University of São Paulo
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Agrochemicals on growth and hormonal relations of 'Micro-Tom' and *Arabidopsis* roots under water deficit conditions

Valdinei Moreira dos Santos

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

Piracicaba
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To my Family, especially my father and my mother who ever believed me and wished me a better life, I dedicate...

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CONTENTS

RESUMO

Efeito de agroquímicos no crescimento e relações hormonais em raízes do tomateiro ‘Micro-Tom’ e *Arabidopsis* sob condições de déficit hídrico

As crescentes preocupações oriundas a partir dos atuais debates sobre mudanças climáticas somadas ao descontrolado crescimento da população mundial têm dado espaço para o uso de insumos agrícolas naturais, objetivando sustentabilidade na agricultura. Diante de tais preocupações, é importante pensar antecipadamente e encontrar maneiras de aumentar a produção e a qualidade de alimentos, em um ambiente de supostas mudanças climáticas. Substâncias naturais, como bioestimulantes à base de alga, estão ganhando relevância como melhoradores da produtividade e tolerância a estresses abióticos com crescentes usos na agricultura. Diante desse cenário, é importante compreender os efeitos e modos de ação dessas substâncias na fisiologia das plantas para permitir o desenvolvimento de produtos consistentes e garantir aos produtores soluções que atendam às suas necessidades. Portanto, este estudo foi realizado com o objetivo de entender o papel de agroquímicos (reguladores vegetais clássicos e biostimulantes à base da alga) sobre o desenvolvimento radicular e relações hormonais do tomateiro ‘Micro-Tom’ e *Arabidopsis* sob condições de estresse hídrico. Foi testada a habilidade de extratos *Ascophyllum nodosum* (ANE) em estimular respostas hormonais em condições de seca e estresse osmótico. Foram realizados experimentos com duas plantas-modelo: *Arabidopsis* que é largamente utilizada em estudos científicos de plantas e o tomateiro ‘Micro-Tom’, uma planta-modelo mais adequada para estudos aplicados à agricultura sob condições tropicais. Foram utilizados mutantes hormonais e linhas-repórter de tomate cultivadas em condições de seca para o estudo dos efeitos dos reguladores vegetias e extratos de alga no crescimento radicular e respostas hormonais, respectivamente. Além disso, foram testados *in vitro* os efeitos dessas substâncias no crescimento radicular de plântulas de *Arabidopsis* em condições de estresse osmótico. O desenvolvimento radicular do tomateiro e *Arabidopsis* foi negativamente afetado pela ocorrência do déficit hídrico causado por seca e estresse osmótico, respectivamente. Inibidores de giberelinas afetaram apenas o crescimento radicular do mutante com baixo nível endógeno giberelinas (*gib-3*). Extratos da mesma espécie de alga apresentaram diferentes efeitos no crescimento radicular de plântulas de *Arabidopsis* e resposta hormonal em raízes de tomateiro. ANE A e B aprensenta efeitos opostos no desenvolvimento de raízes. ANE A promove o crescimento radicular enquanto ANE A inibe. ANEs pouco influenciam as respostas auxínicas, contudo, essas substâncias alteram o balanço entre entre ácido abscísico e etileno sob condições de estresse hídrico por seca. A padronização da composição de extratos de alga somada aos estudos sobre os seus efeitos fisiológicos e moleculares em culturas é crucial para o estabelecimento desses insumos agrícolas como uma das soluções para as necessidades atuais e futuras da produção de alimentos.

Palavras-chave: Estresse hídrico; Hormônios vegetais; Reguladores vegetais; Bioestimulantes

ABSTRACT

Agrochemicals on growth and hormonal relations of 'Micro-Tom' and *Arabidopsis* roots under water deficit conditions

The increasing concerns launched by debates about climate changes added to the uncontrolled growth of world population have opened a market to natural inputs for a more sustainable agriculture. Taking those concerns together, it is very important to think in advance in terms of finding solutions to increase the food production, with improved quality and in a supposed changing environment. Natural substances such as seaweed biostimulants are coming up as inputs for crops yield and abiotic stress tolerance enhancement with increasing use in agriculture. In this scene, it is important to understand effects and mode of action of these substances on plant physiology to permit the development of consistent products and guarantee the delivery of solutions to growers that assist them in solving their needs. Therefore, this research was carried out aiming to understand the role of agrochemicals (classical plant growth regulators and seaweed biostimulants) on root development and hormonal relations of tomato 'Micro-Tom' and *Arabidopsis* under water deficit conditions. *Ascophyllum nodosum* extracts (ANE) ability to stimulate endogenous hormonal responses in conditions of drought and osmotic stress was tested. We carried out experiments with two plant models: one is classically used in plant science studies and the other, a crop-like plant model whose scientific findings can be applied for several crops. We used tomato hormonal mutants and reporter lines grown in conditions of drought stress to study the effects of plant growth regulators and seaweed extracts on root growth and hormonal responses, respectively. Additionally, we tested *in vitro* the effects of these substances on root growth of *Arabidopsis* seedlings under osmotic stress conditions. The root development of tomato and *Arabidopsis* was negatively affected by the presence of water deficit caused by drought and osmotic stress, respectively. Gibberellins biosynthesis inhibitors only affected the root growth of gibberellin-deficient mutant (*gib-3*). Extracts of the same seaweed presented different effects on root growth of *Arabidopsis* seedlings and hormonal responsiveness in roots of tomato, possibly due to variability in their compositions. ANE A and B show opposite effects on root growth. ANE A promotes root growth, whereas ANE B shows inhibitory effects. These substances seem to have little influence on auxin responses in roots, however, they alter the balance between abscisic acid and ethylene under drought conditions. The standardization of the composition of seaweed extracts complemented with studies on their physiological and molecular effects in crops is crucial for the establishment of these agricultural inputs as one of the solutions for current and future requirements of food production.

Keywords: Drought; Plant hormones; Plant growth regulators; Biostimulants

ABBREVIATIONS AND SYMBOLS LIST

ANE	*Ascophyllum nodosum* extract
MT	‘Micro-Tom’
sit	*sitiens*
cv.	cultivar
gib-3	*gibberellin-deficient-3*
dgt	*diageotropica*
Col-0	Columbia-0
PGR	plant growth regulator
ABA	abscisic acid
GA	gibberellins
IAA	indole-3-acetic acid
BA	6-benzylaldenine
RV	root volume
RL	root length
SDM	shoot dry mass
RDM	root dry mass
WR	watering regime
PEG	poly(ethylene glycol)
DF	degrees of freedom
ANOVA	analysis of variance
CV	coefficient of variation
^{ns}	non-significant
P	probability
SD	standard deviation
n	number
g	grams
mg	milligram
mM	millimolar
µM	micromolar
pmol	picomol
cm	centimeter
mm	millimeter

µL	microliter
GUS	β-glucuronidase
MUG	4-methylumbelliferyl glucuronide β-D-glucuronide
MU	4-methylumbelliferyl
ACC	aminocyclopropane-1-carboxylic acid
NH_4NO_3	ammonium nitrate
KNO_3	potassium nitrate
LEA	late embryogenesis abundant

1 INTRODUCTION

The world population is increasing very quickly in the last decades and it seems to do not have any control. Up against this challenge, a question is scaring scientists, agronomists, technicians, and growers worldwide, which is: how to feed around 9 billion people by the year 2050? The situation sounds even worse when we take into account the potential climate changes believed to be stronger in upcoming decades. Agricultural lands, where a regular regime of rainfall was common years before, are facing drying seasons that provoke crops yield loses due to the drought stress caused to plants. The understanding of how plants cope with drought stress is the most important step to find solutions for preventing yield loses or even increasing the crop productivity under drought conditions, more and more frequent in the production fields.

The physiological responses of plants to stresses vary in function of severity and duration of certain stress. The most sensitive mechanisms are altered in the presence of moderate stresses and are intensified while other mechanisms are affected in tandem according to the plant sensibility to the specific stress (BURKE, 2007). Plants have signaling substances named hormones that are the front line of response to any threat to their healthy condition. Therefore, the knowledge of these substances and their relations under adverse environments is the first step to face such conditions and explore the plant productive potential aiming to reach food production with minimal yield loses.

Hormonal mutants are an important approach to be considered in studies of hormones relations in plants. This approach has been underused in such studies. The species *Solanum lycopersicum* (tomato), as *Arabidopsis thaliana*, presents a great diversity of hormonal mutants and traits of a genetic model, making it suitable for this kind of study. *S. lycopersicum* cv. Micro-Tom has being used as a plant model due to its small genome and considerable easiness for genetic manipulation (PERES et al., 2001). The use of this cultivar in plant science presents several advantages, mainly for the research applied to agriculture. It is a plant model closely related to major crops, permitting assessment of the yield, what it is not possible with *Arabidopsis*. ‘Micro-Tom has a life cycle of approximately 70-90 days, produces fruit and seeds in very small pots (50-100 mL), being firstly proposed as a scientific plant model by Meissner et al. (1997).

The use of transgenic plants that express genes of interest is other valuable approach. The GUS gene system is based on β-glucuronidase activity, an enzyme produced by *Escherichia coli* bacteria. This system was developed by Jefferson et al. (1986) at the University of Colorado. A significant amount of *Arabidopsis* reporter lines, and more recently for 'Micro-Tom', carrying this system is available to be used in plant science studies worldwide. A classical approach for plant hormonal studies is the application of exogenous plant regulators. In the last years, biostimulants have emerged and gained importance in such studies due to their reported ability to modify the endogenous hormonal levels, leading to yield increases and improvement of plant tolerance to stresses.

The reduction of abiotic stresses as drought, salinity, cold, and heat are the main arguments for biostimulants use in agriculture. The classification of this category of agricultural inputs is not clear so far. Nevertheless, plant biostimulants have been defined as products that contain substances and/or microorganisms that when applied to plants or their rhizosphere stimulates natural processes that enhance the nutrient uptake and efficiency, tolerance to abiotic stresses, and crop quality (DU JARDIN, 2015). According to this author, biostimulants definition is important for future regulations of the category, since they have no direct effect against pests, and therefore, cannot be treated or ruled by regulatory framework of pesticides.

The increasing use of biostimulants in agriculture has led to interests in regulation of these products. Some countries are looking for a new biostimulants registration category aiming to identify their unique mode of action and consolidate their contribution to crop quality and productivity enhancement.

The knowledge on how biostimulant products work and their impact on plant physiology of crops is still limited. The challenge lays on the complex variety of stimulatory compounds such as carbohydrates, secondary metabolites, proteins, fat acids, and minerals. Another issue is the variation in the concentration of these components that are considerably high between products of the biostimulant class and even between products manufactured from the same natural source. This compositional variation results in large variation in the effects observed in treated plants. Research on the impact of the composition variation of seaweed-based biostimulants on the plant physiological processes is the key point for better understanding of their mode of action and making possible the development of

consistent products that help growers to grow crops in changing environment without affecting the quality of their produce.

This research was carried out with aim of understanding the role of agrochemicals (plant growth regulators and seaweed biostimulants) on root development and hormonal relations of tomato ‘Micro-Tom’ and *Arabidopsis* under water deficit conditions. The seaweed extracts ability to stimulate endogenous hormonal responses in conditions of abiotic stress was tested.

2 REVIEW

2.1 Abiotic stress by drought and hormonal relations in roots

The drought stress is caused by the water deficit inside the plant due to the scarcity of water in the soil in a way that this low water availability does not replace to the plant the water lost by evapotranspiration (WANJURA; UPCHURCH, 2000). The stress caused by drought occurrence leads to the reduction or delay of the plant growth, damaging the distribution of carbohydrates from sources to sinks organs in development (BURKE, 2007). Thus, it is necessary to find strategies to attenuate the negative drought effects to the plant growth and development by the improvement of water deficit stress tolerance (APSE et al., 1999).

To cope with the stress, plants respond with physiological and biochemical changes aiming to improve the water retention in order to maintain the photosynthetic activity. These changes include reduction of the stomatal opening to prevent water losses to the atmosphere and osmotic adjustment in the roots to absorb more water from the soil. The accumulation of compounds such as sugars and proline are mechanisms that balance the water potential of cells during and after the stress (PILON-SMITS et al., 1995). In addition to these osmotic compounds, specific proteins and mRNA are induced by the drought stress presence (REVIRON et al., 1992).

The root development of plants exposed to water deficit is usually less damaged than the shoot growth, and in many cases it can be promoted (SHARP; DAVIES, 1979). This root response to the abiotic stress is regulated by the action and balance among phytohormones, mainly, abscisic acid (ABA), auxin, and ethylene (RIBAUT; PILET, 1994). Phytohormones are involved in multiple processes and are responsible for providing plants with the ability of adapting to changing environments and coping with stresses generating by adverse conditions. The ability of resilience conferred to plants by hormones is through regulation of growth, development, nutrients allocation, source/sink relations, etc. (PELEG; BLUMWALD, 2011).

Ethylene is a gaseous phytohormone involved in fruit ripening, senescence, and leaf abscission (KENDRICK; CHENG, 2008) and is considered the main inhibitor of root growth (STENLID, 1982). This hormone is an important root growth regulator and it can affect different aspects in the root development. Under normal conditions

to the plant growth, the ethylene level is very low in roots, but when under stress conditions, this level can increase considerably leading to huge changes in the root growth (FELDMAN, 1984). Ethylene biosynthesis is increased in response to abiotic stress, including osmotic and drought stress (SPOLLEN et al. 2000), and most of the responses to theses stresses, such as root growth and solute accumulation is mediated by ethylene (CHENG et al., 2013; CUI et al., 2015).

Cytokinins are a class of phytohormones involved in various physiological processes on plant development such as: cellular division, morphogenesis, lateral root growth, leaf expansion, stomatal opening, and chlorophyll biosynthesis (DAVIES, 2010). Cytokinins also are inhibitors of root growth by stimulating the ethylene biosynthesis through positive post-transcriptional action in the biosynthesis of the key enzyme of ethylene biosynthetic pathway, 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) (RASHOTTE et al., 2005).

Gibberellins are a class of phytohormone involved in several features of plant growth and development i.e. seed germination, organ differentiation, stem elongation, leaf expansion, floral development and fruit set (YAMAGUSHI, 2008). Gibberellins are required for the normal root growth. Defective pea mutants in the gibberellin biosynthesis showed lower root growth than the wild type, however, when the hormone was applied, the growth of roots was improved (YAXLEY et al., 2001). The root growth was inhibited with application of uniconazole-P, an inhibitor of gibberellin biosynthesis. That inhibition was diminished with application of an active gibberellin GA_3, indicating the positive action of gibberellins on the root growth (INADA et al., 2000).

Auxins, the first phytohormones identified, regulate plant cell division and expansion and by this way control several aspects involved in plant growth and development, such as root elongation, embryo patterning, and vascularization (MOCKAITIS; ESTELLE, 2008; PERROT-RECHENMANN, 2010; REN; GREY, 2015). Auxins promote the rooting of cuttings and regulates the initiation and development of lateral roots. However, they inhibit the growth of primary roots, mainly if applied at high concentrations. Concomitant to the roots growth, the auxin concentration increases up to levels which inhibit that growth (PILET et al., 1979; REN; GREY, 2015). Auxins have complimentary function in the ethylene biosynthesis, acting in the induction of the ACC synthase enzyme transcription. Therefore, they act in the inhibition of root growth by the ethylene production

(RASHOTTE et al., 2005). Auxins in low concentrations promote the root growth by destabilizing the DELLA proteins (inhibitors of gibberellin action) and thus favor the root growth by the gibberellin action (FU; HARBERD, 2003). According to Zhang et al. (2009), the role of auxins in conferring tolerance to drought is due to enhanced expression of *LEA* (late embryogenesis abundant) genes in function of encoding indole-3-acetic acid (IAA)-amido synthetase (TLD1/OsGH3.13) gene expression.

ABA is also an inhibitor of root growth by promotion of ethylene production (LUO et al., 2014). Several studies have confirmed the inhibitory effect of ABA on the root growth in crops such as maize, but under normal water conditions for the plant development. Under conditions of moderate water deficit, ABA accumulation is necessary to maintain the roots growth. According to Sharp (2002), this ABA accumulation has the function of inhibiting the production in excess of ethylene due to the stress.

A possible interaction between ABA and auxins on the control of root growth has been previously suggested (PILET; SAUGY, 1987). Interactions at the level of signaling transduction between these phytohormones have been demonstrated by some authors (SUZUKI et al., 2001; BRADY et al., 2003). Besides the strong ABA-auxins and ABA-ethylene relations, ethylene also has intense interaction with auxins (MUDAY et al., 2012). Auxins stimulate ethylene biosynthesis and vice versa (TSUCHISAKA; THEOLOGIS, 2004; SWARUP et al. 2007). The model of interaction between ethylene and auxins on root elongation is based on auxins production in meristems induced by ethylene and ethylene-mediated auxins transport from the biosynthesis sites (meristems) to the elongation zones where auxins concomitantly inhibits cell elongation and induce ethylene responsiveness (STEPANOVA et al. 2007; SWARUP et al. 2007; STEPANOVA; ALONSO, 2009). According to Thole et al. (2014), auxins and ethylene may take action in a linear manner to modulate the ABA effect on primary root growth. However, according to Liu et al. (2014), the interplay of ABA and osmotic stress with other hormones still remains poorly understood.

2.2 Plant growth regulators (PGR) and drought stress attenuation

The use of PGRs such as gibberellins inhibitors can lead to root growth enhancement and increase of the root to shoot ratio (STEEN; WÜNSCHE, 1990).

The application of a plant growth inhibitor, chlormequat chloride, has resulted in increased root growth of wheat cultivated under drought stress conditions, improving the efficacy of plants to absorb water from deeper layers of the soil and consequently leading to increased yield under such conditions (DE et al., 1982). Chlormequat chloride, also known as (2-chloroethyl)-trimethylammonium chloride and chlorocholine chloride, is an inhibitor of the gibberellins biosynthesis by inhibiting the activities of two enzymes involved in the early steps of this pathway (RADEMACHER, 2000). According to this author, the enzymes affected by chlormequat chloride are copalyl-diphosphate synthase and *ent*-kaurene synthase, mainly the former than the latter. Copalyl-diphosphate synthase catalyzes the conversion of geranylgeranyl diphosphate into copalyl diphosphate and *ent*-kaurene synthase acts in the conversion of the copalyl diphosphate produced into *ent*-kaurene (RADEMACHER, 2000).

Chlormequat chloride has been largely used in agriculture aiming to reduce the height of crops, mainly cereals, and increase the tolerance to plant lodging (RAJALA; PELTONEN-SAINIO, 2001). According to these authors, the application of chlormequat chloride at the beginning of stems elongation led to decreased biomass of wheat and barley tillers. Rajala and Peltonen-Sainio (2001) found roots with higher volume and length in chlormequat chloride-treated wheat plants, mostly at upper layers of the soil.

Daminozide is a plant growth retardant through inhibition of gibberellins biosynthesis, but different of chlormequat chloride, it acts in the final steps of the pathway (RADEMACHER, 2000). This compound, also known as succinic acid 2,2-dimethyl hydrazide, negatively affects the 2-oxoglutarate-dependent dioxygenase enzyme, inhibiting the conversion of inactive precursors to active gibberellins, mainly the conversion of GA_{20} to GA_1 (RADEMACHER, 2000). According to these author, daminozide competes with 2-oxoglutarate, co-substrate of dioxygenases, and by this action affects the formation of active gibberellins, consequently retarding the growth of plants. Gussman et al. (1993) report that daminozide also affects ethylene biosynthesis by inhibiting the conversion of *S*-adenosylmethionine into 1-aminocyclopropane-1-carboxilic acid. Daminozide has been used in agriculture with the purpose of reducing the excessive shoot growth of crops, but due to toxicological issues its application in edible crops has been considerably decreased (RADEMACHER, 2000).

According to Fletcher et al. (1999), the application of plant growth retardants can alter the endogenous balance of phytohormones, increasing the ABA and cytokinins contents and decreasing ethylene. These changes in the hormones proportions can lead to delayed senescence and increase the tolerance to abiotic stresses (FLETCHER et al., 1999). The inhibition of ethylene biosynthesis by daminozide has been reported as early as 60`s by Looney (1968). According to Rademacher (2000), monooxygenase enzymes are activated in plants growing under well-watered conditions, which results in gibberellins accumulation and decrease of ABA levels, favoring the normal growth of shoots. However, under drought stress conditions, monooxygenases are inactivated, leading to decreased gibberellins contents and accumulation of ABA, which as consequence leads to reductions in shoot growth and transpiration rate of plants (RADEMACHER, 2000). According to Creelman et al. (1990) the biomass allocation in response to ABA accumulation into the roots permits the root growth under abiotic stresses and functions as an adaptive mechanism. Rademacher (2000) reports daminozide, inhibitor of dioxygenases, has shown to have the same effect as monooxygenases-inhibiting compounds. According to Rajala and Peltonen-Sainio (2001), is possible to increase the tolerance to abiotic stresses via improvement of root growth resulting from the control of shoot growth by the use of PGRs. However, the impact of such agrochemicals on environmental and food safety is unclear, which makes more ideal the identification of natural-occurring compounds in aim to improve the abiotic stresses tolerance of crops (NAIR et al., 2012).

2.3 Plant biostimulants

Biostimulants have been defined as any substance, microorganism or their mixtures applied to plants with purpose of improving the nutrients uptake and assimilation, stresses tolerance, and quality traits (DU JARDIN, 2015). According to this author, biostimulants definition is important to make them distinguishable from other agricultural inputs classes such as fertilizers, defensive products and biocontrol agents. Biostimulants have emerged as a class of natural agricultural products with focus on the attenuation of crop stresses, leading to the enhancement of crop yield and quality (GOÑI et al. (2016). According to Du Jardin (2015), biostimulants impart physiological benefits to crops, regardless their nutrients composition. Wally et al.

(2013) quantified the hormonal concentrations in leaves of *Arabidopsis* at up to 144 hours after treatment with ANE and verified increased levels of cytokinins, mainly zeatins. ABA and ABA metabolites levels also were increased but the opposite effect was observed for auxins (WALLY et al., 2013). According to these authors, ANEs treatments led to reduced root growth and decreased expression of the auxin-sensitive promoter *DR5::GUS* in *Arabidopsis* leaves. Goñi et al. (2016) realized microarray assays with samples of *Arabidopsis* leaves collected at one week after ANEs spray and did not find any dysregulation of genes involved in the auxin biosynthesis pathway.

The organic and inorganic compositions of an ANE from seaweed harvested in Canadian Atlantic rocky coast have been already determined (RAYIRATH et al., 2009). Most of polysaccharides found in ANEs (alginates) are structural carbohydrates present in cell walls, but there are also storage carbohydrates like laminarin, fucoidan, and mannitol (SHARMA et al., 2014; GOÑI et al., 2016). Fan et al. (2011) found that the Fe^{2+} chelating ability of plants treated with higher dosages of ANEs was increased compared to untreated or treated with low dosage of ANEs. These authors also found higher antioxidant phenolic compounds levels in ANE-treated plants and assumed that their biosynthesis may be induced by specific systemic physiological responses driven by ANEs. According to Allen et al. (2001), the plant antioxidant system is improved by exogenous application of ANEs via both increasing the levels of antioxidant compounds and activities of enzymes involved in the reactive oxygen species scavenging system.

The improvement of antioxidant system is a very important tool to obtain fresh produce with a better nutritional quality and longer shelf live (FAN et al., 2011). These authors also concluded that the improvement of antioxidant system and increase of Fe^{2+} complexant ability and phenolic content might be due to increased ANE-stimulated hormonal activities. According to Michalak (2006), phenolic compounds are important inactivating agents of reactive oxygen species. Zhang and Schmidt (2000) reported that ANEs have elicitors of cytokinin-like responses which increased the antioxidant activities and promoted stress tolerance in an *Agrostis sp*. grass. According to Stirk et al. (2003), cytokinins and cytokinins-like activities are the most reported effect of ANEs on the phenotype of treated plants.

Holdt and Kraan (2011) reported that seaweeds have high contents of phenolic compounds. Some studies have demonstrated that the polyphenols

contents of different crops has increased in response to treatments with ANEs (FAN et al., 2011; LOLA-LUZ et al., 2013). Fan et al. (2011) found increased activities of the enzyme chalcone isomerase, which is a key enzyme in the biosynthetic pathway of phenylpropanoid plant defense compounds. However, the authors emphasize that the ANEs compounds that can elicit the phenylpropanoids biosynthesis pathway as well as the flavonoids pathway still remain to be discovered.

Goñi et al. (2016) report that ANEs result in significant heterogeneity in terms of mRNA regulation according to the analyzed transcriptome in *Arabidopsis*. These researchers also verified significant increases in biomass, plant height, and number of leaves, as well as earlier flowering and longer stems in ANEs-treated plants compared to those non-treated.

2.4 Seaweed biostimulants use in agriculture

Seaweed extracts have been used in agriculture for centuries with the purpose of improving crops growth and attenuation of stresses (WALLY et al., 2013). Around 30 tons of *Ascophyllum nodosum* are harvested for agricultural purposes every year (CRAIGIE, 2011). These extracts are classified as plant biostimulants and their application aims to improve yield and produce quality (GOÑI et al., 2016). These authors see positive perspectives for agricultural uses of seaweed extracts, since the search for a more sustainable agriculture is required in function of increasing worries concerning growth of population and climate changes. Seaweed extracts have been shown to provide several benefits in crop productivity improvement through stimulation of shoot growth and branching, enhanced biotic stress tolerance, and alleviation of abiotic stresses such as drought, salinity, and frost (FEATONBY-SMITH; VAN STADEN, 1983; TEMPLE; BOMKE, 1989; NABATI et al., 1994; NAIR et al., 2012).

There are several studies in literature reporting the positive stimulatory effect of seaweed extracts on crops yield and nutritional fruit or grain quality. Dobromilska et al. (2008) have found increases of N, P, K, Ca, Zn, and Fe contents in tomato fruit after treatments with ANE. Zodape et al. (2008) also reported the improvement of okra nutritional quality. The contents of carbohydrates, proteins, and minerals as well as the yield were increased in wheat treated with extract of the red alga *Kappaphycus alvarezii* according to a study carried out by Zodape et al. (2009). The

increase of flavonoids biosynthesis, which improve the nutritional quality, in spinach plants treated with *A. nodosum* extracts (ANEs) was observed by Fan et al. (2011). According to Sangha et al. (2014), these studies give strong evidence of the biostimulant action of seaweed extracts on crops and support their use in agriculture aiming to increase yield and improve the quality of the produce.

According to Stirk et al. (2014), the extract of the seaweed *Ecklonia maxima* confers several agricultural benefits such as improving root and shot development, giving tolerance to biotic and abiotic stress, and consequently, increasing the yields of crops. Stirk et al. (2014) report that cytokinins, auxins, and polyamines have been found in *E. maxima* extracts. According to Sangha et al. (2014), it was always suspected that the crop yield increase effect after the application of seaweed extract was due to the presence of phytohormones or growth stimulatory compounds, such as auxins, cytokinins, gibberellins, micronutrients, vitamins, and amino acids, in the composition of these agricultural inputs. Wally et al. (2013) found relatively low concentrations of phytohormones in different commercial ANEs. The authors found concentrations of ABA and ABA metabolites as low as 2 ng g^{-1} dry mass of sample, cytokinins metabolites up to 25 ng g^{-1} dry mass of sample and IAA and IAA precursors ranging from 3 to 50 ng g^{-1} dry mass of sample. According to these authors those concentrations are not able to trigger hormonal responses in plants growth and development and that the responses observed are due to hormonal biosynthesis or signaling activation in the plant by other natural components present in ANEs. Sangha et al. (2014) affirm that the available data on ANEs benefits are significant and support the use these extracts as a biostimulant input aiming to improve plant growth and yield and mitigate abiotic stresses.

A. nodosum is a perennial brown marine macro alga that occurs in the North Atlantic Ocean intertidal zones from Canada to Norway (RAYIRATH et al., 2009). Rayirath et al., (2009) cite that this seaweed has been used in several applications not only in agriculture, but as animals and human nutritional supplement. The agriculture usages of this seaweed are traditionally as fertilizers or soil conditioning agents (RAYIRATH et al., 2009). According to some authors (ZHANG; ERVIN, 2004; SPANN; LITTLE, 2011), commercial seaweed extracts are known to confer abiotic stress tolerance to crops. Nair et al. (2012) report that the lipophilic components present in ANEs are responsible for imparting freezing tolerance to plants. According to these authors, the ANEs lipophilic components increase the cellular soluble sugar,

proline and unsaturated fatty acid contents of freezing-stressed plants. Jithesh et al. (2012), found induction of many positive regulators of tolerance to salt stress following treatment with ANEs, suggesting another characteristic of ANEs in helping plants to cope with abiotic stresses.

The calibration of doses and responses of hormones or hormones-stimulating substances remains a challenge in function of the necessity of keeping the balance between their positive effects on stress tolerance and negative effects on plant growth (PELEG; BLUMWALD, 2011). Goñi et al. (2016) concluded that ANEs composition is significantly variable and the compositional standardization of ANE-based products constitutes a challenge to obtain consistency in gene regulation and biostimulation in crops. The authors found differentiated expressions of genes related to redox, stress, and secondary-metabolism pathways in response to two ANEs manufactured by different processes of extraction. To consolidate these products in the mainstream of agricultural inputs it is first necessary to understand the mode of action, diminish the compositional variability, and obtain consistency of crops responses to their application (BROWN; SAA, 2015).

2.5 Tomato hormonal mutants and reporter lines

Phytohormones are low-molecular-weight signaling compounds naturally produced by plants that coordinate all physiological and developmental processes, from germination to senescence (CAMPOS et al., 2010; WALLY et al., 2013). According to Wally et al. (2013), these compounds are responsible for the regulation of plant responses to environmental stress, such as drought. The phytohormones well known by plant scientists up to date are: auxins, gibberellins, cytokinins, ABA, ethylene, jasmonates, brassinosteroids, polyamines, salicylic acid, nitric oxide, and strigolactones (PELEG; BLUMWALD, 2011). Phytohormones do not act in isolation, but in synergistic or antagonistic interplays between two or more of them, modulating their responses or biosynthesis (PELEG; BLUMWALD, 2011). The network formed by precursors and catabolites of phytohormones metabolisms can be very complex and constitute intricate biological responses (WALLY et al., 2013). According to some authors (WERNER; SCHMÜLLING, 2009; PELEG; BLUMWALD, 2011; SU et al., 2011) there is a complex crosstalk among the hormonal signaling pathways that

regulates their physiological responses to modulate the plant development in response to environmental cues.

Arabidopsis thaliana has been classically used as a plant model for hormonal studies, however, for such studies with applications in agriculture it presents some disadvantages like the lack of fleshy and climacteric fruit (CAMPOS et al., 2010). Tomato (*Solanum lycopersicum* L.) is a plant of economic importance and has the properties that can serve as a dicot model for innumerous other crops, connecting the basic and applied sciences (CAMPOS et al., 2010; CARVALHO et al., 2011). The dwarf cultivar of tomato 'Micro-Tom' has a compact habit and suits well for those needs in function of being a crop-like plant and having short lifespan (70-90 days) and small architecture (grows in high densities), traits required for applied biological researches (MEISSNER et al., 1997; MARTÍN et al., 2006; CAMPOS et al., 2010). According to Martín et al. (2006), 'Micro-Tom' compact habit is due to mutations in *SELF-PRUNING* (*SP*) and *DWARF* (*D*) genes. The mutation (*sp*) imparts a determinate phenotype to 'Micro-Tom' and (*d*) causes mis-splicing, leading to production of small mRNAs, and generates truncated DWARF proteins (MARTÍN et al., 2006). According to these authors, 'Micro-Tom' harbors at least another mutation that negatively affects internodes growth without affecting the levels of active gibberellins.

The tomato mutant *sitiens* (*sit*) exhibits low levels of ABA due to mutations in gene loci that encode the enzyme ABA-aldehyde oxidase, the enzyme that catalyze the conversion of ABA-aldehyde into ABA (LINFORTH et al., 1987). Taylor et al. (1988) observed that the application in *sit* plants of deuterium-labeled ABA-aldehyde led to accumulation of reduced ABA alcohol instead oxidized ABA due to the mutation in ABA-aldehyde oxidase and drew the conclusion that ABA-aldehyde is the immediate precursor of ABA biosynthesis. The tomato mutant *diageotropica* (*dgt*) has low sensitivity to auxins (KELLY; BRADFORD, 1986) and is characterized by horizontal growth in both shoots and roots, lack of lateral roots, thin stems, and epinastic leaves (ZOBEL, 1976). This author reports that *dgt* also exhibits low levels of ethylene because of a failure in auxin-mediated ethylene biosynthesis due to its low sensitivity to auxins. According to Oh et al. (2006), the *Dgt* gene encodes a cyclophylin and the mutation in the coding sequence of *LeCYP1* is important for specific aspects of auxin signaling, plant development, and responses to the environment. The tomato mutant *gibberellin-deficient* (*gib-3*) is deficient in gibberellin

biosynthesis and presents a dwarfed growth habit (BENSEN; ZEEVAART, 1990). According to these authors, *gib-3* has a reduced ability for synthesizing *ent*-kaurene from copalyl diphosphate due to reduced activity of the enzyme *ent*-kaurene synthase, being this enzyme the site of the mutation harbored by *gib-3*.

The tomato reporter line *EBS::GUS* harbors the *uidA* gene which encodes the enzyme β-glucuronidase, an enzyme produced by *Escherichia coli* bacteria (JEFFERSON et al., 1986) driven by a synthetic EIN3-responsive promoter (STEPANOVA et al., 2007). According to these authors, EIN3 is a family of transcription factors whose targets can be turned on and off in response not only to ethylene but to other ethylene-responsive factors, providing specificity in response to ethylene. The tomato reporter line *RD29b::GUS* carries the *uidA* gene fused with the *RD29b* promoter. This promoter region harbors two ABRE sequences and the drought-inducible response by *RD29b* is controlled by ABA (KOORNNEEF et al., 1984; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 1994). The tomato reporter line *DR5::GUS* harbors the fusion of the auxin-responsive synthetic promoter with the *uidA* gen in a construct known as DR5::GUS (BAI; DEMASON, 2008). The auxin responsiveness of this promoter is conferred by a seven-copy in tandem direct repeats of ARF-binding site from the soybean G3 promoter (ULMASOV et al., 1997).

Components of ANEs may stimulate the hormonal biosynthesis in plants (WALLY et al., 2013) and hormonal mutants and specific reporter lines are useful tools for revealing the noticed effects of this kind of biostimulants on important crops for global food production.

3 MATERIAL AND METHODS

3.1 Growth conditions for tomato cv. 'Micro-Tom' and its hormonal mutants

The experiments with 'Micro-Tom' and tomato mutants were carried out at Luiz de Queiroz College of Agriculture - University of São Paulo, Piracicaba-SP, Brazil. The mutations introgression into the background 'Micro-Tom' was carried out by Carvalho et al. (2011). These genotypes were grown under greenhouse conditions. The average temperature inside the greenhouse was 28 ºC, with photoperiod varying from 11.5 hours in the winter to 13 hours in the summer and photosynthetic active radiation ranging from 250 to 350 µmol m^{-2} s^{-1}. The radiation is reduced by a reflective mesh (Aluminet – Polysack Industrias Ltda, Itápolis, Brazil) that covers the greenhouse.

The genotypes used for these experiments were the cv. 'Micro-Tom' and the hormonal mutants: *sitiens* (*sit*), *diageotropica* (*dgt*), and *gibberellin-deficient3* (*gib-3*). The genotypes seeds were previously sown in 250 mL pots filled with a mixture of potting compost (Plantmax HT, Eucatex, São Paulo, Brazil) and vermiculite in a 1:1 ratio, supplemented with NPK 10-10-10 fertilizer (1 g L^{-1}) and lime (4 g L^{-1}). *gib-3* seeds were previously germinated in germinating boxes containing 20 mL 100 µM ProGibb® (Valent BioSciences Corporation, Libertyville, Illinois, USA), a commercial gibberellin-based product. Due to the gibberellin deficiency presented by this mutant, exogenous supply of the phytohormone is required to aid its germination process. As soon as seeds were germinated, *gib-3* seedlings were transferred to the sowing pots (250 mL) and from this step they were grown under the same conditions as the other genotypes. After the appearance of the first pair of true leaves (13 days after sowing) in all genotypes, except *gib-3*, seedlings were individually transplanted into 150 mL pots. *gib-3* seedlings were transplanted into 150 mL pots when they were 19 day-old. Before treatments application, plants were watered with automated irrigation four times a day. The fertilization was supplemented twice: 0.2 g NPK 10-10-10 per pot, one week after transplanting and at the flowering stage.

3.1.1 PGRs spray versus different watering regimes assays

The assays with the tomato 'Micro-Tom' and mutants were carried out in a randomized blocks design with four replications. Four assays were run simultaneously: one for each genotype ('Micro-Tom', *sit*, *dgt*, and *gib-3*). A factorial

scheme 3 x 2 was used for each assay: 3 plant growth regulators (PGR) treatments (chlormequat chloride, daminozide, and control) x 2 watering regimes (well-watered and drought-stressed conditions). Chlormequat chloride and daminozide were sprayed on leaves at concentrations of 500 mg L^{-1} and 1500 mg L^{-1}, respectively. Different dosages of these PGRs were previously tested through visual screening aiming to detect possible phytotoxicity effects. The spray of PGRs was realized simultaneously with the beginning of drought-stressed watering regime at the onset of flowering stage (54 days after sowing). The control plants were sprayed with tap water. All treatments were sprayed until the solutions started to drip from leaves.

As the irrigation of the greenhouse used for these experiments is an automated system in a hydroponic structure, the water withholding was carried out by putting the pots of drought-stressed treatments into plastic bags to prevent water going into the substrate in the pots. Plants from those treatments were rehydrated with 100 mL water per pot just after the appearance of the first wilting symptoms, and then kept without irrigation until the symptoms showed up again. *sit* drought-stressed plants were rehydrated at 7, 13, and 18 days after the watering withholding. *dgt* drought-stressed plants were rehydrated at 7, 13, and 19 days after watering withholding. For *gib-3* drought-stressed plants, the rehydration was done only twice (at 12 and 19 days), due to their small size, which leads to a reduced loss of water. By the other hand, the rehydration of 'Micro-Tom' was done at 6, 12, and 18 days after the irrigation suppression.

At the occasion of harvest, the roots were washed in flowing tap water to remove the particles of horticultural stuck on them. The variables evaluated for these assays were as follow: root volume (RV), root length (RL), root dry mass (RDM), and shoot dry mass (SDM). RV was measured by the volume of water displaced when the root system was immersed in a graduated cylinder. RL data was obtained by measuring the length of the root system with a ruler. RDM and SDM measurements were taken after oven-drying samples at temperature of 105 ºC.

3.2 Assays with *Arabidopsis* seedlings

Arabidopsis seedlings were germinated and grown *in vitro* in the 96-well microphenotyping method developed by Forde et al. (2013). This method consist of 8-tubes PCR strips (FameStrip^{TM}, 4titude, Surrey, England, United Kingdom)

supported by 96-well PCR plates (Thermo Fish Scientific, Waltham, Massachusetts, USA). These tubes were filled under laminar flow hood with approximately 300 µL of sterile medium composed of 0.8% agar Phytagel^{TM} (Sigma-Aldrich Co. St. Louis, MO, USA), 0.5% sucrose, 1/20 Gamborg's B5 medium, 1 mM $MgCl_2$ and $CaCl_2$, and 20 µM NH_4NO_3, except for the assays presented on Figures 9, 11, and 12, where 0.5 mM NH_4NO_3 instead of 20 µM NH_4NO_3 was the amount added to the medium. All operations were performed under sterile conditions. PCR 96-well plates and plastic boxes were disinfected with a solution of 1% Virkon (Day-Impex Ltd. Colchester, Essex, UK) just before pouring the Phytagel^{TM} medium.

Arabidopsis seeds were sterilized prior the sowing on medium with washing in 95% ethanol for 1 minute and then, bleach (20% with 0.01% Triton X-100) for 10 minutes. Seeds were rinsed five times with sterile deionized water and an amount of water from the last rinse was left to make a seed suspension. Seeds were pipetted (6-10 seeds) on the surface of the solid medium in the wells using a 200 µL pipette with a cut-off tip. The plates inside sterile, transparent plastic boxes were moved to a horticultural propagator containing a moistened paper tissue (deionized water) and then moved to a cold room (4 ºC) for 1 day to aiming to overcome the seed dormancy. After 1 day in the cold room, the propagator with the plates were moved into a growth chamber JUMO IMAGO 500 (Snijders Scientific, Tilburg, The Netherlands). The growth conditions inside the growth chamber were temperatures ranging from 21 to 22 ºC, and a light/dark ratio of 16h/8h. After germination and appearance of primary roots through the tubes walls, plates were moved to the laminar flow hood to receive the treatments.

The treatments were prepared under the laminar flow hood with filter-sterilized stock solutions. Treatments were applied in a total volume of 150 µL into new and sterile 96-well V-bottom plates (Anachem, Luton, England, United Kingdom) along with 1/20 B5 medium. Each 8-tubes strip received one treatment, thus, it was considered 8 replications each treatment. The tube ends were cut-off with a paper cutter guillotine to permit the ascension of treatments through the solid medium and reach the roots. The position of root tip in the tube wall at the moment of treatments (time 0) was marked with a felt-tip pen. The plates were put back into the plastic boxes and then, into the propagators and returned to the growth chamber. At the moment of root growth evaluation, the plates were open and the position of root longest root tip position was marked in the tube wall. Each 8-tubes strip (one

treatment) with the marks of root growth were put close to a ruler and pictures were taken from them. The measurements of root length were calculated from the pictures obtained using software ImageJ.

A. nodosum extracts (ANEs) were applied at concentrations of 0.04 weight volume^{-1} in the assays presented in Figures 1 and 8; 0.02 weight volume^{-1} in the assay of Figure 8; and 0.03 weight volume^{-1} in the assays of Figures 8, 9, 10, 11, and 12. The composition of two ANEs (A and B) used in this research was determined by Goñi et al. (2016). Both ANEs are manufactured by proprietary processes at temperatures higher than 100 ºC. ANE A is fabricated in alkaline conditions whereas ANE B is obtained in neutral pH. In the composition analysis made by Goñi et al. (2016), ANE A presented significant higher contents of solids, ash, polyphenols, and uronics compared to ANE B, whereas fucoidan, laminarin, and mannitol contents were higher in ANE B.

IAA was applied in concentrations ranging from 0.001 to 3 µM. ABA was applied in concentrations ranging from 0.5 to 30 µM. 6-Benzyladenine (BA) was applied in 1 and 3 µM concentrations (Figure 9). NH_4NO_3 supplementations in the concentration of 25 mM were carried out along with the treatments application in the assays of Figures 3, 6, 7, 8, 10, and 11. These nitrogen supplementations were realizing with the purpose of growing seedlings for longer periods. Poly(ethylene glycol) (PEG 8000) (Sigma Aldrich, Co., Saint Louis, Missouri, USA) was autoclaved before application. PEG 8000 was applied in a concentration of 40% to the osmotic-stressed seedlings along with the treatments in the assays of Figures 7, 9, 10, 11, and 12.

3.2.1 GUS staining analysis

Histochemical staining assay was carried out according to methodology described by Jefferson et al. (1987) with some modifications. The solution of the chromogenic β-glucuronidase substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) (Sigma Aldrich, Co., Saint Louis, Missouri, USA) was prepared as following: 0.5 mg mL^{-1} X-gluc was dissolved in 200 mM sodium phosphate buffer (pH=7), containing 0.1% Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 10 mM EDTA. Roots and shoots of *Arabidopsis AIR1-8::GUS* seedlings were immersed in X-gluc solution in a 96-well-format plate, covered with aluminum foil, and incubated

overnight at temperature of 37 ºC and shaken with 125 rpm. After the incubation, leaves samples were washed in 70% and 90% ethanol solutions for two hours each, at room temperature, to remove the chlorophyll molecules and permit the visualization of the blue staining. These washing steps were not necessary for roots samples. After this, samples were placed on microscopic slides and pictures were captured by a camera (Leica Microsystems, Wetzlar, Hesse, Germany) attached to the confocal microscope.

3.3 Growth conditions for tomato hormonal reporter lines

Aiming to validate the results of root growth obtained with plant model *Arabidopsis* through hormonal responses, assays with reporter lines in a crop-like plant model background were carried out. The tomato reporter lines were grown in growth room at Shannon Applied Biotechnology Centre - Institute of Technology, Tralee, Ireland. The growing conditions inside the growth room were as follow: temperatures ranging from 21.7 to 33.3 ºC, relative humidity of 24.5-56.6%, and photoperiod of 16 hours by artificial radiation (~160 µmol m^{-2} s^{-1}) using LED lights. Seeds were sown in horticultural trays for germination. The horticultural substrate used to grow these genotypes was composed of the potting compost GroWise (Bord na Móna Horticulture Ltd., Newbridge, Co. Kildare, Ireland), perlite Sinclair Perlite Standard (William Sinclair Horticulture Ltd., Lincoln, England, United Kingdom), and vermiculite in proportions of 6:1:1. The fertilization was done by adding 1.44 g NPK 7-7-7 fertilizer and 4 g lime per liter of substrate. After the appearance of first pair of true leaves, seedlings were transferred to 150 mL pots containing the same composition of the substrate used for seeds germination. Plants of all reporter lines were kept under the same watering regime until the water withholding of plants used for the drought-stressed treatments.

3.3.1 ANEs, ABA, and IAA treatments and watering regimes application

The assays with reporter lines were carried out in a factorial scheme 4 x 2 (4 spray treatments and 2 watering regimes) with three replications. The water withholding of plants of the reporter line *EBS::GUS* in the background ‘Micro-Tom’, herein called (MT) *EBS::GUS*, used for drought-stress treatments was initiated at 39 days after sowing. At five days after watering withholding of drought-stressed plants,

ANEs and ABA were sprayed on leaves of both stress and non-stressed plants. The control plants were sprayed with distilled water. All treatments were sprayed until solutions started to drip from leaves. ANEs were sprayed in the concentration of 0.33 volume volume^{-1}. ABA was sprayed in the of 200 µM concentration made up of a stock solution of 5 mM ABA in 10% ethanol and final volume of 0.4% ethanol following the methodology used by Achuo et al. (2006).

The reporter line (MT) *RD29b::GUS* plants used for the drought-stressed treatments had the watering withheld at 76 days after sowing. The ANEs and ABA treatments were applied at the same concentrations and time after watering withholding used for (MT) *EBS::GUS* assay. The reporter line (MT) *DR5::GUS* plants used for drought-stressed treatments had the watering withheld at 74 days after sowing. For this reporter line, ANEs were sprayed at the same concentrations as the two previous ones, however, IAA in the concentration of 100 mM (WU et al., 2012) was applied instead of ABA. Treatments were also sprayed at five days after watering withholding.

3.3.2 Fluorometric assays for GUS activity

Samples were collected at 24 and 48 hours after treatments spray, six and seven days after water withholding of drought-stressed plants, respectively. The samples collection was a destructive action and, therefore, plants used for analysis at 24 hours after spray of treatments were not the same of 48 hours. Roots were carefully and completely washed under flowing water, removing the particles of horticultural substrate stuck on them. Portions of roots were picked up throughout the root system and placed into 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen.

The fluorometric analyses were carried out according methodology described by Jefferson (1987) and Gallagher (1992), with some modifications. The fluorometric analysis is based on the measurement of β-glucuronidase activity by quantifying, through fluorescence, the product generated from the reaction of this enzyme with the substrate 4-methylumbelliferyl-glucuronide (MUG) (JEFFERSON, 1987; GALLAGHER, 1992). According to these authors, the β-glucuronidase enzyme cleaves MUG generating 4-methylumbelliferyl (MU), which emits fluorescence.

An extraction buffer was made up of 0.1 M sodium phosphate buffer (pH=7.4), 1 mM EDTA, 1 mM PMSF, and 1% PVPP. The extraction buffer (300 µL) was added to 40-50 mg in 1.5 mL microcentrifuge tubes kept on ice. The samples and extraction buffer were mixed in vortex and pulverized with a metal bead for 30 seconds. Samples were moved to a cold room (4 ºC) for one hour and then centrifuged at 10000 g for 15 minutes. The supernatant was collect and transferred to new tubes and centrifuged again (10000 g) for 15 minutes. From each sample, 200 µL were collected and diluted in buffer in a 1:4 proportion for the β-glucuronidase reaction preparation.

The reaction mix was prepared in an dark 96-well-format plate by adding 70 µL sodium phosphate buffer, 40 µL 2 mM MUG solution (Sigma Aldrich, Co., Saint Louis, Missouri, USA), and 40 µL sample. All samples were assayed in duplicates. Autoflorescence reactions were performed for each sample by adding 110 µL sodium phosphate buffer and 40 µL sample. Blanks were prepared by mixing 110 µL sodium phosphate buffer and 40 µL 2 mM MUG solution. The reaction mixes were incubated at 37 ºC and shaken with 125 rpm. The times of incubation were 1.5 hour for *RD29b::GUS* and 1 hour for *EBS::GUS* and *DR5::GUS* reporter lines. After the incubation, the reaction was stopped by the addition of 100 µL 0.2 M Na_2CO_3 and fluorescence measured in a calibrated Varioskan Flash plate reader (Thermo Fish Scientific, Waltham, Massachusetts, USA) at 360 and 460 nm wavelengths. A standard curve was previously determined with different MU concentrations and used to calculate the amount of MU equivalent to fluorescence generated by each sample. The fluorescence values converted to amount of MU produced in pmol through the standard curve were divided by the time of incubation and normalized with the protein content of each sample.

The protein content was determined using the methodology described by Bradford. In a transparent 96-well-format plate it was prepared the samples protein reactions by adding 190 µL Bradford reagent (dilution of 1:4 mL water) to 10 µL sample. It was allowed 20 minutes at room temperature for reaction to take place and then absorbances were measured in the Varioskan Flash plate reader (Thermo Fish Scientific, Waltham, Massachusetts, USA) at 595 nm wavelength. A protein standard curve was done with different concentrations of bovine serum albumin (BSA) protein. The protein concentrations of sample were obtained in mg through BSA standard curve. The normalization of MU concentrations with proteins contents was done to

eliminate differences in weigh and extraction of samples. The final GUS activity was obtained in pmol MU/(minutes of incubation * mg protein)

3.4 Statistical analyses

Statistical analysis carried out for the assays with tomato ‘Micro-Tom’ and mutants were based in the F values of the analysis of variance (ANOVA). The Tukey’s test at the probability level of 5% ($p \leq 0.05$) was applied for those variables with significant differences in ANOVA. The statistical analysis used in tomato reporter lines (GUS activity) assays also consisted of F values of ANOVA and the Tukey’s test at the probability level of 5%. The statistical analyses realized in *Arabidopsis* assays consisted of F values of ANOVA complemented with Tukey’s test (Figures 1, 9, 10, 11, and 12), regression analysis and Dunnett’s test (Figures 2, 3, 5, 6, and 7), and Duncan’s test (Figure 8). These analyses were performed in the following statistical software: XLSTAT®, SAS®, and Sisvar.

4 RESULTS AND DISCUSSIONS

4.1 Effect of PGRs on tomato genotypes development under drought

4.1.1 Effect of PGRs on tomato cv. Micro-Tom development under drought

An experiment with tomato cv. Micro-Tom was carried out aiming to study the effect of drought stress and PGRs foliar spray on the plant developmental traits. The PGRs application did not have any effect on the traits analyzed. But the drought stress (successive watering withholdings and rewaterings) did significant influence on RV and SDM traits (Tables 1 and 2).

Table 1 – Summary of ANOVA analysis (degrees of freedom and F values) for root volume (RV), root length (RL), root dry mass (RDM), and shoot dry mass (SDM) of tomato, cultivar Micro-Tom.

Source	DF	F value			
		RV (cm^3)	RL (cm)	RDM (g)	SDM (x^0,5) (g)
PGR	2	1.81^{ns}	0.53^{ns}	0.10^{ns}	1.21^{ns}
WR	1	13.33^{*}	0.00^{ns}	1.22^{ns}	5.15^{*}
PGR x WR	2	0.10^{ns}	0.05^{ns}	0.22^{ns}	0.20^{ns}
Blocks	3	1.18^{ns}	0.37^{ns}	0.14^{ns}	0.69^{ns}
Residue	15				
Total	23				
CV (%)		10.23	27.95	29.56	9.35

PGR = Plant Growth Regulator (Chlormequat chloride and Daminozide), WR = Watering Regime.
* There is significant difference by the F test at the probability level of 5% (P≤0.05).
ns There is no significant difference by the F test at the probability level of 5% (P≥0.05).

The drought stress impaired the plant growth in both root and shoot development. The traits RV and SDM were smaller in plants submitted to drought watering regime (Table 2). According to Feldman (1984) the ethylene level is low in roots of plants growing in well-watered conditions, but the phytohormone level increases considerably under stressful conditions. Stenlid (1982) reported about ethylene as being the main root growth inhibitor. ABA which is overproduced under abiotic stress conditions also inhibits the growth of roots by promoting ethylene

biosynthesis (LUO et al., 2014). Sharp et al. (1994) reported that root elongation can be promoted by increased endogenous ABA levels in plants under moderate stress conditions. According to Pilet and Saugy (1987), the ABA effect on root inhibition or promotion is dependent of the endogenous levels. It can be concluded from our study that the drought stress regime applied to the plants was severe, since no root promotion was observed for none of the following drought stress experiments. Instead of root elongation, it was observed growth inhibition, possibly due to ABA produced in excess or ABA-mediated ethylene production.

Table 2 – Root volume (RV) and shoot dry mass (SDM) of tomato, cultivar Micro-Tom submitted to two watering regimes (WR).

WR	RV (cm^3)	SDM (x^0,5) (g)
Well-watered	1.81 a	1.68 a
Drought-stressed	1.56 b	1.54 b

Averages followed by distinct letters at the columns are different by the F test at the probability level of 5% (P≤0.05).

Sharp and Davies (1979) reported that roots are generally less damaged by drought stress impact than shoots. McAdam et al. (2016) found reduced biomass accumulation in roots in ABA-biosynthetic mutants due to reduced ABA levels in roots. These authors also verified increased roots biomass in wild-type plants due to normal levels of ABA. RDM was not affected in our ‘Micro-Tom’ experiment, but it was in the shoot, suggesting that ABA accumulated differently in these organs or organs exhibit different sensitivity to the hormone. The decrease in RV under drought stress may be due to reduced water uptake, since RL and RDM were not affected. Creelman et al. (1990) reported that the biomass allocation driven by ABA into the roots has is an adaptive response to the abiotic stresses. According to McAdam et al. (2016), shoot-synthesized ABA has the major role in governing the root architecture in both well-watered and stressed conditions.

4.1.2 Effect of PGRs on tomato mutant *sitiens* (*sit*) development under drought

The mutant *sit* plants, which present low endogenous ABA level, did not show differentiated response to PGRs application, but responded to the watering regime for one of the traits measured (Table 3).

Table 3 – Summary of ANOVA (degrees of freedom and F values) for root volume (RV), root length (RL), root dry mass (RDM), and shoot dry mass (SDM) of the tomato mutant *sitiens* (*sit*).

Source	DF	F value			
		RV (x^0,5) (cm^3)	RL (cm)	RDM (logx) (g)	SDM (x^0,5) (g)
PGR	2	1.23^{ns}	1.25^{ns}	1.95^{ns}	2.67^{ns}
WR	1	1.92^{ns}	0.01^{ns}	1.43^{ns}	5.64*
PGR x WR	2	0.10^{ns}	0.62^{ns}	0.67^{ns}	0.24^{ns}
Blocks	3	0.94^{ns}	0.14^{ns}	0.52^{ns}	0.98^{ns}
Residue	15				
Total	23				
CV (%)		32.19	40.19	24.00	16.42

PGR = Plant Growth Regulators (Chlormequat chloride and Daminozide), WR = Watering Regime.
* There is significant difference by the F test at the probability level of 5% (P≤0.05).
^{ns} There is no significant difference by the F test at the probability level of 5% (P≥0.05).

None of the root traits were different between the watering regimes, but the response to drought was observed in the shoot. Plants submitted to drought accumulated less biomass (SDM) than those maintained in normal watering regime (Table 4). According to Tardieu et al. (2010), the understanding of shoot development inhibition by endogenous ABA seems to be clear and explainable by the reduced stomatal aperture and, consequently, decreased gas exchanges. Achard et al. (2006) report that stress-induced ABA signaling represses plant growth through interactions with gibberellins in a DELLA proteins-dependent manner. However, Nagel et al. (1994) observed significant decrease in relative growth rate of *sit* plants compared to the wild-type tomato cv. Moneymaker. As *sit* plants are deficient in ABA biosynthesis, they show higher transpiration rates and lower root hydraulic conductance in normal watering conditions compared to wild-type tomato plants

(NAGEL et al., 1994). These features of *sit* plants are aggravated under drought stress conditions and affect negatively the shoot growth, as observed in this study. With higher transpiration rates and scarce water in the soil to replace the water lost by leaves, the leaf turgor is lowered, affecting leaf expansion and resulting in smaller growth rates. According to Nagel et al. (1994), the influence of ABA deficiency on biomass allocation is a result of water relations alterations in plant organs. According to Creelman et al. (1990), ABA produced under drought stress permits an adaptive response by allocating biomass into roots and, therefore, increasing the water uptake effectiveness. It can be concluded that the drought stress did not affect the root development of ABA-deficient *sit* plants by allocating biomass from the shoot into the roots what reflected negatively on the shoot development.

Table 4 – Shoot dry mass (SDM) of the tomato mutant *sitiens* (*sit*) submitted to two watering regimes (WR).

WR	SDM (x^0,5) (g)
Well-watered	0.84 a
Drought-stressed	0.71 b

Averages followed by distinct letters at the columns are different by the F test at the probability level of 5% (P≤0.05).

4.1.3 Effect of PGRs on tomato mutant *diageotropica* (*dgt*) development under drought

The tomato mutant *dgt* has low sensitivity to auxins (KELLY; BRADFORD, 1986). No effect of PRGs and watering regime was detected on the evaluated root and shoot traits of this mutant plants (Table 5), suggesting that auxin signaling on root regulation under drought may act in a fine-tuning mechanism. Also, the inhibition of gibberellins biosynthesis to some degree does not affect the growth of the low auxin-sensitive mutant.

Table 5 – Summary of ANOVA (degrees of freedom and F values) for root volume (RV), root length (RL), root dry mass (RDM), and shoot dry mass (SDM) of the tomato mutant *diageotropica* (*dgt*).

Source	DF	F value			
		RV (cm^3)	RL (cm)	RDM (x^0,5) (g)	SDM (g)
PGR	2	0.99^{ns}	3.15^{ns}	0.80^{ns}	0.08^{ns}
WR	1	0.70^{ns}	1.98^{ns}	0.84^{ns}	0.74^{ns}
PGR x WR	2	0.28^{ns}	1.44^{ns}	0.08^{ns}	0.61^{ns}
Blocks	3	0.11^{ns}	0.91^{ns}	0.14^{ns}	0.22^{ns}
Residue	15				
Total	23				
CV (%)				15.01	27.92

PGR = Plant Growth Regulator, WR = Watering Regime.
^{ns} There is no significant difference by the F test at the probability level of 5% (P≥0.05).

4.2.3 Effect of PGRs on tomato mutant *gibberellin-deficient-3* (*gib-3*) development under drought

The PGRs used are inhibitors of gibberellin biosynthesis and they influenced RV and RL of the mutant with deficiency in gibberellin biosynthesis, *gib-3*. The WR influenced the other two traits, RDM and SDM, the ones not affected by PGRs. Moreover, RL was affected by an interaction between PGRs and WR (Table 6).

Table 6 – Summary of ANOVA (degrees of freedom and F values) for root volume (RV), root length (RL), root dry mass (RDM), and shoot dry mass (SDM) of the tomato mutant *gibberellin-deficient-3* (*gib-3*).

Source	DF	F value			
		RV (cm^3)	RL (cm)	RDM (g)	SDM (x^0,5) (g)
PGR	2	4.97^{*}	5.51^{*}	2.14^{ns}	1.59^{ns}
WR	1	0.28^{ns}	0.46^{ns}	6.78^{*}	40.93^{*}
PGR x WR	2	1.58^{ns}	6.92^{*}	3.26^{ns}	0.61^{ns}
Blocks	3	5.61^{**}	1.52^{ns}	0.96^{ns}	0.42^{ns}
Residue	15				
Total	23				
CV (%)			16.66	16.74	10.82

PGR = Plant Growth Regulator, WR = Watering Regime.
* There is significant difference by the F test at the probability level of 5% (P≤0.05).
** There is significant difference by the F test at the probability level of 1% (P≤0.01)
^{ns} There is no significant difference by the F test at the probability level of 5% (P≥0.05).

Chlormequat chloride had a negative effect on RV compared to RV of the control plants, sprayed with water (Table 7). This result indicates that the root system was strongly affected by the PGR-driven gibberellin biosynthesis inhibition combined with the inhibition caused by the mutation. Fu and Harbert (2003) report that gibberellins are regulators of root growth, and that they do so by interacting with auxin. According to these authors, auxins control root growth by modulating cellular responses to gibberellins and the latter permit the growth responses by opposing the DELLA proteins effects. DELLA proteins have growth inhibition effects through repression of gibberellins responses, and gibberellins promote root growth by destabilizing these proteins (FU; HARBERT, 2003).

The mutant *gib-3* is deficient in gibberellin biosynthesis and naturally shows a dwarf habit of growth (BENSEN; ZEEVAART, 1990). Both chlormequat chloride and daminozide are classical PGR with well-stablished effects on gibberellins biosynthesis (RADEMACHER, 2000). The spray of chlormequat chloride solution, in the beginning of flowering stage, led to root growth inhibition, measured by RV, in *gib-3* (Tables 7 and 8), but not in the other genotypes (‘Micro-Tom’, *sit*, and *dgt*).

These results indicate that the root growth is stabilized at the onset of flowering and the inhibition of gibberellins biosynthesis does not affect it anymore. As *gib-3* is a gibberellin-deficient genotype, chlormequat chlolide action was more pronounced in function of the low endogenous gibberellins level of this genotype. According to Bensen and Zeevaart (1990), *gib-3* plants present reduced ability to synthesize *ent*-kaurene from copalyl diphosphate in function of decreased activity of the enzyme *ent*-kaurene synthase, the site of the mutation harbored by this mutant. Rademacher (2000) reports that chlormequat choride action on gibberellins biosynthesis is the activity inhibition of *ent*-kaurene synthetase and copalyl diphosphate synthase enzymes. Thus, the low activity of the *ent*-kaurene synthetase enzyme of *gib-3* was easily inhibited by chormequat chloride.

Daminozide is considered a growth retardant and it acts in the final steps of gibberellin biosynthesis, by inhibiting the conversion of inactive precursors to active gibberellins, like the conversion of GA_{20} into GA_1 (RADEMACHER, 2000). As it acts downstream in the gibberellin pathway, its effect on root development was intermediate between the control and chlormequat chloride (Table 7). However, daminozide resulted in RL inhibition under drought stress to the same dimension as chlormequat chloride did (Table 8). Under drought stress, ABA is overproduced and its inhibitory effects through DELLA proteins stabilization, resulting in repression of root elongation by interaction with gibberellins (ACHARD et al., 2006) may have be an additive effect to the daminozide effect on RL under the abiotic stress.

Table 7 – Root volume (RV) of the tomato mutant *gibberellin-deficient-3* (*gib-3*) sprayed with two plant growth regulators (PGR).

PGR	RV (cm^3)
Control	1.04 a
Chlormequat chloride	0.68 b
Daminozide	0.84 ab

Averages followed by distinct letters at the columns are different by the Tukey test at the probability level of 5% ($P \leq 0.05$).

It is interesting to observe plants not-treated with PGRs (control) presented a positive effect on RL under drought-stress, compared to those well-watered (Table 8). According to some authors (CREELMAN et al., 1990; SHARP et al., 1994), the

root elongation is stimulated by ABA accumulation in response to moderate stress. This effect was observed in this study (Table 8), where plants sprayed with water (control) under drought stress had longer roots than plants sprayed with water (control) but in well-watered condition. As control plants were not treated with gibberellins inhibitors, the root growth of plants under drought was possibly favored by the positive interaction of the overproduced ABA and gibberellins and auxins leading to inhibition of ethylene biosynthesis. According to McAdam et al*.* (2016) the increased ABA levels suppress ethylene biosynthesis, leading to reduction of auxins transport and prevention of their biosynthesis in the root tip, permitting the growth therein. The increased ethylene biosynthesis in absence of ABA has been studied by several researchers (SPOLLEN et al*.*, 2000; CHENG et al., 2002; SWARUP et al., 2007).

Table 8 – Root length (RL) of the mutant *gibberellin-deficient-3* (*gib-3*) sprayed with two plant growth regulators (PGR) and submitted to two watering regimes (WR).

PGR	RL (cm)	
	WR	
	Well-watered	Drought-stressed
Control	18.80 abB	23.78 aA
Chlormequat chloride	16.55 bA	15.65 bA
Daminozide	22.35 aA	15.68 bB

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows are different by the F test at the probability level of 5% (P≤0.05).

RDM and SDM were negatively affected by the drought stress presence (Table 9). These results highlight the growth inhibiting effect of ABA through interaction with gibberellins. As *gib-3* is deficient in gibberellins, there were not enough levels of this phytohormone to antagonize the action of the growth repressors (DELLA proteins). According to Piskurewics et al. (2008), ABA is involved in the transcription of a gene that encodes DELLA proteins. Sun (2010) reports that DELLA proteins stabilization is achieved after ABA treatment because of a reduction in gibberellins accumulation. The mutant *gib-3* has a small ability to produce

gibberellins and thus, the ABA overproduction in function of drought stress can easily stabilize DELLA. Zentella et al. (2007) also report that a mutant with deficiency in gibberellin biosynthesis failed to inhibit the gibberellin-induced degradation of DELLA proteins with the ABA application.

Table 9 – Root dry mass (RDM) and shoot dry mass (SDM) of the tomato mutant *gibberellin-deficient-3* (*gib-3*) sprayed with two plant growth regulators (PGR) and submitted to two watering regimes (WR).

WR	RDM (g)	SDM (x^0,5) (g)
Well-watered	0.09 a	0.83 a
Drought-stressed	0.07 b	0.63 b

Averages followed by distinct letters at the columns are different by the F test at the probability level of 5% (P≤0.05).

4.2 PGRs and ANEs effects on root growth of *Arabidopsis* seedlings

4.2.1 PGRs effect on root growth of *Arabidopsis* seedlings

4.2.1.1 Auxin action on root growth Arabidopsis seedlings

The effect of IAA applied in growth medium on root growth of *Arabidopsis* (Col-0) seedlings is observed in Figure 1. It is noticed that higher the concentration, stronger the inhibition. Both concentrations applied have promoted stronger inhibition than the ANE B, showing that this specific ANE might trigger an auxin signaling on plants, but not at same magnitude as the concentrations utilized. IAA applied in the medium in concentrations of 1 and 3 μM and absorbed by plants through the roots led to inhibitions of 32.99% and 21.93% compared to the control, respectively. Despite having no significant difference from the control, ANE A seems to do not activate such auxin signaling or act in root growth non-inhibition through another pathway.

Rayirath et al. (2008) have reported that high IAA concentrations as 10 and 100 μM applied in the growth medium resulted in inhibition of *Arabidopsis* primary root growth. Our results are showing that smaller concentrations (1 μM) IAA has still a strong inhibitory effect on root growth in length. Stepanova et al. (2007) reports that intact ethylene responses are required to activate the effects of exogenous auxins on root growth. According to Yoshii and Imaseki (1981), IAA increases the content of 1-

aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, leading to correspondent increase of the growth-inhibitor phytohormone. Swarup et al. (2007) report that auxins are involved in the root growth inhibition mediated by ethylene. The strong root growth inhibition by high IAA concentrations found in this study (Figure 1) might be due to the auxin-induced rise in ethylene levels inside the roots.

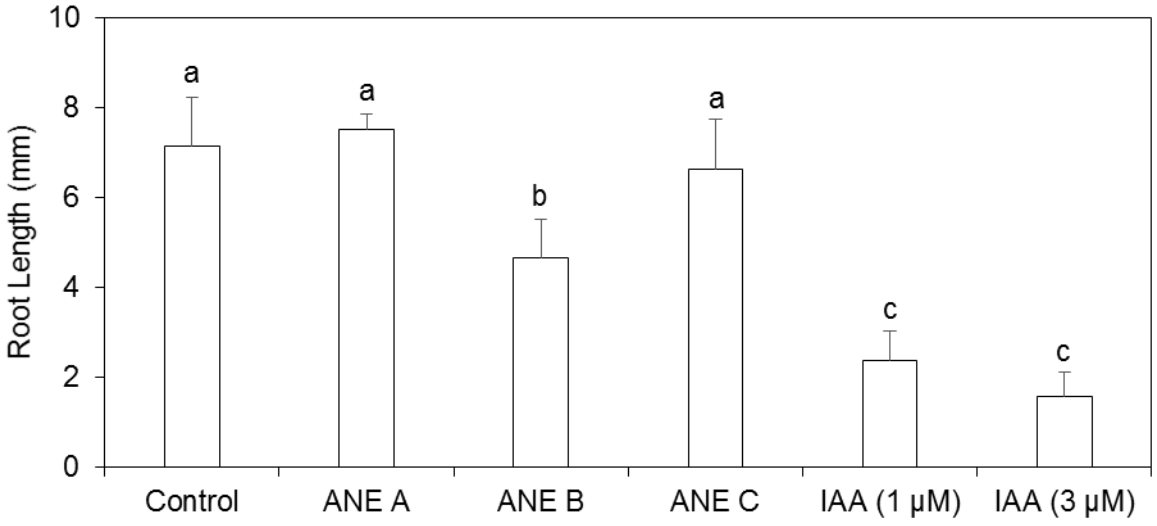

Figure 1 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C) and indole-3-acetic acid (IAA) on the root growth of 7-day-old *Arabidopsis thaliana* (Col-0) seedlings, two days after treatments. Different letters indicate significant differences by Tukey test at the probability level of 5% (P≤0.05). CV=16.36%, n=8 (bars represent ± SD). ANEs (0.04 weight volume^{-1}).

As shown in Figure 1, low IAA concentrations inhibit less the root growth. It is shown in Figure 2 that concentrations as low as 0.01 µM can promote root growth, generating an exponential effect. According to Ioio et al. (2008), auxins have opposing effects in root growth. These phytohormones can either promote root growth through meristem size increase or inhibit root growth by suppressing cell expansion in the elongation zone (IOIO et al., 2008). Ours results clearly show that root growth promotion or inhibition action by auxin is concentration-dependent.

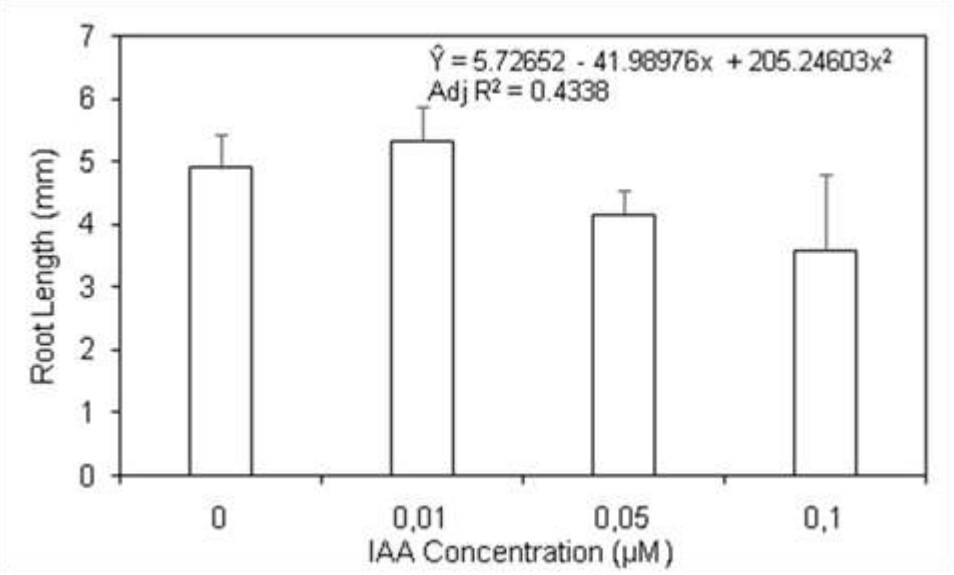

Figure 2 – Effect of the auxin, indole-3-acetic acid (IAA), on the root growth of 7-day-old *Arabidopsis thaliana* (Col-0) seedlings, three days after treatments. CV=17.65%, n=8 (bars represent ± SD).

Figure 3 shows that those low concentrations range from 0.001 to 0.05 µM. In both experiments (Figures 2 and 3), root measurements were taken three days after treatments, but the latter was carried out using the *Arabidopsis* genotype *AIR1-8::GUS*, instead of Col-0. Also, *AIR1-8::GUS* seedlings were 2 days older than Col-0. As the root growth of *AIR1-8::GUS* was negatively affected by the supplementation with 25 mM NH_4NO_3 when the controls are compared (2.76 mm against 4.90 mm), it shows the improved capacity of such low IAA concentrations in promoting root growth in the presence of nitrogen excess. Caba et al. (2000) reported that auxins levels were markedly decreased in soybean roots with the application of 8 mM KNO_3 and their sensitivity was improved. These reports support the improved root growth induction by such low IAA concentrations.

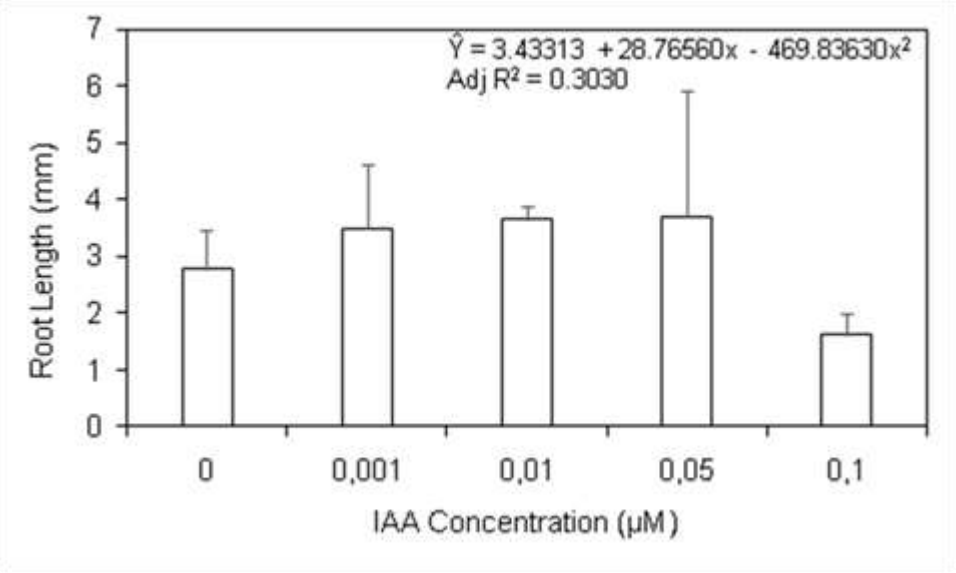

Figure 3 – Effect of the auxin, indole-3-acetic acid (IAA), on the root growth of 9-day-old *Arabidopsis thaliana* seedlings, reporter line *AIR1-8::GUS*, three days after treatments. CV=38.84%, n=8 (bars represent ± SD).

The expression of the auxin-inducible glutathione *S*-transferase gene promoter, *AIR1-8::GUS*, in 14-day-old *Arabidopsis* seedlings was decreased with the smallest IAA concentrations, according to the GUS staining assay (Figure 4). This assay was carried out in both shoots and roots at three days after application of IAA treatments in the root medium. Interestingly, 0.01 µM IAA inhibited considerably the promoter expression indicating that auxins were less produced in shoots and not transported to the roots, once no or very little blue color is perceived in roots. However, the control (0 µM IAA) showed intense blue color in leaves and also in the elongation zone and tip of roots, indicating that the basipetal auxin transport was active. It was observed that the *AIR1-8::GUS* promoter expression decreased with increasing IAA concentrations up to 0.001 µM IAA and increased again with higher concentrations.

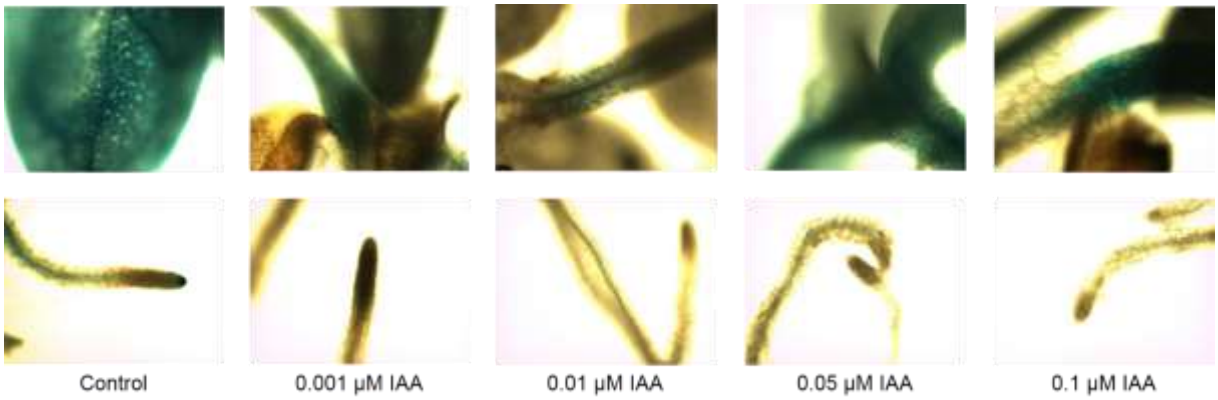

Figure 4 – Effect of the auxin, indole-3-acetic acid (IAA), on the endogenous auxin distribution (GUS staining) of 14-day-old *Arabidopsis thaliana* seedlings, auxin-responsive reporter line *AIR1-8::GUS*, three days after application of treatments in the bottom of the culture medium.

4.2.1.2 ABA action on root growth of Arabidopsis seedlings

The phytohormone ABA inhibits the root growth in a greater the concentration stronger the inhibition basis. This action is confirmed by the results shown in Figure 5, where the lowest concentration (0.5 µM) promoted an inhibition of 37.73% compared to the control whereas the highest (30 µM) showed root growth inhibition of 73.76%. According to Thole et al., (2014), ABA affects root growth through interaction with ethylene and auxin.

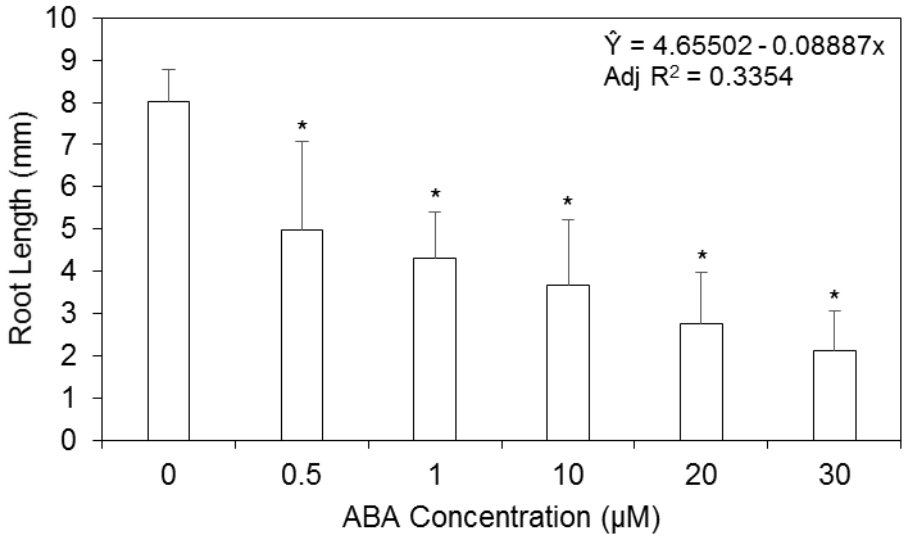

Figure 5 – Effect of abscisic acid (ABA) on the root growth of 10-day-old *Arabidopsis thaliana* (Col-0) seedlings, five days after treatments. *Significant differences by Dunnett test at the probability level of 5% (P≤0.05). CV=31.44%, n=8 (bars represent ± SD).

Analyzing the results presented in Figure 6, one can notice that this root growth inhibition might not exit depending on the time of evaluation after treatment. Instead of inhibition, low ABA concentration can slightly promote root growth. It is important to mention that treatments of the assay in Figure 5 were applied along with nitrogen supplementation of 25 mM NH_4NO_3. This result supports the evidence of root growth promotion by ABA under moderate abiotic stress conditions. According to Forde (2002), high nitrate concentrations in the root zone trigger the lateral root proliferation, especially in the local root zone where nitrate is more concentrated. Caba et al. (2000) reported that nitrate decreased auxins concentrations, but increased their sensitivity and showed little influence on cytokinins levels in soybean roots. According to these authors the root ABA level was decreased with the application of 8 mM KNO_3 compared to 1 mM KNO_3. The root growth inhibition noticed in our assay with nitrogen supplementation might be due to the increased ethylene biosynthesis induced by NO_3 as reported by Caba et al. (1998). It was observed that the root inhibition by the highest ABA concentrations was less pronounced, compared to the assay where the nitrogen supplementation was not realized. Instead of inhibition, ABA 0.5 µM promoted the root growth, suggesting that the root development mediated by ABA is dependent on ABA endogenous levels and interplay with other hormones, mainly ethylene.

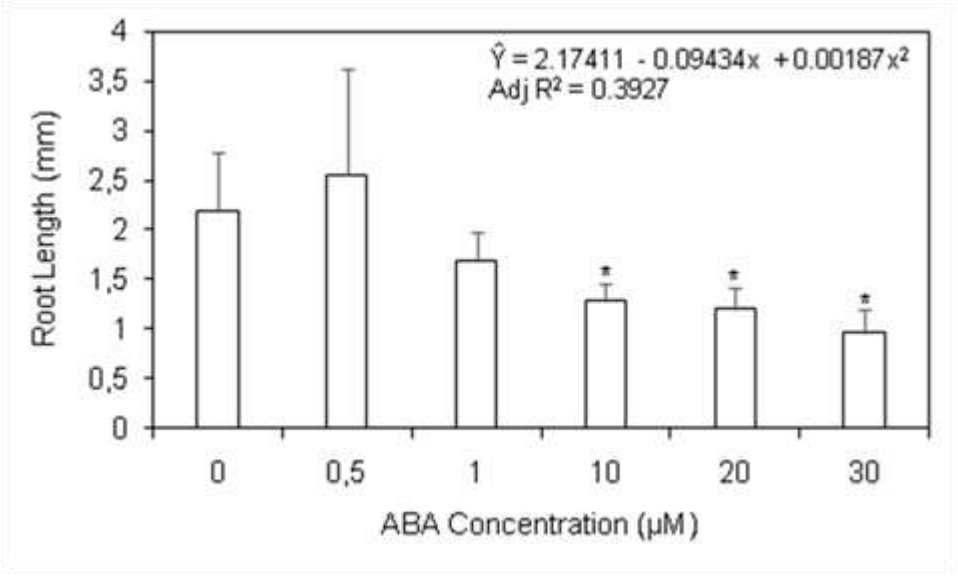

Figure 6 – Effect of abscisic acid (ABA) on the root growth of 10-day-old *Arabidopsis thaliana* (Col-0) seedlings, three days after treatments. *Significant differences by Dunnett test at the probability level of 5% (P≤0.05). CV=32.38%, n=8 (bars represent ± SD).

This evidence is strengthened by the results shown in Figure 7, where it is seen that 0.5 µM ABA led to improved root growth in PEG-induced osmotic stress condition, even at only 2 days after treatments application. However, no effect of ABA on root growth was significant when PEG was not applied. The supplementation with NH_4NO_3 was equally done for both PEG conditions.

According to Tran et al. (2007), low dosages of ABA and mild osmotic stress can stimulate root growth, but high ABA dosages or severe osmotic stress can lead to inhibition. As we show in Figure 7, the lowest ABA concentration applied promoted root growth, and this was observed for the short-term osmotic stress (2 days after treatments), once no difference was detected between ABA concentrations and the control without osmotic stress. According to Rowe et al. (2016), *Arabidopsis* seedlings under osmotic stress show increased ABA responses. These authors report that combined experimental analysis have revealed that ABA regulates root growth under osmotic stress conditions, but in interaction with ethylene, auxins, and cytokinins. According to Yoshii and Imaseki (1981), ABA inhibits ethylene production by inhibiting ACC biosynthesis whilst IAA and BA stimulates the ACC accumulation. Therefore, theses interactions are highly concentration-dependent and the differences found for ABA treatments under osmotic stress is possibly due to the imbalance of the three phytohormones. Thole et al. (2014) have stated that ABA regulates root elongation through the activity of auxin and ethylene and that these hormones act by a linear pathway to modulate the ABA response on the root elongation inhibition. These authors also affirm that auxin responsiveness is required for full ABA and ethylene responses. The root growth mediated by ABA observed for seedlings under osmotic stress effects (Figure 7) might be linked a decrease of endogenous auxin levels as reported by Rowe et al. (2016).

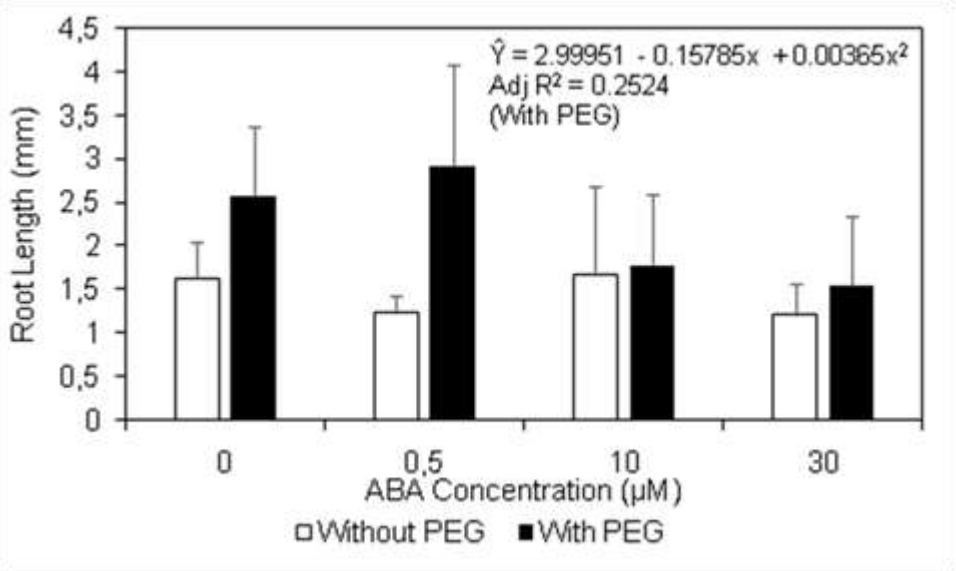

Figure 7 – Effect of abscisic acid (ABA) on the root growth of 7-day-old *Arabidopsis thaliana* (Col-0) seedlings in the presence of osmotic stress induced by poly(ethylene glycol) (PEG), two days after treatments. CV=41.06% and 41.14% (without and with PEG, respectively), n=8 (bars represent ± SD).

4.2.3 Root growth of *Arabidopsis* seedlings in response to ANEs treatments under osmotic stress conditions

The ANEs effect on *Arabidopsis* root growth was pronounced in increased time after treatments. This effect was also dependent on concentrations and numbers of applications. As it can be observed in Figure 8, only ANE B concentrations were different from the control with one application of treatments and evaluated two days later, resulting in an inhibition effect. ANE B 0.04 weight volume^{-1} was the concentration that most inhibited the root growth. For the assay conducted with two applications, root growth measurements taken seven and two days after first and second treatments applications, respectively, revealed the accentuation of ANE A 0.02 and 0.03 weight volume^{-1} on the root growth promotion. In this assay ANE B 0.03 weight volume^{-1} was the ANE concentration that inhibited root growth. The other ANE B treatments did not have significant difference compared to the control, although they have resulted in root growth of 6.70 and 5.72 mm against 7.24 mm of the control. ANE C 0.04 weight volume^{-1} caused the same inhibition effect as ANE 0.03 weight volume^{-1}.

Wally et al. (2013) reported a reduction in root growth of *Arabidopsis* seedlings in response to ANEs treatment. In our study, it was found that this effect is

dependent on the natural composition of the extract, since different extracts manufactured from the same seaweed species presented opposite effects on root growth.

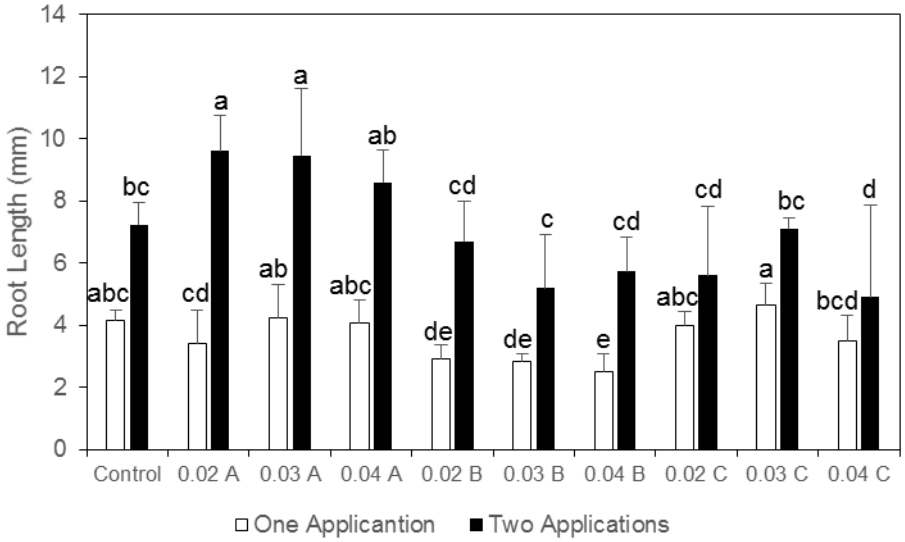

Figure 8 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C) on the root growth of 8-day-old (one application) and 12-day-old (two applications) *Arabidopsis thaliana* (Col-0) seedlings. Measurements were taken at two days after treatments (one application) and seven and two days after first and second application, respectively (two applications). Different letters indicate significant differences by Duncan test at the probability level of 5% (P≤0.05) within each way of ANEs application. CV=19.37% and 23.50% (one and two applications, respectively), n=8 (bars represent ± SD). 0.02 A = ANE A (0.02 weight volume^{-1}), 0.02 B = ANE B (0.02 weight volume^{-1}), and 0.02 C = ANE C (0.02 weight volume^{-1}).

It was carried out an assay aiming to test the effect of osmotic stress induced by PEG 40% on root growth and the action of ANEs and BA treatments in the amelioration of root growth impairment by the stress. No interaction effect between osmotic stress condition and ANEs and BA treatments was found. However, PEG 40% strongly inhibited root growth as shown in Table 10, indicating the osmotic stress generated in the root cells of *Arabidopsis* seedlings treated with the osmotic stress mediator substance. Osmotic stress leads to a crosstalk between the biosynthesis of cytokinins and signaling of auxin and ethylene (ROWE et al. 2016). This interplay between the phytohormones possibly results in altered responses in the whole plant, including the difference in root growth compared to non-stressed plants found in this assay.

Table 10 – Effect of osmotic stress induced by poly(ethylene glycol) 40% (PEG) on the root growth of 12-day-old *Arabidopsis thaliana* (Col-0) seedlings, eight days after treatments. CV=21.90%, n=16.

Osmotic Condition	Root Length (mm)
Without PEG	17.84 a
With PEG	12.87 b

Different letters indicate significant differences by F test at the probability level of 5% (P≤0.05).

In spite of none of the ANEs or BA treatments have present significant difference compared to the control, it is clear the tendency of ANE A in promoting root growth (Figure 9). This ANE effectivelly promoted root growth when compared to the other ANEs as well as the different BA concentrations. These observations were only noticed when PEG was not applied along with the treatments. When the osmotic stress inducer compound was applied, no significant difference among the treatments was found. However, observing the distribution of bars on Figure 9, one can see that the treatments showed the same pattern of root growth for both osmotic stress conditions (without or with PEG).

Small BA concentrations were applied, aiming to estimulate root growth, once high concentrations have resulted in strong growth-inhibiting effects. According to Vogel et al (1998) and Nordstrom et al (2004), cytokinin can promote ethylene and inhibit auxin biosynthesis, respectively. However, Jones et al. (2010) have evidenced that cytokinin also promotes auxin biosynthesis in young developing tissues. These crosstalks mediated by cytokinin may be the cause of non-stimulatory root growth response observed in this study.

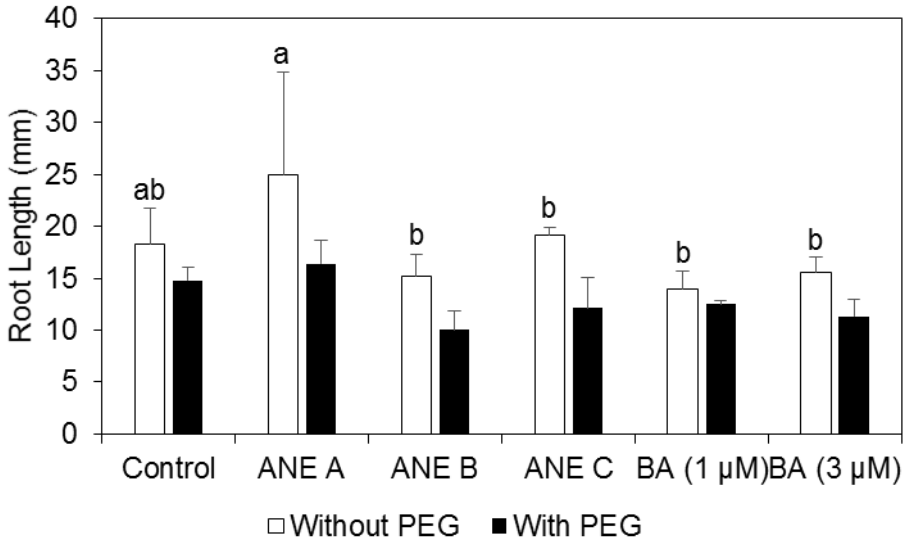

Figure 9 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C) and 6-benzyladenine (BA) on the root growth of 12-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at eight days after treatments application. Different letters indicate significant differences by Tukey test at the probability level of 5% (P≤0.05) for treatments without PEG. CV=21.90%, n=16 (bars represent ± SD). ANEs (0.03 weight volume^{-1}).

The effect of root growth promotion by ANE A when PEG is not present in root zone is confirmed in Table 11, whereas ANE B showed the smallest root growth. These observations were taken three days after treatments in 8-day-old *Arabidopsis* (Col-0) seedlings. No significant difference was found among ANEs and PGRs in the presence of PEG. However, ANE B and ABA showed tendency for inhibition and promotion of root growth, respectively.

Table 11 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 8-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at three days after treatments application. CV=17.16%, n=16. ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

Agrochemical	Root Length (mm)	
	Without PEG	With PEG
Control	6.20 bcA	5.56 aA
ANE A	7.48 aA	5.87 aB
ANE B	5.74 cA	5.10 aA
ANE C	7.32 abA	5.53 aB
ABA	6.76 abcA	6.08 aA
IAA	7.56 aA	5.50 aB

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows are different by the F test at the probability level of 5% (P≤0.05).

Comparing the osmotic conditions (without and with PEG), ANE A, ANE C, and IAA resulted in significant differences in *Arabidopsis* root growth (Figure 10), indicating that these ANEs and PGR promote the root growth but not in the presence of osmotic stress.

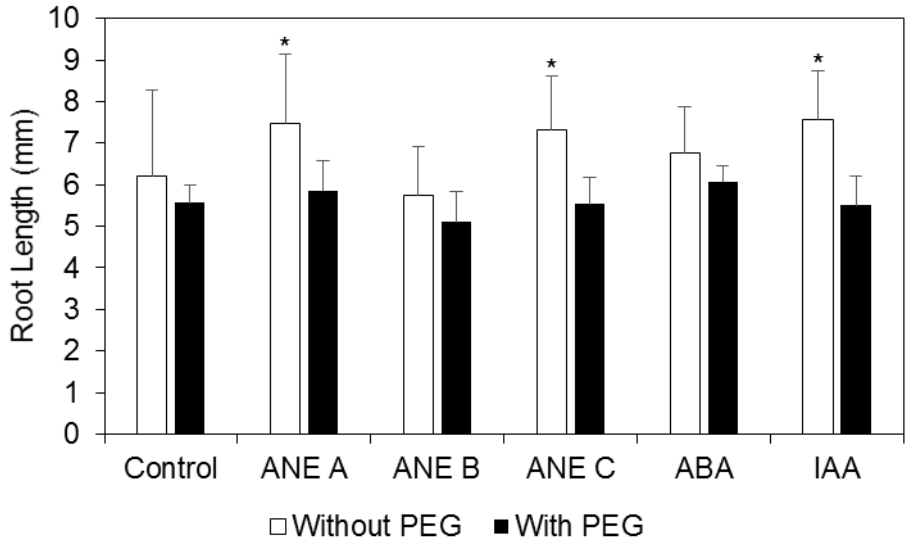

Figure 10 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 8-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at three days after treatments application. *Significant differences by F test at the probability level of 5% (P≤0.05). CV=17.16%, n=16 (bars represent ± SD). ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

The ANE A effect on root growth promotion was overcome by ANE C at six days after treatments when PEG was not applied, according to Table 12. ANE A was not either significantly different from ANE C or control, however, ANE C resulted in significant root growth compared to the control. In this assay, the nitrogen supplementation was realized with 0.5 mM NH_4NO_3 in the medium and also along with the treatments application. This amount of nitrogen may have improved the ABA-induced root growth compared to the previous assay, when 0.25 mM NH_4NO_3 was applied solely in the medium. We have found in our experiments (Figure 6) that ABA induces root growth in higher NH_4NO_3 concentrations. The root growth induction by ABA under such conditions might be through interactions with other hormones which have their levels altered (CABA et al., 1998; CABA et al. 2000). For the PEG-induced osmotic stress condition, no significant difference was observed within treatments with ANEs and PGRs.

Table 12 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 11-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at six days after treatments application. CV=41.32%, n=16. ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

Agrochemical	Root Length (mm)	
	Without PEG	With PEG
Control	9.92 bcA	6.61 aB
ANE A	12.93 abA	7.08 aB
ANE B	6.96 cA	5.11 aA
ANE C	16.04 aA	6.88 aB
ABA	17.53 aA	7.70 aB
IAA	0.97 bcA	8.50 aA

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows are different by the F test at the probability level of 5% (P≤0.05).

Taking into account the comparison of each ANE and PGR within the osmotic stress condition, ANE A, ANE B, and ABA resulted in root growth significantly greater without the osmotic stress induction (Figure 11). This assay was repeated and results were confirmed (Table 13 and Figure 12). The root growth of seedlings treated with ANE B was significantly different of those treated with ANEs A and C. This difference may be due to the differences in their compositions. Goñi et al. (2016) found highly significant differences (p≤0.001) in the contents of components of two ANEs (i.e. polyphenol, uronics, and laminarin). One can note that IAA resulted in similar root growth in both stressed and non-stressed conditions. This observation highlights the involvement of auxin in rescuing root growth under mild osmotic stress reported by Rowe et al. (2016). According to these authors, auxin in small concentrations is able to rescue the root growth under mild osmotic stress and partially under severe stress.

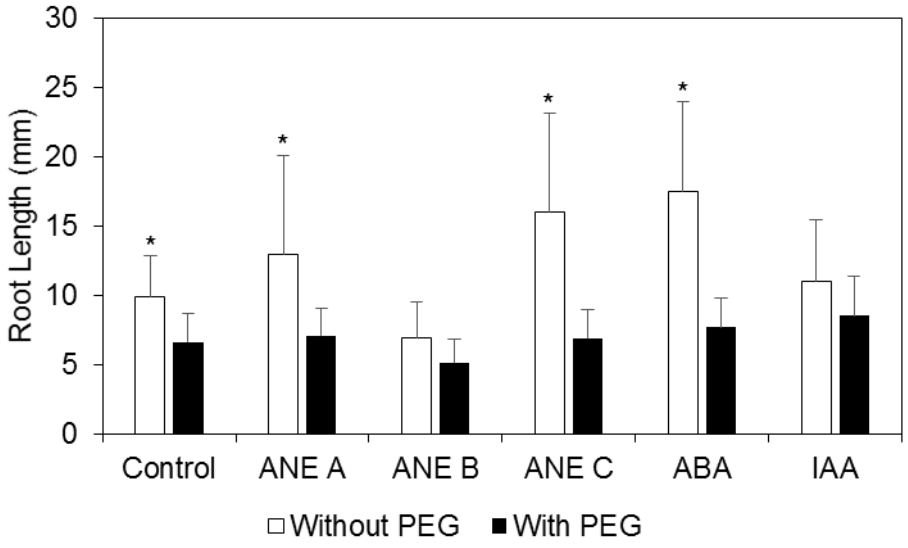

Figure 11 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 11-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at six days after treatments application. *Significant differences by F test at the probability level of 5% (P≤0.05). CV=41.32%, n=16 (bars represent ± SD). ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

The ANE A effect in root growth promotion was confirmed by the assay repetition (Table 13). In this second assay, ANE A effect on root growth was significant higher than the control root growth as well as that of ANE C, the second highest growth, without the osmotic stress treatment. According to Wally et al. (2013) the *Arabidopsis* gibberellins contents increase with ANEs application. Goñi et al. (2016) found up- and down-regulation of gibberellin-responsive genes in response to ANE B treatment, *GASA1* and *GASA4*, respectively.

ABA, in small concentration, has confirmed its tendency to promote root growth. ANE B has also confirmed its effect in root growth inhibition (Table 13). IAA, as seen in the previous assay, did not have effect in neither root growth promotion nor inhibition, once it did not have significant difference from the control. In presence of PEG-induced osmotic stress, none of the ANEs and PGRs showed significant differences among themselves. This result might be due to the degree of osmotic stress generated, once the controls, without and with PEG, were significantly different.

Table 13 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 11-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at six days after treatments application. CV=22.26%, n=8. ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

Agrochemical	Root Length (mm)	
	Without PEG	With PEG
Control	20.15 cdA	15.02 aB
ANE A	33.04 aA	15.98 aB
ANE B	17.16 dA	11.67 aB
ANE C	25.86 bA	14.04 aB
ABA	24.97 bcA	14.48 aB
IAA	18.90 dA	14.60 aB

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows are different by the F test at the probability level of 5% (P≤0.05).

Now, looking at the effect of osmotic stress generated by PEG, it can be observed that all treatments presented significant difference by the comparison of PEG conditions, being the greatest difference observed for ANE A. It is noticeable the tendency of ANE B to inhibit root growth observing only osmotic-stressed treatments in Figure 12. This pattern of treatments response was observed in all assays made with osmotic stress condition. It was also noticed that ANE B effect applied without PEG had similar results as the control with PEG application for all assays. This observation emphasizes the strong ANE B effect on root growth inhibition.

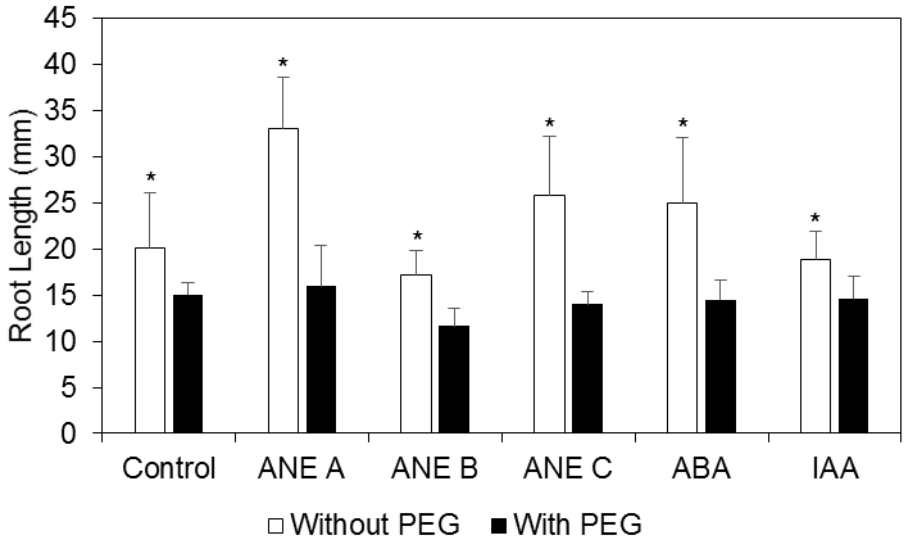

Figure 12 – Effect of different *Ascophyllum nodosum* extracts (ANE A, ANE B, and ANE C), abscisic acid (ABA), and indole-3-acetic acid (IAA) on the root growth of 11-day-old *Arabidopsis thaliana* (Col-0) seedlings, in the presence of osmotic stress induced by poly(ethylene glycol) (PEG). Measurements were taken at six days after treatments application. *Significant differences by F test at the probability level of 5% (P≤0.05). CV=22.26%, n=8 (bars represent ± SD). ANEs (0.03 weight volume^{-1}), ABA (0.5 µM), IAA (0.01 µM).

This research demonstrates that auxin in a small concentration (0.5 µM) initially promotes root growth, but it does not show this effect along the time. As it can be observed in Table 11, auxin treatment led to a significant root growth when the root measurements were taken three days after, but at six days (Tables 12 and 13), no differences from the control were observed, despite of clear trends of root growth inhibition. According to Fu and Harberd (2003), auxin regulates root growth by controlling gibberellin responses and that shoots modulate the root growth in distance through auxin biosynthesis that is transported to the roots. These authors mention that auxin modulates gibberellin responses by destabilizing the nuclei DELLA proteins, the gibberellin responses repressors. Nordstrom et al. (2004) report that auxin can rapidly down-regulate cytokinins biosynthesis, root growth inhibitors, and these are the possible mechanisms by which auxin promoted root growth shortly after the IAA treatment. The endogenous level of auxin in roots might has been attenuated from four days after treatments and at six days after treatment application, when root length measurements were taken, any effect of IAA applied was detected anymore. According to Rosquete et al. (2012), not only the biosynthesis but the endogenous accumulation of auxin is tightly controlled to keep the homeostasis

within the organ. Aiming to inactivate the excess auxin present in roots and alleviate its action, the phytohormone is conjugated, mainly to amino acids and sugars, or degraded (BAJGUZ; PIOTROWSKA et al., 2009). The conjugation or degradation of endogenous auxin present inside the root in function of IAA treatment might be reason of no difference in root growth of IAA-treated plants compared to the those non-treated (control).

4.3 Effect of ANEs on hormonal response in roots of tomato reporter lines

4.3.1 GUS activity in roots of ethylene-responsive (MT) EBS::GUS reporter line plants in response to ANEs and ABA foliar spray

A quick assay was carried out aiming to obtain an overview of the ANEs effect in the ethylene response in roots of the tomato reporter line (MT) *EBS::GUS* (Table 14). The phytohormone action was measured by the GUS activity, which is the amount of 4-methylumbelliferyl (MU) generated by β-glucuronidase enzyme reaction (detected by fluorescence) divided by (incubation time * protein content of sample). Only one plant per plot was used in this assay and GUS activity (fluorometric assay) analysis performed in triplicates. ANE B showed effective in increasing the ethylene-responsive *EBS::GUS* promoter expression indicating possible increase in endogenous ethylene level in roots at 24 hours after foliar spray, in normal conditions of watering. This possible increase in ethylene level in tomato roots may explain the root growth inhibition previously observed in *Arabidopsis*. What should be pointed out from this experiment is the possible influence of ANE B in inhibiting ethylene response or accumulation in response to drought stress. Fan et al. (2011) report that an ANE was found to be effective in improving resistance to drought in several plant species. This resistance might be linked to the increase in bioactive molecules such as antioxidants agents in ANE treated plants (RAYIRATH et al., 2009).

Table 14 – Effect of *Ascophyllum nodosum* extracts (ANE B and ANE C) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 77-day-old tomato ethylene-responsive reporter line (MT) *EBS::GUS* at 24 hours after treatments spray and six days after water withholding. ANE A (0.33 volume volume^{-1}), ANE C (0.5 volume volume^{-1}). CV=7.67%.

Agrochemical	GUS Activity (pmol MU/min*mg protein)	
	Well-watered	Drought-stressed
Control	12.18 bA	11.96 aA
ANE B	21.34 aA	4.90 bB
ANE C	11.46 bA	11.48 aA

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows are different by the F test at the probability level of 5% (P≤0.05).
MU = 4-methylumbelliferyl

ANE B effect on root ethylene-mediated *EBS::GUS* promoter expression was significant different for well-watered and drought-stressed conditions (Figure 13). No significant difference was observed for the control and ANE C treatments. ANE C seems to do not influence the ethylene level in tomato roots.

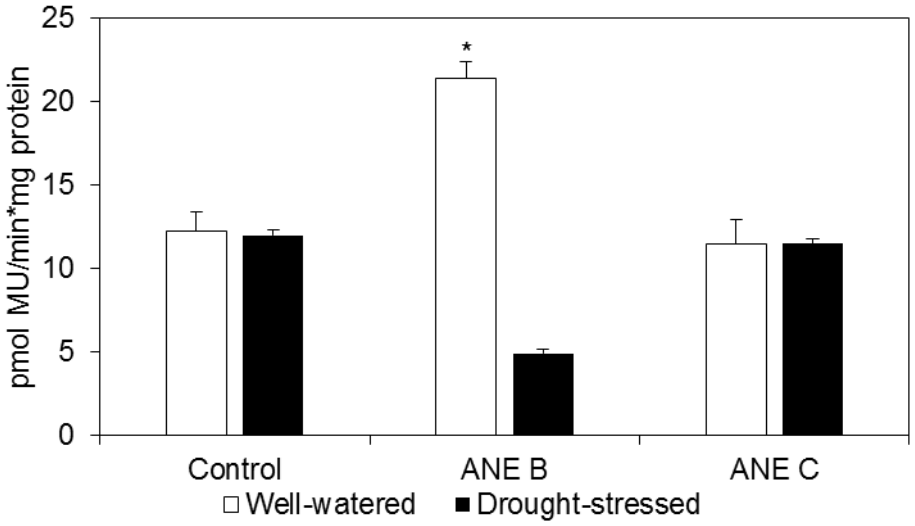

Figure 13 – Effect of *Ascophyllum nodosum* extracts (ANE B and ANE C) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 77-day-old tomato ethylene-responsive reporter line (MT) *EBS::GUS* at 24 hours after treatments spray and six days after water withholding. ANE B (0.33 volume volume^{-1}), ANE C (0.5 volume volume^{-1}). *Significant differences by F test at the probability level of 5% (P≤0.05). CV=7.67%. MU = 4-methylumbelliferyl (bars represent ± SD for n=3 replicates).

There was found no significant effect of ANEs and ABA on the expression of ethylene-responsive *EBS::GUS* promoter in roots of the tomato reporter line in 44-day-old plants at 24 hours after foliar spray. Also, no effect of drought stress on the hormone response was detected, at six days of water withholding (Table 15). Even not resulting in significant differences, it is clear the tendency of increasing ethylene responses for the treatments in the following order: control, ABA, ANE A, and ANE B (Figure 14).

The influence of ANEs and ABA, at 48 hours after foliar spray and 7 days after water withholding, on GUS activity in roots of tomato reporter line (MT) *EBS::GUS*, 44-day-old plants, only had significant results at 48 hours after treatment and in presence of drought stress (Table 15). ABA inhibited whilst ANE A favored the *EBS::GUS* promoter expression. These responses are not different from the control, but they are different of each other. The ethylene response in plants sprayed with ANE B was similar to the control, indicating no effect of this extract on the hormone action in young tomato plants (Figure 15).

Table 15 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 45-day-old tomato ethylene-responsive reporter line (MT) *EBS::GUS* at 24 and 48 hours after treatments spray and six and seven days after water withholding, respectively. CV=16.15%, n=3. ANEs (0.33 volume volume^{-1}), ABA (200 µM).

Agrochemical	GUS Activity (pmol MU/min*mg protein)			
	24 hours		48 hours	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Control	54.46 aA	52.59 aA	77.47 aA	56.35 abA
ABA	64.40 aA	71.46 aA	74.50 aA	51.82 bA
ANE A	72.60 aA	77.98 aA	80.52 aA	88.59 aA
ANE B	60.90 aA	77.71 aA	79.24 aA	74.00 abA

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows for each time course are different by the F test at the probability level of 5% (P≤0.05).
MU = 4-methylumbelliferyl

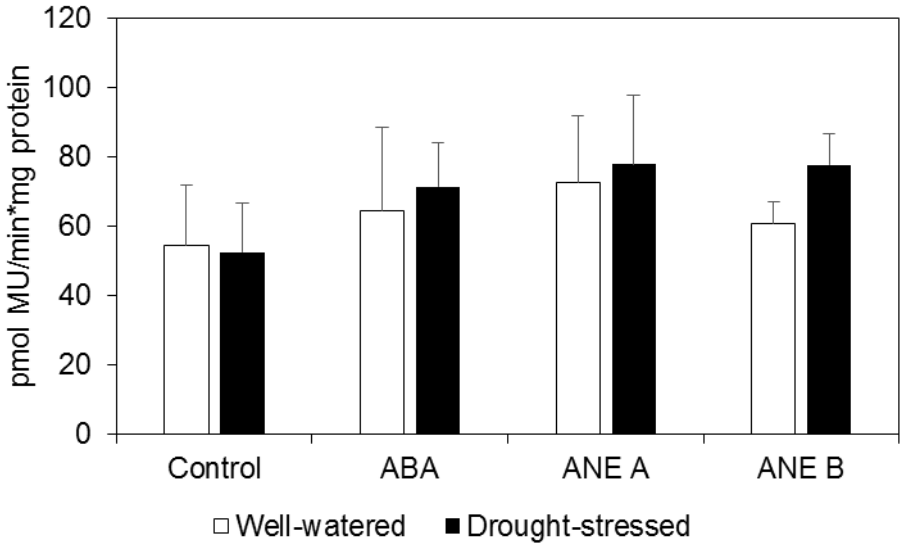

Figure 14 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 44-day-old tomato ethylene-responsive reporter line (MT) *EBS::GUS* at 24 hours after treatments spray and six days after water withholding. CV=20.87%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), ABA (200 µM), MU = 4-methylumbelliferyl.

The fact of ANE A in increasing the ethylene responses, compared to ABA foliar treatment, in roots under drought stress can indicate no plant tolerance to drought conferred by this ANE. Goñi et al*.* (2016) report the ANE-induced up-regulation of two ethylene-responsive transcription factors, *ERF2* and *ERF72*. Additionally, there have been found ANEs-induced up-regulations of two putative 2-oxoglutarate-dependent dioxygenases (*At5g43450* and *At2g25450*) genes, key enzymes in the final steps of ethylene biosynthesis (JANNIN et al*.*, 2013; GOÑI et al*.*, 2016). There are several reports affirming that ethylene is a root growth inhibitor. According to Swarup et al. (2007), ethylene produces this response by stimulating auxin biosynthesis and basipetal transport to the root elongation zone via control of efflux and influx carriers PIN2 and AUX1, respectively. The ABA treatment somehow inhibits the ethylene biosynthesis or its action in roots and hence it confers tolerance to drought stress by permitting the root growth to moister soil layers.

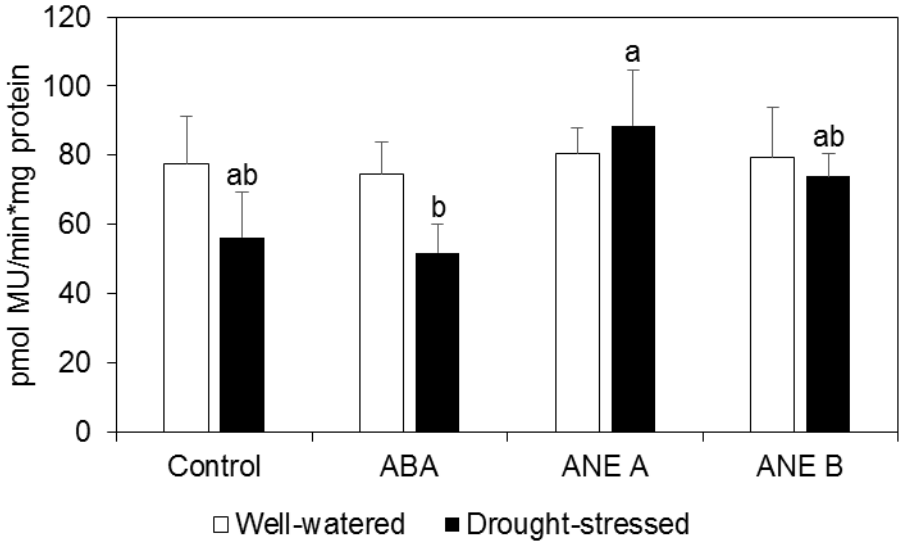

Figure 15 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 45-day-old tomato ethylene-responsive reporter line (MT) *EBS::GUS* at 48 hours after treatments spray and seven days after water withholding. Different letters indicate significant differences by Tukey test at the probability level of 5% (P≤0.05) for the drought-stressed treatments. CV=16.15%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), ABA (200 µM), MU = 4-methylumbelliferyl.

4.3.2 GUS activity in roots of ABA-responsive (MT) RD29b::GUS reporter line plants in response to ANEs and ABA foliar spray

The effect of ANEs and ABA foliar spray on ABA-responsive *RD29b::GUS* promoter in roots was evaluated in an experiment with the tomato reporter line in 81-day-old plants. It was not detected any significant differences among ANEs and ABA treatments within both watering conditions (Table 16).

Table 16 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 81-day-old tomato ABA-responsive reporter line (MT) *RD29b::GUS* at 24 and 48 hours after treatments spray and six and seven days after water withholding, respectively. CV=64.54%, n=3. ANEs (0.33 volume volume^{-1}), ABA (200 µM).

Agrochemical	GUS Activity (pmol MU/min*mg protein)			
	24 hours		48 hours	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Control	0.67 aA	2.30 aA	0.65 aA	1.38 aA
ABA	0.48 aA	1.63 aA	0.53 aA	1.18 aA
ANE A	1.26 aA	2.24 aA	0.64 aB	1.78 aA
ANE B	0.97 aB	4.42 aA	0.56 aA	1.42 aA

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows for each time course are different by the F test at the probability level of 5% (P≤0.05).
MU = 4-methylumbelliferyl

However, by comparing the watering conditions, ANE B foliar spray showed significant difference in the ABA-induced gene expression, leading to greater β-glucuronidase activity in roots under the drought stress condition (Figure 16). Classic researches have demonstrated that ABA biosynthesis increase under drought stress (ZHANG; DAVIES, 1987). Allen et al. (2001) report a significant increase in β-carotene biosynthesis, a precursor of ABA, in plants treated with ANEs. Goñi et al. (2016) results show a five-fold increase in *LEA* genes regulation in *Arabidopsis* plants treated with ANE B compared to ANE A treatment. ANEs A and B studied by Goñi et al. (2016) were manufactured by the same extraction processes as ANEs A and B utilized in our study. According to Hundertmark and Hincha (2008), there are inductions in the expression of more than three-fold for most of *LEA* genes following ABA treatment. These authors report that despite *LEA* proteins accumulate mainly in seeds, they are also found in vegetative tissues in response to environmental stresses such as dehydration. It was found an increased response to ABA in ANE B-treated plants under drought stress (Figure 16), which indicates possible ABA biosynthesis induction by this ANE. The improved-ABA response or possible ABA accumulation can provide plants with drought tolerance via *LEA* proteins production.

Observing Figures 16 and 17 one can note the clear trend of ABA to accumulate in roots of drought-stressed plants. The higher response to ABA in

function of only one of the ANEs treatments reinforces the findings of Wally et al. (2013) that the variability in levels of hormonal biosynthesis-stimulating compounds in ANEs results in variable effects in plant growth and development. Goñi et al. (2016) found that two extracts manufactured by different processes from the same seaweed species show different influences on genes regulation. According to these authors one ANE dysregulated 4.47% of the transcriptome, whereas another one dysregulated 0.87%, however, there were more up-regulated than down-regulated genes in response to ANEs treatments. Additionally, the two ANEs had composition significantly different, especially the polyphenol content, what can be the source of variability in genes regulation in treated plants (GOÑI et al., 2016).

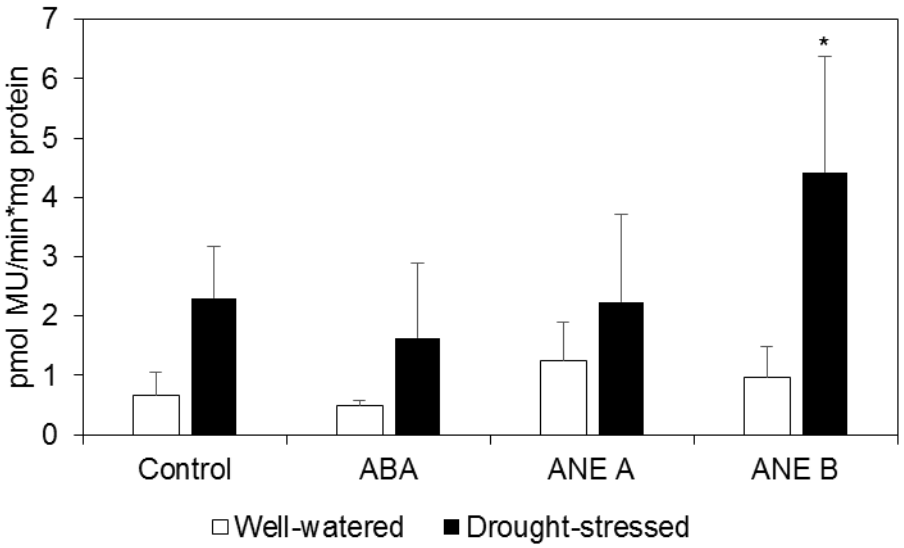

Figure 16 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 81-day-old tomato ABA-responsive reporter line (MT) *RD29b::GUS* at 24 hours after treatments spray and six days after water withholding. *Significant differences by F test at the probability level of 5% (P≤0.05). CV=64.54%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), ABA (200 µM), MU = 4-methylumbelliferyl.

At 48 hours after treatments and seven days after watering withholding, it also was observed no significant effect of ANEs and ABA foliar spray on ABA-responsive *RD29b::GUS* promoter in roots within each drought stress condition (Table 16). However, ANE A led to increased *RD29b::GUS* expression by the comparison of watering conditions. Rayirath et al. (2009) found an increase of two-fold transcription of the drought-responsive gene *RD29a*. Wally et al. (2013) verified

significant increase in ABA content in *Arabidopsis* ANE-treated plants and noticed an up-regulation of the gene of the key enzyme in the ABA biosynthesis pathway, *NCED3*. Goñi et al. (2016) also detected influence of ANE B on regulation of *NCED* genes family, *NCED4* herein. ANE B was similar to the control in both watering conditions (Figure 17). At 24 hours after spray, ANE B showed effect in increasing the *RD29b::GUS* promoter expression in drought stress condition, but this effect was not observed at 48 hours. In contrast, ANE A that did not had significant difference on the expression of the ABA-responsive promoter at 24 hours, showed to have this effect after 48 hours. This result suggest that the interplay of hormonal-stimulating compounds of ANEs is very complex and can lead to different results upon the time.

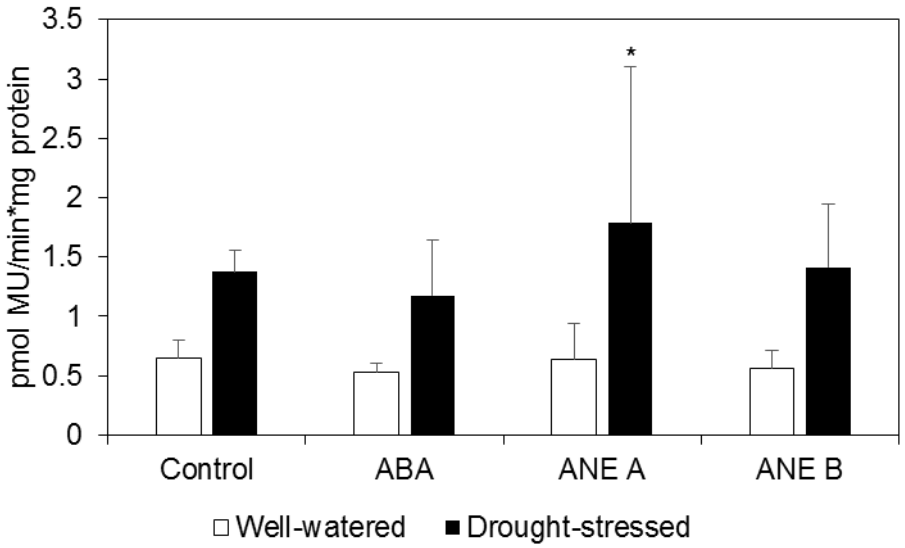

Figure 17 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and abscisic acid (ABA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 82-day-old tomato ABA-responsive reporter line (MT) *RD29b::GUS* at 48 hours after treatments spray and seven days after water withholding. *Significant difference by F test at the probability level of 5% (P≤0.05). CV=49.13%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), ABA (200 µM), MU = 4-methylumbelliferyl.

4.3.3 GUS activity in roots of auxin-responsive (MT) DR5::GUS reporter line plants in response to ANEs and IAA foliar spray

There was no effect of ANEs on IAA-inducible *DR5::GUS* promoter expression in roots of 79-day-old tomato at 24 hours after spray and six days after watering withholding, compared to the control (Table 17). This reporter line carries a high active synthetic promoter responsive to auxin and poses as a very useful tool to

evaluate auxin-induced responses of plants treated with ANEs (RAYIRATH et al., 2008). This research shows that ANEs do not trigger any significant auxin response in tomato roots in a short period (24 hours) after the foliar spray, once no difference in β-glucuronidase enzyme activity in roots of plants sprayed with ANEs and the control was detected. According to Rayirath at al. (2008), ANEs have shown to be effective in activating the DR5 promoter in *Arabidopsis* plants. This study shows that ANEs activation of the synthetic auxin-responsive promoter located in cells of tomato roots is slow, detected 48 hours after treatment, and only for one of two ANEs tested (Figure 19). But the foliar application of a high IAA concentration (100 mM) is strongly effective to activate the promoter in roots after 24 hours, compared to the control (Figure 18).

Kingman and Moore (1982) have reported that the concentration of IAA in ANEs is estimated to reach 50 mg g^{-1} ANE dry mass as early as 80's. More recently, Wally et al. (2013) found that levels of auxins and their precursors present in commercial seaweed extracts are relatively very low. The auxin IAA, for example, ranges from 3 to 35 ng g^{-1} extract dry matter, depending on the seaweed source. According to the authors, these auxin levels are not able to influence the development or growth of plants. It was verified in our experiments that any auxin response was activated in tomato root system after the application of ANEs on leaves. This finding comes up as an evidence that ANEs neither influence plant auxin-mediated responses by stimulating auxin biosynthesis nor its compositional auxin metabolites is detected by the DR5 promoter as an auxin response in tomato roots.

Table 17 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) and indole-3-acetic acid (IAA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 79-day-old tomato auxin-responsive reporter line (MT) *DR5::GUS* at 24 and 48 hours after treatments spray and six and seven days after water withholding, respectively. CV=88.02%, n=3. ANEs (0.33 volume volume^{-1}), IAA (100 mM).

Agrochemical	GUS Activity (pmol MU/min*mg protein)			
	24 hours		48 hours	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Control	2.21 bA	0.86 bA	2.81 bA	4.06 aA
IAA	69.62 aA	51.32 aA	69.89 aA	11.46 aB
ANE A	3.12 bA	1.94 bA	11.90 bA	1.41 aB
ANE B	2.73 bA	2.87 bA	1.74 bA	1.00 aA

Averages followed by distinct lower-case letters at the columns are different by the Tukey test at the probability level of 5% (P≤0.05).
Averages followed by distinct upper-case letters at the rows for each time course are different by the F test at the probability level of 5% (P≤0.05).
MU = 4-methylumbelliferyl

The effect of IAA foliar spray on IAA-induced response in roots was significantly different of other treatments in both watering conditions (Figure 18). The application of a 100 mM IAA solution on leaves of well-watered plants led to β-glucuronidase activity in roots of 3146.65% higher than that of the control. In spite of resulting in a slightly small β-glucuronidase enzyme activity, the auxin response in roots of drought-stressed plants to 100 mM foliar spray was not significantly different of that of well-watered plants.

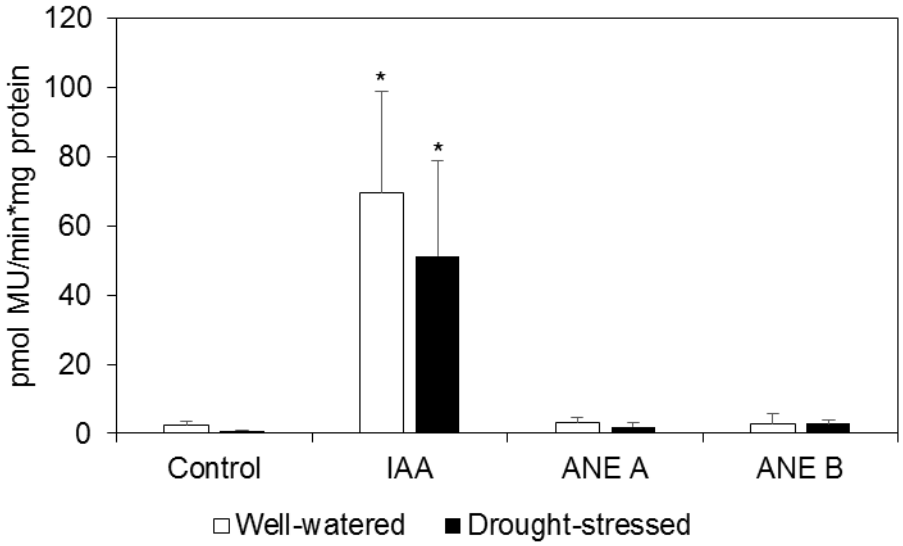

Figure 18 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) indole-3-acetic acid (IAA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 79-day-old tomato auxin-responsive reporter line (MT) *DR5::GUS* at 24 hours after treatments spray and six days after water withholding. *Significant differences by Tukey test at the probability level of 5% (P≤0.05) within both watering regime treatments. CV=88.02%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), IAA (100 mM), MU = 4-methylumbelliferyl.

At 48 hours after foliar spray and seven days after watering withholding, the root auxin-induced β-glucuronidase activity due to IAA foliar spray decreased significantly in drought stress condition (Table 17).

By doing comparisons of each treatment within the watering conditions, IAA resulted in significant difference, due to the decreased auxin-induced *DR5::GUS* promoter expression (measured by the β-glucuronidase activity) in roots of drought-stressed plants (Figure 19). According to Rowe et al. (2016), osmotic stress results in decrease of root auxin concentration, by regulating auxin transporter levels and localization. These authors observed that the PIN proteins levels are reduced under stress in an ABA-dependent way. This finding may explain the significant auxin decrease in root auxin level of (MT) *DR5::GUS* plants at seven days under drought stress and 48 hours after spray with 100 mM IAA, compared to IAA-treated but well-watered plants (Figure 19).

Interestingly, ANE A led to increased IAA-induced DR5::GUS expression in well-watered plants compared to those drought-stressed (Figure 19). This increase

was of 8.78 pmol MU/min*mg protein, ranging from 3.12 pmol MU/min*mg protein at 24 hours after spray to 11.90 pmol MU/min*mg protein at 48 hours. By quantifying the hormone levels in *Arabidopsis* rosette leaves at 24, 96, and 144 hours after treatment with ANE, Wally et al. (2013) reported that the auxin level was decreased compared to the control. Our study reveals a slight increase in root auxin level in response to ANE A foliar spray at 48 hours following treatment in well-watered plants, but any increase was observed for ANE B (Figure 19). This fact show how the hormonal responses or biosynthesis induction by ANEs is variable depending on the ANE source and also on the time after spray, once it was not found any difference in the auxin-induced promoter expression at 24 hours (Figure 18). According to Wally et al. (2013) the variability in concentration of hormonal biosynthesis-stimulating compounds results in variable effects in plant growth and development.

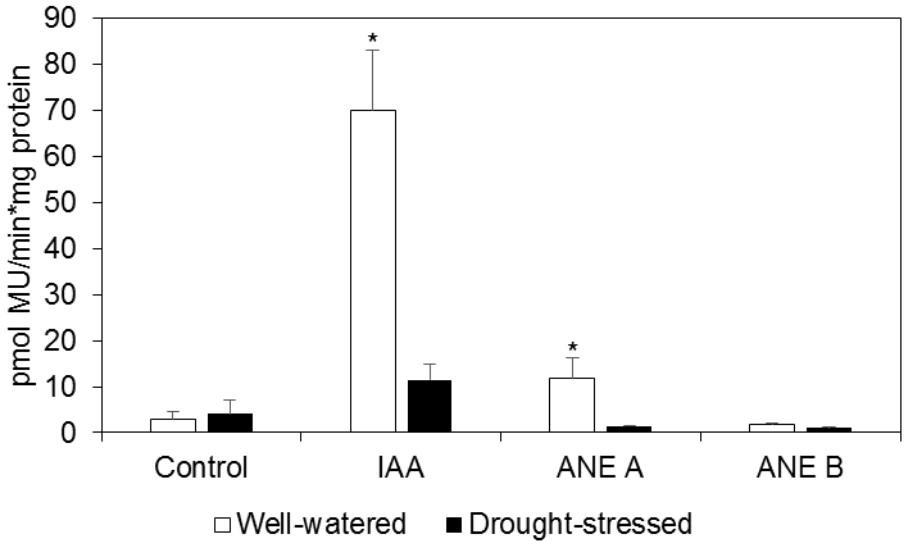

Figure 19 – Effect of *Ascophyllum nodosum* extracts (ANE A and ANE B) indole-3-acetic acid (IAA) on the GUS activity (β-glucuronidase fluorometric assay) in roots of 80-day-old tomato auxin-responsive reporter line (MT) *DR5::GUS* at 48 hours after treatments spray and seven days after water withholding. *Significant differences by F test at the probability level of 5% (P≤0.05). CV=40.23%, n=3 (bars represent ± SD). ANEs (0.33 volume volume^{-1}), IAA (100 mM), MU = 4-methylumbelliferyl.

5 GENERAL DISCUSSION

The future of humanity has been thrown in a scene of uncertainties with recent debates concerning climate changes and growth of world population. Governments, scientists, agronomists, farmers, and consumers are more than ever assuming some compromises and habits aiming to guarantee the future of next generations. There are some catastrophic assumptions that the food production will not be enough for feeding around 9 billion people by 2050's. The first challenge is producing sufficient amounts of food to guarantee the food security of such massive global population in a supposed changing environment. We need to be concerned not only about the huge amounts expected to be required, but producing food with quality and economically affordable to everyone.

Classical agricultural inputs are not well-seen by consumers anymore, who are demanding produce obtained from environmentally friendly production systems. In addition, the increasing tolerance of weeds, pests, and pathogens to classical agrochemicals is opening a space for new and natural products, aiming to improve the produce quality and provide healthier growth conditions to the crops, such as tolerance to stresses. In this study we searched for the understanding of how seaweed-based biostimulants can fit to those needs mentioned above.

We grew tomato and *Arabidopsis* plants in stressful conditions (drought and PEG-induced osmotic stress) treated with classical plant growth regulators and seaweed extracts biostimulants and looked at the effects of these substances on root traits and hormonal responses in those organs. We have verified that drought stress damages the root development, compromising the growth of shoots, except in auxin low-sensitivity tomato mutant, suggesting a minor role of this hormone in plant stresses responses. We also verified that gibberellin-biosynthesis inhibition by classical PGRs does not affect the root system growth of tomato genotypes in well-watered conditions and neither helps plants to cope with the stress generated by drought. Gibberellin inhibition by the PGRs used in this study just affected the root growth of the gibberellin-deficient genotype with mutation at the same site of action of these compounds.

We tried to find exogenous dosages of PGRs (IAA, ABA, and BA) that can match the effects of ANE treatment on root growth of *Arabidopsis* seedlings. Here, we can draw the conclusion that it is strictly hard to attribute ANEs effects on root growth to each class of phytohormone isolated because of the lack of consistency in

the results, in function of the possible multi-hormonal-stimulatory action of these substances. It was found that ANE B has the property of inhibiting the root growth of *Arabidopsis* seedlings. It was initially thought that this inhibition effect was by means of its supposed action on endogenous auxin-stimulatory biosynthesis. However, it was found no effect of this ANE on the auxin-responsive promoter *DR5::GUS*, compared to untreated plants. Instead of auxin response, it was found variable responses to ethylene and ABA, evaluated with the genetic constructs carrying the promoter *EBS::GUS* and *RD29b::GUS*, responsive to ethylene and ABA, respectively. These findings indicate the possible roles of ANEs on abiotic stress attenuation by modulating ethylene and ABA responses and/or accumulation. However, these responses were reversed between ANE A and ANE B from 24 to 48 hours after treatment, indicating the possible impact of their composition on plant biostimulation.

We noticed that the plant hormonal responses to ANE treatment are intricate and influenced by several factors of variation during the growth of plants possibly due to complex crosstalk networks among the phyhormones. Therefore, elucidating these networks in the molecular levels is the crucial next step to form a system view that aids in the understanding of plant responses to ANE application, both under abiotic stress and normal conditions for plant development. After progress in unveiling the molecular mechanisms behind the biostimulatory actions of these natural substances, it is possible to deliver to growers consistent biostimulant products that attend their needs in the mission of feeding the world.

6 CONCLUSIONS

This research is an important step in the understanding of how seaweed biostimulants can act in the hormonal status of plants growing in conditions of abiotic stress by water deficit.

We conclude that the root system development of the tomato ‘Micro-Tom’ and *Arabidopsis* is negatively affected by the occurrence of water deficit caused by drought and osmotic stress, respectively. Classical gibberellins biosynthesis inhibitors only affect the root development of the tomato gibberellin-defficient mutant, *gib-3*.

Treatment of plants with seaweed extracts manufactored from the same brown macroalga *A. nodosum* (ANEs) by different processes of extraction results in different responses on the root growth of *Arabidopsis* seedlings. Those differences are influenced by the composition of extracts and plant growing conditions. ANE A promotes the root growth whereas ANE B shows an inhibitory effect. The effect of ANEs treatment on the root growth is more pronounced over the time. ANEs show no significant role on the attenuation of root inhibition or rescue of root growth of *Arabidopsis* seedlings under osmotic stress conditions. However, the same standard of responses observed for root growth of seedlings without the presence of osmotic stress are also observed for those under osmotic stress.

The seaweed extracts application on leaves leads to little influence on responsiveness to auxins in tomato roots, but these biostimulants increase the responsiveness to the hormones ABA and ethylene. However, these responses seem to be variable over the time and in function of the composition of the extracts applied.

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APPENDICES

Statistical analysis:

Auxin action on root growth of *Arabidopsis* seedlings

Analysis of variance (Results presented in Figure 1).

Source	DF	SS	MQ	F value	P > F
Trat.	5	260.09	52.02	78.38	0.0001
Error	42	27.87	0.66		
Total	47	287.97			

Analysis of variance (Results presented in Figure 2).

Source	DF	SS	MQ	F value	P > F
Trat	3	12.56	4.19	6.67	0.0015
Error	28	17.56	0.63		
Total	31	30.12			

Parameters estimate (PE) of regression analysis (Results presented in Figure 2).

Source	DF	PE	SE	t value	P > t
Intercept	1	5.72652	0.42	13.58	0.0001
Trat	1	-41.99976	20.01	-2.10	0.048
$Trat^2$	1	205.24603	174.76	1.17	0.2534

Analysis of variance (Results presented in Figure 3).

Source	DF	SS	MQ	F value	P > F
Trat	4	25.05	6.26	4.49	0.0049
Error	35	48.82	1.40		
Total	39	73.87			

Parameters estimate (PE) of regression analysis (Results presented in Figure 3).

Source	DF	PE	SE	t value	P > t
Intercept	1	3.43313	0.38	9.11	0.0001
Trat	1	28.76560	23.07	1.25	0.2224
$Trat^2$	1	-469.83630	221.24	-2.12	0.0423

ABA action on root growth of *Arabidopsis* seedlings

Analysis of variance (Results presented in Figure 5).

Source	DF	SS	MQ	F value	P > F
Trat	5	174.53	34.90	19.07	0.0001
Error	42	76.87	1.83		
Total	47	251.39			

Parameters estimate (PE) of regression analysis (Results presented in Figure 5).

Source	DF	PE	SE	t value	P > t
Intercept	1	4.65502	0.38	14.23	0.0001
Trat	1	-0.08887	0.02	-4.55	0.0001

Analysis of variance (Results presented in Figure 6).

Source	DF	SS	MQ	F value	P > F
Trat	5	15.45	3.09	10.88	0.0001
Error	42	11.93	0.28		
Total	47	27.38			

Parameters estimate (PE) of regression analysis (Results presented in Figure 6).

Source	DF	PE	SE	t value	P > t
Intercept	1	2.17411	0.16	13.73	0.0001
Trat	1	-0.09434	0.03	-3.05	0.0042
$Trat^2$	1	0.00187	0.001	1.82	0.0762

Analysis of variance for treatments without PEG (Results presented in Figure 7).

Source	DF	SS	MQ	F value	P > F
Trat	3	1.42	0.47	1.36	0.2742
Error	28	9.73	0.35		
Total	31	11.15			

Analysis of variance for treatments with PEG (Results presented in Figure 7).

Source	DF	SS	MQ	F value	P > F
Trat	3	10.08	3.36	4.08	0.0160
Error	28	23.08	0.82		
Total	31	33.16			

Parameters estimate (PE) of regression analysis for treatments with PEG (Results presented in Figure 7).

Source	DF	PE	SE	t value	P > t
Intercept	1	2.99951	0.36	8.40	0.0001
Trat	1	-0.15785	0.07	-2.20	0.0390
$Trat^2$	1	0.00365	0.002	1.67	0.1106

Root growth of *Arabidopsis* seedlings in response to ANEs treatments under osmotic stress conditions

Analysis of variance for one application of ANEs (Results presented in Figure 8).

Source	DF	SS	MQ	F value	P > F
Trat	9	35.30	3.92	7.97	0.0001
Error	70	34.44	0.49		
Total	79	69.74			

Analysis of variance for two applications of ANEs (Results presented in Figure 8).

Source	DF	SS	MQ	F value	P > F
Trat	9	215.73	23.97	8.82	0.0001
Error	70	190.29	2.72		
Total	79	406.02			

Analysis of variance (Results presented in Table 10 and Figure 9).

Source	DF	F value	P > F
Trat	5	12.91	0.0001
PEG	1	52.38	0.0001
Trat x PEG	5	2.28	0.0544
Rep	7	1.51	0.1773
Error	77		
Total	95		

Analysis of variance (Results presented in Table 11 and Figure 10).

Source	DF	F value	P > F
Trat	5	6.37	0.0001
PEG	1	64.20	0.0001
Trat x PEG	5	3.01	0.0126
Rep	15	2.20	0.0080
Error	165		
Total	191		

Analysis of variance (Results presented in Table 12 and Figure 11).

Source	DF	F value	P > F
Trat	5	10.87	0.0001
PEG	1	87.87	0.0001
Trat x PEG	5	5.96	0.0001
Rep	15	1.91	0.0254
Error	165		
Total	191		

Analysis of variance (Results presented in Table 13 and Figure 12).

Source	DF	F value	P > F
Trat	5	21.77	0.0001
PEG	1	223.76	0.0001
Trat x PEG	5	11.36	0.0001
Rep	15	0.97	0.4849
Error	165		
Total	191		

GUS activity in roots of ethylene-responsive (MT) *EBS::GUS* reporter line plants in response to ANEs and ABA foliar spray

Analysis of variance (Results presented in Table 14 and Figure 13).

Source	DF	F value	P > F
Trat	2	4.78	0.0350
WR	1	157.58	0.0001
Trat x WR	2	151.72	0.0001
Rep	2	0.43	0.6601
Error	10		
Total	17		

Analysis of variance (Results presented in Table 15 and Figures 14 and 15).

Source	DF	24 hours		48 hours	
		F value	P	F value	P > F
Trat	3	2.65	0.0893	4.05	0.0288
WR	1	1.46	0.2470	4.55	0.0510
Trat x WR	3	0.46	0.7147	2.29	0.1231
Rep	2	4.01	0.0421	0.94	0.4156
Error	14				
Total	23				

GUS activity in roots of ABA-responsive (MT) *RD29b::GUS* reporter line plants in response to ANEs and ABA foliar spray

Analysis of variance (Results presented in Table 16 and Figures 16 and 17).

Source	DF	24 hours		48 hours	
		F value	P	F value	P > F
Trat	3	2.28	0.1245	0.52	0.6750
WR	1	15.30	0.0016	17.19	0.0010
Trat x WR	3	1.51	0.2551	0.29	0.8319
Rep	2	0.25	0.7814	2.73	0.0997
Error	14				
Total	23				

GUS activity in roots of auxin-responsive (MT) *DR5::GUS* reporter line plants in response to ANEs and ABA foliar spray

Analysis of variance (Results presented in Table 17 and Figures 18 and 19).

Source	DF	24 hours		48 hours	
		F value	P > F	F value	P > F
Trat	3	23.14	0.0001	75.13	0.0001
WR	1	0.73	0.4068	63.81	0.0001
Trat x WR	3	0.53	0.6712	42.83	0.0001
Rep	2	0.44	0.6525	0.98	0.3986
Error	14				
Total	23				