azevedo et al., 2006], [Hichem et al., 2009].

Under salt stress conditions, many changes can be observed in the metabolism of the maize plant such as osmotic adjustment, maintaining of homeostatic balance, stimulation antioxidant, and hormonal system to tolerate the stress situation [Gong et al., 2014], [Farooq et al., 2015]. The high concentration of Na^+ ions is excluded or transported into vacuoles by antiporters. This strategy is not only critical to protect maize from of Na^+ ion accumulation but also important for osmotic adjustment to balance the water potential. In addition, salt tolerant maize crops can possess higher K^+/Na^+ ratio as a result of enhancing K^+ uptake while inhibiting Na^+ uptake [Neubert et al., 2005], [Wakeel et al., 2011a], [Akram et al., 2007].

Antioxidant defense system are activated as a response to salinity stress in maize plants [de Azevedo et al., 2006]. Production of polyphenols, upregulated stress-related genes and expressed proteins and also enzymatic antioxidants such as SOD, CAT, APX, GR, guaiacol peroxidase (GPX) are some examples. These enzymatic or non-enzymatic antioxidant molecules can scavenge highly toxic ROS and protect maize against oxidative stress [Rios-Gonzalez et al., 2002], [de Azevedo et al., 2006], [Hichem et al., 2009].

Improvement of maize species, which can adapt to undesirable environmental conditions such as salinity stress, is becoming more and more important. Breeding of crops with genetic engineering by means of screening of genotypes, utilization of specific markers, desirable genes selection may be developed salt tolerance maize crops [Giaveno et al., 2007], [Gosal et al., 2009], [Li et al., 2010], [de Azevedo et al., 2004]. On the other hand, treatment of various microorganism such as beneficial soil bacteria and fungi, seed treatment or foliar application with various priming agents, which can also be classified as biostimulant, can be used as easily applicable, cheap and effective management strategies for production of salinity-tolerant maize plants [Yang and Lu, 2005], [Janmohammadi et al., 2008], [Feng et al., 2002], [Nadeem et al., 2009].

1.4. Seed Treatment

In the very early stages of their life cycle plants are often subject to abiotic stresses during their seed germination and seedling establishments. These stages are vital stages for the development of plants, healthy growth as well as high yielding capacity. Unfortunately, at these early stages, plants are very susceptible to different stresses including salinity [Hubbard et al., 2012], [Patade et al., 2011]. Low cost, efficient and eco-friendly seed treatment strategies can improve seed performance, enhance germination capacity and seedling vigor which are crucial for healthy growth and development of plants under both control and stress conditions [Afzal et al., 2016].

Seed soaking, seed priming and seed coating are different seed treatments techniques [Halmer, 2004], [Jisha et al., 2013]. In the seed soaking method, which is a simple and commonly used method in crop production, seeds are hydrated for a certain period of time before being sowing [Pan et al., 2017]. A positive influence of seed soaking with several agents are documented in many studies. In these studies, pre-treated seeds demonstrate higher germination potential, biomass, yield, quality and stress tolerance [Roychoudhury et al., 2016], [Tian et al., 2014], [Nathawat et al., 2007]. However in seed priming, the seeds which are soaked in the specific solution for given period are dried before usage [Rajjou et al., 2012]. Primed seeds have a developed physiologic status than the other ones. When compared to non-primed seeds, primed seeds have rapid radical emergence, higher germination rate and yield, greater seed vigor and seedling establishment, and homogeneity of seedlings [Bewley et al., 2013], [Basra et al., 2005]. In seed coating, specific machinery is required to coat the outer layer of seeds with the desired materials and the seeds can be stored like this for a long time. This seed treatment can increase growth and development by providing growth regulators and nutrients [Farooq et al., 2012], [Halmer, 2004]. In addition, seed coating using plant-based protein hydrolyzate has been reported to increase biomass, root, shoot and chlorophyll content, but cause a decrease in germination rate [Amirkhani et al., 2016].

Pre-sowing application of different compounds by using these methods are possess a considerable high economic value for the agrochemical sector [Sharma et al., 2015]. With seed treatments sowing process becomes easier, seeds can be protected for a longer time during storage and by enhancing pre-germination metabolism, post-germination performances can be higher [Sharma et al., 2015], [Paparella et al., 2015], [Halmer, 2004].

Plus, by using these treatments plants can be protected against biotic (pathogen, pests, weed, etc.) and abiotic stress (salinity, drought, low temperature, etc.) factors [Amirkhani et al., 2016].

In literature, many studies have shown different seed treatment agents including water, micronutrients like Zn, B, Mo, Mn, Cu and Co [Farooq et al., 2012], osmopriming agents such as KNO_3 [Yan, 2015] and PEG [Amini, 2013], compatible solute like glycine betaine [Cheng et al., 2018] and proline [Hua-long et al., 2014]. Moreover, different phytohormones such as JA [Sharma et al., 2018], SA [Tahjib-Ul-Arif et al., 2018], [Yang et al., 2016], IAA [Iqbal and Ashraf, 2007], GA [Siadat et al., 2011], CK [Iqbal et al., 2006] are also used as seed treatments agents for different purposes in agriculture practices. Various chemical compounds such as hydrochloric acid (HCl), sodium hypochlorite (NaOCl) are also used as seed treatment agents particularly for pest and diseases management, but while providing some tolerance to stress conditions, these chemicals can negatively affect germination and seedling establishment [Taylor and Salanenka, 2012], [Khah, 1992].

Under saline conditions, seed priming is reported to decrease the negative effects of stress on germination and seedling establishment by increasing uptake of K+ and Ca^{+2} and decreasing Na^+ and Cl^- accumulation and thus contributed to osmotic adjustment and water uptake [Bakht et al., 2011], [Ashraf, 2004]. In another study where seeds are soaked with SA improved germination, growth, proline metabolism, antioxidant activity and better water uptake, higher photosynthetic activity and less accumulation of toxic ions such as Na^+ and Cl^- ions are observed [Misra and Saxena, 2009], [Kaydan et al., 2007], [Gunes et al., 2005].

Usage of biostimulants as seed treatments agents are another emerging application strategy for food safety and sustainable agriculture due to usage of safe and renewable resources [Sharma et al., 2015]. A wide variety of biostimulants can be used as seed treatment agents, for example, inorganic compounds [Abdel et al., 2016], [Jerše et al., 2017], humic acid [Mereddy, 2015] seaweed extracts [Rady et al., 2018]; [Kasim et al., 2016], animal-based protein hydrolysate [Wilson et al., 2018], plant-derived protein hydrolysates [Amirkhani et al., 2016], beneficial microorganism including fungi [Gómez-Muñoz et al., 2018] and bacteria [Disi et al., 2018], chitosan [Orzali et al., 2014] and botanicals [Ahmad et al., 2016], [Panuccio et al., 2018], [Ashraf et al., 2018].

Seed treatments with botanical extract, which is a sustainable, green, promising approach in agriculture, are becoming increasingly important. [Kumar et al., 2017], [Panuccio et al., 2018]. Botanical-priming using various plant extracts such as the mulberry, eucalyptus, curry, brassica, aloe vera and sorghum leaf are reported to increase seed performance, quality and vigor, enhance growth parameters like root and shoot lengths, fresh and dry weights and provide rapid and higher germination and optimize nutrient-use-efficiency under both control and stress conditions [Mehta et al., 2010], [Masuthi et al., 2015], [Rafi et al., 2015].

One of the most commonly seed treatment agent among other botanical biostimulants is the extracts of the moringa plant which is rich in natural bioactive compounds. In many studies, it has been documented that when applied to the maize seeds moringa extracts affects the plant metabolism, enhance yield, biomass, seedling vigor and germination performance and also improve growth and development by increasing enzyme activity involved in carbohydrate metabolism and photosynthetic pigment [Basra et al., 2011], [Afzal et al., 2012], [Bakhtavar et al., 2015], [Bibi et al., 2016]. Moreover, seed treatment with this extract can alleviate negative effects of abiotic stress on maize crops by improving antioxidant systems [Foidl et al., 2001], [Basra et al., 2011].

1.5. Salicylic Acid (SA)

SA, which is considered a phytohormone, acts both as a natural phenolic and as an important signaling molecule in plants [Gunes et al., 2007], [Jini and Joseph, 2017]. The first commercial production of synthetic salicylic acid was made in Germany in 1874 by Hermann Kolbe, however, after 20 years Felix Hoffman converted salicylic acid into acetylsalicylic acid due to the side-effects (ASA) [Mahdi, 2010]. In 1899, the German company Bayer produced the commercially ASA the close analog of SA and in a short time it has become the best selling and quite successful plant-derived drug in the world which is named as "ASPIRIN" [Drew et al., 2016].

In plants, SA is synthesized in two different ways: 90% or more of it are synthesized by isochorismate (IC) pathway in stressed plants, and the rest is synthesized by PAL pathway under control conditions [Kumar, 2014]. After synthesis, SA is transported in plants via the phloem channel and its levels can change substantially according to tissues and species. Moreover, this phytohormone is intensely present in the infected or/and

necrosis areas and active in the event of possible stress conditions [Hayat et al., 2010], [Chen et al., 2001]. Under control conditions, SA is usually found in low amounts (more or less one µg/g fresh weight) in different plant species such as soybean, tomato, rice, tobacco and barley [Raskin et al., 1990], [Yang et al., 2004], [Methenni et al., 2018].

Salicylic acid plays a critical role in various physiological and biochemical processes in plants [Nazar et al., 2011]. The application of SA may increase growth and development [Eraslan et al., 2008], synthesis of compatible solutes including proline and glycine betaine [Loutfy et al., 2012], chlorophyll content [Singh and Usha, 2003], flowering [Kim et al., 2018], photosynthesis [Khan et al., 2014], seed germination [Rajou et al., 2006] and also ion uptake and transportation [Belkhadi et al., 2010]. Moreover, depending on the concentration, method of application and developmental stage of different plant species, SA can act differently in plants [Horváth et al., 2007]. Low SA levels may have a beneficial impact on stress tolerance; however, as the level of SA increases, oxidative stress is induced and thus can result in cell damage [Miura and Tada, 2014], [Hara et al., 2012].

Salicylic acid may act as a non-enzymatic antioxidant and enhance resistance to abiotic stress [Miura and Tada, 2014], therefore the protective impacts of SA against various abiotic stresses have extensively been investigated [Fardus et al., 2018]. It was observed that application of SA can help plants withstand various abiotic stress conditions such as salinity [Khan et al., 2014], [Gunes et al., 2007], [Palma et al., 2013], [Idrees et al., 2011], heavy metals [Moussa and El-Gamel, 2010], [Belkhandi et al., 2010], [Zhang et al., 2015], drought [Azooz and Youssef, 2010], [Yazdanpanah et al., 2011], [Fayez and Bazaid, 2014], heat [Khan et al., 2013], boron toxicity [Eraslan et al., 2008] and osmotic stress [Singh and Usha, 2003], [Al-Hakimi, 2006], [Alavi et al., 2014]. In these studies, the exogenous application of SA has also been exhibited to regulate accumulation of osmolytes [Liu et al., 2016], antioxidants and pigments [Fardus et al., 2018] and stimulate defense-related genes [Dixon et al., 1995], enzyme activities involved in nitrogen metabolism [Hayat et al., 2010], secondary metabolite synthesis [Zhao et al., 2005] and antioxidative metabolism [Noreen et al., 2009].

Under the salt stress, SA applications was shown to mitigate the harmful effects of salinity by enhancing osmotic adjustment, photosynthesis, plant growth and development, acitivating defense systems and reducing uptake of Na and Cl ions whereas improving

uptake of Mg and N ions [Pirasteh-Anosheh et al., 2017], [Yan et al., 2018], [Horváth et al., 2007], [Grzeszczuk et al., 2018].

1.6. Willows (*Salix* spp.)

Willows (*Salix* spp.) which are a member of the Salicaceae family, are deciduous trees or shrubs as well as are dioecious species. They may reaches 30 meters in height and their life span is approximately 100 years. With their elongated leaves and high evapotranspiration rates, willows can easily adapt to their region and grow very quickly and provide a huge biomass [Guo et al., 2015], [Wiesneth et al., 2018], [Popova and Kaleva, 2015]. Willows are naturally observed in moist soils like river banks because they need high moisture as well as high water tolerance but can also grow almost everywhere and are widely used in parks and gardens for ornamental purposes [Kenstavičienė et al., 2009].

Although it is difficult to determine the number of willow species correctly due to the complexity of the genus, there are approximately 450 species worldwide [Mabberley, 2008], [Shah et al., 2016]. Turkey has a rich diversity in terms of natural willow species. There are 28 willow species grow in Turkey 4 of which are peculiar to our country. They are S. anatolica (anatolian willow), S. purpurea (purple willow or Denizli willow), S. rizeensis (Rize willow), S. trabzonica (Trabzon willow) [Arihan and Güvenc, 2011].

The utilization of this tree dates back to about 6000 years ago and it is well-known as an important medicinal plant. For many years, the bark and leaves of the willow tree were used by the ancient civilizations for the treatment of various diseases without knowing the active ingredients. Many ancient civilizations, such as Assyrians, Sumerian, Egyptians used the extracts of willow bark and leaves due to their analgesic, antipyretic, and anti-inflammatory properties [Noleto-Dias et al., 2018], [Mahdi, 2010]. Before the 19th century, the ingredients of willow extracts were unknown but later studies documented the presence of many bioactive secondary compounds such as polyphenols (proanthocyanidins (PAs), phenolic acids, flavonoids, tannins, lignans), terpenoid and many salicylate compounds (salicin, saligenin, and salicylic acid) [Khan et al., 2015], [El-Sayed et al., 2015], [El-Shazly et al., 2012]. In willow, these substances play a critical role not only as a part of defense mechanism and signaling molecule but also as therapeutic properties especially due to salicin content.

Salicin, which is the main ingredient in willow bark and leaves, is a prodrug and at the same time it is the precursor of aspirin [Alamgir, 2017], [Wiesneth et al., 2018]. Salicin was first isolated in 1828 by Johann Buchner in low amounts from the willow tree bark. About 10 years later in 1838, salicylic acid was first isolated by Raffaele Piria and its name was derived from willow *(Salix alba* L.) [Popova et al. 1997], [Arteca 1996].

When salicin is absorbed, it turns into glucose and saligenin (salicylic alcohol). Afterward, saligenin is easily oxidized to SA in the blood, tissue and in the liver and thus, it can ensure its therapeutic effects [Rappoport, 2004], [Schrör, 2016]. When the hydroxyl group of salicylic acid is acetylated and thus aspirin is obtained [Roberts and Marrow, 2001]. On the other hand, when aspirin contacts with water it is rapidly hydrolyzed and converted into SA. According to the literature, 0.5 gram aspirin is equivalent to approximately 800 mg of salicin which be obtained from about 90 grams of dry willow bark [Schulz et al., 2001].

Besides the medicinal properties of the willows, they are extensively used for phytoremediation, soil erosion and flood control, basketry and fence, biodiesel fuel, wood supply, cellulose and paper production. In addition, willow trees have a rich habitat and food source for various living organisms [Kuzovkina and Quigley, 2005].

The chemical composition and amount of Salicylates compounds may change with age, seasonal, tissue, genotype, species and various environmental factors. For example, salicylates amount in bark and leaves is higher in spring when compared to autumn [Arimura and Maffei, 2016].

1.6.1. *Salix Babylonica* (Weeping Willow)

Due to the very this structure of shoots and branches, Salix babylonica, which is also known as a weeping willow, leaves look as if they are hanging down. Weeping willow trees can grow up to 15 m with leaves of about 8 to 15 cm. Yellowish white flowers bloom between March and April. [Eminagaoglu et al., 2014]. Weeping willow is commonly used as an ornamental tree and distributed all over the world. It is generally found in moist places and grown virtually anywhere in Turkey [Elghandour et al., 2015].

Like other Salix species, weeping willow has a rich content of bioactive compounds such as flavonoid (catechins, kaempferol-7- O-glucoside, luteolin, luteolin-7-O-β-D-

glucopyranoside, luteolin-4'-O glucoside, apigenin-7-O-galactoside, chrysoeriol), phenolic compounds (lignan), alkaloids, terpenoid, saponins and salicin [Ruuhola and Julkunen-Tiitto, 2000], [Jiménez-Peralta et al., 2011], [Khatoon et al., 1988].

1.7. What was this MSc Thesis Project about?

In this thesis, it is aimed to test the potential of willow bark and leaf extracts as new plant-based biostimulant to improve the growth of maize (*Zea mays* cv. Caramelo) in the absence and in the presence of salinity stress. Maize is selected as a model plant for this project as it is one of the most important cereal sources for human diet due to its rich nutrients and at the same time it is moderately sensitive to salt stress. Salinity is one of the most commonly observed abiotic stresses threatening crop production all over the world including Turkey. For maize plants, even minor increases in salinity stress tolerance could have a substantial effect on crop production, food safety and sustainable agriculture all around the world.

For this study experiments were conducted on three different growth media (perlite, soil and solution culture) under growth chamber conditions.

In perlite experiments, the impacts of willow bark and leaf extracts were investigated as a seed treatment agent in the very early growth stages of maize under both control and salt stress conditions. As the first step, the effect of salt stress on germination and seedling establishment which are considered to be the most sensitive stages to salinity stress were investigated and clear effects of these extracts on the seed performance were documented.

In soil experiments again the performance of maize seeds which were treated with willow bark and leaf extracts as well as SA were tested under both control and salt stress condition. In this growth media, plants had a longer time to grow when compared to perlite experiments. Various parameters related to germination, growth, stress tolerance and mineral homeostasis were measured to explain the physiological basis of the observed effects.

In hydroponic experiments, beneficial effects of willow tree extracts and SA on the vegetative growth and development were investigated in maize seedlings which were

treated with these compounds via nutrient solution. Again, various parameters related to germination, growth as well as stress tolerance were measured.

Plant-based biostimulants, also called botanicals, are attractive options to address current challenges to sustainable agriculture due to their safety, renewability and low cost however, the mechanisms of their action are not fully explored. When the literature analysis is performed, the thesis study is unique in many respects. To our knowledge, this will be the first study to investigate the biostimulant potential of willow tree extracts and also the effects of the willow extracts, a natural source of salicylic acid will be compared with the chemical compound SA for the first time.

2. MATERIALS AND METHODS

2.1. Materials and General Information

2.1.1. Plant Material

The maize seeds (*Zea mays* cv. Caramelo F1) which were used in all experiments of this study were obtained from May Seed, Bursa, Turkey. Caramelo species are an early, dwarf, hybrid sweet maize and their germination percentage is about 90%. Caramelo is suitable for both fresh consumption and industrial usage.

2.1.2. Sodium Chloride (NaCl)

In all experiments salt stress conditions were created by using sodium chloride (NaCl) with a molecular weight of 58,44 g/mol and obtained from Merck.

2.1.3. Salicylic Acid

Salicylic acid($C_7H_6O_3$) with a molecular weight of 138.12 g /mol was used as the chemical compound of Sigma Aldrich (France).

2.1.4. Preparation of Willow Tree Extracts

For preparation of plant extracts, fresh willow leaves and barks were randomly collected from a mature willow tree (*Salix babylonica*) from Tuzla region of Istanbul. For obtaining 2% and 4% willow extracts 15 and 30 g of willow leaves (or barks) were chopped and the total volume was adjusted to 750 ml by using dH_2O. This mixture is kept at 90°C for 30 minute and by using a stirrer, stirred at 400 rpm during this process. Using a cheese cloth, the solution is filtered and stored at -20°C until applications. For seed treatment experiments which were conducted in perlite or soil media, 2 or 4% solutions were directly used. For solution culture experiment, the solution was diluted and 0.1% or 0.2% willow extracts were applied to hydroponic pots as described below (Figure 2.1).
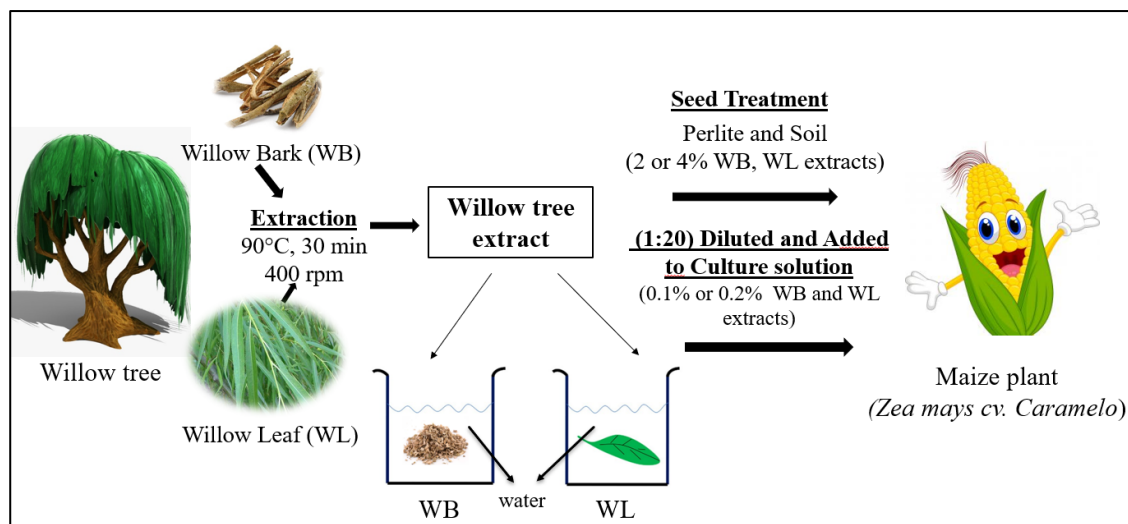
Figure 2.1: Schematic representation of willow tree extracts preparation and application

2.1.5. Nutrient Solution

Nutrient solution was used for all perlite and hydroponic experiments. The full strength nutrient solution contained 0.6 mM K_2SO_4, 2 mM $Ca(NO_3)_2.4H_2O$, 0.2 mM KH_2PO_4, 0.75 mM $MgSO_4.7H_2O$, 0.1 mM KCl, 75 µM Fe (in the form of FeEDTA), 2 µM H_3BO_3, 2 µM $MnSO_4.H_2O$, 3 µM $ZnSO_4.7H_2O$, 0.6 µM $CuSO_4.5H_2O$, 0.50 µM $(NH_4)_6Mo_7O_{24}.4H_2O$. All the chemicals used for his purpose was analytical grade.

2.1.6. Growth Chamber Conditions

All the experiments were conducted in a growth chamber under controlled climatic conditions with the properties of light / dark periods: 16 / 8 h; temperature (light / dark): 25 / 20°C; relative humidity (light / dark): 60 / 70.

2.2. Plant Growth Methods and Media

2.2.1. Perlite Experiments

Perlite has a porous structure and it provides effective ventilation, water and supporting media for germinating seeds. Since perlite has a strong capillary attraction, water and nutrients can easily be taken by plant roots. Perlite can successfully be used as

a soil regulator in gardens and greenhouses, a growing medium in soilless agriculture and seed germination media.

2.2.1.1. First Perlite Experiment Methods

2.2.1.1.1. Preparation of Salt Solution

Four different salt doses were tested as a preliminary test to determine the optimal salt dose. 0, 50, 100, 150 mM NaCl solutions were prepared separately in half-strength nutrient solution (as described at 2.1.5). Before sowing the seeds, perlite was washed in these solutions.

2.2.1.1.2. Experimental Design

In this experimental design 48 (4 replication × 4 salt doses × 3 harvest time) small plastic boxes were used with dimensions 110 x 120 x 55 mm (WxLxH). For the determination of salinity effect, 4 different salt solutions (0, 50, 100, 150 mM NaCl) were used.

Maize seeds were germinated in moistened perlite for 3, 5 or 7 days in growth chamber conditions. At different harvest times (3 days after sowing (DAS), 5 (DAS), and 7 (DAS)) germination rate, shoot and root fresh weight, shoot length and and total fresh weight of maize seeds were measured for all the plants grown in a single germination box.

2.2.1.2. Second Perlite Experiment Methods

2.2.1.2.1. Preparation of Salicylic Acid Solution

To obtain 1mM of SA solution, 0.138 g of SA was mixed with 100 ml water and stirred at 40°C for 1 hour. The final volume was completed to 1 liter with pure water. This heating step was critical to solubilize SA. From this stock solution various dilutions were prepared and applied as seed soaking agent. This stock solution was stored in the refrigerator at +4 °C until usage.

2.2.1.2.2. Seed Soaking with Salicylic Acid and Sowing

In this experiment, three different doses of salicylic acid were used to determine the optimum salicylic acid dose as seed treatment agents. The maize seeds were selected and soaked with 0.25, 0.5 or 1 mM SA for 16 h. During seed soaking, seeds were placed between filter papers which were wet with 4 different (0, 0.25, 0.5, and 1 mM) SA solutions.

Soaked seeds were sowed in small plastic boxes which include perlite that is treated with a half-strength nutrient solution (described at 2.1.5). For this experiment 96 boxes (2 salt treatment (0 and 100 mM NaCl) x 4 replicates x 3 harvest times (3, 5 and 7 DAS) x 4 salicylic acid treatments) were used. The selected salinity level (100 mM NaCl) used in the 2nd and 3rd experiments was determined according to the results of the first experiment. The experiment was conducted under growth chamber conditions.

2.2.1.3. Third Perlite Experiment Methods

2.2.1.3.1. Seed Soaking with Willow Bark and Leaf Extracts and Sowing

The willow leaf and willow bark extracts which prepared as described 2.1.4 were tested as seed treatment agents to determine their effects on seed performance under control and saline conditions.

One group of maize seeds were left untreated as control whereas the rest of the seeds were soaked between filter papers in big plastic boxes for 16 hours at room temperature. For seed treatment agents:

- Water,
- Salicylic acid (0.5 mM),
- Willow bark Extract (2%) and
- Willow leaf Extract (2%) was used

In the experiment design maize seeds were subjected to germination test in perlite by using 80 pots (4 replicates x 5 treatment agents including non-soaked control plants x 2 salt doses (0 and 100 mM NaCl) x 2 different harvest time (3 and 6 DAS). As described in the previous experiment, the perlite was washed with half-strength nutrient solution in

the presence or absence of selected salt applications. The experiment was conducted under growth chamber conditions.

In order to determine the effects of seed treatment agents, in addition to the parameters measured for the first and second perlite experiment, the total root area of the germinated seeds was calculated by using a computer program as described below.

2.2.1.3.2. Total Root Area Determination

For calculating the root area of maize seedlings, a program called image J was used. After opening the image file for the root area analysis, first the desired area to be measured was selected and cropped. A line of a certain length was drawn between the two points to adjust the measurement scale (Figure 2.1A). The scale was then cropped (Figure 2.1B). Analysis → Set Scale were clicked and known distance and unit of length were filled. Then, Image → Color → Split Channels were clicked respectively and red pop-up window was selected. After that, Adjust → Threshold → Fig C → Apply → Fig D was obtained. Analyze → Analyze Particles were selected and total root area were calculated by the program.
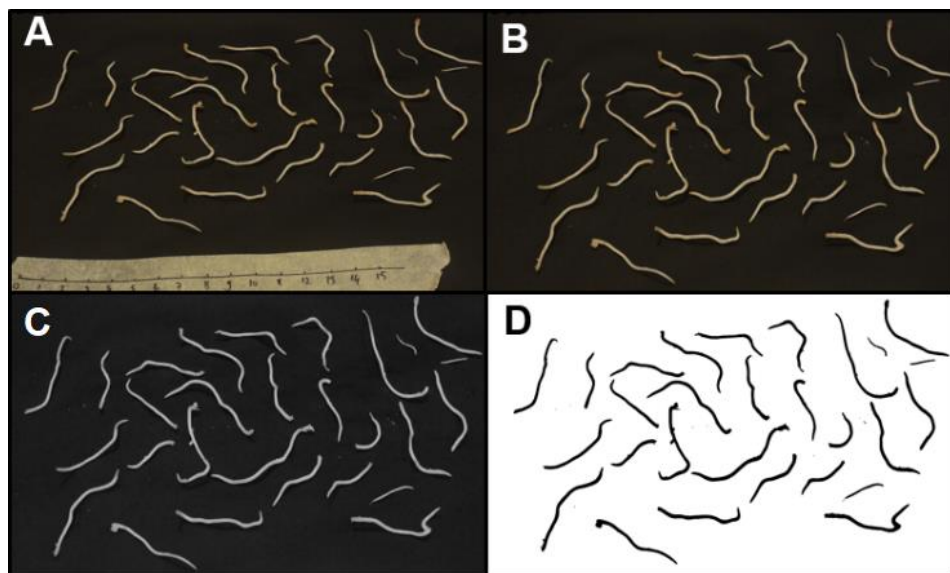
Figure 2.2: Representation of analysis of total root area, (A) determination of scale, (B) removing of scale, (C) adjustment of colour, (D) convert a picture to black and white.

2.2.2. Soil Experiment

The primed or soaked seeds were sown in plastic pots (no:4) containing 1.25 kg soil. Pots of all treatments were fertilized with 100 ppm P (in the form of KH_2PO_4) and 250 ppm N (in the form of $Ca(NO_3)_2$) before sowing. The soil characteristics were clay-loam texture where EC_e was measured as 3.3 ds.m^{-1}. After fertilization, the pots were irrigated with 50 ml distilled water regularly until the experiment finished. The experimental design was a randomized complete block with four (first experiment) and five (second experiment) replicates. 15 maize seeds were sowed in each pot for the two soil experiments. Both experiments were conducted in a growth chamber under controlled climatic conditions as described above.

2.2.2.1. First Soil Experiment Method

To determine the optimal priming time, hydropriming was performed for 0, 4, 8, 12 and 16 h. Seeds for sowing were kept between filter papers which were moistened with water in plastic boxes. When the priming times were completed, the seeds were dried at room temperature for 2 days and used for sowing.

Before sowing the seeds, salt and fertilizers were mixed with the soil. It was calculated that 0.375 g of salt was required to increase the EC_e of 1 kilogram of soil by 1 ds/m. The amount of salt required to increase 1.5 ds/m for 1.25 kilograms of soil was determined. For this experiment soil EC values were adjusted to 3.3 (Control), 4.8, 6.3, 7.8 and 9.3 ds/m.

Per each pot 15 seeds were sown in soil and the pots were irrigated with 50 ml dH_2O daily for 14 days. The germination rate of seeds was determined for days 5, 7 and 10. Plant heights was measured at day 7, 10 and 14 (before harvest). At the end of 14 days, the plants were harvested and their fresh weights were measured. The harvested plant shoots were dried at 60º C for 3 days and afterwards their dry weights were determined.

2.2.2.2. Second Soil Experiment Method

In this experiment, the selected concentrations of SA (0.5 mM) willow bark (2%) and willow leaf (2%) which were used for perlite experiment were again used. In addition to these selected rates, concentrated willow extracts (4% both for bark and leaf) was tested

as seed treatment agents. For control purposes, a group of seeds were left untreated and for another group water was used as seed treatment agent. The selected EC_e (7.8) and soaking times (16 h) levels were used in this second experiment. Instead of priming, soaking was preferred as the seed treatment method due to some unexpected damages observed as the first soil experiment as a result of seed priming.

Selected seeds for soaking application were exposed to 7 different seed treatment agents (no treatment, water, SA, willow bark (2 or 4%) and willow leaf (2 or 4%)) in big plastic boxes between impregnated filter paper with agents solutions for 16 hours at the room temperature. Soaked seeds were sown into pre-prepared pots. In total 70 pots were used (7 seed treatment x 5 replicates x 2 salinity levels).

In addition to the measurements made for the first soil experiment, antioxidative enzyme activities, tissue mineral and protein concentrations were analyzed. For enzyme and protein determination fresh samples were used. From each pot replicate, 0.5 grams of fresh samples were taken and after shocking by using liquid nitrogen the samples were stored at -80°C until they were used for extraction. The remaining plant samples were placed in the drying oven for determination of dry weight and mineral concentration and were dried at 60° C for 2 days.

2.2.2.2.1. Element Analysis

Dried shoot samples were finely ground with coffee milling machine. Approximately 0.2 g of the dried and ground plant samples were weighed and placed in digestion tubes. The samples which were treated with 2.0 ml of 30% hydrogen peroxide (H_2O_2) and 5.0 ml of nitric acid (HNO_3) were digested in a closed vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). After cooling down sufficiently, total sample volume was finalized to 20 ml by adding double-deionized water and filtered through filter papers (Macherey-Nagel, Ø125 mm, blue band). Inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian, Australia) was used to determine the concentrations of macro and micronutrient in digested plant samples. The accuracy of element analyses was checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.2.2.2.2. Preparation of Crude Plant Extracts for Enzyme and Protein Analyses

By mixing 50 mM KH_2PO_4 and 50 mM K_2HPO_4 in a 2:1 volume ratio, a potassium phosphate (K-P) buffer with a pH of 7.6 was prepared. The extraction buffer was prepared daily by adding 0.1 mM Titriplex III to this K-P buffer and kept on ice. 0.5 grams of frozen fresh leaf samples were homogenized with the help of a mortar and pestle by using liquid nitrogen and 5 ml of K-P buffer. The homogenates were centrifuged at 4600 min^{-1} for 15 min at 4°C, and the supernatants were transferred to Eppendorf tubes, which were centrifuged again at 15.000 min^{-1} for 15 min at 4°C. These supernatants were used for determination of antioxidants enzymes (SOD, GR, AP, CAT) and proteins concentrations.

2.2.2.2.3. Antioxidant Enzyme Analysis

2.2.2.2.3.1. Superoxide Dismutase (SOD) Assay

For the SOD assay, 2.95 ml of K-P buffer was mixed with 0.5 ml of Na_2CO_3, L-methionine, NBT, 0.05 ml of crude sample extract (1:10 diluted) and 0.5 ml riboflavin, respectively in glass tubes. Since the chemicals used are light sensitive, they were kept under dark during preparation. After riboflavin addition, glass tubes were placed in the growth chambers and kept under light for 8 min. The measurements were performed by using a spectrophotometer (Cary 300 Bio, Varian, Australia) at 560 nm wavelength by using a sipper.

2.2.2.2.3.2. Glutathion Reductase (GR) Assay

To determine the activity of the GR enzyme, 0.7 ml of K-P buffer, 0.1 ml of Oxidized Glutathion (GSSG), 0.1 ml of 0.45 mM H_2O_2 and 0.1 ml of crude sample extract (1:40 diluted) were mixed and finally 0.1 ml of NaDPH-Na_4 was added to this mixture. The activity was determined spectrophotometrically by using 340 nm wavelength and absorbance values were monitored for 2 min to find the average depletion rate of NADPH-Na_4.

2.2.2.2.3.3. Ascorbate Peroxidase (AP) Assay

To measure AP activity, 0.7 ml of K-P buffer was mixed with 0.1 ml of 12 mM H_2O_2 and 0.1 ml of crude sample extract (1:40 diluted), and then 0.1 ml of ascorbic acid ($C_6H_8O_6$) was added. To calculate the average depletion rate of L-ascorbic acid, absorbance values at 290 nm were measured spectrophotometrically.

2.2.2.2.3.4. Catalase (CAT) Assay

The catalase activity was also determined spectrophotometrically. 0.8 ml of K-P buffer was mixed with 0.1 ml of 110 mM H_2O_2 and 0.1 ml of crude sample extract (1:40 diluted). The change in the absorbance of this mixture was followed for 2 min at 240 nm to calculate the average rate of H_2O_2 breakdown.

2.2.2.2.4. Total Protein Analysis

To prepare Bradford reagent, 0.1 g Coomassie Brilliant Blue G-250 was dissolved in 50 ml ethanol and was mixed with 100 ml 85% ortho-phosphoric acid. This mixture was filtered and after filtration 100 ml glycerin was added to the reagent and the volume was brought to 1000 ml with deionized water. The reagent was kept at 4°C for 24 h and then used for the assay. Protein standards (0, 100, 200, 400 and 800 ppm) were prepared by dissolving bovine serum albumin in K-P buffer. 5 ml of reagent was added to 0.1 ml sample (1:10 diluted) or standard and vortexed. After 10 minutes, the reading of the protein concentration was performed at 595 nm by sipper for standards and samples.

2.2.3. Solution Culture Experiments

Maize seeds were germinated in moistened perlite containing 1 mM $Ca(NO_3)_2$ for 7 d in growth chamber before being transferred to nutrient solution. After germination, plants were transferred to hydroponic culture pots which were filled with 4.5 L of half-strength nutrient solution which was prepared according to the procedure described at 2.1.5. Nutrient solutions were continuously aerated. After 5 day, solution culture is refreshed with full-strength nutrient solution and it was renewed weekly. One week later, for half of the pots 60 mM NaCl was mixed with the nutrient solutions. At the same time

SA (2.5 μM), low (0.1%) and high (0.2%) levels of willow bark and leaf extracts were added to the pots which belong to that treatments group. In total, two stress and treatment application were performed. The experiment was designed as a total of 48 pots (4 replicate x 6 treatment agents x 2 salt levels). There are 5 maize plants in each pot.

Plant height measurements were performed on 19th (right before starting the stress application), 26th and 30th DAS. 30th DAS, plants were harvested and the weight of fresh samples (shoot) were determined. Roots were washed with distilled water. Both the root and shoot samples were dried in an oven for 3 days at 60°C. The dry weight of shoot and root samples were also measured.

2.2.4. Statistical Analysis

For statistical analysis The JMP software (14.0.0) was used. The significance of the effects of the treatments and their interactions on the reported traits for each experiment was evaluated by using analysis of variance (ANOVA). Where ANOVA revealed a significant effect, post-hoc tests at 5% significance were used to determine significant differences between means. In cases where there was only one source of variation, Fisher's protected least significant difference (LSD) test was preferred, however when there were more than one sources of variation, Tukey's honestly significant difference (HSD) test was used.

3. RESULTS

3.1. Perlite Experiment Results

3.1.1. First Perlite Experiment Results

The average germination percentages of maize seeds grown in perlite treated with control and 3 different NaCl solutions (50, 100 and 150 mM) was shown in Figure 3.1. The germination percentage was not affected by the NaCl treatment on 3 DAS (Figure 3.1A), 5 DAS (Figure 3.1B) and 7 DAS (Figure 3.1C). Since according to ANOVA test there was no statistically significant difference between treatments, the same letter was written on related bars (Table 3.1A).
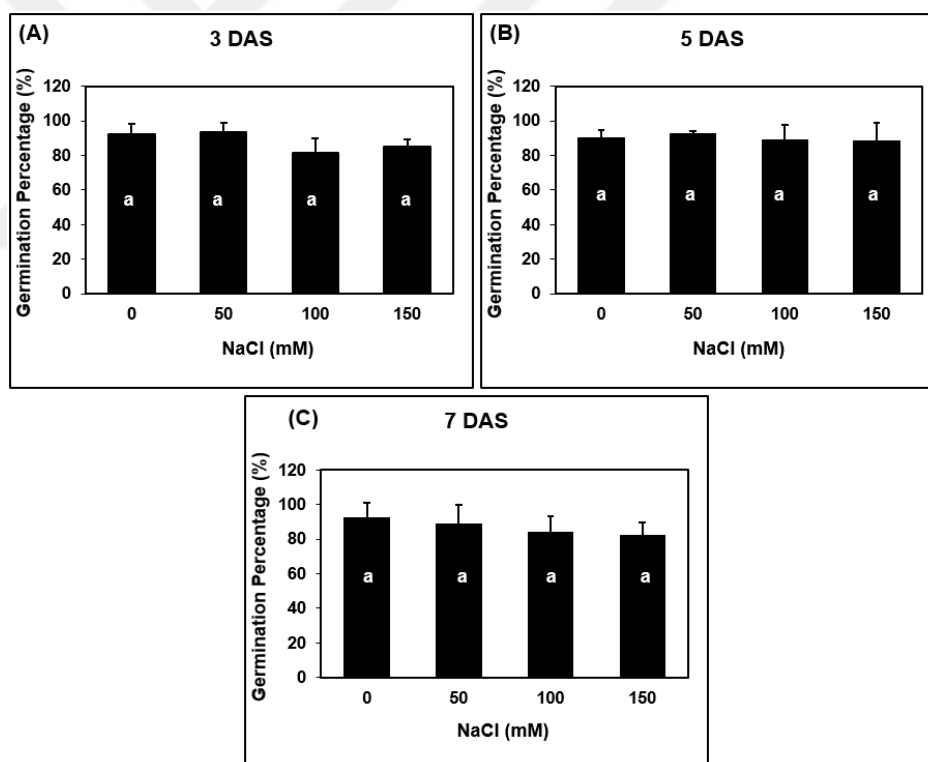
Figure 3.1: Effect of salinity treatments on germination percentage (%) of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Bars represent standard deviations.

Table 3. 1: One-way ANOVA of the effect of salinity on seed germination percentage (%), shoot, root and total FW and shoot length for 3 DAS, 5 DAS and 7 DAS of maize (*Zea mays* cv. Caramelo) plants grown in perlite for the first perlite experiment.

		Germination Percentage		
(A)	**Source of Variation**	**3 DAS**	**5 DAS**	**7 DAS**
	Salinity	*n.s.*	*n.s.*	*n.s.*
		Shoot FW		
(B)	**Source of Variation**	**3 DAS**	**5 DAS**	**7 DAS**
	Salinity	***	***	*
		Root FW		
(C)	**Source of Variation**	**3 DAS**	**5 DAS**	**7 DAS**
	Salinity	*	**	*n.s.*
		Total FW		
(D)	**Source of Variation**	**3 DAS**	**5 DAS**	**7 DAS**
	Salinity	*n.s.*	*	*n.s.*
		Shoot Length		
(E)	**Source of Variation**	**3 DAS**	**5 DAS**	**7 DAS**
	Salinity	***	***	**

n.s. Not significant; * $0.01 \leq F\ Pr. < 0.05$; ** $0.001 \leq F\ Pr. < 0.01$; *** $F\ Pr. < 0.001$

The salinity stress had a highly significant effect on shoot fresh weight (Shoot FW) of maize seedlings at 3, 5 and 7 DAS (Figure 3.2; Table 3.1B). Under all salt stress conditions, maize plants had lower shoot FW than the control plants. When compared to control treatments, the shoot FW was decreased by approximately 25, 50 and 60 % in 3-day-old seedlings, 20, 40 and 50 % in 5-day old seedlings, 10, 15 and 30 % in 7-day-old seedlings under 50, 100 and 150 mM NaCl treatment, respectively.

Salinity also significantly affected root fresh weight for 3 and 5-day-old seedlings but these significant effects were lost when the seedlings grow for 7 days (Table 3.1C). The concentration of 100 and 150 mM NaCl significantly reduced the root weights of the 3- and 5-day-old seedlings compared to the control, whereas the effect of 50 mM had a significant effect only on 5-day-old seedlings (Figure 3.2).
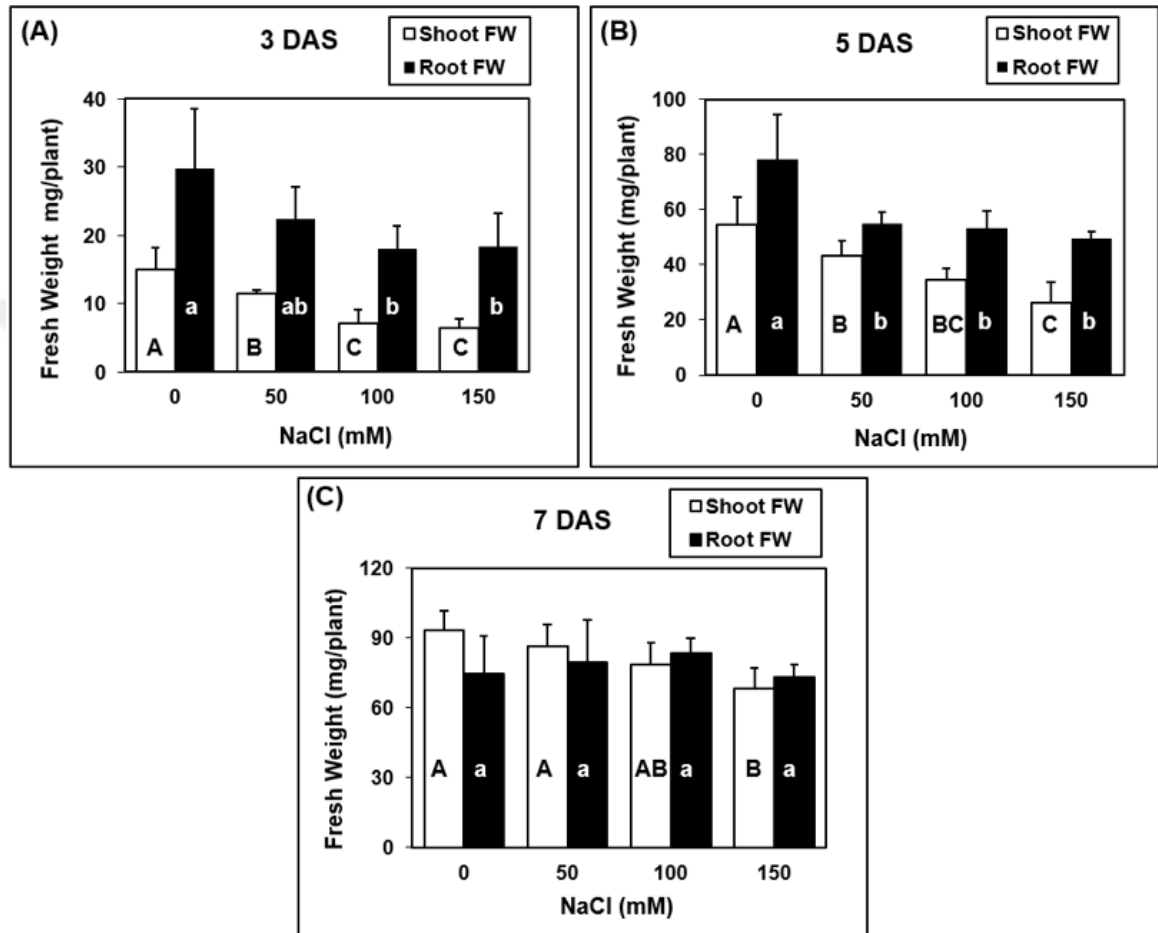
Figure 3.2: Shoot and root FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and subjected to salinity stress at different levels. Values are measured for plants harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Different upper- and lower-case letters indicate significant differences between means according to Fisher's protected LSD test.

When compared to control condition, applied salt doses had no significant effect on the total weight of the maize seedlings (Figure 3.3) except for on 5-day-old-seedlings grown in perlite treated with 150 mM NaCl solutions (Figure 3.3B, Table 3.1D). As shown in Figure 3.3A and Figure 3.3B, the total fresh weight of the 3 and 5 day seedlings shows a decrease as the salt concentration increases.
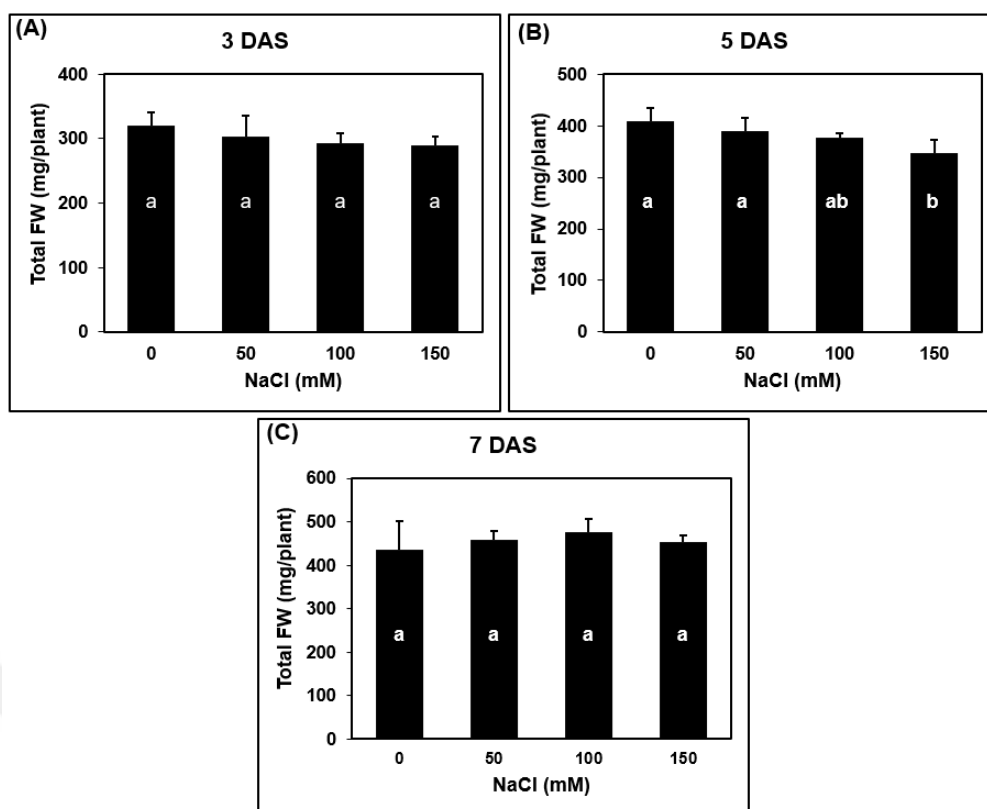
Figure 3.3: Total FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and subjected to salinity stress at different levels. Values are measured for plants harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent box replicates, each containing 30 seeds. Different lower-case letters indicate significant differences between means according to Fisher's protected LSD test.

Shoot length was also significantly affected by salt treatment (Table 3.1E; Figure 3.4). Plants had the highest shoot length value under the control conditions while plants grown at 150 mM had the lowest shoot length. As salt concentration increased, the shoot length of 5-day-old plants significantly decreased. The negative effect of 50 mM NaCl on shoot length of maize seedlings was observed when the plants were 5-days-old but this effect was lost when after 2 days. Although 150 mM NaCl further reduced the ,shoot length at 7DAS, effect of 100 mM and 150 mM NaCl was not significantly different.
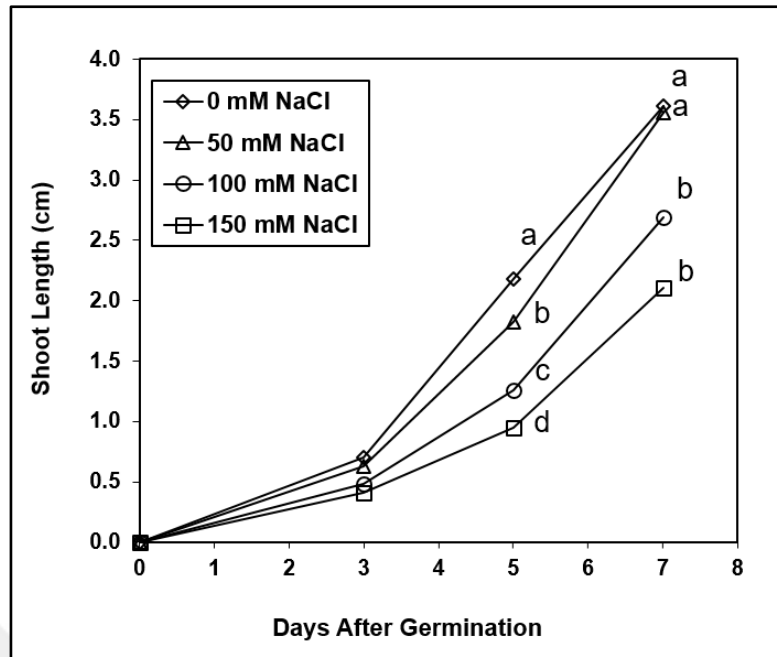
Figure 3.4: Shoot Length of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and subjected to salinity stress at different levels. Values are measured for plants harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Different lower-case letters indicate significant differences between means according to Fisher's protected LSD test.

3.1.2. Second Perlite Experiment Results

In this second perlite experiment, where the aim was to determine the optimum SA dose under control and saline conditions, interaction of the variables (salinity (EC_e) x seed treatment (SA)) did not have a significant effect on germination percentage (Table 3.2A; Figure 3.5). Germination percentage was significantly affected by seed treatment with SA but not salinity except for 3 DAS. Under the control conditions, germination percentage measured was not significantly changed by SA treatment. In salinity treated case, low SA dose increased germination rate at 3 and 5 DAS but this effect was lost for 7 day-old-seedlings (Figure 3.5).
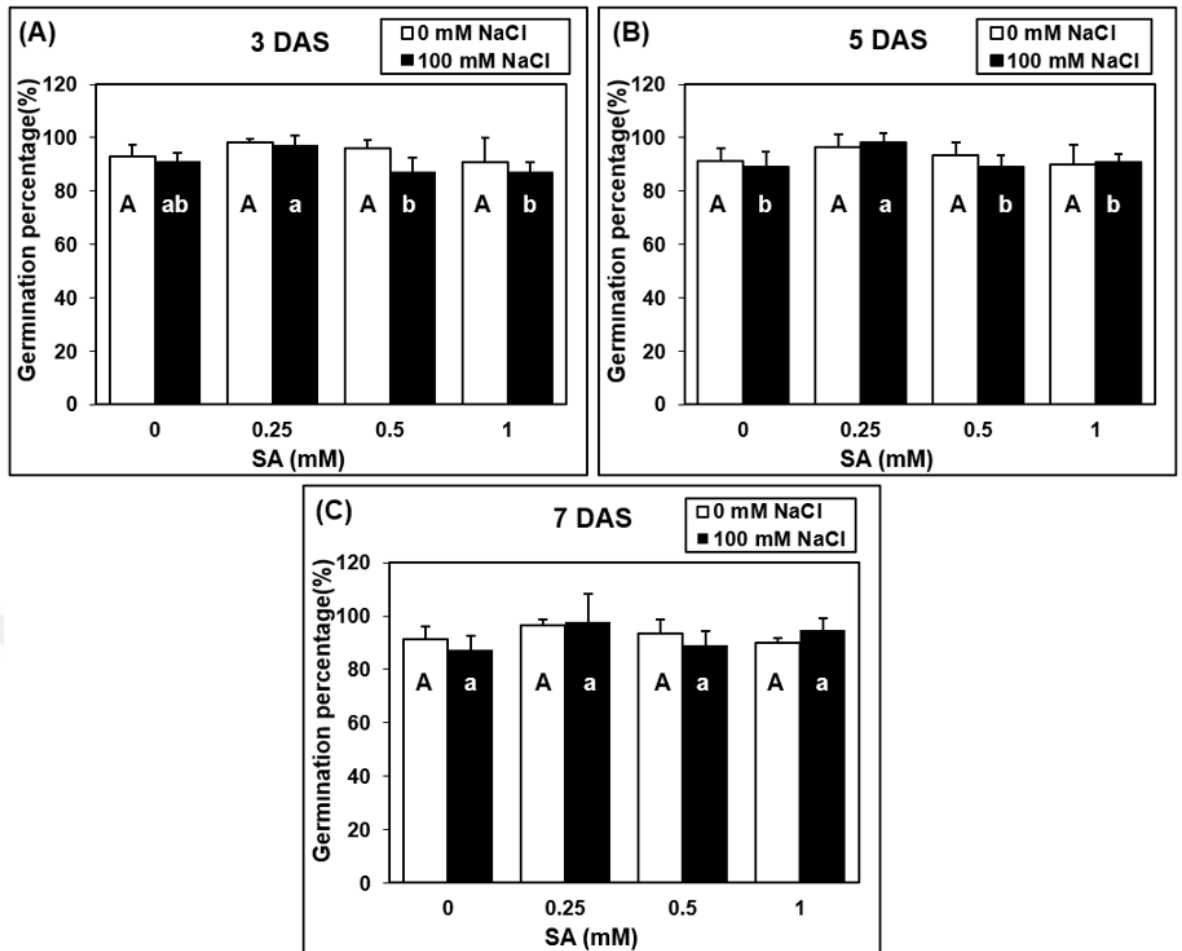
Figure 3.5: Effect of seed treatment with SA on germination percentage of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Different upper- and lower-case letters indicate significant differences between means according to Fisher's protected LSD test.

Table 3. 2: Two-way ANOVA of the effects of salinity (EC_e) and seed treatment with Salicylic acid (SA) on (A) germination percentage, (B) shoot, (C) root and (D) total FW and (E) shoot length for maize *(Zea mays cv. Caramelo)* plants grown in perlite in the second experiment and harvested 3 DAS, 5 DAS or 7 DAS.

| | Source of Variation | Germination Percentage | | |
		3 DAS	5 DAS	7 DAS
(A)	EC_e	*	*n.s.*	*n.s.*
	SA	*	*	*
	EC_e*SA	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Shoot FW**		
		3 DAS	5 DAS	7 DAS
(B)	EC_e	***	***	**
	SA	**	*	*n.s.*
	EC_e*SA	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Root FW**		
		3 DAS	5 DAS	7 DAS
(C)	EC_e	***	***	***
	SA	*n.s.*	*n.s.*	*
	EC_e*SA	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Total FW**		
		3 DAS	5 DAS	7 DAS
(D)	EC_e	***	*	*
	SA	*n.s.*	*n.s.*	*n.s.*
	EC_e*SA	*	*n.s.*	*n.s.*
	Source of Variation	**Shoot Length**		
		3 DAS	5 DAS	7 DAS
(E)	EC_e	***	***	***
	SA	***	*n.s.*	*n.s.*
	EC_e*SA	*n.s.*	*n.s.*	*n.s.*

n.s. Not significant; * $0.01 \leq$ F Pr. < 0.05; ** $0.001 \leq$ F Pr. < 0.01; *** F Pr. < 0.001

At 3 DAS harvest time, 0.5 mM SA treatment increased the shoot fresh weights of maize seedling by approximately 25 % under control and 50% under stress conditions (Figure 3.6 A). Higher SA dose did not provide extra benefit under these conditions. These positive effects of SA treatments at higher doses was lost at 5 and 7 DAS harvest time (Figure 3.6; Table 3.2B). Salt applications dramatically reduced the shoot FW at 3 DAS however, when the seedlings got bigger, this effect was reduced or nearly lost (Figure 3.6).
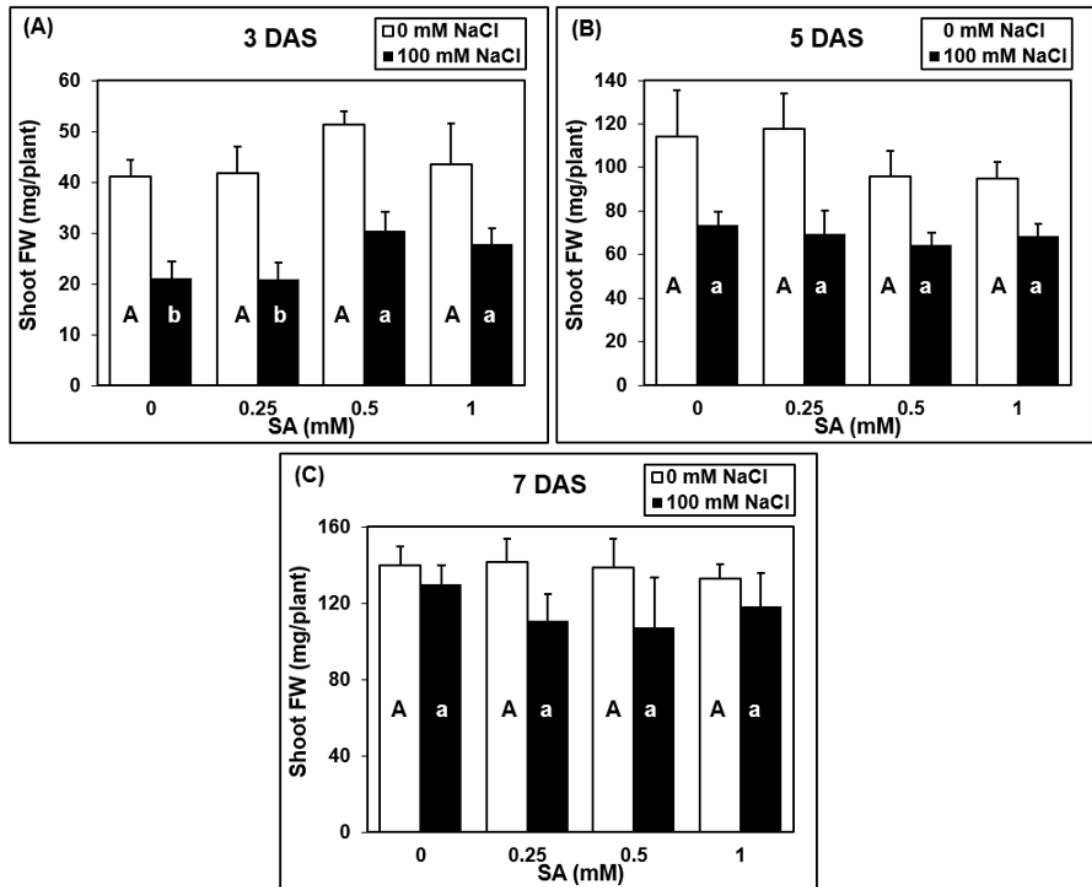
Figure 3.6: Effect of seed treatment with SA on shoot FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

Salinity reduced root growth of maize seedlings at all harvest times (Figure 3.7; Table 3.2C). The application of SA had a significant effect on the root FW of maize seedlings only at 7-days-old plants (Table 3.2C). Seed treatment with 0.5 mM SA gave the highest root FW for 3-day-old-seedlings both under control and salinity stress conditions (Figure 3.7A). However, for the 5 and 7 day-old-maize seedlings salicylic acid applications did not provide any significant difference (Figure 3.7 B, C).
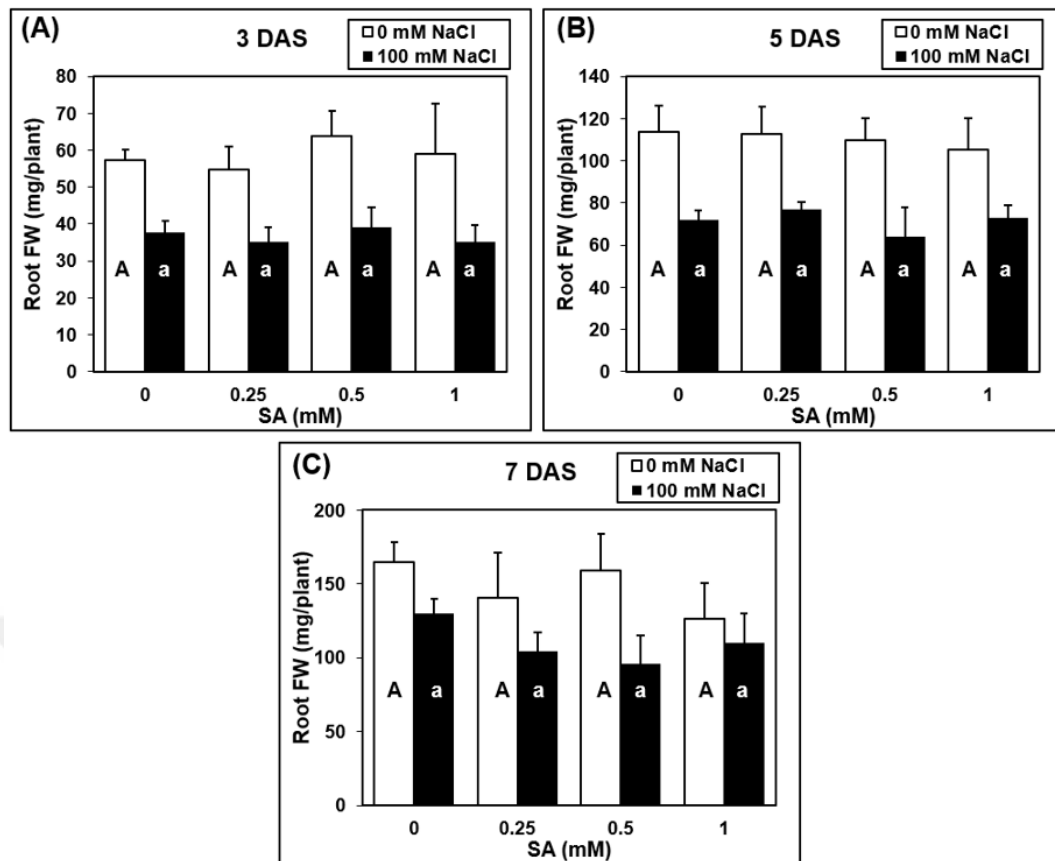
Figure 3.7: Effect of seed treatment with SA on root FW of of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

The interaction of salinity and SA application had a significant effect on the total weights of 3-day-old-maize seedlings, whereas this effect was not observed in 5 and 7-day-old maize seedlings (Table 3.2D; Figure 3.8). In general salinity applications reduced the Total FW of maize plants at early stages of their growth (Figure 3.8 A, B). The application of 0.5 mM SA caused the least reduction in total fw of 3-day-old-seedling among all other SA-treated seedlings under stress condition (Figure 3.8A).
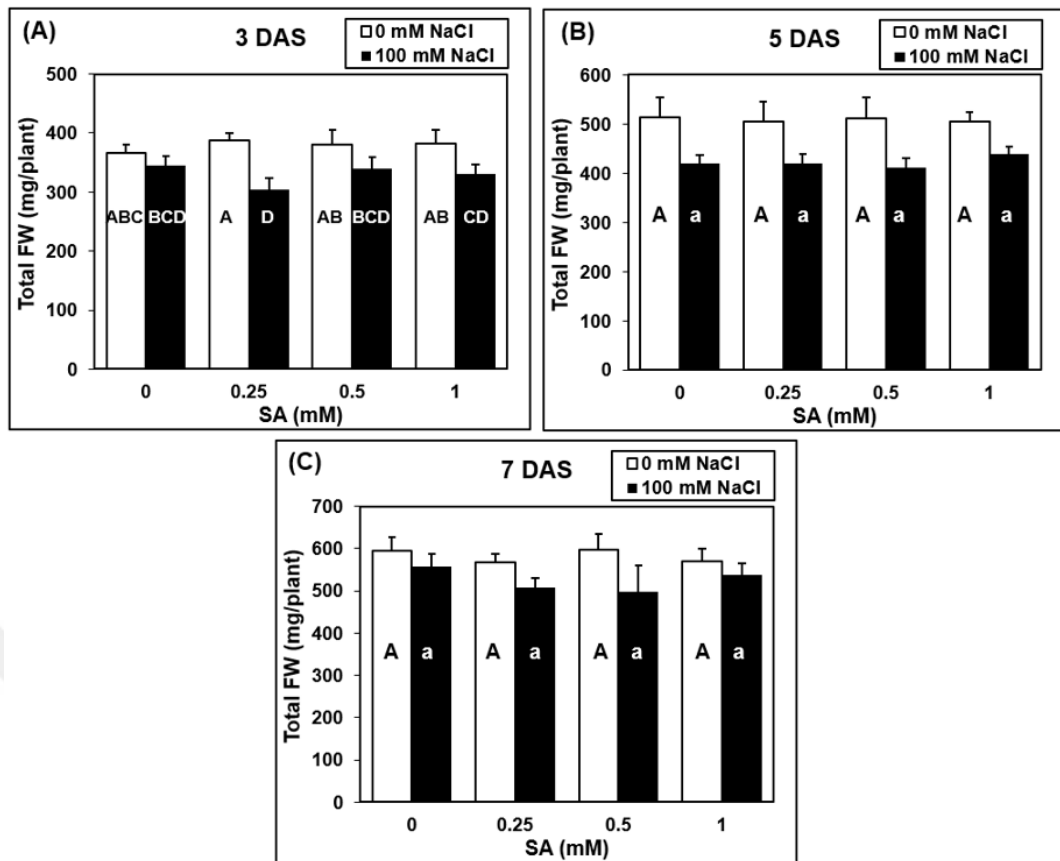
Figure 3.8: Effect of seed treatment with SA on total FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS, (B) 5 DAS and (C) 7 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

A significant effect of the SA application on the shoot length of maize seedlings was not observed for 7-day-old-seedlings under all conditions. (Table 3.2E, Figure 3.9A) On the other measurement dates, the application of 0.5 and 1 mM SA indicated a similar positive effect on shoot length of seedlings under the stress condition at both 3 and 5 DAS (Figure 3.9B). At all harvest times, shoot length was reduced by salinity treatment. Plants grown from seeds which were not treated with SA had the lowest shoot height under both control and saline conditions (Figure 3.9; Table 3.2E).
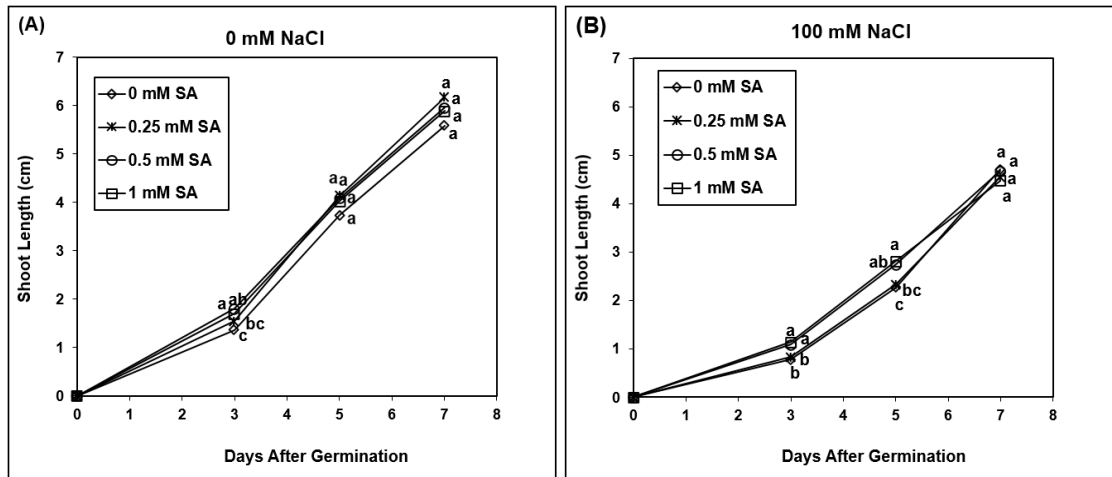
Figure 3.9: Effect of seed treatment with SA on shoot length of maize (*Zea mays* cv. Caramelo) plants grown for 7 days in perlite under growth chamber conditions. Values are means of 4 independent replicates, each containing 30 seeds. The lower-case letters were given according to the Fisher's protected LSD test results in the control condition and salt stressed condition.

For the rest of this study 0.5 mM SA was used for seed treatment agent. Since it was observed that seed treatment of 0.5 mM SA gave some advantage to maize seedlings in respect to measured growth parameters however 1 mM SA did not provide extra benefit.

3.1.3. Third Perlite Experiment Results

In the last perlite experiment, seed treatments of SA and the willow extracts were tested under control and saline stress conditions. As shown in Figure 3.10 and Figure 3.11, salinity application adversely affected shoot and root development of maize seedlings. Seeds which germinated in the presence of 100 mM NaCl produced shorter and weaker shoot and roots.
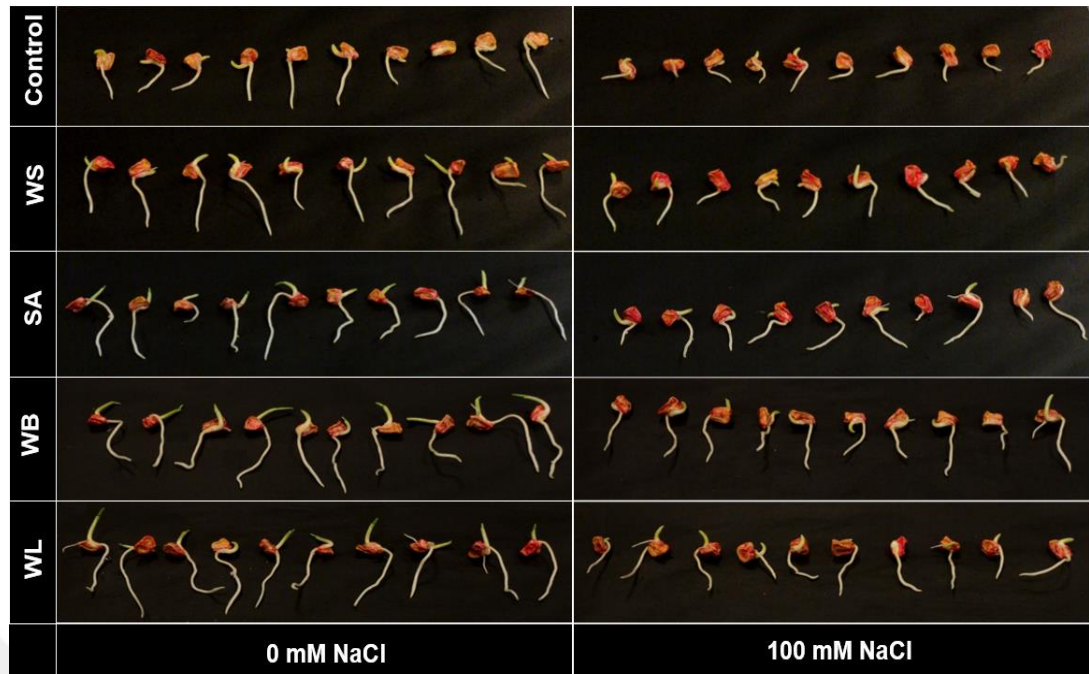
Figure 3.10: Effect of seed treatment with various agents (WS: Water Soaking, SA: Salicylic acid (0.5 mM), WB: Willow Bark (2%), WL: Willow Leaf (2%)) on 3-day-old maize (*Zea mays* cv. Caramelo) seedlings grown in perlite under growth chamber conditions.

When compared to seedlings which did not receive any seed applications, all the seedlings which were treated with any agents (water, SA, WB or WL) showed a better seedling performance, produced longer shoots and roots both in the presence and absence of salinity stress (Figures 3.10-11). Among others, both 3- and 6-days-old maize seedlings which were willow bark and leaf extracts exhibited better growth and development under the stress conditions (Figures 3.10-11). Visually, the differences were more obvious at 6-days-old seedlings when compared to 3-days-old ones.
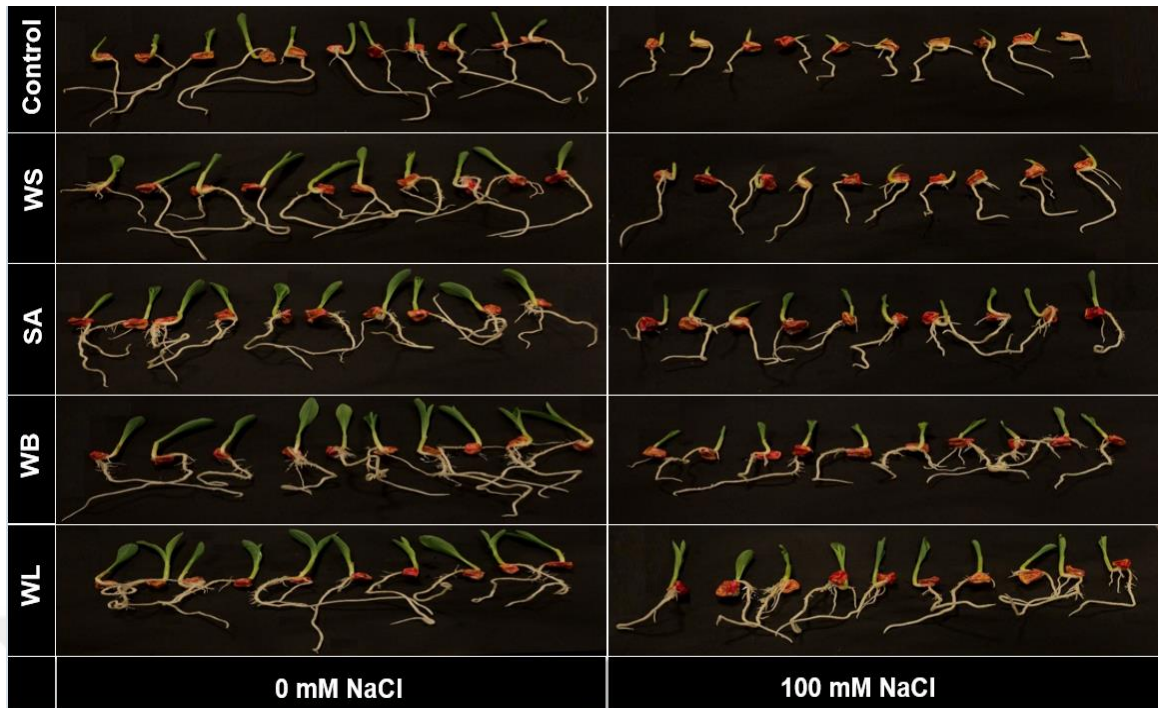
Figure 3.11: Effect of seed treatment with various agents (WS: Water Soaking, SA: Salicylic acid (0.5 mM), WB: Willow Bark (2%), WL: Willow Leaf (2%)) on 6-day-old maize (*Zea mays* cv. Caramelo) seedlings grown in perlite under growth chamber conditions.

Table 3.3: Two-way ANOVA of the effects of salinity (EC_e) and seed treatment (ST) on (A) germination percentage, (B) shoot, (C) root and (D) total FW, (E) total root area and (F) shoot length for maize *(Zea mays cv. Caramelo)* plants grown in perlite in the third experiment and harvested 3 DAS and 6 DAS.

	Source of Variation	Germination Percentage	
		3 DAS	6 DAS
(A)	EC_e	*n.s.*	*n.s.*
	ST	*	*n.s.*
	EC_e*ST	*n.s.*	*n.s.*

	Source of Variation	Shoot FW	
		3 DAS	6 DAS
(B)	EC_e	***	***
	ST	***	***
	EC_e*ST	*	**

	Source of Variation	Root FW	
		3 DAS	6 DAS
(C)	EC_e	***	***
	ST	***	***
	EC_e*ST	*n.s.*	*n.s.*

	Source of Variation	Total FW	
		3 DAS	6 DAS
(D)	EC_e	***	***
	ST	***	**
	EC_e*ST	*n.s.*	*n.s.*

	Source of Variation	Total Root Area	
		3 DAS	6 DAS
(E)	EC_e	***	***
	ST	***	***
	EC_e*ST	***	*n.s.*

	Source of Variation	Shoot Length	
		3 DAS	6 DAS
(F)	Salinity Treatment (0 mM)	***	***
	Salinity Treatment (100 mM)	***	***

n.s. Not significant; * $0.01 \leq$ F Pr. < 0.05; ** $0.001 \leq$ F Pr. < 0.01; *** F Pr. < 0.001

Neither salinity stress nor any of the seed treatments did not cause a significant effect on seed germination rate for 3- or 6-days-old maize seedling (Figure 3.12; Table 3.3).
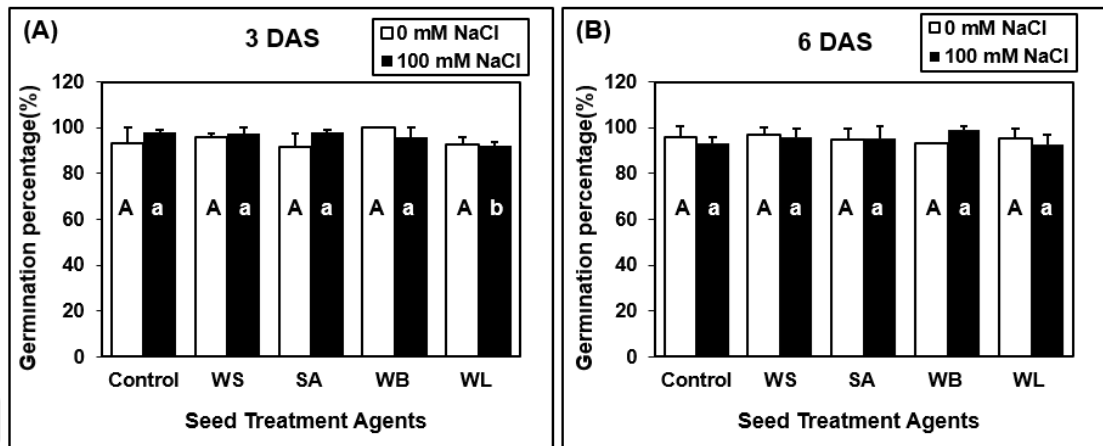
Figure 3.12: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on germination percentage of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

The interaction of salinity and seed treatment was found to be significant on the shoot fresh weight of all seedlings (Table 3.3B; Figure 3.13). Salinity treatment reduced the shoot FW at both harvest stages (Figure 3.13). The reduction which is observed due to salinity was reversed significantly with all seed treatments. At 3 DAS, maximum shoot fresh weight was measured in maize seedlings treated with willow bark or willow leaf under both control and saline conditions (Figure 3.13A). Although the difference was not that severe, at 6 DAS the highest shoot FW was observed in maize seedlings which were pre-treated with willow leaf extract (Figure 3.13B).
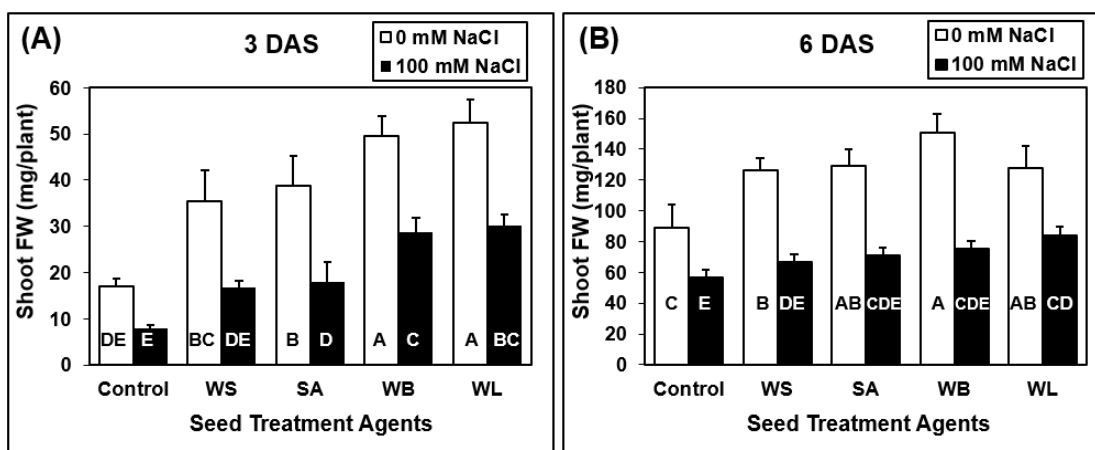
Figure 3.13: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on shoot FW of maize plants (*Zea mays* cv. Caramelo) grown in perlite under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters were given according to Tukey's protected HSD test.

Like shoot FW, root FW was also significantly reduced by salt treatment (Table 3.3C; Figure 3.14). The average root weights of the 3-day-old plants grown under both stress and control conditions were half of the root weights of seedlings treated with willow extracts (Figure 3.14A). The application of SA and willow extracts were significantly enhanced root FW in all seedlings when compared to control treatment (Figure 3.14). The highest root fresh weights were observed in plants which were treated with willow extracts as seed applications at both harvest stages. In salt treated plants which were harvested at 6 DAS, the water application did not cause a significant increase in the roots FW of the maize plants, however, the applications of willow extracts as well as SA caused a significant increase (Figure 3.14B).
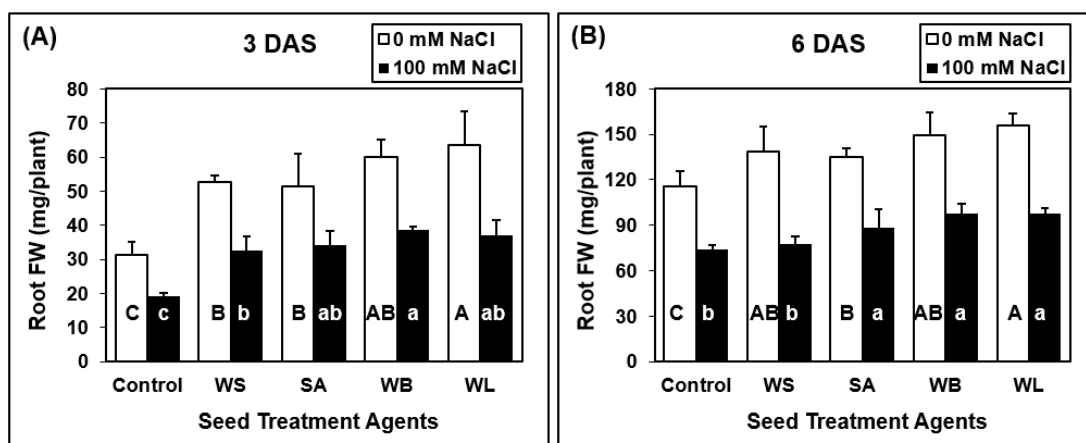
Figure 3.14: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on root FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

Salinity also had a negative effect on total FW of maize plants (Table 3.3D, Figure 3.15). At both harvest stages seed treatments of willow leaf and willow bark extracts significantly increased total FW of maize plants under both control and saline conditions (Figure 3.15) The positive effect of salicylic acid and water application was similar on the and when compared to willow extracts these treatments caused a lower increase in total FW.
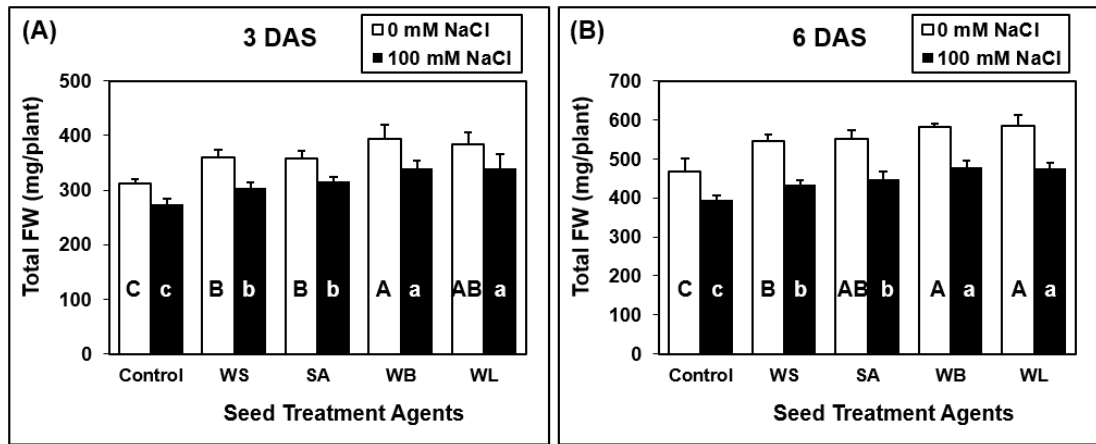
Figure 3.15: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on total FW of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.
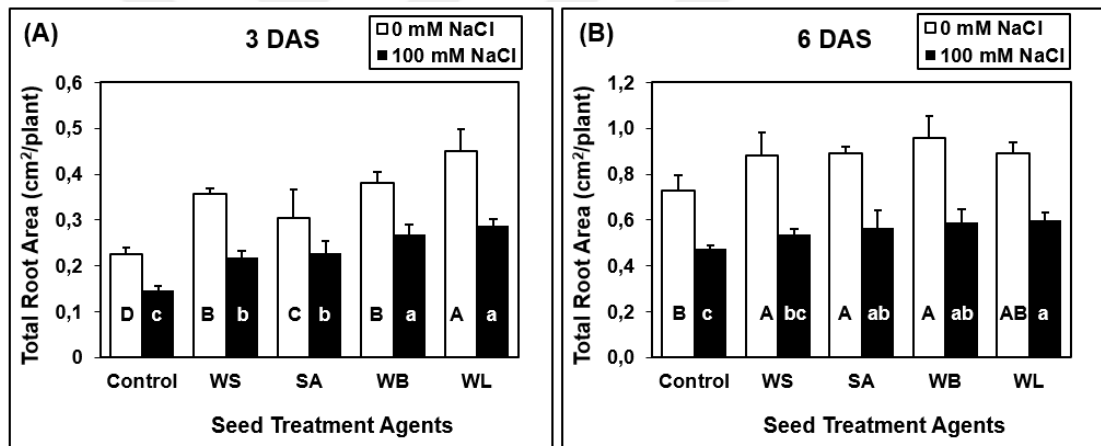
Figure 3.16: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on total root area (calculated by Image J) of maize (*Zea mays* cv. Caramelo) plants grown in perlite under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

It was determined that salt and seed treatment had a significant effect on total root area (Table 3.3E). As expected, salinity stress reduced the total root area drastically (Figure 3.16). Seed treatment agents increased the root area at both harvest times and the best results were obtained in response to willow extracts, particularly willow leaf. Compared to the control seedlings, the total root area of the 3-day-old maize seedlings

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treated with willow leaf extract increased more than 100% in the control state and almost 100% in the case of salinity stress (Figure 3.16A).

Salinity treatment reduced the shoot length of maize seedlings at both 3 and 6 DAS harvest stages (Figure 3.17). At 3 DAS and 6 DAS, shoot length was reduced by 44 % and 43%, respectively. Control seedlings had the lowest shoot length under the all conditions. Shoot length was significantly affected by different seed treatment applications (Table 3.3F). In the case of non-saline conditions, willow bark and leaf applications showed a similar significant increment on the shoot length, while the positive effect of willow leaf application was more pronounced under saline conditions (Figure 3.17). When compared with other seed treatment agents, the plants which were treated with willow leaf extracts as seed applications showed the highest shoot length at both day 3 and day 6 under saline conditions and when compared to control plants, the average shoot length of these plants were 100 % higher at 6 DAS (Figure 3.17B).
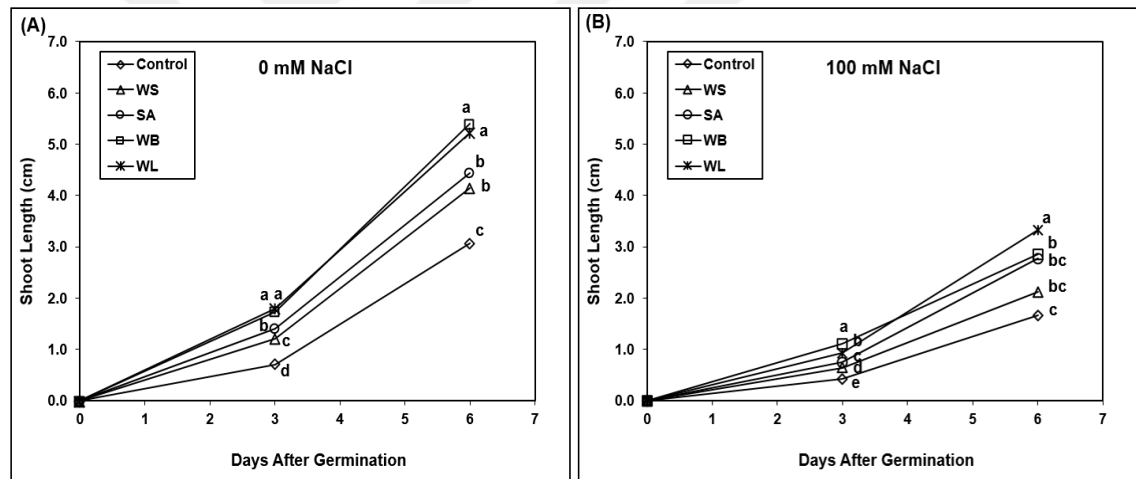
Figure 3.17: Effect of seed treatment with various agents (WS, SA (0.5 mM), WB (2%), WL (2%)) on shoot length of maize (*Zea mays* cv. Caramelo) plants grown in perlite media under growth chamber conditions and harvested (A) 3 DAS or (B) 6 DAS. Values are means of 4 independent replicates, each containing 30 seeds. The lower-case letters were given according to the Fisher's protected LSD test results in the control condition and salt stressed condition.

3.2. Soil Experiment Results

3.2.1. First Soil Experiment Results

The maize plants grown from seeds subjected to different hydropriming applications and were grown under different soil ECs for 14 days are given at Figure 3.18. At all

priming applications, maize plants were adversely affected by the salinity applications. Step by step as soil EC value increased, plant growth and development decreased. Seed priming with water improved the homogeneity and vigor of the maize seedlings under saline conditions.

Figure 3.18: Effect of priming times and salinity stress on maize plants (*Zea mays* cv. Caramelo) grown for 14 days under growth chamber conditions by using salt treated soils which had different soil EC_e values.
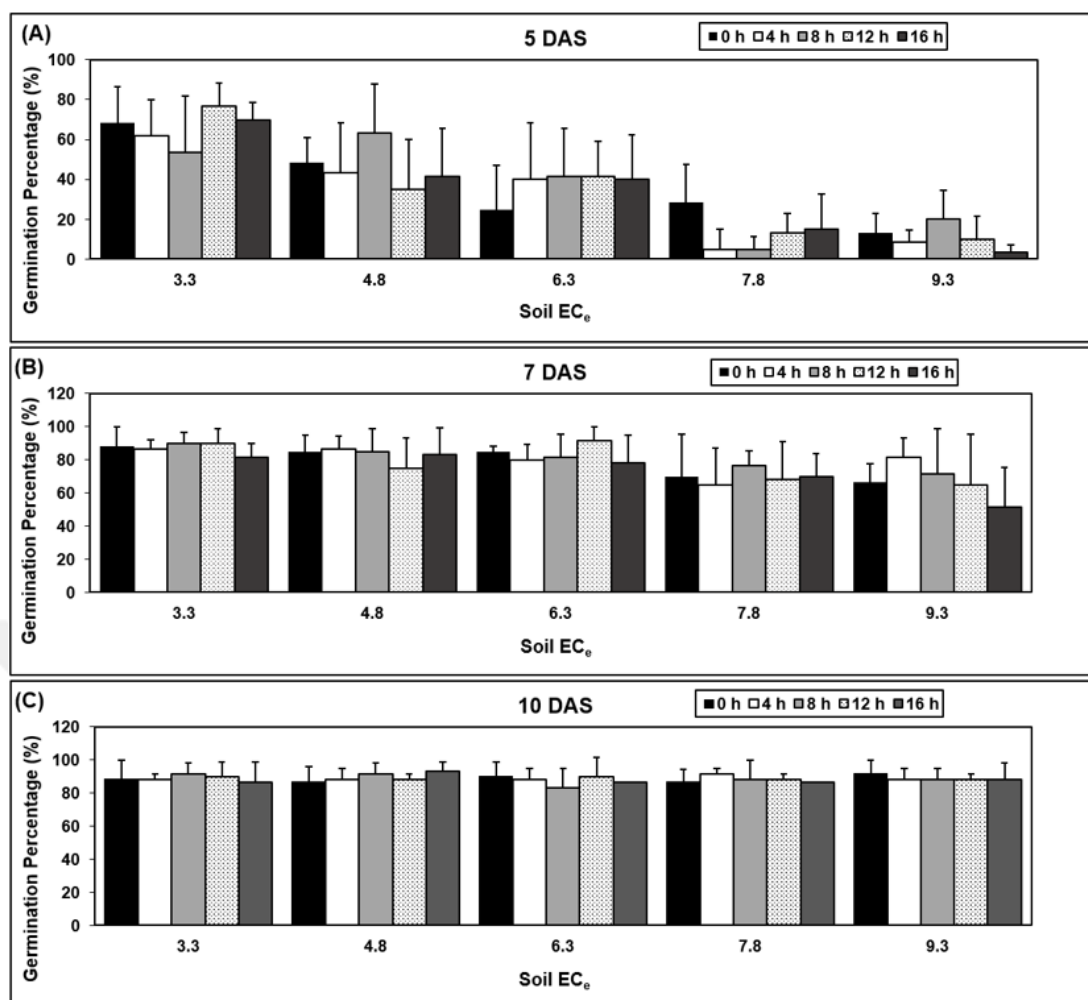
Figure 3.19: Effect of priming times and salt stress on germination percentage of (A) 5 DAS, (B) 7 DAS and (C) 10 DAS maize plants (*Zea mays* cv. Caramelo) grown under growth chamber conditions by using salt treated soils which had different soil EC_e values. Values are means of 4 independent pot replicates, each containing 15 seeds.

There was no significant effect of different priming times on seed germination of maize plants. The effect of salinity on germination percentage of 5-days-old maize seedlings was clearly observed, whereas this effect was totally lost on 10th day (Table 3.4A; Figure 3.19). Salinity treatments reduced the germination percentage of maize plants almost 60 % (Figure 3.19A) at 5 DAS. None of the hydropriming times had a consistent effect on the germination percentage.

Table 3.4: Two-way ANOVA of the effects of salinity (EC$_e$) and priming time (PT) on (A) germination percentage (5 DAS, 7 DAS and 10 DAS), (B) plant height (7 DAS, 10 DAS and 14 DAS), (C) shoot fresh and (D) dry weight (14 DAS) of soil grown maize under control and salt stress.

	Source of Variation	Germination Percentage		
		5 DAS	7 DAS	10 DAS
(A)	EC$_e$	***	**	*n.s.*
	PT	*n.s.*	*n.s.*	*n.s.*
	EC$_e$*PT	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	Plant Height		
		7 DAS	10 DAS	14 DAS
(B)	EC$_e$	***	***	***
	PT	*n.s.*	*n.s.*	*n.s.*
	EC$_e$*PT	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	Shoot Fresh Weight		
		14 DAS		
(C)	EC$_e$	***		
	PT	*n.s.*		
	EC$_e$*PT	*n.s.*		
	Source of Variation	Shoot Dry Weight		
		14 DAS		
(D)	EC$_e$	***		
	PT	*n.s.*		
	EC$_e$*PT	*n.s.*		

While salinity had a significant negative effect on plant height, the priming time did not have a significant effect on this trait (Table 3.4B). Compared with control plants, salt applications (with EC values: 4,8; 6,3; 7,8; 9,3) reduced plant length of 14-day-old maize by 10, 20, 30 and 40%, respectively (Figure 3.20). As expected, maize plants grown at the highest salt dose were the shortest. Under non-saline conditions the tallest plants were obtained from seeds primed with water for 12 or 16 h, whereas at the highest salinity case 8 or 16 h treatment gave the best results.
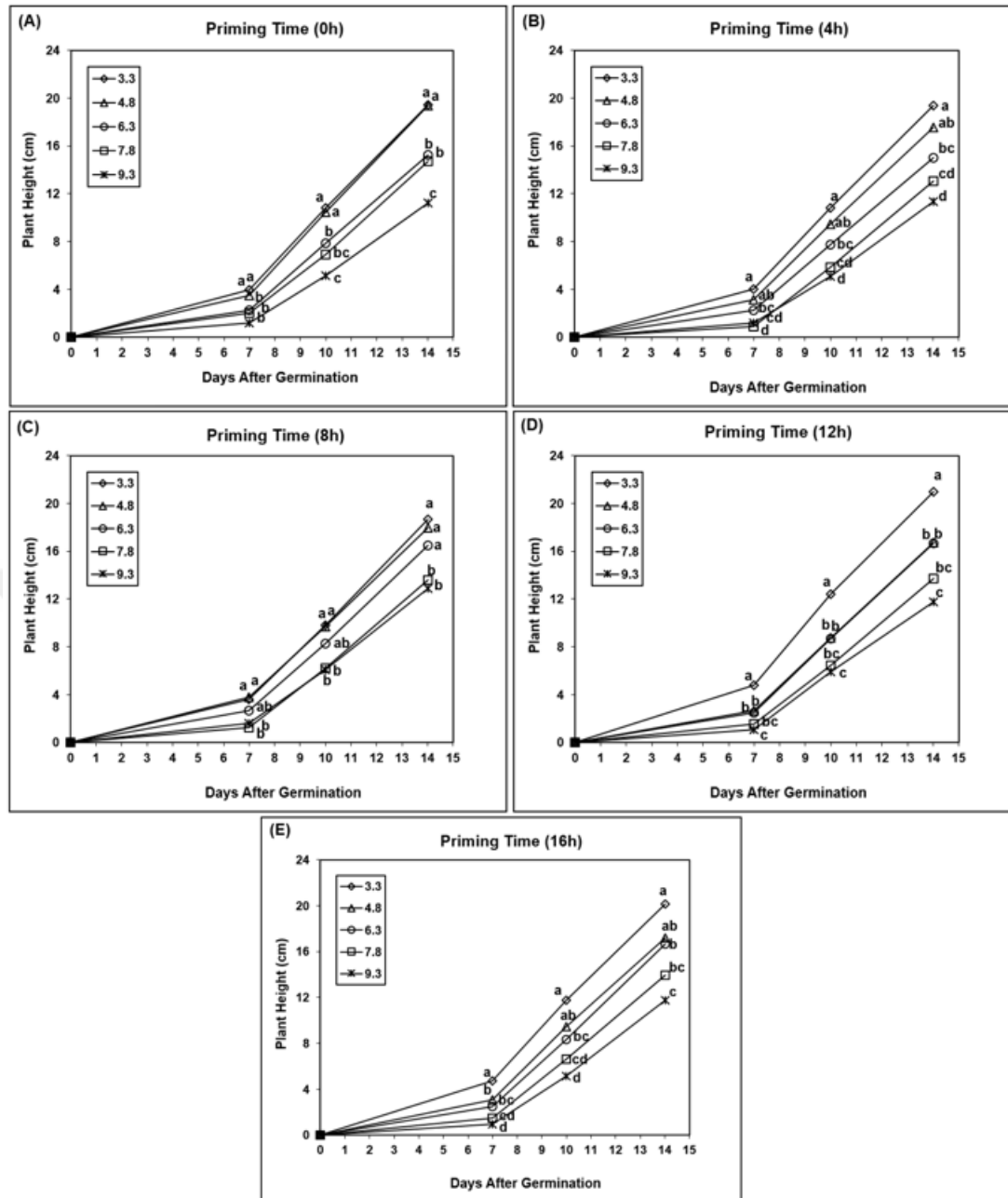
Figure 3.20: Effect of (A) 0h, (B) 4h, (C) 8h, (D) 12h and (E) 16h priming time on plant height of maize (*Zea mays* cv. Caramelo) plants grown under growth chamber conditions by using salt treated soils which had different soil EC_e values. Values are means of 4 independent pot replicates, each containing 15 seeds.

According to statistical calculations, it was observed that shoot fresh weight of maize plants was only affected by salinity treatments (Table 3.4C). In respect to increasing salt applications, shoot FW was reduced by approximately 20, 30, 40 and 50% when to the control plants (Figure 3.21). For the control EC level and EC level of 7.8 (which was selected for the following experiment), 16 h priming time gave the highest shoot FW.
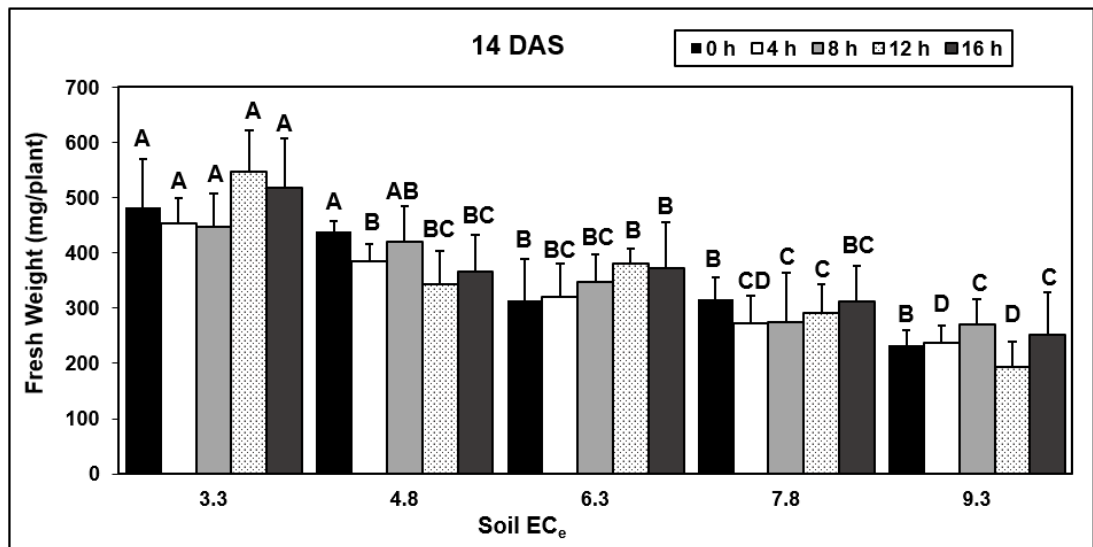
Figure 3.21: Effect of priming time and salinity stress on fresh weight of maize (*Zea mays* cv. Caramelo) plants grown for 14 days under growth chamber conditions by using salt treated soils which had different soil EC_e values. Values are means of 4 independent pot replicates, each containing 15 seeds. Uppercase letters were given according to the Fisher's protected LSD test results according to the priming time.
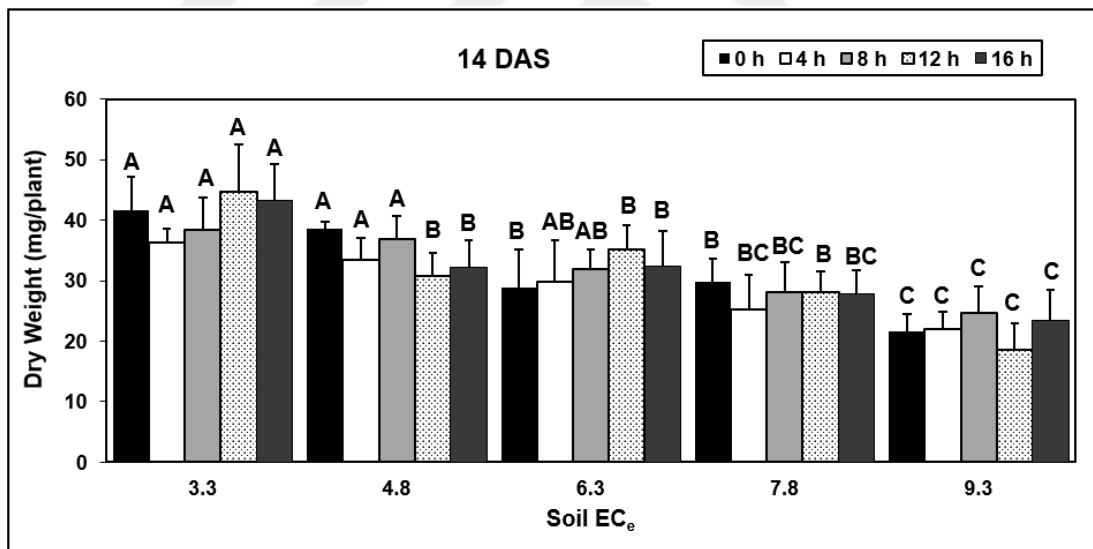
Figure 3.22: Effect of priming time on dry weight 14 DAS of maize (*Zea mays* cv. Caramelo) plants grown for 14 days under growth chamber conditions by using salt treated soils which had different soil EC_e values. Values are means of 4 independent pot replicates, each containing 15 seeds. Uppercase letters were given according to the Fisher's protected LSD test results according to the priming time.

In parallel to the FW results, the DW of maize seedlings decreased significantly with increasing salinity (Table 3.4D; Figure 3.22). Seed treatment of water with different priming times could not prevent this reduction. In the case of control and highest salt treatments, the effect of seed treatments at different times with water was identical (Figure 3.22).

3.2.2. Second Soil Experiment Results

In the second soil experiment maize seeds which were soaked by using SA, WB and WL as seed treatment agents were grown in soil medium under either control or saline conditions where the selected EC dose of 7.8 ds/m was used for salinity treatment. Compared to the non-soaked control plants, all the seed applications were found to have a positive effect on the growth and development of 14 day-old maize plants under both control and stress conditions (Figure 3.23). In the case of saline conditions, the positive effects of water soaking and SA looked limited when compared to seed treatments of WB and WL.
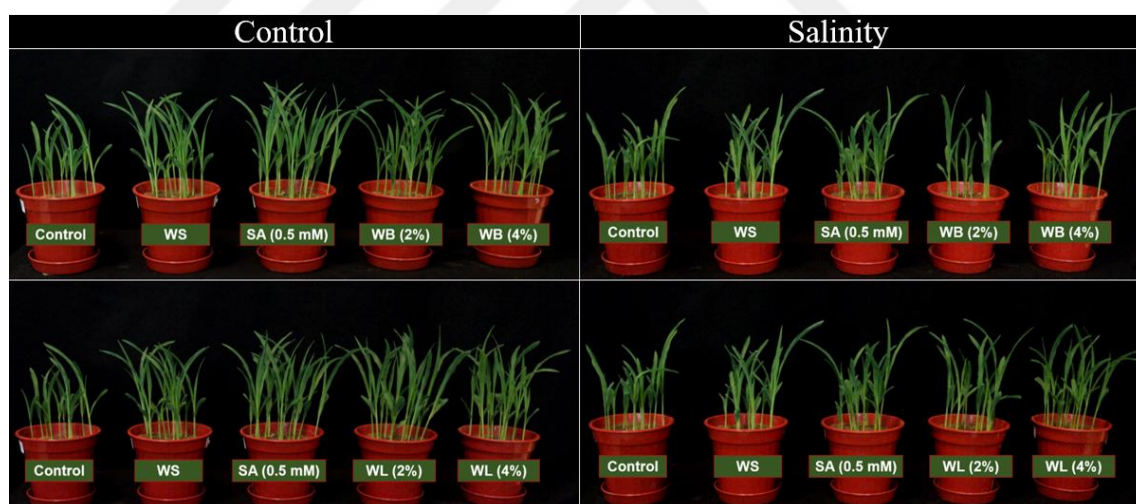
Figure 3.23: Effects of different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) on maize (*Zea mays* cv. Caramelo) plants grown for 14 days under growth chamber conditions under control and saline soil.

Salinity reduced germination percentage significantly only on the 5th day after sowing, while seed applications did not have a significant effect on germination percentage under these conditions (Table 3.5A, Figure 3.24A). On the 7th and 10th day germination percentage results, the highest germination was recorded for the high

concentration willow leaf application under both control and saline status. (Figure 3.24B, C).
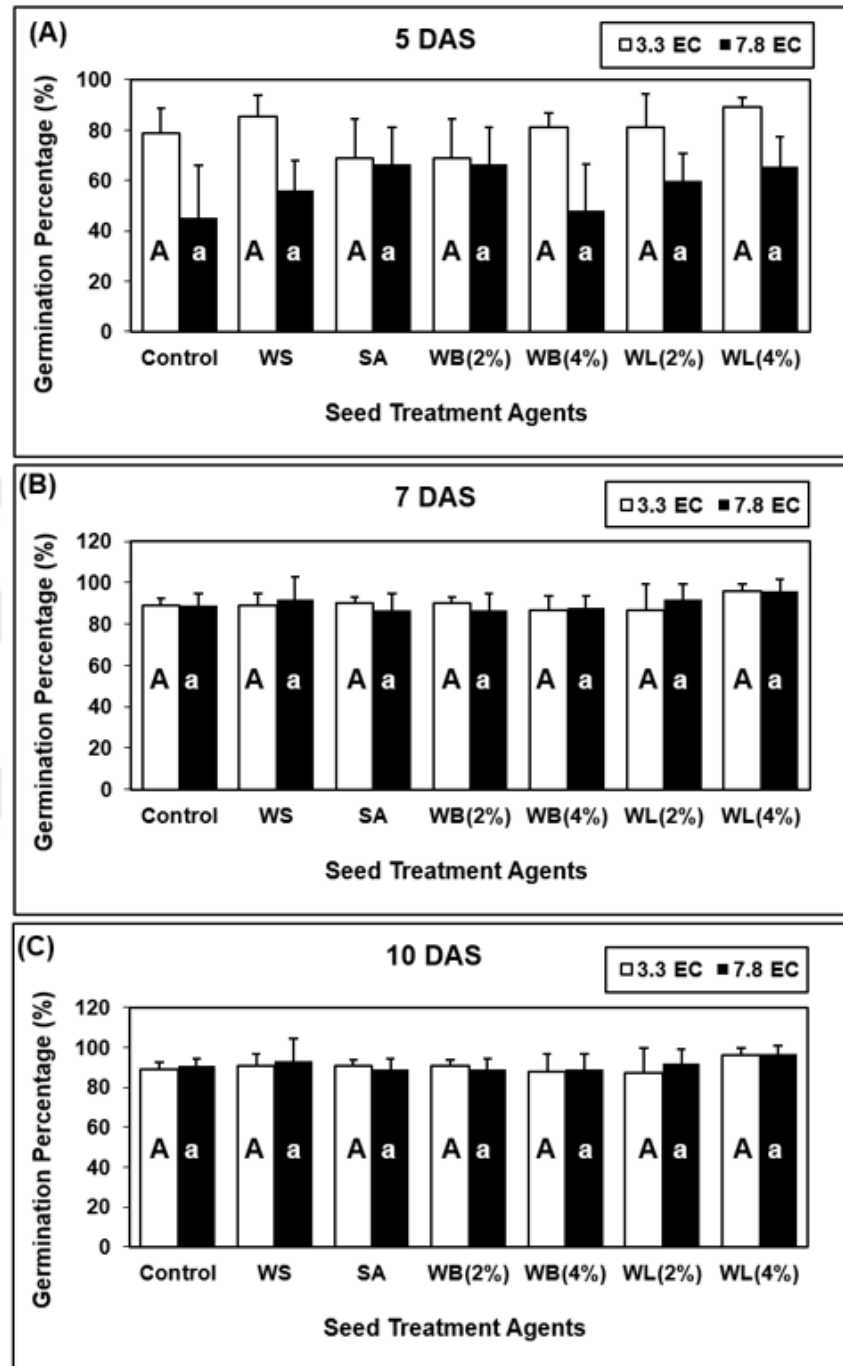
Figure 3.24: Effects of different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) on germination percentage of maize (*Zea mays* cv. Caramelo) plants ((A)5 DAS, (B)7 DAS and (C)10 DAS) grown under control and saline soil conditions. Values are means of 5 independent pot replicates, each containing 15 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

Table 3.5: Two-way ANOVA of the effects of salinity (EC_e) and seed treatment (ST) on (A) germination percentage (5 DAS, 7 DAS and 10 DAS), (B) plant height (7 DAS, 10 DAS and 14 DAS), (C) shoot fresh and (D) dry weight (14 DAS) of soil grown maize under control and salt stress.

	Source of Variation	Germination Percentage		
		5 DAS	7 DAS	10 DAS
(A)	EC_e	***	*n.s.*	*n.s.*
	ST	*n.s.*	*n.s.*	*n.s.*
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Plant Height**		
		7 DAS	10 DAS	14 DAS
(B)	EC_e	***	***	***
	ST	***	***	*
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Shoot Fresh Weight**		
		14 DAS		
(C)	EC_e	***		
	ST	***		
	EC_e*ST	*n.s.*		
	Source of Variation	**Shoot Dry Weight**		
		14 DAS		
(D)	EC_e	***		
	ST	***		
	EC_e*ST	*n.s.*		

When compared to control conditions, under saline conditions plant height was reduced irrespective of the seed treatment (Table 3.5B; Figure 3.25). Under both control and saline conditions, maximum length was observed in plants which were treated with willow leaf at low concentration as a seed soaking agent (Figure 3.25). All the seed treatment agents increased plant height when compared to non-soaked control plants. In saline soil, the heights of the plants treated with water, salicylic acid, willow bark extract and high dose willow leaf extract were similar (Figure 3.25B). Under saline conditions plant height was increased by 12% with watersoaking, 15% with SA, 13% with WB and 17% with WL treatments (Figure 3.25B).
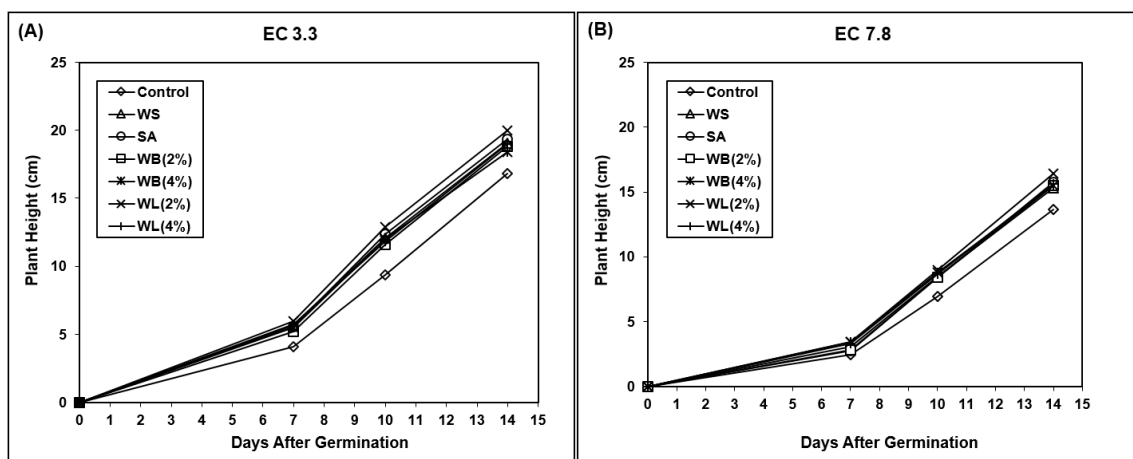
Figure 3.25: Effect of seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) on plant height of soil grown maize (*Zea mays* cv. Caramelo) plants under control and saline conditions. Values are means of 5 independent pot replicates, each containing 15 seeds.
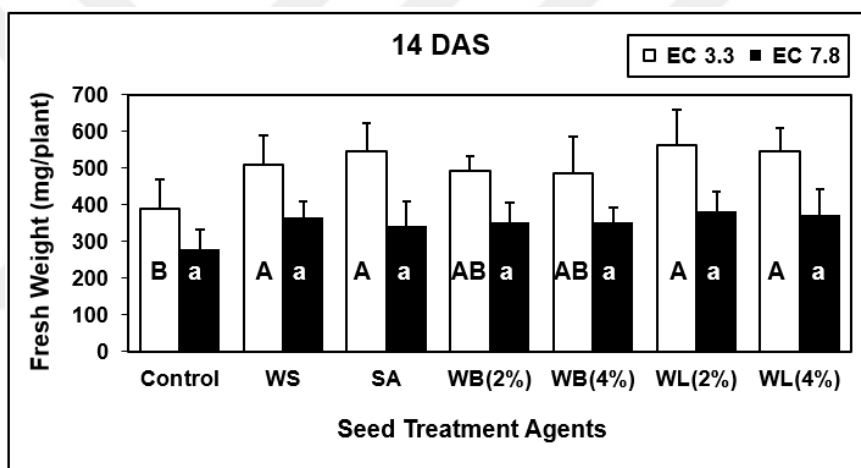
Figure 3.26: Effect of seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) on shoot fresh weight of soil grown maize (*Zea mays* cv. Caramelo) under control and salinity stress. Values are means of 5 independent pot replicates, each containing 15 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

Salinity application significantly reduced the shoot FW of maize plants (Table 3.5C; Figure 3.26). Seed treatments significantly increased fresh weight in the absence of salt, while the same effect was not observed in the saline state (Figure 3.26). In general, all seed treatments positively affected the shoot FW but among others, WL treatments provided the highest increase under both control and saline conditions (Figure 3.26). In the absence of salinity, the increase caused by WL applications in shoot FW was 42 %

and in the presence of salinity it was 37%. However, the increase caused by WB applications was limited to 25% under control and 26% under saline conditions.

In parallel to the shoot FW results, the dry weight of the seed treated plants was significantly higher than the dry weight of the control plant under saline conditions (Table 3.5D; Figure 3.27). In the absence of salinity, water treatment did not provide a significant increase in dry weight however all other seed treatment agents including SA, willow bark and leaf extracts caused a significant increase (Figure 3.27). Under non-saline conditions, according to shoot DW measurements the positive effect of willow leaf (54%) was greater than that of bark extract (%38) application when compared to non-treated control plants (Figure 3.27).
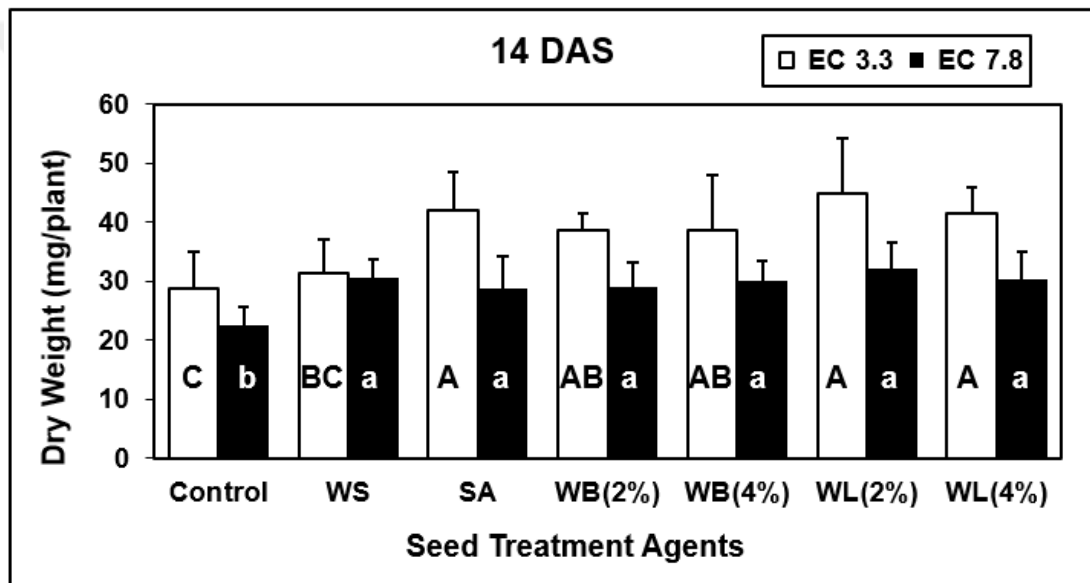
Figure 3.27: Effect of seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) on shoot dry weight of soil grown maize (*Zea mays* cv. Caramelo) under control and salinity stress. Values are means of 5 independent pot replicates, each containing 15 seeds. Uppercase letters and lower-case letters indicate significant differences according to the Fisher's protected LSD test in the absence and presence of salinity stress, respectively.

Table 3.6: Two-way ANOVA of the effects of salinity (EC_e), seed treatment on mineral concentrations of maize grown in soil in the second experiment.

	Shoot K and Na Elements Concentrations				
	Source of Variation	[K]		[Na]	
(A)	EC_e	***		***	
	ST	**		*n.s.*	
	EC_e*ST	*n.s.*		*n.s.*	

	Shoot Macroelements Concentrations				
	Source of Variation	[Ca]	[Mg]	[P]	[S]
(B)	EC_e	**	*n.s.*	*	***
	ST	*n.s.*	***	***	*
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*	*n.s.*

	Shoot Microelements Concentrations					
	Source of Variation	[Fe]	[Zn]	[Mn]	[Cu]	[Mo]
(C)	EC_e	*n.s.*	***	***	***	***
	ST	**	***	*	**	*n.s.*
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

Due to salinity application shoot K concentration was significantly reduced by 10 % irrespective of the seed treatment application (Table 3.6A; Table 3.7). Salinity increased the Na concentration approximately 5-fold in the absence of seed applications (Table 3.7). Water soaking slightly reduced the increase in Na concentration, however SA and willow bark applications did not have any effect. Interestingly under saline conditions Na concentrations was further increased by willow leaf applications by approximately 15%.

Table 3.7: K and Na concentrations of maize (*Zea mays* cv. Caramelo) leaves in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Treatments	Soil EC_e	K (%)			Na(%)		
Control	3.3	3.9	±	0.1	0.06	±	0.02
	7.8	3.5	±	0.1	0.38	±	0.08
WS	3.3	3.8	±	0.1	0.06	±	0.01
	7.8	3.6	±	0.1	0.33	±	0.07
SA (0.5 mM)	3.3	4.0	±	0.1	0.05	±	0.01
	7.8	3.6	±	0.1	0.39	±	0.07
WB (2%)	3.3	3.8	±	0.1	0.05	±	0.01
	7.8	3.7	±	0.2	0.39	±	0.05
WB (4%)	3.3	3.8	±	0.3	0.05	±	0.01
	7.8	3.5	±	0.1	0.39	±	0.04
WL (2%)	3.3	3.8	±	0.2	0.04	±	0.01
	7.8	3.5	±	0.1	0.44	±	0.04
WL (4%)	3.3	3.7	±	0.1	0.06	±	0.01
	7.8	3.4	±	0.2	0.43	±	0.02

Shoot K Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.07; 0.2; *n.s.*)
Shoot Na Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.02; *n.s.* ; *n.s.*)

While the salinity significantly decreased Ca concentration, the effect of seed treatment was not significant (Table 3.6B). Willow bark applications slightly increased the Ca concentration of maize shoots in the absence of salt stress (Table 3.8). Compared to the control plant, it was observed that 4% willow bark and leaf extracts significantly decreased Mg concentration. The highest phosphorus concentration was observed in the control plants and all the seed treatment applications significantly reduced shoot P concentration of maize plants. Salt treatments caused a reduction in S concentrations under all seed applications.

Table 3.8: Shoot macroelement concentrations of maize (*Zea mays* cv. Caramelo) leaves in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Treatments	Soil EC_e	[Ca]		[Mg]		[P]		[S]	
Control	3.3	0.66	± 0.06	0.47	± 0.02	0.84	± 0.07	0.42	± 0.01
	7.8	0.61	± 0.05	0.47	± 0.02	0.91	± 0.04	0.37	± 0.02
WS	3.3	0.67	± 0.03	0.46	± 0.02	0.77	± 0.09	0.41	± 0.01
	7.8	0.59	± 0.03	0.46	± 0.01	0.76	± 0.07	0.35	± 0.01
SA (0.5 mM)	3.3	0.66	± 0.06	0.47	± 0.02	0.78	± 0.09	0.40	± 0.01
	7.8	0.63	± 0.02	0.46	± 0.02	0.80	± 0.06	0.35	± 0.01
WB (2%)	3.3	0.70	± 0.09	0.45	± 0.02	0.73	± 0.06	0.40	± 0.02
	7.8	0.65	± 0.06	0.46	± 0.01	0.79	± 0.03	0.36	± 0.02
WB (4%)	3.3	0.74	± 0.10	0.45	± 0.02	0.73	± 0.08	0.40	± 0.01
	7.8	0.64	± 0.03	0.43	± 0.04	0.74	± 0.07	0.35	± 0.02
WL (2%)	3.3	0.65	± 0.03	0.45	± 0.03	0.70	± 0.06	0.41	± 0.02
	7.8	0.69	± 0.04	0.45	± 0.01	0.75	± 0.04	0.35	± 0.01
WL (4%)	3.3	0.66	± 0.05	0.42	± 0.02	0.72	± 0.05	0.39	± 0.02
	7.8	0.66	± 0.05	0.43	± 0.02	0.75	± 0.07	0.35	± 0.01

Shoot Ca Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (0.03; *n.s.* ; *n.s.*)
Shoot Mg Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (*n.s*; 0.03; *n.s.*)
Shoot P Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (0.03; 0.09; *n.s.*)
Shoot S Conc. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (0.01; 0.02; *n.s.*)

All seed treatment agents except water significantly reduced Fe concentration particularly under saline conditions (Table 3.6C; Table 3.9). On the other hand, Zn concentration was reduced by all treatment agents including water. Salinity caused an increase in Mn concentration, while seed application did not influence it. In contrast to Mn concentrations, shoot Cu and Mo concentrations were reduced by salt treatment.

Table 3.9: Shoot microelement concentrations of maize (*Zea mays* cv. Caramelo) leaves in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Shoot Microelements Concentrations (mg.kg^{-1})						
Treatments	**Soil EC$_e$**	**[Fe]**	**[Zn]**	**[Mn]**	**[Cu]**	**[Mo]**
Control	3.3	150 ± 20	80 ± 9	101 ± 7	16.5 ± 0.5	5.8 ± 1.3
	7.8	171 ± 28	95 ± 11	121 ± 13	15.8 ± 0.8	2.1 ± 0.3
WS	3.3	147 ± 28	67 ± 5	93 ± 11	16.0 ± 0.6	6.4 ± 1.8
	7.8	137 ± 12	77 ± 10	100 ± 7	14.4 ± 0.4	2.5 ± 0.5
SA (0.5 mM)	3.3	136 ± 8	65 ± 6	92 ± 10	15.3 ± 0.4	5.3 ± 1.3
	7.8	136 ± 10	76 ± 5	110 ± 6	14.4 ± 0.7	2.2 ± 0.4
WB (2%)	3.3	135 ± 8	63 ± 5	85 ± 7	15.3 ± 0.7	6.5 ± 1.7
	7.8	130 ± 12	75 ± 5	110 ± 10	14.5 ± 0.8	2.3 ± 0.1
WB (4%)	3.3	148 ± 42	66 ± 8	101 ± 15	15.1 ± 1.1	5.2 ± 2.2
	7.8	119 ± 7	69 ± 6	102 ± 16	14.6 ± 2.0	2.3 ± 0.3
WL (2%)	3.3	138 ± 6	58 ± 5	94 ± 6	14.8 ± 0.4	5.3 ± 1.2
	7.8	129 ± 13	71 ± 4	115 ± 9	14.6 ± 1.9	2.3 ± 0.4
WL (4%)	3.3	139 ± 8	62 ± 5	104 ± 9	15.1 ± 0.3	4.0 ± 0.4
	7.8	127 ± 4	72 ± 7	115 ± 18	13.7 ± 0.4	2.2 ± 0.4

Shoot Fe Conc. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (*n.s.* ; 24; *n.s.*)
Shoot Zn Conc. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (3; 9; *n.s.*)
Shoot Mn Conc. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (5; 15; *n.s.*)
Shoot Cu Conc. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (1; 1; *n.s.*)
Shoot Mo Conc. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (0.5; *n.s.* ; *n.s.*)

Table 3.10: Two-way ANOVA of the effects of salinity (EC_e) and seed treatment on mineral content of maize (*Zea mays* cv. Caramelo) grown in soil under growth chamber conditions.

	Shoot K and Na Elements Content			
(A)	**Source of Variation**	**K**	**Na**	
	EC_e	***	***	
	ST	***	**	
	EC_e*ST	*n.s.*	*	

	Shoot Macroelements Content			
	Source of Variation	**Ca**	**Mg**	**P**	**S**
(B)	EC_e	***	***	***	***
	ST	***	**	**	**
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*	*n.s.*

	Shoot Microelements Content				
	Source of Variation	**Fe**	**Zn**	**Mn**	**Cu**	**Mo**
(C)	EC_e	***	**	**	***	***
	ST	*n.s.*	*n.s.*	***	**	*n.s.*
	EC_e*ST	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

Like K concentrations, the shoot K content of maize plants was also significantly reduced by salinity stress (Table 3.10A; Table 3.11). Seed treatments of SA and willow extracts increased the K content of maize leaves under all conditions, whereas seed soaking with water only caused an increase under saline conditions (Table 3.11). The highest K content was observed in plants treated with 2% leaf extract in both control and stress conditions. Compared to the control plants, K content was increased by approximately 40 % with SA, 30% with WB and 40% with WL seed treatments.

Na content was significantly affected by the interaction between salinity and seed treatments (Table 3.10C). As expected, the Na content of the plants growing under saline condition was higher than that of plants grown under non-saline soil (Table 3.11). Under stress, seed treatments significantly increased the Na content compared to the control plant. The effects of watersoaking, bark and SA treatments on Na content were very similar and they were slightly higher than 4% WL application and slightly lower than 2% WL application.

Table 3.11: K and Na content of maize (*Zea mays* cv. Caramelo) shoots in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Treatments	Soil EC_e	K ($\mu g.plant^{-1}$)			Na ($\mu g.plant^{-1}$)		
Control	3.3	1113	±	253	15	±	2
	7.8	800	±	87	87	±	27
WS	3.3	1193	±	206	17	±	4
	7.8	1101	±	106	100	±	26
SA (0.5 mM)	3.3	1670	±	243	18	±	2
	7.8	1035	±	195	110	±	21
WB (2%)	3.3	1481	±	131	17	±	1
	7.8	1070	±	145	113	±	22
WB (4%)	3.3	1445	±	265	18	±	2
	7.8	1060	±	116	117	±	14
WL (2%)	3.3	1704	±	346	18	±	2
	7.8	1121	±	170	139	±	19
WL (4%)	3.3	1524	±	173	23	±	5
	7.8	1020	±	179	104	±	60

Shoot K Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (0.1; 0.3; *n.s.*)
Shoot Na Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST) = (1; 2; 3)

Under saline conditions Ca, Mg, P and S content of maize shoots were significantly reduced in the absence of any seed treatment agent (Table 3.10B; Table 3.21). Water soaking application slightly increased Ca, Mg, P and S content under saline conditions, however in the absence of stress conditions, the microelement contents were not affected by this treatment. SA, WB and WL seed treatment agents increased the macroelement contents under both control and saline conditions.

Table 3.12: Shoot macroelement content of maize (*Zea mays* cv. Caramelo) plants in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Shoot Macroelement Content (mg.plant^{-1})				
Treatments	Soil EC$_e$	Ca	Mg	P	S
Control	3.3	0.19 ± 0.053	2.91 ± 0.70	0.024 ± 0.003	0.012 ± 0.003
	7.8	0.14 ± 0.019	2.76 ± 0.56	0.021 ± 0.003	0.008 ± 0.001
WS	3.3	0.21 ± 0.032	2.89 ± 0.65	0.024 ± 0.004	0.013 ± 0.003
	7.8	0.18 ± 0.024	3.07 ± 0.41	0.023 ± 0.003	0.011 ± 0.001
SA (0.5 mM)	3.3	0.28 ± 0.058	3.87 ± 0.66	0.032 ± 0.003	0.017 ± 0.003
	7.8	0.18 ± 0.037	3.13 ± 0.46	0.023 ± 0.003	0.010 ± 0.002
WB (2%)	3.3	0.27 ± 0.051	3.29 ± 0.43	0.028 ± 0.002	0.016 ± 0.001
	7.8	0.19 ± 0.041	3.21 ± 0.61	0.023 ± 0.003	0.010 ± 0.001
WB (4%)	3.3	0.28 ± 0.054	3.82 ± 0.71	0.027 ± 0.004	0.015 ± 0.004
	7.8	0.19 ± 0.024	3.03 ± 0.45	0.022 ± 0.001	0.010 ± 0.001
WL (2%)	3.3	0.29 ± 0.059	4.18 ± 0.69	0.031 ± 0.004	0.018 ± 0.003
	7.8	0.22 ± 0.032	3.68 ± 0.50	0.024 ± 0.003	0.011 ± 0.002
WL (4%)	3.3	0.27 ± 0.022	4.32 ± 0.73	0.030 ± 0.004	0.016 ± 0.002
	7.8	0.20 ± 0.026	3.45 ± 0.67	0.023 ± 0.004	0.010 ± 0.002

Shoot Ca Cont. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (0.02; 0.06; *n.s.*)
Shoot Mg Cont. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (0.013; 0.04*; n.s.*)
Shoot P Cont. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (0.0015; 0.0045; *n.s.*)
Shoot S Cont. HSD$_{0.05}$ (EC$_e$; ST; EC$_e$xST) = (0.001; 0.0031; *n.s.*)

Salinity significantly reduced the content of Fe, Zn, Mn, Cu and Mo microelements in the absence of seed treatment applications (Table 3.10C; Table 3.13). In the absence of salinity treatment Fe and Zn contents were enhanced with seed applications. Under both control and salinity stress, Mn and Cu contents were enhanced by seed applications. The highest Mn content was seen in plants treated with willow leaf extracts.

Table 3.13: Shoot microelement content of maize (*Zea mays* cv. Caramelo) plants in response to different seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and soil salinity.

Treatments	Soil EC_e	Fe			Zn			Mn			Cu			Mo		
		Shoot Microelement Contents ($\mu g.plant^{-1}$)														
Control	3.3	4.30	±	1.10	2.25	±	0.32	2.91	±	0.70	0.47	±	0.10	0.17	±	0.05
	7.8	3.80	±	0.50	2.13	±	0.25	2.76	±	0.56	0.36	±	0.04	0.05	±	0.01
WS	3.3	4.60	±	1.20	2.10	±	0.43	2.89	±	0.65	0.50	±	0.09	0.20	±	0.06
	7.8	4.30	±	0.50	2.41	±	0.33	3.07	±	0.41	0.44	±	0.05	0.08	±	0.01
SA (0.5 mM)	3.3	5.70	±	0.80	2.71	±	0.24	3.87	±	0.66	0.65	±	0.11	0.23	±	0.07
	7.8	3.90	±	0.60	2.16	±	0.30	3.13	±	0.46	0.41	±	0.07	0.06	±	0.01
WB (2%)	3.3	5.20	±	0.40	2.39	±	0.26	3.29	±	0.43	0.59	±	0.04	0.26	±	0.06
	7.8	3.70	±	0.10	2.19	±	0.36	3.21	±	0.61	0.42	±	0.04	0.07	±	0.01
WB (4%)	3.3	5.50	±	1.00	2.46	±	0.45	3.82	±	0.71	0.58	±	0.12	0.21	±	0.01
	7.8	3.60	±	0.40	2.05	±	0.16	3.03	±	0.45	0.44	±	0.07	0.07	±	0.01
WL (2%)	3.3	6.20	±	1.40	2.57	±	0.34	4.18	±	0.69	0.66	±	0.12	0.23	±	0.08
	7.8	4.10	±	0.70	2.27	±	0.27	3.68	±	0.50	0.47	±	0.09	0.07	±	0.02
WL (4%)	3.3	5.80	±	0.90	2.56	±	0.18	4.32	±	0.73	0.63	±	0.08	0.17	±	0.03
	7.8	3.80	±	0.60	2.17	±	0.33	3.45	±	0.67	0.41	±	0.06	0.07	±	0.01

Shoot Fe Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.4; *n.s.* ; *n.s.*)
Shoot Zn Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.15; *n.s.* ; *n.s*)
Shoot Mn Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.28; 0.82; *n.s*)
Shoot Cu Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.04; 0.11; *n.s*)
Shoot Mo Cont. $HSD_{0.05}$ (EC_e; ST; EC_exST)= (0.02; *n.s.* ; *n.s*)

Table 3.14: Two-way ANOVA of the effects of salinity (EC_e) and seed treatment (ST) on protein concentration of maize (*Zea mays* cv. Caramelo) plants grown in soil under growth chamber conditions.

Source of Variation	Protein Concentration
EC_e	*
ST	*
EC_e*ST	*n.s.*

Although the interaction was not significant, both salinity and seed treatments had a significant effect on protein concentration of maize leaves (Table 3.14). The lowest protein concentration was measured in the absence of salinity and seed treatment (Table 3.15). Salinity caused an increase of 24% irrespective of the seed treatment. The seed treatment application enhanced protein concentrations when compared to control plants. Under saline condition, the protein concentration was increased by 3%, 7% and 27% with SA, WB and WL seed applications, respectively.

Table 3.15: Effect of seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and salinity on protein concentration of maize (*Zea mays* cv. Caramelo) grown in soil under growth chamber conditions.

Seed Treatment Agent	Soil EC_e	Protein concentration ($mg.g^{-1}$ FW)		
Control	3.3	6.3	±	0.8
	7.8	7.8	±	0.6
Watersoaking	3.3	7.7	±	2.2
	7.8	8.8	±	1.1
SA (0.5 mM)	3.3	7.5	±	1.2
	7.8	8.0	±	2.4
Willow bark (2%)	3.3	7.8	±	1.0
	7.8	9.0	±	0.9
Willow bark (4%)	3.3	7.8	±	1.4
	7.8	7.8	±	3.0
Willow leaf (2%)	3.3	8.4	±	2.2
	7.8	9.8	±	0.7
Willow leaf (4%)	3.3	8.8	±	1.4
	7.8	10.0	±	0.9

Protein Concentration $HSD_{0.05}$ (EC_e; ST; EC_e*ST) = 0.8; 2.2; *n.s.*

Table 3.16 : Two-way ANOVA of the effects of salinity (EC_e) and seed treatment (ST) on antioxidative enzyme of maize (*Zea mays* cv. Caramelo) grown in soil under growth chamber conditions.

Source of Variation	SOD	GR	APX	CAT
EC_e	*n.s.*	*n.s.*	***	**
ST	***	***	*n.s.*	***
EC_e*ST	*n.s.*	*	*n.s.*	***

According to ANOVA results, specific SOD activity was not affected by salinity, but varied with seed treatments (Table 3.16). Although bark applications did not have a consistent effect, seed treatments of willow leaf extracts significantly reduced SOD activity (Table 3.17). The interaction of salinity and seed application significantly affected GR enzyme activity (Table 3.16). In general, all the seed applications reduced specific GR activity (Table 3.17). Among others, application of 4% leaf extract caused the lowest GR activity under both control and saline conditions. Specific APX activity was not affected by salinity, but seed treatment had a significant effect on it (Table 3.16). The treatments that caused the greatest decrease in APX activity were leaf, bark and SA seed treatments. (Table 3.17) The specific CAT activity was also significantly affected by the interaction of salinity and seed treatments (Table 3.16). Salinity caused an increase in the specific CAT activity in the absence of seed treatments. All the seed treatment agents also increased the specific CAT activity under control conditions. Under saline conditions, SA and high WB and all WL applications reduced the specific activity of CAT enzyme.

Table 3.17: Effect of seed treatment agents (WS, SA (0.5 mM), WB (2% and 4%), WL (2% and 4%)) and salinity on specific activity of antioxidative enzymes of maize (*Zea mays* cv. Caramelo) plants grown in soil under growth chamber conditions.

Specific Activity		SOD			GR			APX			CAT		
Seed Treatment Agents	Soil EC_e	(U mg^{-1} Prt.)			(-nmol [NADPH] mg^{-1} Prt. Min^{-1})			(-μmol H$_2$O$_2$ mg^{-1} Prt. Min^{-1})						
Control	3.3	28	±	6	76	±	11	2.0	±	0.3	44	±	5
	7.8	25	±	5	74	±	10	1.9	±	0.4	72	±	9
WS	3.3	21	±	1	58	±	10	1.7	±	0.2	55	±	6
	7.8	28	±	1	66	±	19	1.5	±	0.3	72	±	8
SA (0.5 mM)	3.3	30	±	6	68	±	13	1.4	±	0.5	77	±	13
	7.8	31	±	6	47	±	10	1.5	±	0.3	65	±	12
WB (2%)	3.3	21	±	9	51	±	5	1.7	±	0.3	67	±	8
	7.8	27	±	3	55	±	12	1.2	±	0.2	71	±	8
WB (4%)	3.3	29	±	5	48	±	8	1.3	±	0.2	76	±	6
	7.8	29	±	8	49	±	4	1.3	±	0.2	59	±	7
WL (2%)	3.3	23	±	5	43	±	5	1.3	±	0.3	51	±	14
	7.8	24	±	2	51	±	5	1.2	±	0.3	57	±	11
WL (4%)	3.3	21	±	3	37	±	8	1.3	±	0.1	53	±	5
	7.8	21	±	3	42	±	2	1.2	±	0.2	65	±	7

SOD HSD $_{0.05}$ (EC_e; ST; EC_exST) = *n.s.* ; 7; *n.s.*

GR HSD $_{0.05}$ (EC_e; ST; EC_exST) = *n.s.* ; 0.013; 0.02

APX HSD$_{0.05}$ (EC_e; ST; EC_exST) = 0.4; *n.s.* ; *n.s.*

CAT HSD $_{0.05}$ (EC_e; ST; EC_exST) = 4; 11; 17

3.3. Hydroponic Experiment Results

Maize plants grown for 30 days in the presence or absence of salinity treatment and subjected to SA, WB and WL applications from solution culture can be seen at Figure 3.28. According to this photo it can be observed that the shoot and root growth of the plants was affected negatively from the salinity treatment. Under saline conditions, plants

which received different doses of willow extracts looked better when compared to control plants or to the ones which received SA from solution culture.
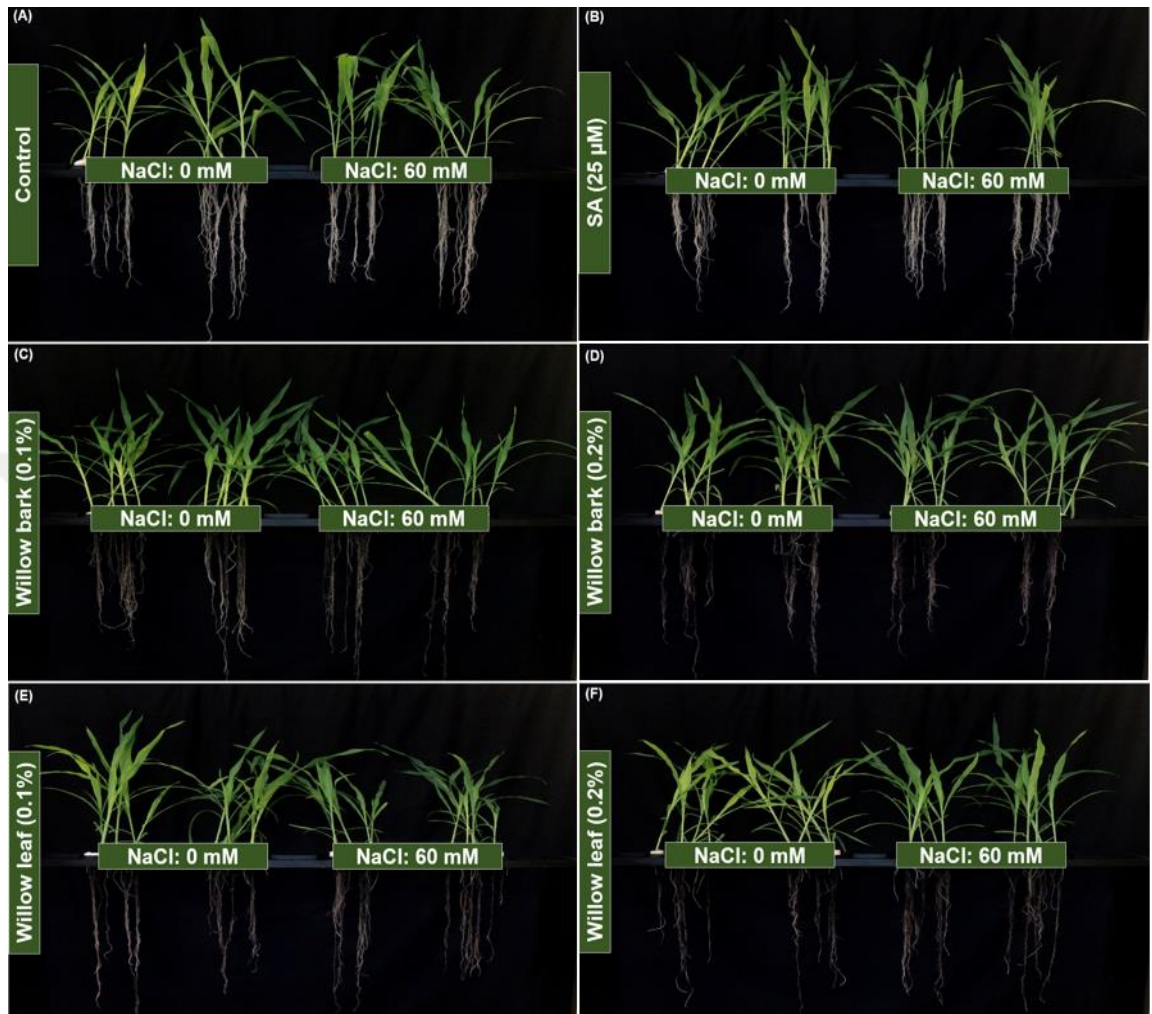
Figure 3.28: Effect of salinity and salicylic acid, willow bark and leaf extracts on maize (*Zea mays* cv. Caramelo) plants grown for 30 days under growth chamber conditions by using hydroponic culture.

Table 3.18: Two-way ANOVA of the effects of salinity (EC_e) and treatment agents (A) on (A) plant height (19 DAS, 26 DAS and 30 DAS), (B) shoot fresh and (C) shoot and (D) root dry weight (30 DAS) of hydroponicaly grown maize plants (*Zea mays* cv. Caramelo) under control and salt stress conditions.

	Source of Variation	Plant Height		
		19 DAS	26 DAS	30 DAS
(A)	EC_e	*n.s.*	***	***
	A	*n.s.*	*n.s.*	*
	EC_e*A	*n.s.*	*n.s.*	*n.s.*
	Source of Variation	**Shoot Fresh Weight**		
		30 DAS		
(B)	EC_e	***		
	A	*n.s.*		
	EC_e*A	*n.s.*		
	Source of Variation	**Shoot Dry Weight**		
		30 DAS		
(C)	EC_e	*******		
	A	*n.s.*		
	EC_e*A	*n.s.*		
	Source of Variation	**Root Dry Weight**		
		30 DAS		
(D)	EC_e	*n.s.*		
	A	*n.s.*		
	EC_e*A	*n.s.*		

19 DAS Plant Height:
$HSD_{0.05}$ (EC_e; A; EC_exA) = *n.s.* ;*n.s.* ; *n.s.*
26 DAS Plant Height:
$HSD_{0.05}$ (EC_e; A; EC_exA) = 3; *n.s.* ; *n.s.*
30 DAS Plant Height:
$HSD_{0.05}$ (EC_e; A; EC_exSA) = 2; 6; *n.s.*
30 DAS Shoot Fresh Weight:
$HSD_{0.05}$ (EC_e; A; EC_exA) = 1; *n.s.* ; *n.s.*
30 DAS Shoot Dry Weight:
$HSD_{0.05}$ (EC_e; A; EC_exA) = 70; *n.s.* ; *n.s.*
30 DAS Root Dry Weight:
$HSD_{0.05}$ (EC_e; A; EC_exA) = *n.s.* ; *n.s.* ; *n.s.*

In the absence of salinity stress, none of the applied treatment agents had any significant effect on plant height of maize plants at 19, 26 or 30 days (Table 3.18A; Figure 3.29). Salinity treatments started at day 19 and reductions in plant heights in respect to salinity stress was observed on day 26 and 30 (Figure 3.29 B). On day 30 the effect of treatment agents was observed under saline condition. The lowest shoot length was measured for control plants, whereas the highest shoot length was observed at WB treated plants with the higher dose. The effect of low concentration willow bark and high concentration willow leaf applications was similar and was right below the high willow bark application. Similarly, the lengths of the plants treated with SA and low leaf extracts were almost the same and although it was statistically not significant when compared to control plants they were still taller (Figure 3.29).
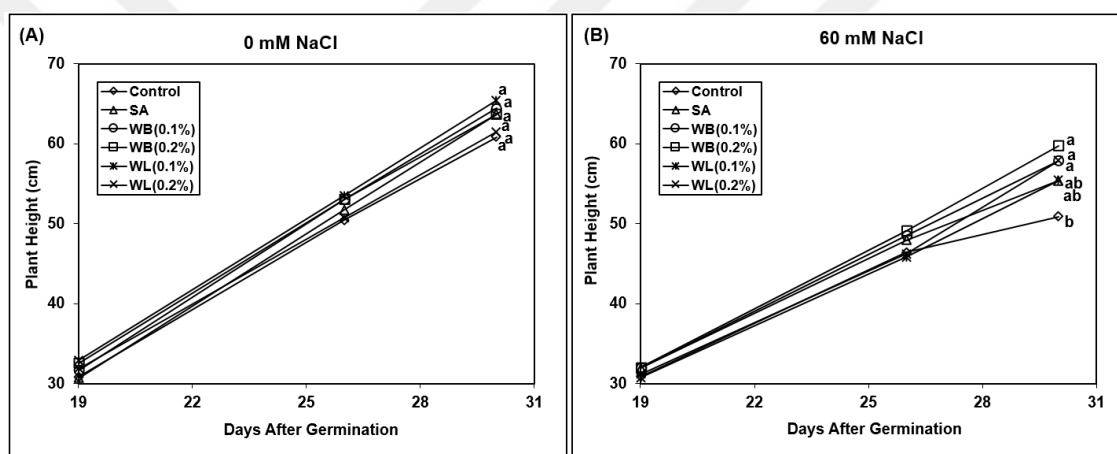
Figure 3.29: Effect of salicylic acid, willow bark and leaf extracts on plant height 30 DAS of hydroponically grown maize plants (*Zea mays* cv. Caramelo) under (A) control and (B) salt stress conditions. Values are means of 4 independent pot replicates, each containing 5 plants. The lowercase letters were given according to the LSD results under the control and salinity conditions.

Shoot fresh weight was significantly decreased by salinity stress (Table 3.14B). The treatments agents increased the fresh weight under both control and saline conditions, but this effect was not statistically significant (Table 3.14 B; Figure 3.30). Under saline conditions, the lowest shoot fresh weight was observed in the absence of any treatment agent, whereas the highest FW were obtained in plants treated with high concentrations of WB or WL extracts (Figure 3.30). Application of WB extract at higher concentration increased shoot FW by 55% and similarly, higher concentration of WL extract increased the measured trait by 50% when compared to control plants (Figure 3.30).
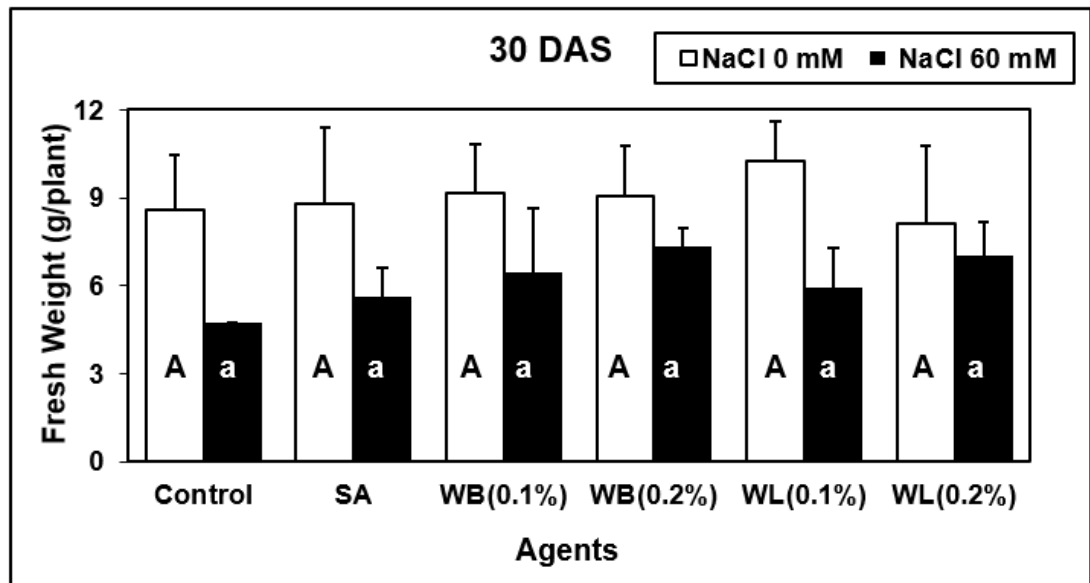
Figure 3.30: Effect of salinity stress and salicylic acid, willow bark and leaf extracts on shoot fresh weight of maize (*Zea mays* cv. Caramelo) plants grown for 30 days under growth chamber conditions by using hydroponic culture. Values are means of 4 independent pot replicates, each containing 5 plants. In cases where the interaction had no significant effect, the uppercase letters were given according to the Fisher's protected LSD test results in the control condition and the lower-case letters were given for to the salt stressed condition.

When compared to control plant, the high concentration of bark and leaf extracts significantly increased the shoot dry weight of the maize plants under saline conditions (Figure 3.31A). The highest shoot dry weight was observed in plants treated with 0.1% of leaf extract application under control conditions, whereas under stress conditions application of 0.2% of willow leaf or willow bark extract resulted with the highest shoot DW (Figure 3.31A). In contrast, the treatments did not have a significant effect on the root dry weight of plants under control or stress conditions (Table 3.18D; Figure 3.31 B). Still, plants which were treated by high concentration of willow leaf extracts had the highest root dry weight under stress conditions when compared to other treatments (Figure 3.31B).
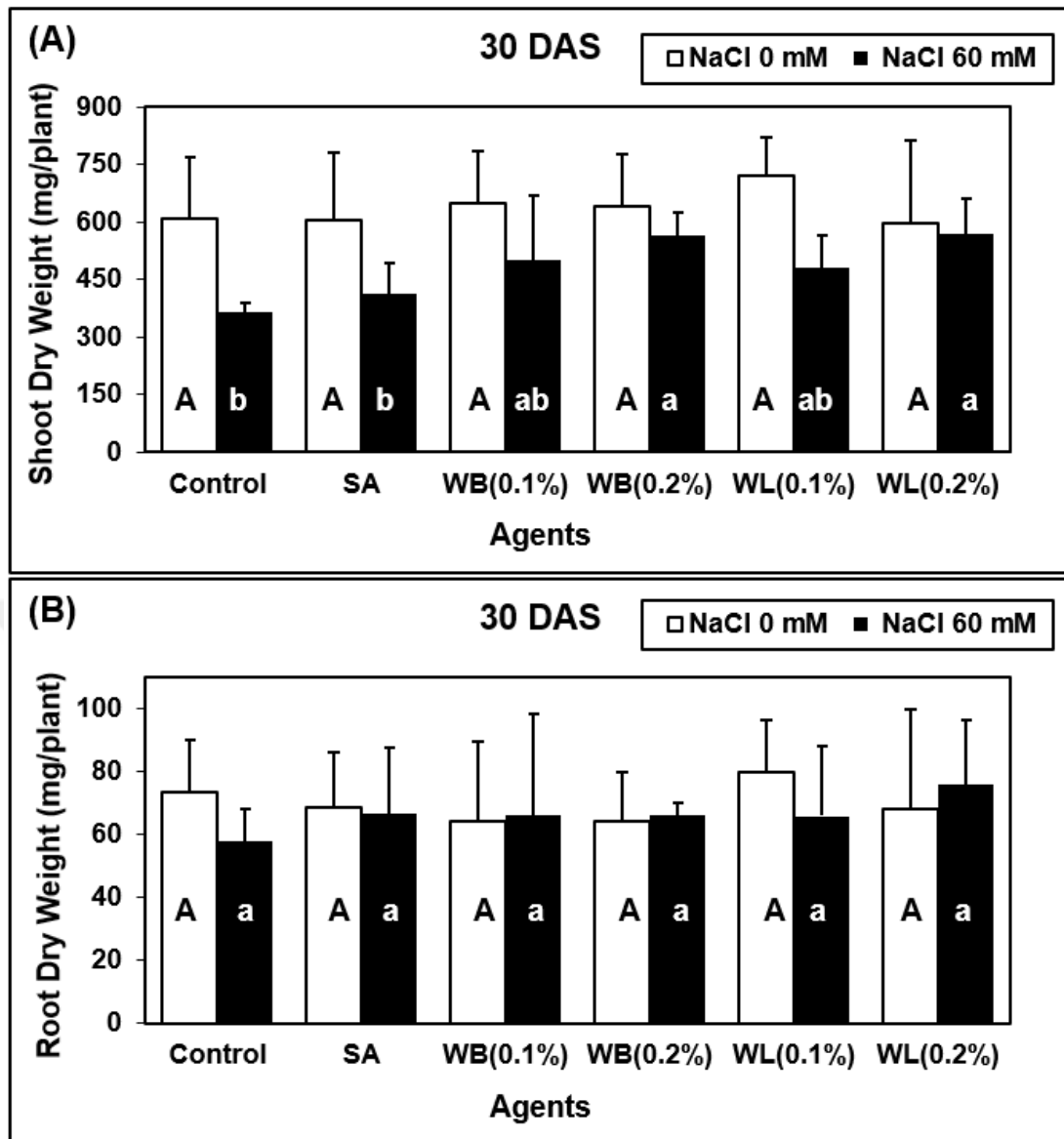
Figure 3.31: Effect of salinity and salicylic acid, willow bark and leaf extracts on shoot (A) and root (B) dry weight of maize (*Zea mays* cv. Caramelo) plants grown for 30 days under growth chamber conditions by using hydroponic culture. Values are means of 4 independent pot replicates, each containing 5 plants. In cases where the interaction had no significant effect, the uppercase letters were given according to the Fisher's protected LSD test results in the control condition and the lower-case letters were given for to the salt stressed condition.

4. DISCUSSION AND CONCLUSION

Seed germination is a vital stage for the lifecycle of plants and this stage is considered to be the most susceptible one to salinity stress. The germinating capacity of seeds is impaired by salinity stress due to inhibition of water uptake or negative effects of excess salt ions (Na^+ and Cl^-) on seeds [Khajeh-Hosseini et al., 2003]. If seeds can germinate well under saline conditions and plantlets can tolerate salinity during seedling establishment, they have a better chance to tolerate salinity in later stages of their development [Jisha et al., 2013]. For this reason, the strategies which can help the seeds to overcome stress conditions are of great importance. One of the most important of these is seed treatments with various agents. Through these applications, plant metabolism is stimulated and prepared for stress conditions, thus improving the ability of plants to tolerate stress [Beckers and Conrath, 2007].

According to the results obtained from the first experiment in perlite media, it was observed that although the highest salinity level (100 and 150 mM NaCl) significantly decreased the shoot and root growth of 3, 5 and 7 days-old maize seedlings in the early period (Figure 3.2-3.4), no adverse effects of salinity was observed on seed germination (Figure 3.1, Table 3.1A) The adverse effects of salinity were more pronounced on the 3-day maize plants, while the negative effects on the 7-day shoots were reduced (Figure 3.2). Therefore, the medium salinity of 100 mM NaCl was selected for the second and third experiments. Moreover, the efforts of using willow extracts as novel plant-based biostimulants were focused on reducing losses on the plant growth related instead of increasing the germination percentage.

In conformity with the results reported in this study, in the literature it has been shown that low levels of salinity had no negative effect on maize growth whereas at high rates, shoot and root growth are inhibited [Turan et al., 2010]. In another study, Khodary (2004) reported that as the salinity level increased, the negative effects of stress on maize plant growth parameters, including shoot and root fresh weight, shoot length, became more severe. When the shoot and root growth is compared it was observed that shoots of maize seedlings were more sensitive to increasing NaCl levels than roots (Figure 3.2; 3.6; 3.7). Ertani et al. (2013) also documented similar results in maize plants.

Salicylic acid is an important phytohormone for plant growth and development and regulates various metabolic process when plants are exposed to salt stress. Treatment with SA can reduce mitigate the adverse effects of stress and enhance plant growth [Hayat et al., 2010]. The effects of salicylic acid on plant metabolism are known to vary depending on applied the dose. It was observed that the most effective salicylic acid dose was 0.5 mM in many applications carried out in different plants and stresses [Tufail et al., 2013], [Gautam and Singh, 2009], [Gunes et al., 2007], [Zanganeh et al., 2018], [Baninasab, 2010]. At this concentration, it was recorded that SA applications decreased negative effects of stress, increased growth and development, and improved stress tolerance. According to the results of second perlite experiments, at 3 DAS, 0.25 mM SA did not show any beneficial effect of shoot FW of maize seedlings, whereas 0.5 mM significantly increased the shoot growth of maize plants under salt stress conditions (Figure 3.6A, 3.9B). It was also observed that 1.0 mM SA did not provide any extra benefit under these conditions. For this study the most appropriate dose was also selected as 0.5 mM from different concentrations of SA tested and only that dose was used for the rest of perlite and soil experiments. The selected concentration was consistent with salicylic acid concentrations in the literature [Khodary, 2004], [Zanganeh et al., 2018].

In plant-based biostimulants applications, improvements in different traits including plant growth and development, nodule development, defense response, root growth and callus growth have been reported for many different plant species [Yakhin et al., 2017]. Usage of plant extracts containing many bioactive substances as seed treatment agents have positive effects on plant growth parameters and improve seedlings establishment under control and stress conditions [Rafi et al., 2015]. In the third perlite experiment, seed treatments of SA and willow extracts were showed to have a positive effect on maize seedlings grown in perlite media (Figures 3.10-3.17). Under both control and salinity conditions, maize seedlings grown from seeds treated with willow extracts showed a significant improve seed performance and vigor (Figures 3.10-3.11), shoot and root biomass (Figures 3.13-3.14), total root area (Figure 3.16) as well as shoot length (Figure 3.17), however the germination percentage or rate was not affected by seed applications (Figure 3.12). Since the germination percentage was not significantly reduced by salinity treatment in this experimental condition, it is not surprising to see a positive response for that treatment. When the positive effects of seed treatment agents were compared, for many measured traits including shoot FW, root FW, total root area and shoot height, in

general willow extracts were reported to be more effective when compared to SA application (Figures 3.10-3.17). For 3-days-old seedlings shoot FW was enhanced by 225 % with willow extracts and 130 % with SA treatment (Figure 3.13A). Also, in total root area calculations, the positive effect of SA was 43% however willow extracts provided 87% increase (Figure 3.16A). Willow extracts may contain higher concentrations of SA derivatives or the other compounds which are present in these crude extracts of willow can provide extra benefit for plants.

When the effects of seed treatments on shoot FW was compared at 2 different harvest times (3 and 6 DAS), it was observed that the differences were more pronounced at earlier stages (Figure 3.13). For example, when compared to water application, WB seed treatment caused an increase of 50% in the shoot FW of 3-days-old seedlings, however this increase was limited to 15% for 6-days-old seedlings. When the shoot lengths were compared, the length of 3-day-old seedlings treated with willow extracts was 2.5 times the length of the control plants (Figure 3.17). However, the height of 6-days-old maize seedlings were about %80 higher when they were treated with willow extracts. The effects of seed applications may be reduced in time but still the positive effects observed in the very early stages of a seedlings life can be very critical and may determine its survivability in nature.

Similar to the results obtained, Afzal et al. (2012) reported that seed treatments using Moringa olifera tree extract, another important botanical biostimulant, increased the growth of maize plant in chilling stress and had a positive effect on root and shoot weight and length of plants. In another study using the same tree extract, it was reported that seed applications with this extract had a positive effect on wheat seedling growth parameters [Yasmeen et al., 2013].

Like the reported biostimulants, which are safe, natural and renewable sources which stimulate the metabolism in plants by affecting each other with endogenous phytohormone [Narwal, 2004], willow extracts may have a potential to be utilized for the same purposes.

The effect of seed treatment may vary depending on various factors such as priming time, priming agent, temperature, plant species [Parera and Cantliffe, 1994]. While seed treatment can simply be conducted by using water, the addition of nutrients, plant growth regulators or biostimulants may be used to enhance its benefits on seed germination and

seedling vigor. In studies conducted with various plants, it has been shown that hydropriming as seed treatment has a great potential for positively affecting events in seed metabolism under stress conditions, increasing germination time and rate, producing more healthy and viable seedlings [Singh, 1995], [Harris et al., 1999]. In contrast, in the first soil experiment, there was no statistically significant effect of different hydropriming time on germination percentage, fresh weight, dry weight and plant height of soil grown maize under control and salt stress conditions (Table 3.4). The lack of a significant effect of hydropriming on maize grown under saline conditions may be due to some unexpected damage which might took place during drying. It has been mentioned in many studies that there may be a decrease in the performance of the seeds subjected to drying or storage for a certain period after hydropriming application [Adetumbi et al., 2009].

Since no critical differences were observed among the tried priming times (Table 3.4; Figures 3.20-22), 16 hours priming time was selected in accordance with the literature to be used in the second soil experiment. Chivasa et al. Reported (2000) that between 12 and 24 hours of hydropriming applications applied to maize seed had positive effects on plant growth. In another study, Nagar et al. (1998) recorded a positive improvement in plants after hydropriming for 16 hours. Other studies showed that hydropriming obtained the best results from maize seeds for 18 hours. This was close to the priming time chosen as the appropriate time in the first experiment. Also, instead of priming, soaking was preferred for the second soil experiment to minimize the unexpected negative effects which could be observed during drying step of seed priming.

Another important parameter determined in the first experiment was the soil EC_e value of the second experiment. The EC_e value of 7.8 was selected for use in the second experiment, since this salinity level cause a reduction of approximately 30% in shoot dry weight and height and 40% in fresh weight of the 14-day-old maize plant (Figures 3.20-3.22). It looked as a relatively high salinity level to effect the growth parameters significantly and a relatively low salinity level not to kill the treated plants and cause irreversible damage.

In the second soil experiment, the negative effects of salinity was observed in growth related parameters including shoot fresh and dry weights and shoot heights (Fig 3.23-3.27) which is in conformity with the literature [Cicek and Cakırlar, 2002], [Menezes-Benavente et al., 2004], [El-Sayed 2011], [Wakeel et al., 2011b].

As in the case of third perlite experiment, the positive effect of seed treatment with willow extracts as botanical biostimulant was observed in maize plant grown under both control and saline conditions (Figures 3.13-3.17; Figure 3.23). The rich bioactive substance content of the willow extracts can be transferred to the embryo growing in the lag stage of priming through seed treatment and these active compounds including SA can increase the seedling stress tolerance and early vigor.

According to the reported results, it has been found that the low dose (2%) of willow leaf extract may be the best treatment in many growth parameters such as fresh weight, dry weight, plant length (Figures 3.25-3.27). However, for medicinal purposes bark is the preferred part for applications [Chrubasik et al., 2000]; [Biegert et al., 2004]. Since it is much easier to collect willow leaves when compared to bark samples, usage of leaf samples as plant-based biostimulants can be an easier strategy for application and production purposes.

Moreover, in some cases it was observed that the high concentration (4%) of leaf and bark extract applications increased the growth compared to control plant but when compared to lower willow extract dose (2%) they did not provide any extra benefit or even reduced the observed positive effects (Figures 3.25-3.27). This can be explained by the fact that the positive or negative effect of the same extract may vary depending on the concentration [Ullah et al. 2014]. High concentrations of plant extracts may be unnecessary or even toxic for plant and have been reported to have a negative effect on plant metabolism and the secondary metabolite amount [Khan et al., 2011].

When the element concentrations were measured, it was observed that salinity stress decreased K concentration and increased Na concentration in maize shoots (Table 3.9). Due to ionic stress, which is an important component of salinity stress, high Na concentration can inhibit K uptake due to chemical similarities [Chinnusamy and Zhu, 2006]. However, it was observed that willow extracts had no positive effect on K uptake or did not decrease Na uptake. This means that the protective role of willow extracts or SA can not be explained by a direct effect on ionic toxicity.

In contrast to the K concentrations, it was documented that the seed treatment with willow leaf and bark extracts caused an increase in the K content of the maize shoots (Tables 3.11-3.13). Especially, 2% leaf extract application increased the K content by

almost 50%. This increase in content may be attributed to the fact that seed treatment with leaf extract increased shoot biomass and/or translocation of K from the roots to shoots.

Salinity and seed treatments also increased or reduced the concentrations and contents of other macro- or micro-elements (Table 3.8; Table 3.9; Table 3.12; Table 3.13). But none of the observed effects could explain the beneficial effects of willow extracts. So, it can be concluded that the positive effects of willow extracts could not be explained by the correction of a nutrient deficiency or toxicity observed in maize samples.

Seed treatment with SA and willow extracts increased protein concentration of maize plant grown in saline soil (Table 3.13). This may be attributed to the fact that nitrogen uptake may be increased by seed treatment of these compounds. According to the literature, it was observed that pre-treatment with SA enhanced nitrate reductase activity, nitrogen uptake as well as nitrogen use efficiency [Singhand Chaturverdi, 2012], [Hayat et al., 2005], [Jain and Srivastava, 1981], [Fariduddin et al., 2003]. This interesting finding should be confirmed with further experiments.

Abiotic stress including salinity stress also leads to increasing level of ROS in peroxisomes, chloroplast and mitochondria. It is important for the plant to control the ROS level with enzymatic (SOD, CAT, GR, APX) or non-enzymatic antioxidants (ascorbate, flavonoids carotenoids, phenolic compounds) in order to withstand oxidative stress [Schutzendubel and Polle, 2002]. SOD enzyme plays a role in the first step of the defense mechanism and converts superoxide to O_2 and hydrogen peroxide (H_2O_2). AP and GR involved in ascorbate–glutathione pathway play a critical role in scavenging of ROS in chloroplast. H_2O_2 is also scavenged by catalase in peroxisomes [Asada, 1999].

In this study, specific SOD activity was increased with seed treatment of SA and bark extract, while specific GR and APX activities decreased with SA as well as willow extracts (Table 3.17). The SA treatment significantly increased specific CAT activity and treatment willow extracts reduced the specific CAT activity under saline conditions (Table 3.17). In some studies, it has been shown that pretreatment with SA increased antioxidant enzyme activity [Yusuf et al., 2008] and decreased it in some studies [Choudhury and Panda, 2004]. The decrease in antioxidant enzyme activity may be thought to be due to the rich bioactive substance content of willow extracts. Non-enzymatic antioxidants such as phenolic agents may be thought to decrease reactive oxygen levels especially, in chloroplast and thus enzyme activity reduces.

Salinity stress does not adversely affect plant growth only in soil condition or germination stage. It also has negative effects on vegetative growth in solution culture (Figures 3.28-3.31). A study by Bose et al. (2018) showed that the salinity level of 8 dsm^{-1} (equivalent to 80 mM) reduced shoot and root growth and development of the maize plant in solution culture. Another study examined the effect of 60 mM NaCl on pepper and cucumber plants grown in hydroponics. This salinity level has been shown to cause negative results in plant metabolism [Kaya et al., 2001].

In the last part of this thesis, the effect of SA and willow extracts on maize plants grown in hydroponics were investigated (Figure 3.28). In contrast to previously reported results, these agents were not applied as seed treatment agents, instead they were added to nutrient solutions at low concentrations as liquid biostimulants. Many studies have reported that salicylic acid is an effective phytohormone in increasing root length and growth [Hayat et al., 2010]. It is also reported that aspirin, a close analogue of salicylic acid, improves rooting in bean plants [Larque-Saavedra et al., 1975]. In agreement with the literature, SA and willow extract applications increased the root dry weight under saline conditions (Figure 3.31B). Moreover, these positive effects were not only limited to root growth, in the shoot growth even a higher positive response was observed in response to willow extract applications (Figure 3.29; Figure 3.30; Figure 3.31A) The salinity treatments as well as biostimulant applications were started when the plants were 19 days old to have uniform plantlets. If these applications would have been applied since the beginning of experiment, the differences could be more dramatic. If the positive effects of SA and willow extracts are compared, willow extracts performed better under these conditions.

It can be concluded that willow bark and leaf extracts as a seed treatment could improve the seedlings performance and reduce the negative effects of salinity in the early periods of plant growth. Nevertheless, seed treatment with willow tree extracts at optimized doses can be a promising, sustainable and innovative approach. Usage of willow leaves instead of willow barks can be an easier strategy for the applicability and production of a potential product. It is thought that the biostimulant effect of willow extracts may be related to the rich bioactive substance content. It was also shown that willow extracts can be used as natural SA sources instead of chemical SA to increase the growth and development of plants. Results suggest that aqueous extracts of willow tissues may be used as biostimulants to improve crop performance although effects may not be

salinity-specific. Further studies are needed to determine the compositions of extracts, their effects on other crops under different stress conditions and the best method of application.

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BIOGRAPHY

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