Ascophyllum nodosum-derived biostimulants modulate plant development through the regulation of hormones signaling

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1. Chapter I
General Introduction

1.1. Biostimulants: Definition and industry forecast

Biostimulants are a promising class of crop management products that can improve crop yield and quality sustainably (el Boukhari et al., 2020). Biostimulants emerge as an environmentally friendly alternative that can improve plant nutrient efficiency and resilience against different stresses, allowing to reduce the use of inorganic fertilizer, and therefore, can work complementary to conventional agrochemicals (Shukla et al., 2019). Until today there has been no specific legislation for biostimulants and no legal definition or classification. This is in part due to the difficulties in determining a mode of action and the active compound of the formulation (du Jardin, 2015; Yakhin et al., 2017). In an effort to better describe this type of product, du Jardin (2015) defined biostimulants as "any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content. By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms».

Biostimulants differ from fertilizers and pesticides since they do not contain essential nutrients or pesticide compounds. Instead, according to the European Biostimulants Industry Council (EBIC), biostimulants: "contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality" (http://www.biostimulants.eu). Biostimulants can be classified into seven groups: (i) humic and fulvic acids, (ii) protein hydrolysates and other N-containing compounds, (iii) seaweed extracts and botanicals, (iv) chitosan and other biopolymers, (v) inorganic compounds, (vi) beneficial fungi and (vii) beneficial bacteria (du Jardin, 2015). Among the products derived from algae extracts, most of them are manufactured from the brown macroalgae *Ascophyllum nodosum* (Oosten et al., 2017).

The global biostimulant market has been growing gradually over the years and is projected to reach USD 4.6 billion by 2027 (https://www.reportlinker.com). Among the different categories of biostimulants, seaweed extracts biostimulants represented in 2018 more than 33% of the global market (el Boukhari et al., 2020) and are expected to account for USD 2.7 billion by 2029 (https://www.databridgemarketresearch.com). It is projected to be the top of the investment pocket in the biostimulant market due to the high demand for more sustainable products in the agriculture sector in Asia-Pacific (Shina, 2017).

1.2. *Ascophyllum nodosum*-derived biostimulants

Ascophyllum nodosum is a brown macroalgae that belongs to the Fucaceae family, being the only species of the genus *Ascophyllum*. This seaweed grows in the northern Atlantic Ocean and can be commonly found on the northwestern coast of Europe and the north-eastern coast of North America. In agriculture, *A. nodosum* extracts (ANE) are widely used for the formulation of commercial plants biostimulants, being one of the most studied biostimulants derived from algae extracts. They are commonly used as a growth stimulant and to protect crops against biotic and abiotic stresses such as freezing, salinity, and drought (Oosten et al., 2017; Shukla et al., 2019). Despite little is known about the mode of action of ANE biostimulants, *A. nodosum* extracts are known to have a phytohormone-like effect on plants (Khan et al., 2011; Rayorath, Khan, et al., 2008; Subramanian, Sangha, Gray, et al., 2011; Wally et al., 2013).

1.2.1. *A. nodosum* benefits on plant development: improve abiotic and biotic stress tolerance

One of the most promising aspects of ANE biostimulants is their ability to enhance plant growth under abiotic stress, such as drought and salinity (Oosten et al., 2017). Crop production is threatened by the increase in the occurrence of drought and events of high temperatures associated to climate change (Giorgi & Lionello, 2008; IPCC, 2022; Polade et al., 2017; Trnka et al., 2014; Vogel et al., 2019). Also, salt accumulation in the soil is one of the major contributors to the loss of productivity in cultivated soils and has been accelerated by climate change (Corwin, 2021). Among the causes of soil salinization are the use of groundwater close to the sea or of low-quality water for irrigation, and the tendency to increase the irrigation water-use efficiency in intensive

farming, mainly due to water scarcity. Low-quality water and reduced irrigation cause salt accumulation in soils due to poor drainage (Machado & Serralheiro, 2017) (https://www.ucsusa.org/global-warming/). Drought and salinity can negatively affect plant growth, development, and crop yield (Xiong and Zhu, 2002; Zörb et al., 2019; Gupta et al., 2020). It has been proved that ANE-biostimulant application can increase stress tolerance and improve stress recovery rates in *A. thaliana* and several crops (Table 1). Among the demonstrated benefits of ANE on plants subjected to stress are the increment of fresh and dry weight (Jithesh et al., 2018; Shukla et al., 2018; X. Zhang & Ervin, 2004), improvement Na+/K+ ratios (Ikuyinminu et al., 2022; Shukla et al., 2018), improves osmotic adjustment (Elansary et al., 2017; Ikuyinminu et al., 2022), increased photosynthetic pigment content and photosynthetic rate (Elansary et al., 2016; Ikuyinminu et al., 2022; Santaniello et al., 2017), enhanced root development(Shukla et al., 2018) , improvement in fruit yield and quality (Carmody et al., 2020; Di Stasio et al., 2018; Ikuyinminu et al., 2022; Karunatilleke, 2014; C. Xu & Leskovar, 2015; X. Zhang & Ervin, 2004), and modulation of stress-responsive genes (Goñi, Quille, & Connell, 2018; Jithesh et al., 2012, 2018; Santaniello et al., 2017; Shukla et al., 2018) (Table 1).

Table 1: Publications where was reported that *A. nodosum*-derived biostimulants improve abiotic stress tolerance

Extract	Crop /Specie	Stress type	Function/Benefits	References
PSI-475, Brandon Bioscience	*A. thaliana*	Salinity	Increased primary root growth and photosynthetic pigments content	(Ikuyinminu et al., 2022)
A. nodosum extracts, Acadian ®	*A. thaliana*	Salinity	Increased fresh and dry weight, improved root development, increased phosphorous and decreased Na+ content, modulates the expression of salinity responsive miRNA	(Shukla et al., 2018)
A. nodosum extracts, Acadian ®	*A. thaliana*	Salinity	Methanolic-subfraction improved growth, increased biomass by modulating the expression of stress-responsive genes	(Jithesh et al., 2018)
PSI-475, Brandon Bioscience	*Lycopersicon esculentum*	Salinity	Improves fruit yield, decreases NA+ content on leaf. Alleviated negatives of salinity stress by improving osmotic adjustment and ion homeostasis markers	(Ikuyinminu et al., 2022)
A. nodosum extracts	*P. americana Mill.*	Salinity	Increased plants heigh, increased potassium, calcium, and nitrogen content in leaves	(Bonomelli et al., 2018)
Stella Maris ™	*Paspalum vaginatum*	Salinity	Higher plant growth under prolonged irrigation and saline conditions by regulating osmotic adjustment and antioxidant defense system	(Elansary et al., 2017)
Rygex ®, Super fifty ®	*S. lycopersicum*	Salinity	Increased plant growth and fruit quality and mitigates salinity stress in tomato plants	(Di Stasio et al., 2018)
A. nodosum extract, Algae, Valagro S.p.a.	*A. thaliana*	Drought	Increased photosynthetic performance, improves stomatal control and water use efficiency, by activation of ABA-responsive genes and antioxidant system pathways	(Santaniello et al., 2017)
Phylgreen®	*A. thaliana*	Drought	Mitigate drought stress by priming. Accumulation of anthocyanin content and reduction of MDA content	(Fleming et al., 2019)
A. nodosum extract, Acadian ®	*Agrostis palustris*	Drought	Increased quality, shoot and root weight under drought conditions. Improves phytochemical efficiency	(X. Zhang & Ervin, 2004)
A. nodosum extract, Acadian ®	*Glycine max*	Drought	Improve drought tolerance by modulating the expression of stress-responsive gene	(Shukla et al., 2017)
A. nodosum extract	*Phaseolus vulgaris*	Drought	Increased tolerance to drought stress by affecting proline metabolism	(Carvalho et al., 2018)
A. nodosum extracts	*S. lycopersicum*	Drought	Enhanced tolerance to drought stress in tomato plants by modulating the expression of dehydrins	(Goñi, Quille, & Connell, 2018)

A. nodosum extract, Acadian ®	*S. lycopersicum*	Drought	Mitigated drought stress in tomato during the early vegetative stage and during the reproductive stage. Increased number of both fruit sets and harvested fruits	(Karunatilleke, 2014)
A. nodosum extracts, Acadian ®	*S. lycopersicum*	Drought	Enhanced tolerance to drought stress in tomato plants by modulating the expression of dehydrins	(Shukla et al., 2017)
Seaweed extract	*Spinacia oleracea*	Drought	Improve growth, quality, and nutritional value of spinach grown under drought conditions	(C. Xu & Leskovar, 2015)
Stimplex ®, Acadian Seaplants	*Spiraea nipponica, Pittosporum eugenioides*	Drought	Improve drought tolerance by inducing phytochemical and antioxidant contents and improving water status, stomatal conductance, and photosynthetic rate	(Elansary et al., 2016)
Stimplex ®, Acadian Seaplants	*Sweet orange*	Drought	Improves drought stress tolerance and maintains shoot growth under drought conditions	(Spann et al., 2011)
A. nodosum extracts, Acadian ®	*A. thaliana*	Freezing	Lipophilic component of ANE enhanced freezing tolerance by protecting membrane integrity and modulating the expression of freezing stress-responsive genes	(Nair et al., 2012; Rayirath et al., 2009)
Acadian®, alkaline extracts	*Nicotiana tabacum*	Freezing	Increased cell viability and biomass of suspension cells of tabaco in the recovery time after freezing	(Zamani-Babgohari et al., 2019)
A. nodosum extract, Acadian ®	*Agrostis stolonifera*	Heat	Increased heat stress tolerance by seaweed-extract-based cytokinin	(X. Zhang & Ervin, 2008)
A. nodosum extract	*Lycopersicon esculentum*	Heat	Improves flower development, pollen viability, and fruit production. Increased accumulation of soluble sugars, and gene transcription of protective heat shock proteins (HSPs) in flowers	(Carmody et al., 2020)
SuperFifty	*A. thaliana*	Oxidative stress	Improves oxidative damage by upregulation of several carbohydrate metabolism genes, growth, and hormone signaling, as well as antioxidant-related genes	(Omidbakhshfard et al., 2020)
A. nodosum extract, Acadian ®	*Ulva lactuca*	Oxidative stress	Reduces ionic liquid-induced oxidative stress	(Kumar et al., 2013)
Super Fifty, Ecoelicitor	Lettuce; oilseed rape	Biotic and abiotic stress	Enhanced plant growth and tolerance to biotic and abiotic stresses	(Guinan et al., 2013)

Studies have shown that ANE biostimulants protect plants against several biotic stresses through a priming mechanism. Plants have evolved inducible defense mechanisms against pathogens such as bacteria, fungi, and viruses. It has been reported two types of disease resistance mechanisms in plants: systemic acquired resistance (SAR) and induced systemic resistance (ISR). In SAR, salicylic acid (SA) mediates the pathogenesis-related (PR) gene activation, while in ISR, jasmonic acid (JA) and ethylene (ET) pathways play a crucial role in the induction of broad-spectrum disease resistance (Shukla et al., 2019). In general SA-responsive pathways regulates the defense against biotrophic pathogens, while JA- and ET- responsive pathways regulate the response to necrotrophic pathogens, herbivory, and wounding (Stroud et al., 2022). There are compounds of biological origin, known as elicitors, that can induce a defense response through SAR and IRS against several plant pathogens. Primed plants with the elicitors induced a greater preventive response against the pathogen infection progression. Examples of elicitors are lipo-polysaccharides, chitin, and bacterial flagella (Shukla et al., 2019). In seaweed, some elicitors are being identified that trigger biotic stress tolerance in plants. Among them, the major cell polysaccharides of brown algae such as alginates and fucans, laminarin and their derived oligosaccharides have been shown to induce an oxidative burst and defense signaling pathways mediated by SA and JA/ET in plants (De Saeger et al., 2019). It has been reported that the bioactive compounds found in ANEs induced a defense response against several pathogens (Table 2), by activating phytohormones-related signaling pathways, SAR-related genes, ROS production, and ROS-associated gene expression (Islam et al., 2021; Subramanian, Sangha, Gray, et al., 2011).

Table 2: Publications where was reported that *A. nodosum*-derived biostimulants improve biotic stress tolerance

Extract	Crop /Specie	Pathogen	Function/Benefits	References
Seasol ™	*A. thaliana*	*P. cinnamomi*	Induced priming by up-regulating genes related SAR and ROS production. After infection, induced ROS production and expression of SA-related genes	(Islam et al., 2021)
A. nodosum extract, alkaline	*A. thaliana*	*Myzus persicae*	Improved recovery rates after infestation, increased seed yield, reduced tissue damage, and delayed senescence	(Weeraddana et al., 2021)
A. nodosum extract, alkaline	*A. thaliana*	*P. cinnamomi*	Reduced pathogen growth, induced SAR, and the expression of SA-related genes.	(Islam et al., 2020)
Stella Maris™	*A. thaliana*	*P. syringae* DC3000, X. *campestris* BP109	Inhibited the growth of multiple bacterial pathogens by inducing the expression ofWRKY30, CYP71A12 and PR-1 gene	(Cook et al., 2018)
A. nodosum extracts, Acadian ®	*A. thaliana*	*P. syringae* pv.tomato DC3000	Reduced the development of disease symptoms on the leaves by increasing expression of JA-related gene	(Subramanian, Sangha, Gray, et al., 2011)
MaxicropR©Original	*A. thaliana*	*Meloidogyne javanica*	Reduces the number of females of *M. javanica*	(Wu et al., 1998)
A. nodosum extract, Acadian ®	Carrot	-	Increased soil microbial colony counts, respiration, and metabolic activity, which correlates with increased carrot growth	(Alam et al., 2014)
A. nodosum extract, Acadian ®	Carrot	*Alternaria radicina and Botrytis cinerea*	Induces the expression of defense-related genes or proteins	(Jayaraj et al., 2008)
Marmarine	Cucumber	*Phytophthora melonis*	Induces defense-related enzymes	(Abkhoo & Sabbagh, 2016)
Stimplex ® , Acadian Seaplants	Cucumber	*Alternariacucumerinum, Didymella applanata, Fusarium oxysporum, Botrytis cinerea*	Stimplex reduces the disease by activating different-related enzymes and accumulation of secondary metabolites	(Jayaraman et al., 2011)
AMPEP	*K. alvarezii*	*Neosiphonia apiculata*	Reduces the biotic stress caused by endophytes	(M. K. M. Ali et al., 2018)
A. nodosum extract, Acadian ®	*K. alvarezii*	*Polysiphonia subtilissima*	Reduces the growth of the epiphyte	(Loureiro et al., 2010)

A. nodosum extract, alkaline	*Medicago truncatula*	*Rhizophagus irregularis*	Enhanced mycorrhization through both direct stimulation of arbuscular mycorrhizal fungus growth and through stimulation of the plant's accommodation of the symbiont	(Hines et al., 2021)
Seasol ™	*S. lycopersicum*	-	Induced priming by up-regulating genes related to SAR and ROS production	(Islam et al., 2021)
A. nodosum extract, Acadian ®	*S. lycopersicum*	*A. solani, X. campestris pv vesicatoria*	Reduces the incidence of diseases in plants by the upregulation of JA/ethylene pathway	(N. Ali, Ramkissoon, et al., 2016)
DalginR©	*S. lycopersicum*	*Phytophthora capsici*	Induces the expression of defense-related genes or proteins	(Panjehkeh & Abkhoo, 2016)
A. nodosum extract, Acadian ®	Strawberry		Increased colony counts in greenhouse and field soil samples, as well as metabolic activity of soil microbes, increasing root and shoot growth and berry yield.	(Alam et al., 2013)
MaxicropR©Triple	Strawberry	*Tetranychus urticae*	Reduces the population of two-spotted red spider mites on treated plants	(Hankins & Hockey, 1990)

1.2.2. Benefits on plant development: growth-promoting activity

In addition to improving the tolerance to variable stresses, ANE biostimulants have also been used as growth stimulants. The application of ANE has been reported to impact plant performance, such as improved plant vigor, increased root yield (e.g., carrots), fruit yields, increased chlorophyll content, sugar content, enhanced germination, seedling vigor, and nutrient-use efficiency, among others (Table 3). A number of publications report that ANE promotes plant/crop performance in different species, including the plant model organism *Arabidopsis thaliana* (Goñi et al., 2016; Rayorath, Jithesh, et al., 2008; Wally et al., 2013), and crops such as tomato (N. Ali, Farrell, et al., 2016), maize (Ertani et al., 2018; Shukla & Prithiviraj, 2021), wheat (Łangowski et al., 2022), soybean (Łangowski et al., 2021), barley (Goñi et al., 2021; Rayorath, Khan, et al., 2008), alfalfa (Khan et al., 2012), oilseed rape (Łangowski et al., 2019), cherry (Macdonald et al., 2014), grapevine (Gutiérrez-Gamboa et al., 2020; Gutierrez-Gamboa & Moreno-Simunovic, 2021; Norrie, 2006; Norrie & Branson, 2002; Salvi et al., 2019), strawberry (Alam et al., 2013), carrots (Alam et al., 2014), spinach (Fan et al., 2013), and others (Shukla et al., 2019).

In *Arabidopsis*, only a few studies are available where the effects of ANE plant growth have been analyzed (Table 3). Studies with two different ANEs (aqueous solutions at 0.01 g L^{-1} and 0.1 g L^{-1}) have shown that the biostimulant treatment stimulated primary root growth of plants in vitro culture conditions, and increased plant height and leaf number in plants grown in greenhouse conditions (Rayorath, Jithesh, et al., 2008). On the contrary, another study in *Arabidopsis* grown *in vitro* showed that ANE (aqueous solution 0.01% w/v) reduced primary root length and lateral root number (Wally et al., 2013). A foliar spray of ANE (0.2% v/v) in *Arabidopsis* grown in pots incremented the plant's height and rosette leaf number (Goñi et al., 2016).

Table 3: Publications where was reported that *A. nodosum*-derived biostimulants improve plant growth

Extract	Crop /Specie	Function/Benefits	References
A. nodosum extract, neutral and alkaline	*A. thaliana*	Increased biomass, plant height, and rosette leaf number. Transcriptomic analysis	(Goñi et al., 2016)
A. nodosum extract, Acadian ®	*A. thaliana*	Reduced root development, increased total CKs, ABA, and reduced AUX	(Wally et al., 2013)
A. nodosum extract	*A. thaliana*	Improved plant growth by modulation of concentration and localization of auxin	(Rayorath, Jithesh, et al., 2008)
Premium liquid seaweed	*Allium cepa*	Improved vegetative growth and yield of onion	(Hidangmayum & Sharma, 2017)
Goëmar BM 86R©	Apple	Improved the fruit quality of apples, and increased nitrogen content	(Basak, 2008)
A. nodosum extract	Barley	Induced gibberellic-acid-independent amylase activity in barley and promote seed germination	(Rayorath, Khan, et al., 2008)
PSI-362, Brandon Bioscience	*Barley, A. thaliana*	Increased nitrogen use efficiency in 75% nitrogen input, by increasing nitrate accumulation. Improved content of free amino acids, soluble proteins, and photosynthetic pigments. Improved yield similar to control in low nitrogen	(Goñi et al., 2021)
Sealicit ™	*Brassica napus*	Reduced pod shattering and increased yield by regulation of the expression of major regulator of pod shattering, and disrupted auxin maximum	(Łangowski et al., 2019)
AZAL5	*Brassica napus*	Promotes plant growth and nutrient uptake	(Jannin et al., 2013)
AZAL5	*Brassica napus*	Stimulated root growth and macronutrient uptake (N, S, K, and P). Increased Mg, Mn, Na, and Cu plants concentrations and root-to-shoot Fe and Zn translocation	(Billard et al., 2014)
AlgaeGreenR	*Brassica oleracea*	Enhanced biosynthesis of secondary metabolites	(Lola-Luz et al., 2013)
A. nodosum extract	Cherry	Increased rooting in seedling establishment	(Macdonald et al., 2014)
Goemar	*Citrus unshiu*	Early maturation of fruit	(Fornes et al., 1995)
Goemar ®	Clementine Mandarin and Navelina Orange	Increased productivity and yield	(Fornes et al., 2002)

Acadian ®, alkaline extracts	*Daucus carota*	Promoted plant growth and root yield in carrots associated with increased root-zone soil microbial activity	(Alam et al., 2014)
Seasol ®	*Fragaria ananassa*	Increased growth response of strawberry root	(Mattner et al., 2018)
Acadian ®, alkaline extracts	Fragaria ananassa	Improved plant growth, fruit quality, and microbial growth	(Alam et al., 2013)
Actiwave ®	*Fragaria ananassa*	Increased fruit yield and quality and acts as iron chelator	(Spinelli et al., 2010)
A. nodosum extract	*Medicago sativa*	Improved root colonization of rhizobia symbionts	(Khan et al., 2012)
A. nodosum extract	*Olea europaea*	Showed increased tree productivity and improved their nutrition status and oil quality parameters	(Chouliaras et al., 2009)
Acadian ®, alkaline extracts	*S. lycopersicum*	Increased plant height, fruit yield, and quality. Improved root system and concentrations of minerals in shoots. Reduced electrolyte leakage, and lipid peroxidation by an improved membrane stability	(N. Ali, Farrell, et al., 2016)
Acadian®, Stimplex	*S. lycopersicum*	Increased chlorophyll content, plant height, root length, and plant biomass. Increased micro and macro-nutrient content, majorly calcium. Increased levels of cytokinin, auxin, and gibberellins.	(O. Ali et al., 2022)
Sealicit ™	*Glycine max*	Improved pod shattering resistance and seed productivity	(Łangowski et al., 2021)
Acadian ®, alkaline extracts	*Spinacia oleracea*	Pre-harvest treatment of spinach with Ascophyllum nodosum extract improved post-harvest storage and quality	(Fan et al., 2014)
A. nodosum extract, Acadian ®	*Spinacia oleracea*	Increased biomass, chlorophyll, and antioxidant activity	(Fan et al., 2013)
A. nodosum extract, Acadian ®	*Spinacia oleracea*	Enhanced phenolic antioxidant content of Spinach	(Fan et al., 2011)
A. nodosum extract, alkaline	*Vitis vinifera*	Increased photosynthesis and stomatal conductance. Improved secondary metabolism and grape quality	(Salvi et al., 2019)
Acadian ®	*Vitis vinifera*	Foliar spray had a positive effect on ripening dynamics and fruit quality	(Frioni et al., 2018)
A. nodosum extract	*Vitis vinifera*	Improved growth, yield, berry quality attributes, and leaf nutrient content of grapevines	(Sabir et al., 2014)
Alga Special	*Vitis vinifera*	Improved vegetative growth	(Cristian Popescu & Popescu, 2014)
A. nodosum extract, Acadian ®	*Vitis vinifera*	Improved fruit quality and yields. Treated fruit also performed better in storage	(Norrie, 2006)
MaxicropR©, ProtonR©, AlgipowerR©	*Vitis vinifera*	Improved copper uptake of grapevine	(Turan & Köse, 2004)

Acadian ®	*Vitis vinifera*	Improved yield and fruit quality	(Norrie & Branson, 2002)
PSI-362, Brandon Bioscience	Wheat	Increased biomass by increasing nitrogen uptake, in nitrogen deficiency conditions	(Łangowski et al., 2022)
A. nodosum extract, Acadian ®	*Zea mays*	Improved root and shoot fresh and dry weight, in phosphorous-limited conditions	(Shukla & Prithiviraj, 2021)
A. nodosum extract	*Zea mays*	Promotes root morphology and plant nutrition	(Ertani et al., 2018)

1.2.3. *A. nodosum*-derived biostimulants effects on root development

The plant root system is essential for plant growth as is responsible for nutrient and water acquisition, anchorage, and interaction with soil micro-organisms to enhance nutrient absorption. Overall root architecture is determined by root branching, root angle, and growth rates of individual root parts. The plasticity of the plant root system is paramount for the plant's ability to explore the soil and the plant's adaptation to the habitat (Duque & Villordon, 2019) and, for example, enhancing root branching and root hair development could improve nutrient efficiency acquisition (Li et al., 2016). Analysis of the root system architecture of plants with the biostimulant treatment can provide a quantitative readout for identifying genes and signaling pathways that enable the plant to perceive changes in the environment and to integrate them into adaptative responses induced by the product (Joshi et al., 2022; Kellermeier et al., 2014). ANE treatment has been previously reported that alters root development and improve nutrient uptake and nitrogen use efficiency (N. Ali, Farrell, et al., 2016; Basak, 2008; Billard et al., 2014; Goñi et al., 2021). However, there is little information regarding the effects of ANE on root system architecture, lateral roots, and root hair development. In this work, we will do a detailed analysis of the effect of ANE-derived biostimulants on root development.

1.2.4. Variable composition of the extracts

One key issue with this kind of biostimulant is that the activity reported for ANE treatments in different studies varies significantly depending on the formulation, despite the fact that they use the extract of the same seaweed (A. *nodosum)* (Goñi, Quille, & Connell, 2018). The composition of the extracts varies depending on the methodology used for the extraction (e.g. water based, alkaline, or acid hydrolysis, among others), the manufacturing process, and the environmental conditions where the algae were harvested (i.e., season, location, or water salinity) (Craigie et al., 2008; Shukla et al., 2019). Therefore, the fabrication methodology influences the bioactivity of the product finally obtained (Goñi et al., 2016; Goñi, Quille, & Connell, 2018; Wally et al., 2013). For example, two commercial ANE extracted using different methods (one under high temperature and neutral pH, the other under high temperatures and alkaline pH) were used to analyze the phenotypes and transcriptional changes in *Arabidopsis*. The composition of both extracts was

significantly different, and one of them showed better results in terms of plant growth performance. The number of gene transcripts up- and down-regulated was also significantly different (501 vs. 348 and 29 vs. 5, respectively) (Goñi et al., 2016). Further evidence that supports the different effects of ANE depending on the formulation was obtained from tomato plants treated with three different commercial ANE under drought stress. The results indicated that the three products showed a different ability to maintain crop productivity and drought tolerance (Goñi, Quille, & Connell, 2018). Also, studies with AZAL5, an aqueous solution prepared by micro-rupture under acidic conditions from freshly harvested *A. nodosum,* it has been shown that increased root and shoot growth, and stimulated macronutrient uptake (N, S, K, and P) and micronutrient concentrations (Billard et al., 2014; Jannin et al., 2013). Analysis of the biostimulants composition showed that the hormone and nutrient levels of AZAL5 were too low to induce the observed effect on rapeseed. It has been suggested that macromolecules, such as the polysaccharides, laminarin or fucoidan, found in the extract or synergistic activity of various compounds might trigger the responses observed (Billard et al., 2014; Jannin et al., 2013).

The known bioactive compounds include poly- and oligosaccharides that are not found in plants, including laminarin, mannitol, fucoidan, and alginate; polyphenols such as phlorotannins; betaines; sterols; vitamins; amino-acids; macro- and micronutrients; phytohormones, such as auxin, cytokinins, and abscisic acid (De Saeger et al., 2019). *A. nodosum* extracts have certain phytohormone levels (Wally et al., 2013). However, due to the relatively low concentrations of phytohormone present in the biostimulant extract, it is highly likely that other compounds within the algae (non-hormone) could be responsible for the biostimulant effects on plants. It has been proposed that ANE could also contain unidentified compounds with “hormone-like effects”. Molecules such as amino acids, polysaccharides, and organic acids may act as precursors or activators of endogenous plant hormones (De Saeger et al., 2019; Shukla et al., 2019; Yakhin et al., 2017).

1.2.5. Role of the main organic components

The variable nature of these extracts makes it difficult to identify with precision, which are the key components that induce biostimulant activity, and, therefore, the mode of action of these

types of products. For these reasons, it is crucial to characterize the extract and biostimulant composition in future studies in an effort to correlate bioactive compounds with the mechanism of action. Understanding the mode of action could allow the manufacturers to improve and optimize the product for different purposes.

Only a few researchers have measured the phytohormone and other component content of the extract or biostimulant they used for the study (De Saeger et al., 2019). The major organic component of *A*. *nodosum* extracts are polysaccharides and polyphenols, and it has been reported that these compounds can show biostimulant activity in plants (Goñi et al., 2016; Goñi, Quille, & Connell, 2018). In an effort to better understand the role of the major organic components of ANE, researchers have been testing subfractions of the extracts. The methanol fraction and subfractions of ANE, stimulated seed emergence, shoot length, and shoot biomass in barley (Rayorath, Khan, et al., 2008). The ethyl acetate subfraction extracts of *A. nodosum* extracts can enhance cold and salt tolerance in *Arabidopsi*s. For example, Rayirath et al. (2009) tested the organic sub-fractions of ANE on *A*. *thaliana* under freezing stress (-2.5 to -5.5 °C) and found faster recovery rates, 70% less damage in chlorophyll, better membrane integrity, and overexpression of key freezing tolerance genes (*RD29A, COR15A,* and *CBF3*). This fraction was rich in fatty acids and sterols (Rayirath et al., 2009). Later studies have proposed that the lipophilic fraction (LPF) induced priming of tolerance genes and increased proline content and total soluble sugars, which contributes to increased freezing tolerance (Nair et al., 2012). Also, Jithesh et al., (2018) have shown that the ethyl acetate subfraction is also the major responsible for salt stress alleviation in Arabidopsis.

1.2.6. *A. nodosum* biostimulants effects on endogenous phytohormones homeostasis

The mechanism of action of biostimulants has been associated with phytohormones, the named "phytohormone-like effect", due to the similarity of the phenotypes with that of the phytohormones themselves (Khan et al., 2011). It is also possible that the phytohormones present in the extract could be contributing to the biostimulant effect of the extract. However, even though phytohormones can promote growth at relatively low concentrations (~0.01% w/v), their relatively low concentration in the extract is not likely to explain the growth-promoting

activity of ANE (Wally et al., 2013). In fact, evidence has shown that the phytohormones content found in brown algae extracts is not high enough and unlikely to cause significant effects in plants taking into consideration the dilution necessary for application (Rayorath, Khan, et al., 2008; Stirk et al., 2003; Wally et al., 2013). However, it has been reported that ANE treatment could be triggering endogenous phytohormone accumulation in plants (Khan et al., 2011; Wally et al., 2013) and altered regulation of hormone-related genes (Jithesh et al., 2018; Santaniello et al., 2017; Wally et al., 2013).

The effect of ANE on the accumulation of plant hormones and the transcriptional regulation of key biosynthesis and metabolism genes has been evaluated. Interestingly, ANE treatment in *Arabidopsis* plants increased the concentration of cytokinins (CK) in rosette leaves (*trans*-zeatin-type the first 96 h after application, and *cis*-zeatin type at 144 h), along with an increment in transcript level of CK biosynthetic genes (*IPT3, 4* and *5*) and repression of CK catabolic genes (*CKX4*) (Wally et al., 2013). Moreover, Khan et al. (2011) reported an endogenous cytokinin-like signaling using a GUS-activity assay in *Arabidopsis* under the *ARR5* promoter, a response regulator gene induced by CK. This is consistent with the upregulation of *ARR5* in rosette leaves observed by Wally et al. (2013). Besides cytokinin, it was also shown that ANE treatment increased abscisic acid (ABA) levels along with increased transcript levels of ABA biosynthetic genes. On the other hand, auxin levels were reduced, and the transcript levels of AUX biosynthetic genes were downregulated (Wally et al., 2013). More recent studies have shown that ANE treatment on tomato plants significantly increased levels of auxin, cytokinins, and gibberellins compared to control plants, which was correlated with the upregulation of auxin-responsive genes (O. Ali et al., 2022).

It needs to be considered that mainly leaf tissue was used for the evaluations, and phytohormones are transported throughout the plant (Kieber & Schaller, 2014; Michniewicz et al., 2007). Also, there is a complex crosstalk between phytohormones to regulate plant development and stress responses. Thus, a more detailed study is needed in order to fully understand how algae extracts interact with plant phytohormones metabolism.

1.3. Principles of root development

The regulation of the growth rates of the different parts of the roots determines the root system architecture. The Arabidopsis root has a hierarchical tree structure with one main primary root producing lateral roots, which in turn produce higher-order lateral roots. The histological structure comprises three external layers, i.e., epidermis, cortex, and endodermis, surrounding a single-layered pericycle and the inner vasculature (Fig. 1.1A, B). Along the longitudinal axes of the root, the structure from root tip to rosette comprises the columella, lateral root cap, quiescent center (QC) and initials stem cells, proximal meristem, transition zone, and elongation zone (Fig. 1.1A, B) (Lee et al., 2012). The QC consists of pluripotent cells and is the source of the stem cell initials. Upon developmental cues, stem cells undergo asymmetric divisions to form transit-amplifying cells at the boundary of the proximal meristem; they exit the cell cycle at the transition zone to start differentiating into the specific root tissues in the elongation/differentiation zone (Lee et al., 2012).

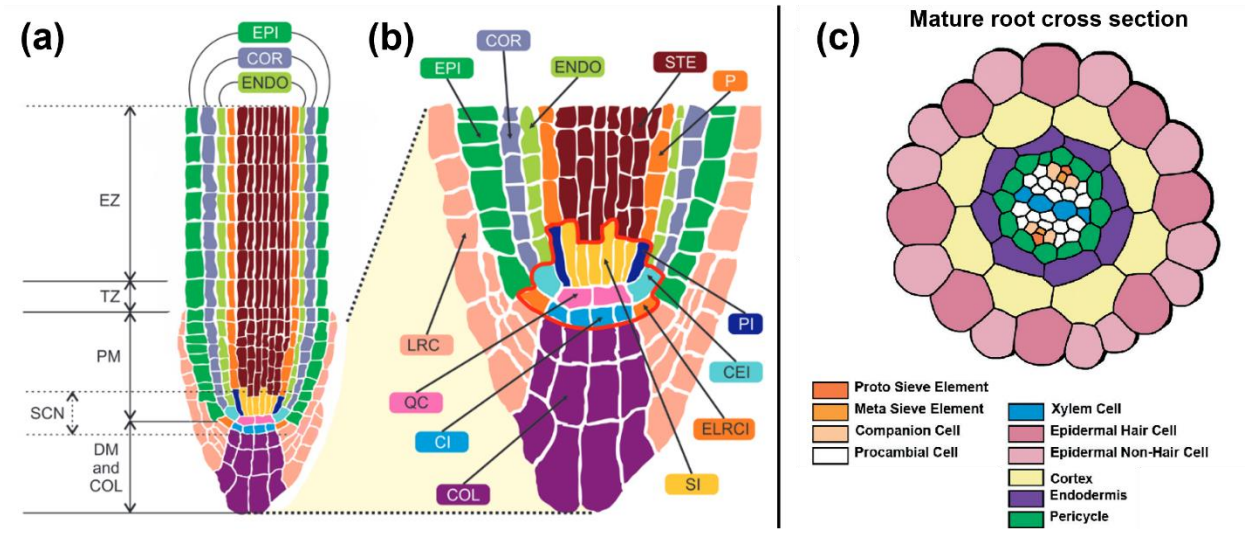

Figure 1.1. Structure of the Arabidopsis root. (a), Schematic longitudinal section of the Arabidopsis root. There are three distinct developmental zones: the meristematic zone (MZ), the transition zone (TZ), and the elongation zone (EZ). The meristematic zone can be divided into the distal meristem (DM) and the proximal meristem (PM). In the meristematic zone, there is a 'stem cell niche' (SCN) that consists of the QC and initials (stem cells). **(b),** Schematic longitudinal section of the Arabidopsis root tip. The area enclosed with the red line shows the SCN. Around the QC, there are four initials (root stem cells). QC,

quiescent centre (purple); CEI, cortex/endodermis initials (light green); ELRCI, epidermis/lateral root cap initials (light brown); CI, columella initials (sky blue); SI, stele initials (light ochre); LRC, lateral root cap (pink); EPI, epidermis (green); COR, cortex (light sky blue); ENDO, endodermis (dark ochre); P, pericycle (orange); STE, stele (dark brown). (Lee et al., 2012). **(c),** schematic cross section of the mature root, the xylem cells determined the xylem axis, and therefore, the xylem poles. Adapted from (Bonke, 2004).

1.3.1. Primary root growth

Phytohormones control root growth by balancing cell division, differentiation, and expansion. Cytokinin (CK), auxin (AUX), and ethylene play a crucial role in the regulation of these processes, maintenance of the meristem size, and, therefore, the regulation of root growth and development (Dello Ioio et al., 2007; Lee et al., 2012) . The interaction between these hormones forms a complex crosstalk network at all levels, including metabolism, signaling, and gene expression (Liu et al., 2017). In general terms CK and AUX have antagonistic interactions; on one hand, CK stimulates cell differentiation at the cell division and elongation zone by suppressing auxin signaling and transport. Auxin, on the other hand, promotes cell division by inactivating CK signaling (Lee et al., 2012). The key genes related to cytokinin and auxin pathways that participate in the regulation of primary root development are described in Fig. 1.2A.

CK and AUX activity can be visualized in root tissues using reporter gene systems. The reporter gene system consists of a gene construct with a promoter of a gene of interest that controls the expression of a gene that codifies for a fluorescent protein (e.g. GFP) or an enzyme that catalyzes a reaction that produces a colored product (e.g. GUS or β-glucuronidase). Examples of these reporter gene systems are the *DR5::GUS* and *TCSn::GFP* constructs; *DR5* is a synthetic auxin-responsive promoter that contains several auxin response factors binding sites and therefore reflects AUX accumulation (Brunoud et al., 2012). *TCSn* (Two Component signal Senser new) is a synthetic sensor of cytokinin that reflects the activity of type-B response regulators (Zurcher et al., 2013).

Gradients of different factors correlate to spatiotemporal successive waves of gene expression changes between single cells, which controls cell division in the meristematic zone (MZ) and cell

differentiation in the elongation/differentiation (EZ/DZ) zone (Fig. 1.2A). Among these factors, auxin and PLETHORA (PLT) transcription factors are necessary to specify the root meristem and stem cell niche (SCN). The auxin gradient is maximum in the SCN, declines along the MZ, and reaches a minimum in the transition zone (TZ). This distribution depends on the polar cell auxin efflux mediated by PIN-FORMED (PIN), local auxin biosynthesis and degradation, and tissue-specific signaling. PLTs gradients match the auxin gradient with a maximum in the SCN and a minimum in the TZ. Cytokinins have a primary role in the spatiotemporal coordination between cell division, expansion, and differentiation that controls the meristem size. CKs are involved in the transcriptional regulation of genes that control auxin transport and its irreversible conjugation to aspartate and glutamate acids, shaping the auxin gradient (Fig. 1.2A) (Svolacchia et al., 2020).

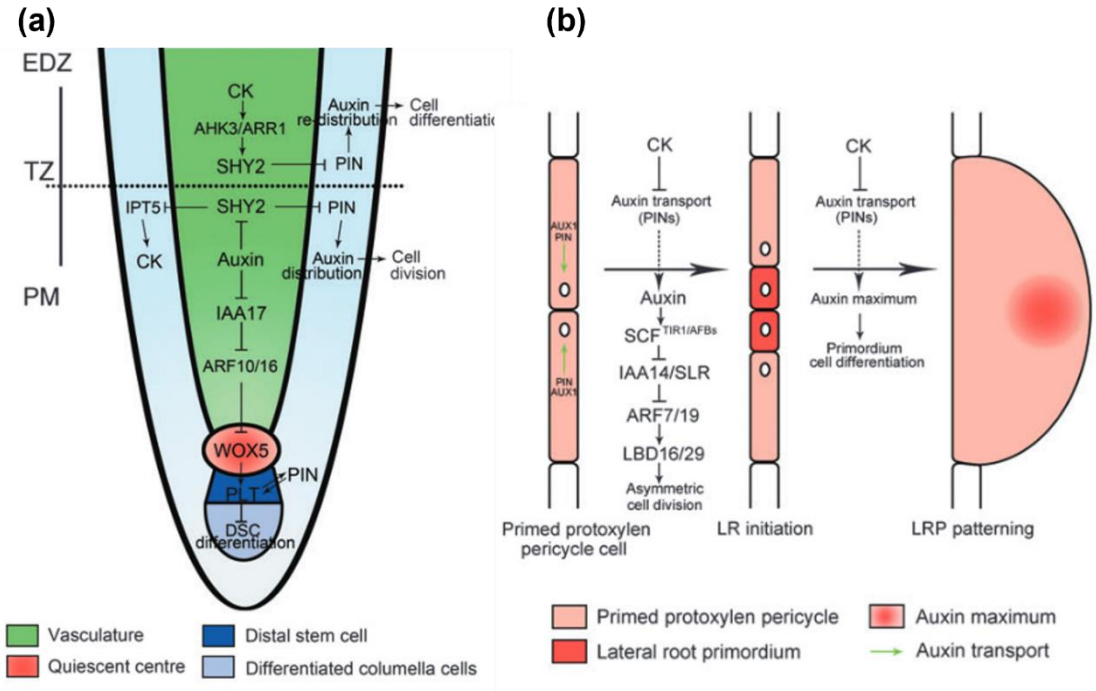

Figure 1.2. Hormonal regulation of root growth. (a), In the root meristem, auxin promotes the expression of PINs through the degradation of SHORT HYPOCOTYL 2 (SHY2) proteins, resulting in the maintenance of an auxin gradients and cell division. In contrast, cytokinin impedes the expression of PINs by stimulating the expression of *SHY2*, leading to auxin redistribution and cell differentiation. Auxin also plays an

important role in the differentiation of root DSC by mediating the expression of *WUSCHEL RELATED HOMEOBOX 5* (*WOX5)* and PLT. PM, proximal meristem; EDZ, elongation differentiation zone; TZ, transition zone; DSC, distal stem cell. **(b),** In primed xylem pole pericycle cells, the transport and perception of auxin trigger an asymmetric cell division critical for the lateral root initiation and lateral root primordium patterning. By contrast, cytokinin negatively regulates the lateral root initiation and lateral root primordium patterning by inhibiting the expression of PINs and the auxin distribution gradients. Adapted from (Su et al., 2011).

1.3.2. Lateral root development

Lateral roots initiate at regular intervals along the primary root exclusively from pericycle founder cells located opposite xylem poles (Fig. 1.2C). Auxin primes pericycle founder cells, giving the signal to undergo several rounds of anticlinal divisions to create a single layered primordium of cells of equal length, forming the lateral root primordium (Stage I, Fig. 1.2B). Later steps of lateral root primordium development have been classified into eight stages until lateral root emergence, detailed in Fig. 1.3 (Péret et al., 2009). Under standard *in vitro* growth conditions, lateral roots of Arabidopsis distribute evenly along the primary root and follow a right-left alternating pattern. This phenomenon is controlled by an endogenous oscillatory mechanism in the basal meristem that generates oscillations of the auxin response in the protoxylem cell within the oscillation zone. An auxin response maximum in these cells primes the neighboring xylem pole pericycle cells, which marks the founder cells and lateral root initiation sites. Auxin maximum is visualized by the synthetic auxin reporter *DR5::GUS* in the protoxylem cells adjacent to those pericycle cells that will be able to form a lateral root (Fig. 1.3) (de Smet, 2012; Lavenus et al., 2013). After lateral root initiation, auxin transporters (*PINs)* are responsible for the formation of an auxin concentration gradient from the lateral root primordium base to the tip that is required for the proper organization and development of the lateral root primordium (Lavenus et al., 2013).

Cytokinins also have a crucial role, mainly during lateral root organogenesis, as they act antagonistically with auxin, inhibiting lateral root formation. Cytokinin may be involved in the control of lateral root primordium spacing by preventing new lateral roots from initiating proximal to already existing lateral root primordium and therefore, has a significant role in

shaping the root system. Cytokinin response is repressed during early lateral root development and is induced in the xylem pole pericycle cells located in between developing lateral roots primordium, inhibiting a lateral root primordium formation in those cells (Bielach et al., 2012). Cytokinins also have a stage-dependent effect on primordium development; enhancement of the cytokinin activity during early stages strongly interferes with the auxin gradient formation, whereas at later phases, cytokinin only mildly affects the established auxin gradients (Bielach et al., 2012).

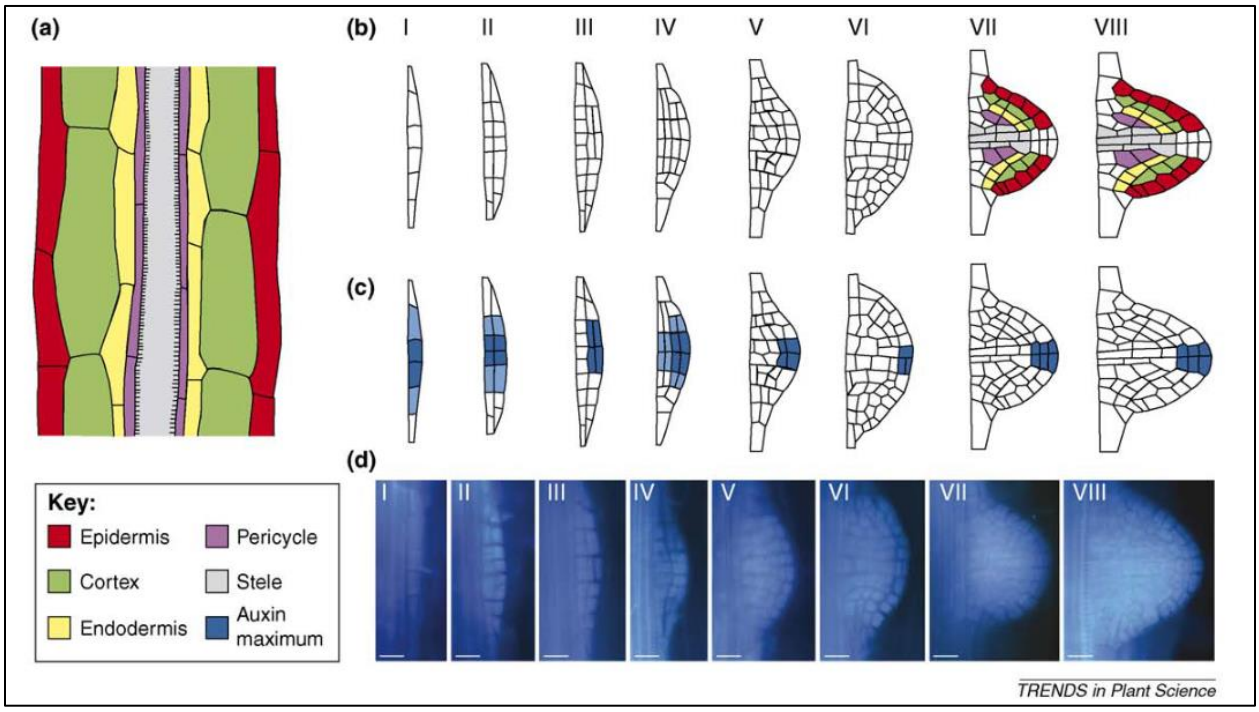

Figure 1.3. Stages of lateral root primordium development. **(a),** Lateral roots originate deep within the primary root from the pericycle cells. **(b),** the eight stages of primordium development (roman numbers), and the establishment of the auxin signaling maximum as demonstrated with the *DR5::GUS* reporter gene system (blue gradient, **(c)**). **(d),** microscopy images of the eight stages of lateral root primordium development. The scale bars represent 20µm. (Péret et al., 2009).

1.3.3. Root hair development

The root hair is a cylindrical extension of a single root epidermal cell, though not essential for plant viability. However, root hairs vastly increase the root surface area and root diameter, which facilitates water and nutrient uptake, soil anchorage, and interaction with the soil microbiome. Therefore, root hair development is constantly adapted to changes in the root's surroundings, allowing the plant to meet the nutrient demand in heterogenous soil environments (Grierson & Schiefelbein, 2002; Vissenberg et al., 2020).

Root hair development starts with the determination of whether an epidermal cell becomes a root hair. The root epidermis of members of the family Brassicaceae, such as Arabidopsis, possesses a distinct position-dependent pattern of root hair cells and non-hair cells (Fig. 1.1C). Epidermal cell fate determination depends on a position-dependent signal originating from the underlying cortical cells, which triggers a transcription factor cascade that leads to the initiation of root hair bulge. The bulge then begins tip growth before ultimately maturing (Vissenberg et al., 2020). The genetic regulation of root hair development is controlled by hormonal cues, and crosstalk between them is essential to the plant's ability to dynamically regulate root hair form and function in response to changing soil environment (Fig. 1.4).

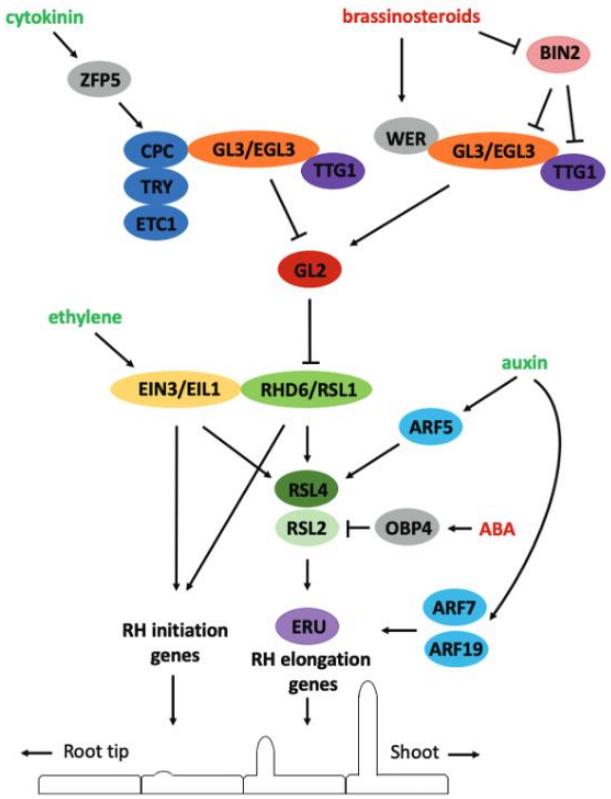

Figure 1.4. Hormonal regulation of root hair development. Hormones stimulating root hair growth are shown in green, those repressing are shown in red. Root tip is at the left side in the schematic longitudinal section through a single epidermal cell layer with consecutive root hair developmental phases. ARF5/7/19, AUXIN RESPONSE FACTOR 5/7/19; BIN2, BRASSINOSTEROID-INSENSITIVE 2; CPC, CAPRICE; EGL3, ENHANCER OF GLABRA 3; EIN3, ETHYLENE INSENSITIVE 3; EIL1, ETHYLENE INSENSITIVE 3-LIKE 1; ETC1, ENHANCER OF TRY AND CPC1; GL2/3, GLABRA2/3; OBP4, OBF BINDING PROTEIN 4; RHD6, ROOT HAIR DEFECTIVE 6; RSL1–4, ROOT HAIR DEFECTIVE 6-LIKE 1–4; TRY, TRYPTICHON; TTG1, TRANSPARENT TESTA GLABRA 1; WER, WEREWOLF; ZFP5, ZINC FINGER PROTEIN 5 (Vissenberg et al., 2020).

Auxin and cytokinin are known to enhance root hair elongation but had no influence on root hair distribution patterns. Ethylene, on the other hand, stimulates root hair growth and regulates the epidermal cell fate determination pathway (Vissenberg et al., 2020). Supplementation or overproduction of auxin leads to longer roots hair. Cytokinin supplementation also induces root hair elongation, and lowering endogenous cytokinin levels results in a shorter root hair phenotype. Evidence showed that auxin, cytokinin, and ethylene target similar genes in the

regulation of root hair elongation. However, they can regulate root hair independently, suggesting that elongated root hairs could benefit plants in different environments (S. Zhang et al., 2016). The regulation of root hair development is governed by multiple transcription factors. Among them, the ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4) appears to be a common target of auxin, cytokinin, and ethylene, suggesting a role in the integration of multiple phytohormones signals on root hair development (Fig. 1.4) (S. Zhang et al., 2016).

1.4. Cytokinin and auxin signaling pathways

The phytohormones cytokinin and auxin have a major role in the regulation of plant development, as described in the previous section. Also, the biostimulants that would be studied in this work are described to have a "cytokinin-" and "auxin-like" effect on plants. For these reasons, we would analyze the interaction of the biostimulants with the biosynthesis and signaling pathways of these two hormones.

1.4.1. Cytokinin

Cytokinin is synthesized from an intermediate compound of the MEP/MVA pathways mainly by ISOPENTENYL TRANSFERASE (IPT) and LONELY GUY (LOG) enzyme families (Figure 1.5a). The first step is to convert ATP and dimethylallyl pyrophosphate (DMAPP) into iPRTP and iPRDP (isopentenyl-adenosine-5'-triphosphate and isopentenyl-adenosine-5'-diphosphate, respectively) by IPT enzymes. Subsequently, the CYTOCHROME P450 enzyme (e.g., CYP735A) catalyzes the formation of cytokinin ribosides to be finally converted to the active forms (tZ, iP, cZ, and DZ) by LOG enzymes.

The cytokinin signaling pathway is a multistep phosphorelay system similar to the bacterial two-component response system (Fig. 1.5b). Cytokinin binds to histidine kinase receptors (AHKs) localized at the plasma membrane, which auto-phosphorylates. The phosphate is then transferred to cytosolic histidine phosphor-transmitter proteins (AHPs). AHPs are translocated to the nucleus and transfer the phosphate to type-B Arabidopsis response regulators (ARRs), which modulates the expression of type-A ARRs, which can also be phosphorylated through AHPs.

Cytokinin signaling can be tuned down through negative regulation mediated by type-A ARRs. Moreover, cytokinins can suffer inactivation through the conjugation with glucose or through irreversible oxidative cleavage by the cytokinin oxidases CKXs, which leads to degradation (Kieber & Schaller, 2014). Also, cytokinin levels can be decreased through conjugation with glucose (Schaller et al., 2015a).

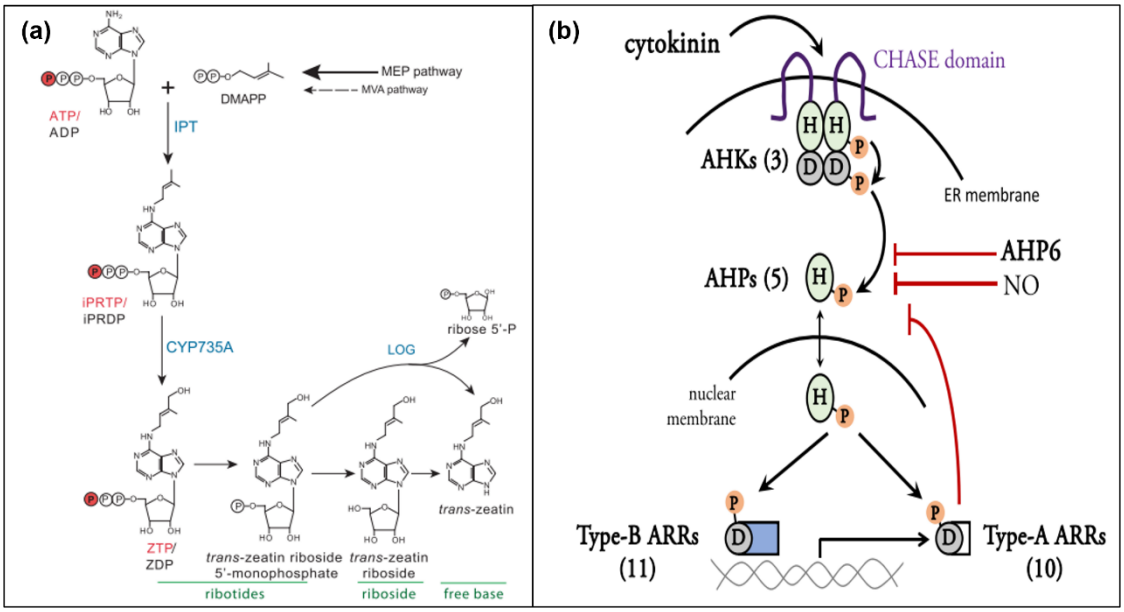

Figure 1.5. Cytokinin biosynthesis and signaling pathways. (a), Cytokinin biosynthesis pathway. The proposed biosynthesis of trans-zeatin tri-/diphosphate in Arabidopsis. Both ADP and ATP are likely substrates for the plant IPT enzyme, and these and their di- and tri-phosphate derivatives are indicated together (e. g. ATP/ADP). **(b)**, Cytokinin signaling pathway. See text for details. (Kieber & Schaller, 2014).

1.4.2. Auxin

The most abundant auxin is indole-3-acetic acid (IAA) and is primarily synthesized in a two-step process from the amino acid tryptophan, known as the tryptophan-dependent pathway. In the first step, tryptophan is converted to indole-3-pyruvate by the TRYPTOPHAN AMINOTRANSFERASE OF the ARABIDOPSIS (TAA) family of aminotransferases. Indole-3-pyruvate is then converted to IAA by the YUCCA family of flavin monooxygenases (Fig. 1.6a) (Schaller et al., 2015a). In the tryptophan-independent pathway, indole-3-glycerol phosphate (IGP) is

converted to IAA through a process that remains mostly uncharacterized. In the tryptophan-dependent pathway, IGP is converted to Indole, the precursor of tryptophan, by the TRYPTOPHAN SYNTHASE α (TSA) in the chloroplast. It has been proposed that the first step of the tryptophan-independent pathway is the conversion of IGP to Indole by the enzyme INDOLE SYNTHASE (INS), a homologous of TSA, but in the cytosol (B. Wang et al., 2015; R. Zhang et al., 2008). IAA can also be obtained via the β-oxidation of IBA in the peroxisomes (Fig. 1.6a) (Schaller et al., 2015a).

In the regulation of gene expression by auxin, the auxin-inducible genes have Auxin Response Elements (AREs) in the promoters, which are normally repressed by the interaction of members of three protein families: AUXIN RESPONSE FACTORS (ARF), Aux/IAA transcriptional repressor family, and TOPPLES (TPL) (Fig. 1.6b). Dimers of ARFs are bound to specific AREs in auxin-inducible genes. In the canonical auxin signaling pathway, ARFs recruit Aux/IAA, which subsequently recruits TPLs corepressors to prevent gene expression. In the presence of auxin, auxin brings together F-box proteins of the TRANSPORT INHIBITORRESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) family and the Aux/IAA transcriptional repressor family. The dimmer transfers activated ubiquitin from a ubiquitin-activating enzyme to the Aux/IAA, resulting in their degradation and subsequent transcription activation of auxin-inducible genes (Leyser, 2018). In the non-canonical signaling pathway, the ETTIN (ETT), also known as ARF3, interacts with an alternative set of transcriptional regulators: INDEHISCENT (IND), REPLUMLESS (RPL), and BREVIPEDICELLUS (BP). ETT interacts with the transcription factors such as IND to form a repressive complex that is released by high IAA concentrations, activating the auxin-inducible genes transcription (Kubeš & Napier, 2019).

Auxin levels can be regulated by the conjugation of active auxin to sugars and to various amino acids, some of which can be reversible. Active IAA levels can also be decreased by the degradation of IAA to 2-oxindole-3 acetic acid through an unknown enzyme (Schaller et al., 2015a).

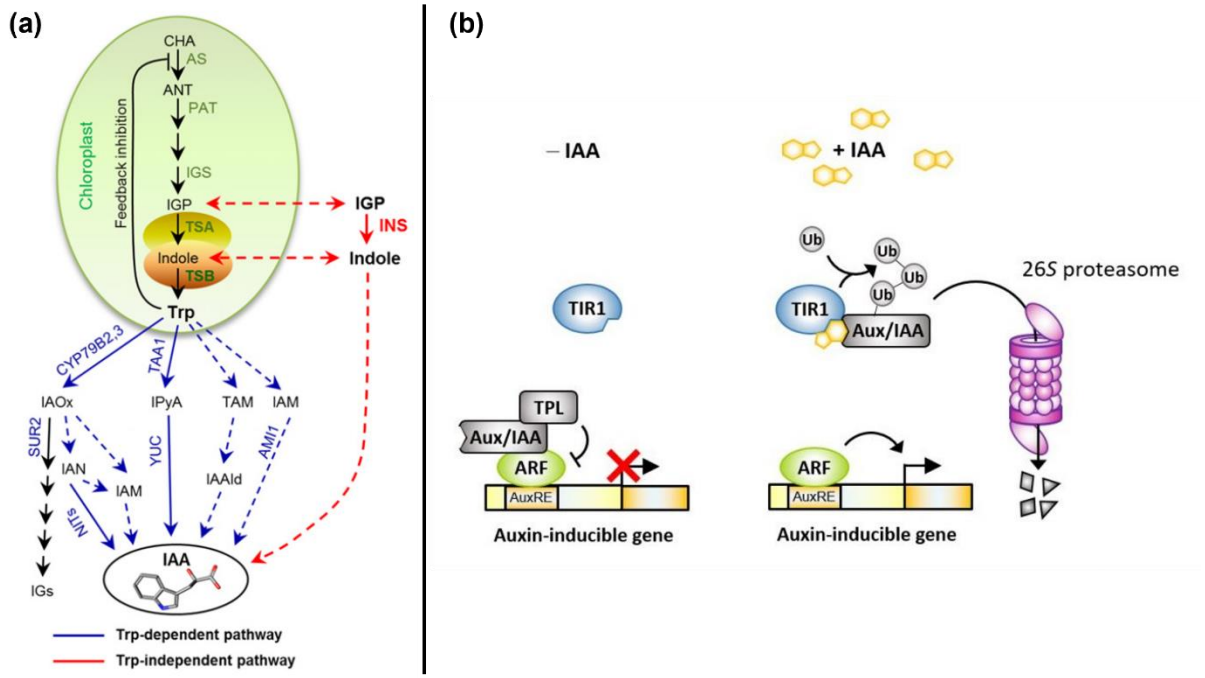

Figure 1.6. Auxin biosynthesis and signaling pathways. (a), model of the Trp-dependent and Trp-independent IAA biosynthetic pathways. Solid arrows refer to pathways with identified enzymes, and dashed arrows to undefined ones. ANT, anthranilate; AS, ANTHRANILATE SYNTHASE; CHA, chorismic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IGP, in-dole-3-glycerol phosphate; IGs, indole glucosinolates; IGS, INDOLE-3-GLYCEROLPHOSPHATE SYNTHASE; IPyA, indole-3-pyruvic acid; PAT, PHOSPHORRIBOSYL ANTHRANILATE TRANSFERASE; TAA1, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1; TAM, tryptamine; TSA, TRYPTOPHAN SYNTHASEα; TSB, TRYPTOPHAN SYNTHASEβ; YUC, YUCCA (B. Wang et al., 2015)**. (b),** the canonical auxin signaling pathway. At low auxin concentrations, Aux/IAA and TPL repress the activity of ARFs. At high auxin concentrations, IAA is bound by a TIR-Aux/IAA co-receptor complex, which triggers ubiquitination and subsequent degradation of the Aux/IAA repressor, thereby enabling ARF activity (Kuhn et al., 2020).

1.5. Approach

Taken all together, the beneficial effects of the seaweed appear to be modulated through the stimulation of the endogenous phytohormone signaling and biosynthesis in the plant, probably by other components within the extract, but not phytohormones themselves. Also, how this seaweed extract interacts with the hormonal metabolism within the plant has not been well established yet. The main objective of this study is to understand the mechanism of action of two commercial ANE biostimulants on plants by characterizing the phytohormone-like effect and identifying the hormone pathways that are being affected by the biostimulant treatment. For the industry, a knowledge of the mechanism of action of this type of product is of critical importance in order to reach new markets and improve product sales.

1.6. Hypothesis and Objectives

Hypothesis:

"*Ascophyllum nodosum-derived* biostimulant regulates plant development through the regulation of hormone signaling, orchestrated by cytokinin and auxin."

General aim:

To characterize the cytokinin and auxin-mediated regulation of plant development induced by *Ascophyllum nodosum*-derived biostimulants.

Specific objectives:

1. Characterize the phenotypic changes induced by the cytokinin and auxin-like biostimulants in *Arabidopsis thaliana* grown under standard *in vitro* conditions.
2. Evaluate the modulation of cytokinin and auxin signaling in response to cytokinin and auxin-like biostimulants and the downstream hormonal responses in *Arabidopsis thaliana.*
3. Evaluate the expression of cytokinin, auxin and other hormone-related genes induced by cytokinin and auxin-like biostimulants in *Arabidopsis thaliana.*

1.7. References

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2. Chapter II
Phenotypic characterization of the effect of two commercial *Ascophyllum nodosum* biostimulants treatment on *Arabidopsis thaliana in vitro*

2.1. Introduction

The term "biostimulants" refers to any substance or microorganism used to enhance plants' nutritional use efficiency, abiotic stress tolerance, and/or quality traits, irrespective of their nutrient composition (du Jardin, 2015). Biostimulants operate through a different mechanism than fertilizer and do not contain pesticide compounds. According to the European Biostimulants Industry Council (EBIC), biostimulants promote natural processes to enhance/benefit nutrient uptake, nutrient efficiency, biotic/abiotic stress tolerance, and crop quality (http://www.biostimulants.eu/). In a world scenario where erosion and inadequate agricultural practices negatively affect soil fertility every year, biostimulants arise as an emerging class of crop management products that can improve crop yield and quality and, at the same time, improve soil health in a sustainable manner (el Boukhari et al., 2020). Moreover, biostimulants are complementary to traditional crop nutrition and crop protection products. Among the different categories of biostimulants, seaweed extracts biostimulants represented in 2018 more than 33% of the global market (el Boukhari et al., 2020).

Ascophyllum nodosum is a brown macroalga from the northern Atlantic Ocean, commonly found in Europe's northwestern coast and North America's north-eastern coast. In agriculture, *A. nodosum* extracts (ANE) are widely used as a growth stimulant and to protect crops against stress (Shukla et al., 2019). Algae biostimulants from *A. nodosum* extracts are known to have a phytohormone-like effect on plants (Khan et al., 2011; Rayorath, Khan, et al., 2008; Subramanian, Sangha, & Gray, 2011; Wally et al., 2013). The application of ANE has been reported to impact plant performance, such as improved plant vigor, increased root yield (e.g., carrots), fruit yields, increased chlorophyll content, sugar content, enhanced germination, and seedling vigor, among others. This has been reported not only in *Arabidopsis thaliana* (Goñi et al., 2016; Rayorath, Jithesh, et al., 2008; Wally et al., 2013) but also in several crops such as maize, wheat, soybean, barley, grapevine, and others (Shukla et al., 2019).

The plant root system is essential for plant growth as it is responsible for nutrient and water acquisition, anchorage, and interaction with soil micro-organisms to enhance nutrient absorption. Overall root architecture is determined by root branching, root angle, and root growth rates. The plasticity of the plant root system is paramount for the plant's ability to explore the soil and the plant's adaptation to the habitat (Duque & Villordon, 2019). Enhancing root branching and root hair development could improve nutrient efficiency acquisition (Li et al., 2016). ANE treatment has been previously reported that alters root architecture and improve nutrient uptake (Shukla et al., 2019). However, there is little information regarding the effects of ANE on root system architecture, lateral roots, and root hair development. In this work, we will do a detailed analysis of the effect of ANE-derived biostimulants on root development.

In *Arabidopsis*, only a few studies are available where the effects of ANE plant growth have been analyzed. Treatment with two different ANEs (aqueous solutions at 0.01 g L^{-1} and 0.1 g L^{-1}) has shown that the biostimulant stimulated primary root growth of plants *in vitro* culture conditions and increased plant height and leaf number in plants grown in greenhouse conditions (Rayorath, Jithesh, et al., 2008). On the contrary, another study in *Arabidopsis in vitro* showed that ANE (aqueous solution 0.01% w/v) reduced primary root length and lateral root number (Wally et al., 2013). Other studies have shown that foliar spray treatment with ANE (0.2% v/v) in *Arabidopsis* grown in pots incremented the plant's height and rosette leaf number (Goñi et al., 2016).

The mechanism of action of ANE biostimulants has been associated with phytohormones due to the similarity of the effects on plant phenotype with that of the phytohormones themselves (Khan et al., 2011). However, studies have shown that the phytohormone content found in ANE is not high enough to induce the phenotypic changes in plants and most likely does not explain the growth-promoting ability of ANE (Rayorath, Khan, et al., 2008; Stirk et al., 2003; Wally et al., 2013). Thus, other components within the seaweed could trigger endogenous phytohormone accumulation in plants by altering the regulation of hormone-related genes (Jithesh et al., 2018; Khan et al., 2011; Santaniello et al., 2017; Wally et al., 2013).

One of the most critical challenges of the biostimulant industry is to standardize the production process to obtain consistent and efficient products. Parameters such as environmental

conditions where the raw material was taken and the manufacturing process affect the extract's composition and, therefore, the reported biostimulant activity even between products of the same algae specie (Craigie et al., 2008; el Boukhari et al., 2020; Goñi et al., 2016; Goñi, Quille, & O'Connell, 2018; Wally et al., 2013). However, this problem could be seen as an opportunity for the industry to obtain more than one product from the same raw material. In this study, we developed an extensive phenotypic characterization of the effect of two commercial *A. nodosum* biostimulants, Exelgrow and Exelmax (ADAMA Ltd.), in *Arabidopsis thaliana in vitro* culture conditions. Both products were produced with different manufacturing methodologies from the same raw material. The main objective of the study was to differentiate the products' biostimulant activity and characterize the hormone-like effect induced in plants. Due to the conditions of the assays, the most extensive analysis was done on root development. Here we report that both products have different biostimulant activity. Moreover, Exelgrow could be associated with inducing a cytokinin-like, and Exelmax having an auxin-like effect. However, some of the phenotypes observed could be related to more than one hormone action, which leads us to believe that biostimulants probably act holistically on plants, altering more than one pathway. Further genetic analysis of cytokinin, auxin, and other phytohormone-related pathways needs to be done to elucidate the action mechanism of EG and EM biostimulants.

2.2. Materials and methods

2.2.1. Plant material and growth conditions

Arabidopsis thaliana (Col-0) seeds were surfaced sterilized with 50% v/v NaClO (commercial chlorine, 4.9%) and 10% v/v Triton X-100 at 0.1% for 10 min, and washed five times with sterile distilled water, 2 min each time. Sterile seeds were germinated in ½ MS medium salt (Murashige & Skoog, 1962) supplemented with 1% (w/v) sucrose, 0.05% MES sodium salts, 0.8% agar, pH 5.9. Seven days after sowing, seedlings with consistent growth were selected and transferred to square plates with ½ MS medium supplemented with the corresponding biostimulants treatment. Control plants were transferred to a new plate with only ½ MS medium. Plates were sealed with microporous tape to allow gas exchange. Each plate contained 5-6 seedlings to

enable roots to develop properly without intercepting the next plant. Plates were maintained vertically oriented in a growth chamber at 21±2°C with a day/night photoperiod of 16/8 h with a light intensity of 100 µmol $m^{-2}s^{-1}$.

2.2.2. Commercial biostimulant treatments

Two commercial *A. nodosum* biostimulants from ADAMA Ltd. were used: Exelgrow (EG) and Exelmax (EM). Several concentrations were tested to determine the concentration of biostimulants to be applied in an *in-vitro* culture system with *A. thaliana*, starting with the recommended concentration for field conditions (CF) and between 1/10 and 1/100 of FC. With this starting curve, phenotype was analyzed, and then, if necessary, new concentrations in between those were added to the curve until it reached a pick on some of the parameters examined. Thus, finally, for the EG biostimulant concentrations were 0.0001, 0.0005, 0.001, 0.003, and 0.005% v/v, and for the EM biostimulant were 0.001, 0.01, 0.02, and 0.1% v/v. The biostimulants did not need to be sterilized. The corresponding amount of biostimulants was mixed by inversion with the melted ½ MS media in a 50 ml falcon tube and then added to the plate. Each experiment comparing the biostimulants at different concentrations and the control, was repeated at least three times (replicates).

2.2.3. Phenotypic root analysis

Plates with *A. thaliana* Col-0 seedlings were scanned on the day of transfer (day 0) and at three and seven days of treatment. ImageJ (1.53f51) software was used for primary and lateral root quantification. Primary root length was measured on the day of transfer and at seven days of treatment. Then, primary root growth (PR) was determined as $length_{day7} - length_{day0}$. The lateral root number was determined by visual quantification in photos. Lateral root density (D) was calculated as LR/PR $length_{day7.}$ To measure the length of lateral root (LR) and be comparable, all lateral roots were measured at the end of treatment (seven days). Then, total lateral root length (TLRL) was calculated as the summation of all lateral roots of the plant. The TLRL of each treatment was obtained as the average TLRL of all the plants per treatment. Data were expressed as relative values between treatment and control plants, and 15 plants distributed in 3 plates were evaluated per treatment and replicate. In addition, fresh and dry weight of shoot and roots

was measured at the end of the assay. For dry weight, the material was dehydrated in an oven for three days at 37°C. Dry weight was measured three times, and the value of each replicate corresponded to the mean of 3 measurements.

2.2.4. Root hair quantification

A. thaliana Col-0 seedlings were transferred to the corresponding treatment seven days after sowing. Photos of each plant were taken with a ZEISS Stemi 305 stereo microscope and camera Axiocam 105 after seven days of treatment (objective 1X, optovar 2X, exposure time 159.6 ms, dark filed reflector). The quantification area was a 5 mm section of the zone of transfer to treatment. To identify the zone of transfer to treatment, the root tip position was marketed on the plate on day 0 of treatment. Root hair length and number were quantified using ImageJ (1.53f51) software. Root hair density was then calculated as the root hair number per length of the root section measured. Ten plants were analyzed per treatment (2 plates) and replicate (3).

2.2.5. Microscopic analysis of lateral roots primordium

Microscopic analysis of lateral roots primordia was done in the transgenic Col-0 line with the DR5::GUS construct (Ulmasov et al., 1997) plants. Plants were transferred to treatment seven after sowing and maintained for three days. For root clearing, the roots were fixed in 70% ethanol overnight. Then, roots were incubated in two solutions: first, a solution of 0.24 N HCl in 20% methanol at 62°C for 40 min, and second, a solution of 7% NaOH in 60% ethanol for 20 min at room temperature. Roots were hydrated in 40%, 20%, and 10% ethanol for 20 minutes for each step. Then roots were incubated in 50% glycerol (25% glycerol in 5% ethanol, final concentration) for 2 hr. Plants were mounted in glycerol 25%. Root primordium was observed and photographed with ZEISS Axio Scope.A1 microscope, and camera Axiocam 208. All lateral root primordium were counted and classified from stages I-VII, Emerging (VIII), and lateral root completely developed (LR) (Malamy & Benfey, 1997). Evaluations were performed in 8 plants per treatment (1 plate) and replicate (3).

2.2.6. Phenotypic rosette analysis

Col-0 seedlings were transferred to the corresponding treatments seven days after sowing. After transfer to treatment, plates were maintained horizontally for ten days. Photos of the plate were taken next to a ruler to scale. The rosette leaf number of each plant was counted and recorded. ImageJ (1.53f51) software was used to quantify the rosette projected area, perimeter, and convex hull area. Details of each parameter measurement are in Supplementary Figure S1. Rosette's compactness and rosette´s stockiness were calculated as follows:

$$Compactness = \frac{ProjectedArea}{ConvexHullArea}$$

$$Stockiness = \frac{4 * \pi * ProjectedArea}{(Perimeter)^2}$$

2.2.7. Statistical analysis

Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$). Relative to control values of each replicate were calculated as $Mean_{treatment}$ / $Mean_{control}$ *100.

2.3. Results

2.3.1. Standardization of the methodology for biostimulants treatment *in vitro* conditions

The biostimulants availability is different in the solid medium than in soil. Therefore, it was necessary to test various concentrations of each product to determine the optimum dose of each biostimulant for Arabidopsis *in vitro* culture conditions detailed in "Materials and Methods".

Preliminary assays were developed to determine the age of the plant for the treatment application and to define the treatment period. First, an assay was performed to test whether to start the biostimulant treatment 4 or 7 days after sowing (DAS). The application of the biostimulant 7 DAS resulted in a less detrimental effect on root development compared to the

treatment administered four 4 DAS (data not shown). To determine the appropriate treatment period, PR, LR number, and LR density were analyzed at 3 and 7 days after transfer to treatment (DAT) (Supplementary Figure S2). As the differences between concentrations were more distinct at 7 DAT, we chose this time point for the subsequent experiments.

2.3.2. Biostimulants promote lateral root elongation

Different dilutions of EG (10^{-4}-10^{-2} v/v) and EM (10^{-3}-10^{-1} v/v) were used under the experimental setting standardized above to evaluate the biostimulant concentration. Interestingly, high EG and EM biostimulant concentrations result in a strong inhibitory effect of primary root length (Fig. 2.1). However, at lower concentrations, both EG and EM promoted lateral root development. For EG biostimulant, while no differences were observed for the number and density of lateral roots, a concentration of 0.0005% v/v seems to have longer lateral roots. The same was observed for EG at a concentration of 0.01% v/v. To quantify this, we measured the TLRL of each treatment (Fig. 2.1C, D), which represents the total length of all lateral roots if they were placed aligned and could be understood as the total LR surface. Thus, EG showed a trend to promote lateral root development by increasing TLRL by ~35% at 0.0005% v/v, whereas the EM biostimulant promoted TLRL at medium concentrations (0.01%-0.02% v/v) (Fig. 2.1C). Indeed, in EM-treated plants the TLRL at 0.01% v/v were approx. 70% greater than control plants and two times higher than at 0.02% v/v. Interestingly, the promoting effect on lateral root development was observed in the PR section developed before the transfer to treatment (Fig. 2.1A). The TLRL followed a similar trend to the LR number in plants with EG treatment. Thus, the increment on the total LR surface at low concentrations could be associated with the increase in LR (Fig. 2.1C). With EM, instead, the peak on TLRL was not associated with the LR trend (Fig. 2.1D).

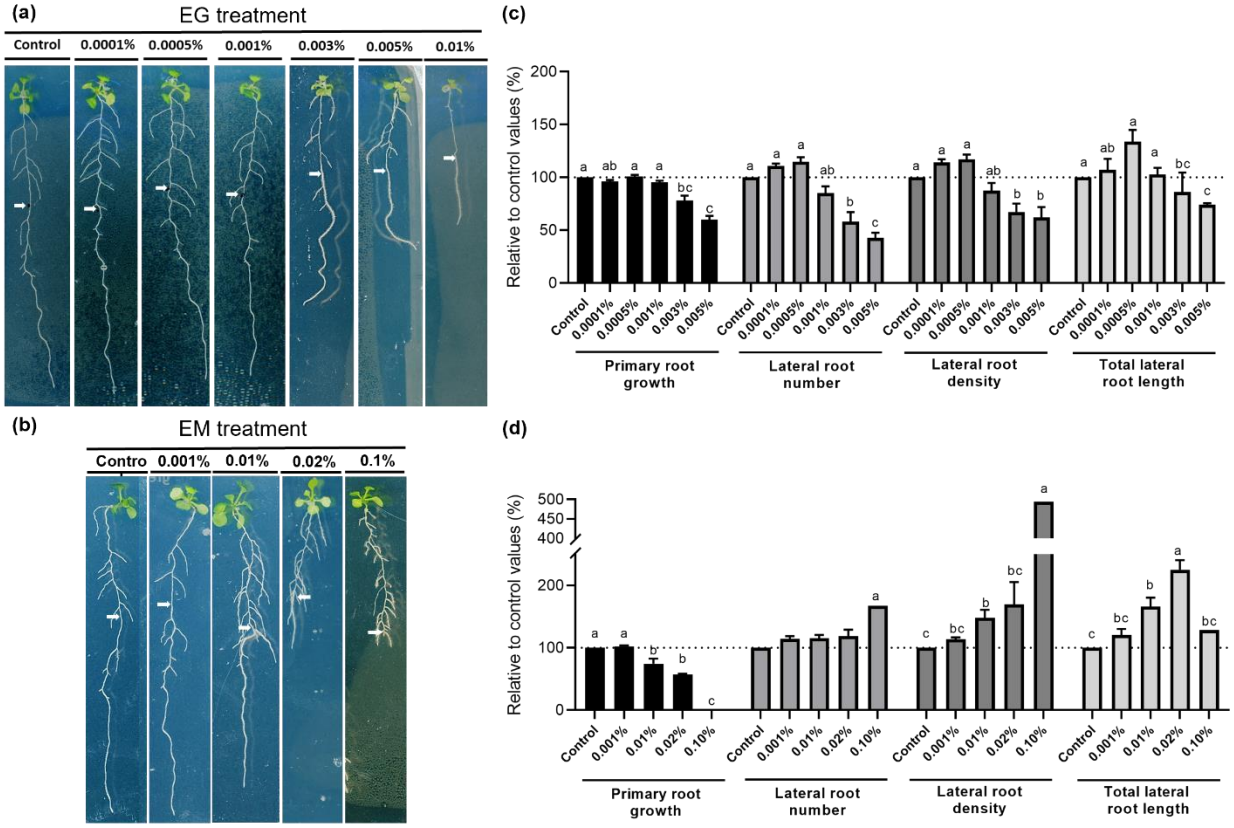

Figure 2.1. Effects of different concentrations of Exelgrow (EG) and Exelmax (EM) biostimulants on root growth of *A. thaliana* Col-0. Seedlings. Plants were transferred to treatment seven days after sowing. Photos for phenotypic analysis were taken seven days after transfer to treatment. Photos **(a & b)** are representative phenotypes of plants of *A. thaliana* Col-0 growing under the different treatments. White arrows indicate the position of the primary root at the time of transfer to treatment. **(c & d)**, relative values (treatment/control*100) for primary root growth, lateral root number, lateral root density (lateral root number per cm of primary root), and total lateral root length of plants growing with EG and EM treatment, respectively. 15 plants were measured per condition, and at least three independent experiments were done with each concentration (exception with 0.02% two replicates, and 0.1% v/v one replicate). Bars represent the mean values (N=3) and standard error (SEM). Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences.

To characterize these results in more detail, we analyzed the distributions of the lateral roots according to their length (Fig. 2.2). Thus, plants treated with EG had a significantly higher proportion of lateral roots of size > 1 cm than the control plants (Fig. 2.2A). In control plants, nearly 30% of the lateral roots had a size < 0.1 cm; ~ 65% were distributed between 0.1-1 cm, and the remaining 5% were lateral roots higher than 1 cm. With increasing EG concentration, there was a trend towards increasing the percentage of RLs >1 cm, and towards decreasing the percentage of RLs <0.1 cm, compared to control plants. Interestingly, the EM biostimulant had similar effects than EG (Fig. 2.2B). As the concentration of EM increased, plants had more developed lateral roots, as they had a greater percentage of longer lateral roots (>1cm), reaching a peak at 0.02% v/v. However, EM > 0.02% v/v concentrations had a strong inhibitory effect, and lateral root growth was arrested. Together, these results show that the biostimulants treatment promotes lateral root elongation.

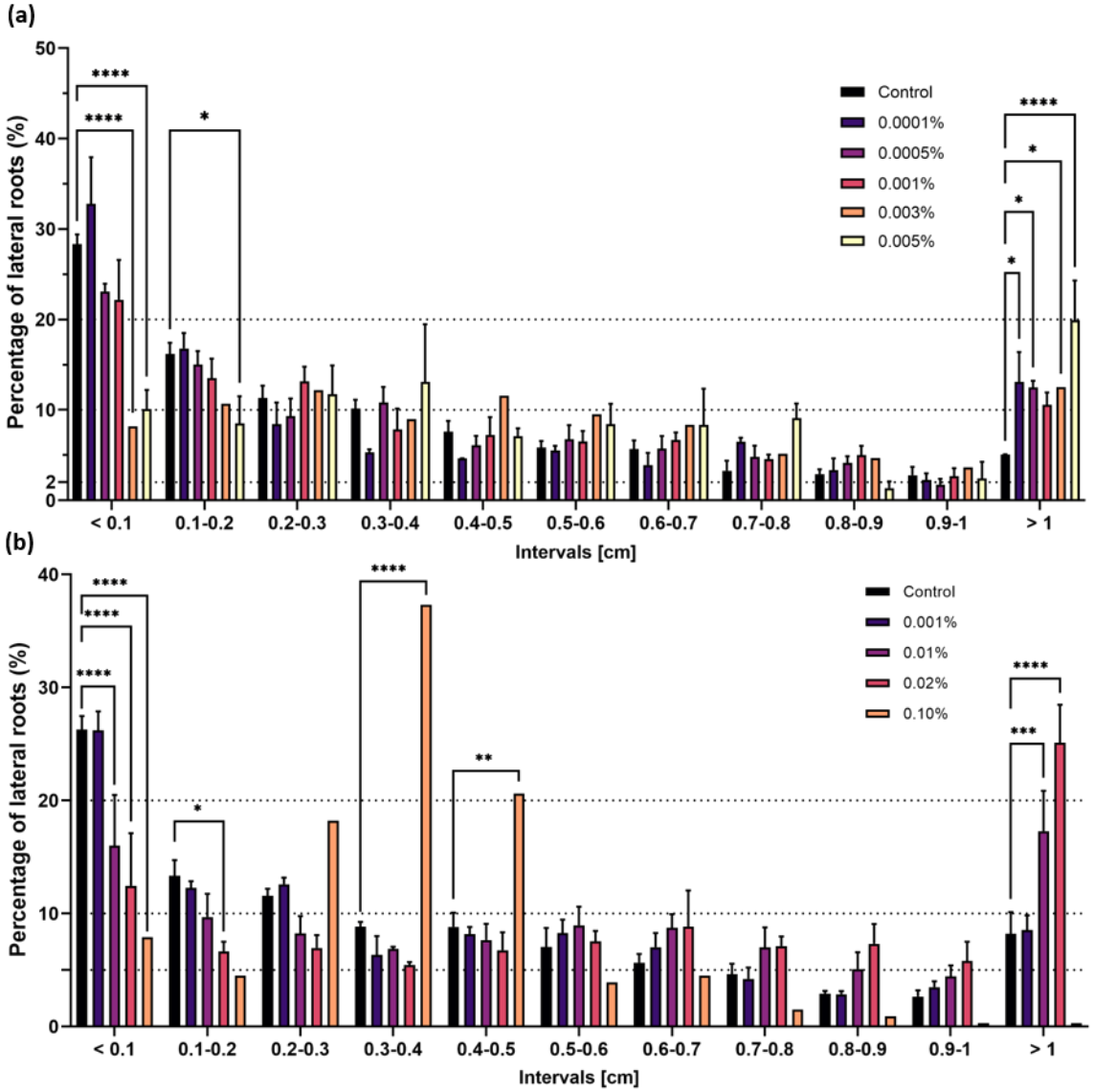

Figure 2.2: Distribution of lateral roots per length intervals of plants in Figure 2.1. Plants with EG (a) and EM (b) treatments, respectively. Results are expressed as the percentage of the total lateral root number per treatment. Measurements were performed on 15 plants per condition in each replicate. Bars represent the mean values and (SEM) of three replicates (N = 3). Mean significant differences in each interval were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$; 0.03(*), 0.002(**), 0.0002(***), <0.0001(****).

We also measured roots' fresh and dry weight at the end of the assay. Interestingly, despite the negative effect on root development with the biostimulants treatment at higher concentrations, roots fresh weights do not follow the same trend. The root fresh weight under EG treatment was moderately higher than control plants independently of the concentration and was significantly greater with EG 0.001% v/v treatment (Fig. 1.3A). Furthermore, in EM-treated plants, the fresh weight increased by over 20% in all concentrations, having a peak at 0.01% v/v with an increment of ~50%. In both EG- and EM-treated plants, root dry weight values were similar to control plants at all concentrations tested (Fig. 1.3B). Although EM treatment inhibits PR development, it did not negatively affect the LR number and, in fact, had a positive effect on LR development, which could explain the results obtained on root weight (Figs. 1.1 and 1.2).

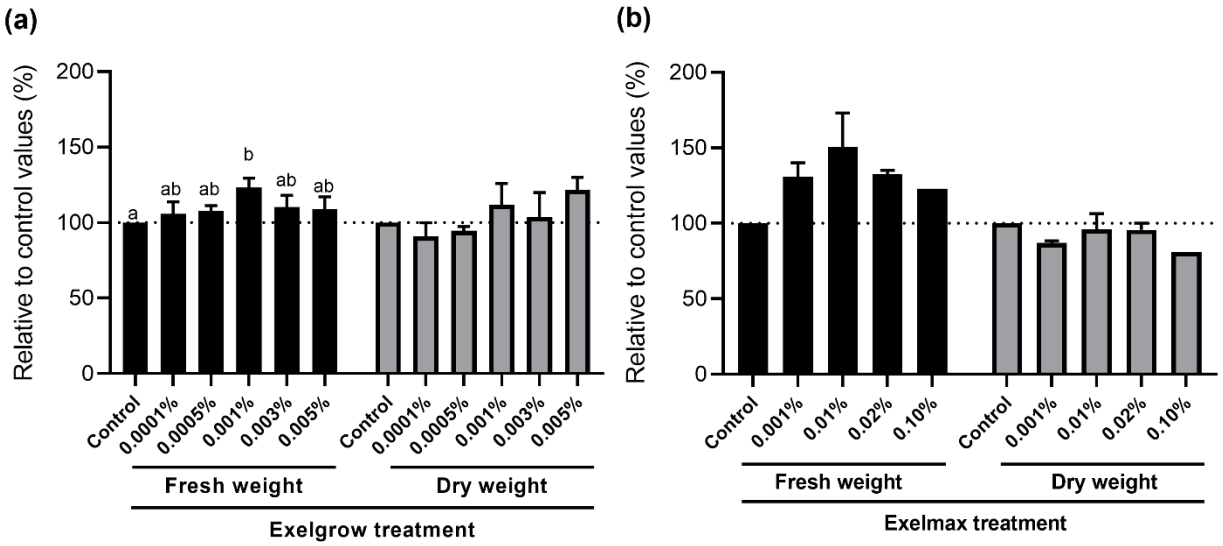

Figure 2.3. Fresh and dry weight of roots of *A. thaliana* Col-0 seedlings growing for seven days at different concentrations of Exlegrow **(a)** and Exelmax **(b)** biostimulants. Values are means ± standard deviation of treatment relative to the control of > 10 plants per treatment and at least three biological replicates*. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); Different letters represent significant statistical differences. No letters mean there are no significant differences in that group. *Exception of EM 0.1% v/v, only 1 replicate.

2.3.3. Lateral root primordium development and organization

With the EM biostimulant at concentrations above 0.01% v/v lateral roots were mainly concentrated on the upper part of the root, which corresponds to the primary root section that was developed before the transfer to treatment (Fig. 2.1B). To have a better understanding of the effect of the biostimulants on lateral root initiation and development, we analyzed lateral root primordium (LRP) in plants at earlier times of the treatments. Seedlings were observed in the microscope 3 days after transfer to treatment, and LRP were analyzed and classified according to their stage of development. Interestingly, there was no significant difference in the total LRs between biostimulants and control treatment. Nevertheless, EG-treated plants had moderately fewer total lateral roots than the control (Fig. 2.4A, B); the LRP proportion was slightly higher, and the lateral root proportion was reduced (Fig. 2.4D).

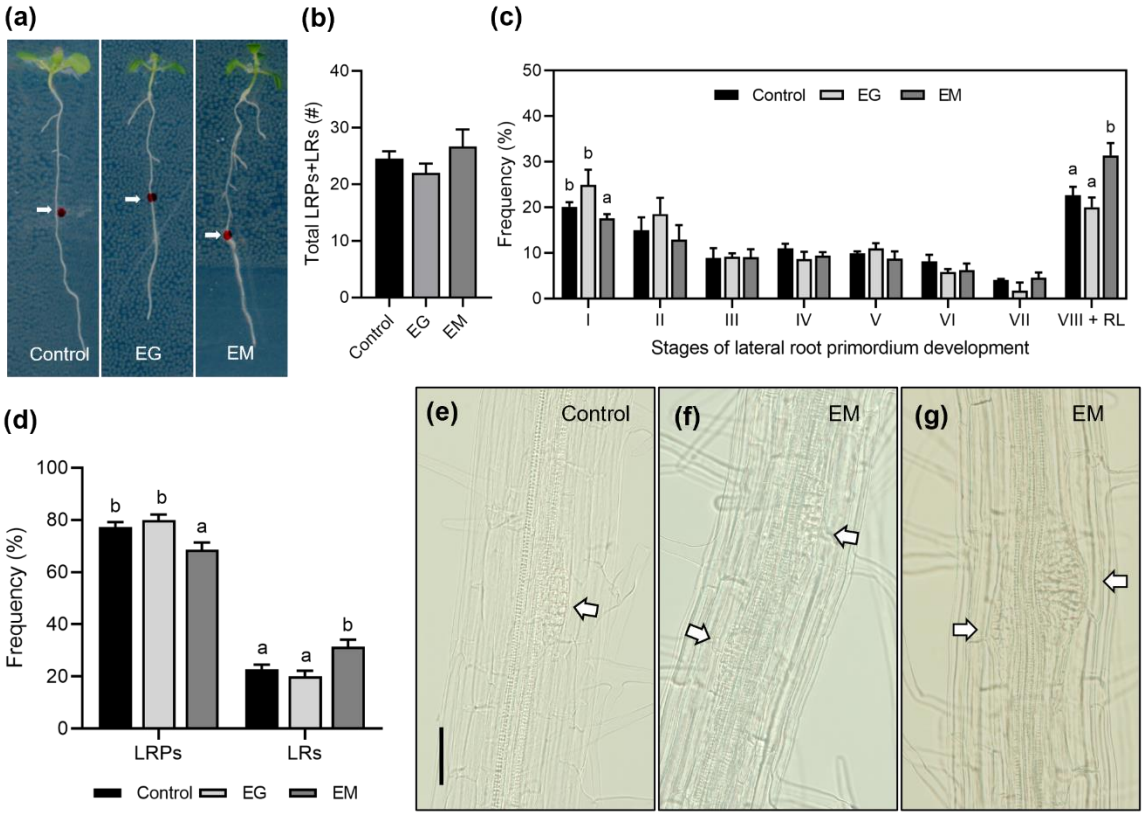

Figure 2.4. Primordium development at early stages of biostimulants treatment. **(a)** photo of representative plants of transgenic *A. thaliana* DR5::GUS, treated for 3 days with EG 0.001% v/v and EM

0.01%v/v biostimulants and control. White arrows indicate the position of the primary root at the time of transfer to treatment; **(b)**, total number of lateral roots (LR), lateral root primordium (stages I-VII) + lateral roots (stages VIII, emerging, and LRs, fully developed); **(c)** distributions of lateral root primordium of plants in **(a)** per stage of development. Stage VIII and LR represent primordium in the emergence and fully developed lateral roots, respectively. **(d),** frequency of LRPs and LRs. **(e-f),** representative phenotype of abnormal primordium organization on the root; a similar effect is observed on plants treated with auxin. Plants with control **(e)** and EM treatment **(f-g)**. White arrows indicated the primordium position. Bars represent the mean values and SEM of 3 replicates (N=3) of 8 plants per treatment and replicate. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences. No letters mean there are no significant differences in that group. For **c and d**, the comparison was made between treatments in each group (stage).

Plants treated with EM biostimulant followed the same trend that was observed previously with lateral roots but attenuated; total lateral roots (LRs+LRPs) were 8% higher than in control plants (Fig. 2.4B), in contrast with the 70% increment in total LR number at the same biostimulants concentration (Fig. 2.1B). The EM-treated plants showed a reduced number of LRPs, which could be indicated that EM treatment disrupted lateral root initiation. However, plants showed an increased proportion of emerging and fully developed lateral roots, which could suggest that EM enhances the development of pre-existing LRPs (Fig. 2.4D).

The distributions of primordium per stage of development showed that plants with EM treatment had a reduced proportion of LRPs in early stages (I-II) compared with plants with no biostimulants treatment and an enhanced proportion of lateral roots in advanced stages (VIII+LR). EG-treated plants, on the other hand, had more lateral roots in early stages (I and II) and less primordium in advanced stages of development, although the difference was not significant (Fig. 2.4C). Moreover, the microscopic examination of EM-treated plants showed events of abnormal primordium orientation. It was frequently observed primordium growing opposite or adjacent to each other (Fig. 2.4E). This phenomenon was not observed in EG-treated nor control plants.

These results suggest that EM causes the disorganization of the development of LRPs at the primary root.

2.3.4. Biostimulants enhanced root hair development

Interestingly, while higher biostimulant concentrations negatively affect root length, it also seems to regulate root hair development. This could be easily observed at higher concentrations in the newly developed root (Fig 2.1A-B, Fig 2.5). Thus, to characterize this response in more detail, EG 0.001% v/v and EM 0.01% v/v were used for further analysis.

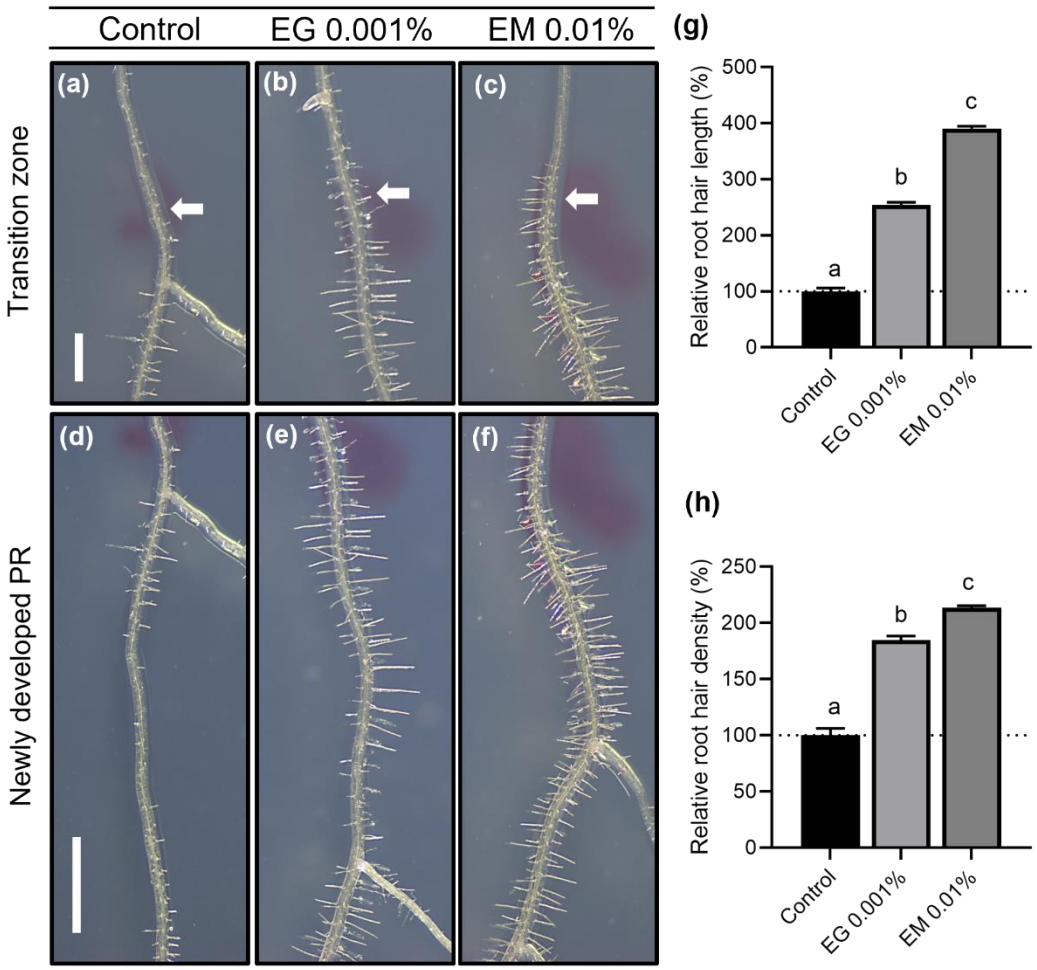

Figure 2.5. Root hair development of seedlings of *A. thaliana* Col-0 with the biostimulants treatment. **(a-f)** photos of representative phenotypes of seedlings treated for 7 days with EG 0.001% v/v and EM 0.01% v/v. **(a-c)**, the transition zone between the primary root developed before and after transfer to treatment. The white arrow indicated the point where the root tip was at the moment of transfer to

treatment Scale bar= 500 µm. **(b-f)** 5 mm section of the primary root below the transition point. Scale bar= 1000 µm. **(g)** root hair length. **(h)** root hair density, calculated as root hair number in a ~ 5 mm section of the primary root. All root hairs in a ~ 5mm section of the root just below the point of transfer to treatment were counted and measured. Bars represent relative to control mean values and SEM of 3 independent experiments, with n~10 plants per assay. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences.

To quantify root hair development, root hair number and length were measured in a 5 mm section of the primary root right after the point of transfer to treatment (Fig. 2.5A, B). Remarkably, both EG and EM biostimulants increase root hair length and density, being even more exacerbated with EM treatment (Fig. 2.5C, D).

2.3.5. Biostimulant effect on rosette development

To analyze how the biostimulant treatment in the media impacts the aerial tissues, we evaluated different parameters of rosette development, such as leaf number, leaf area, compactness, and stockiness. Thus, rosettes were apparently larger in plants with EM ≤ 0.001% v/v and EG at low concentrations (≤ 0.0005% v/v) (Fig. 2.1A).

To allow better rosette development, plants were grown on horizontally oriented plates during the treatment period (Fig. 2.6). Pictures of the plate were taken on the 10^{th} day of treatment, and the rosette projected area, perimeter, convex hull area, and leaf number were measured. Then, two morphological parameters were calculated: rosette compactness and stockiness. These parameters are related to the rosette's leaf density and shape. Under these growth conditions, plants with EM treatment had a significantly increased rosette area, reduced compactness, and similar stockiness value compared to control plants (Fig. 2.6). Rosettes of EG-treated plants had a slightly reduced area, compactness value similar to control plants, and stockiness value slightly higher than control but not significant (Fig. 2.6). No major differences were observed in leaf number between treatments.

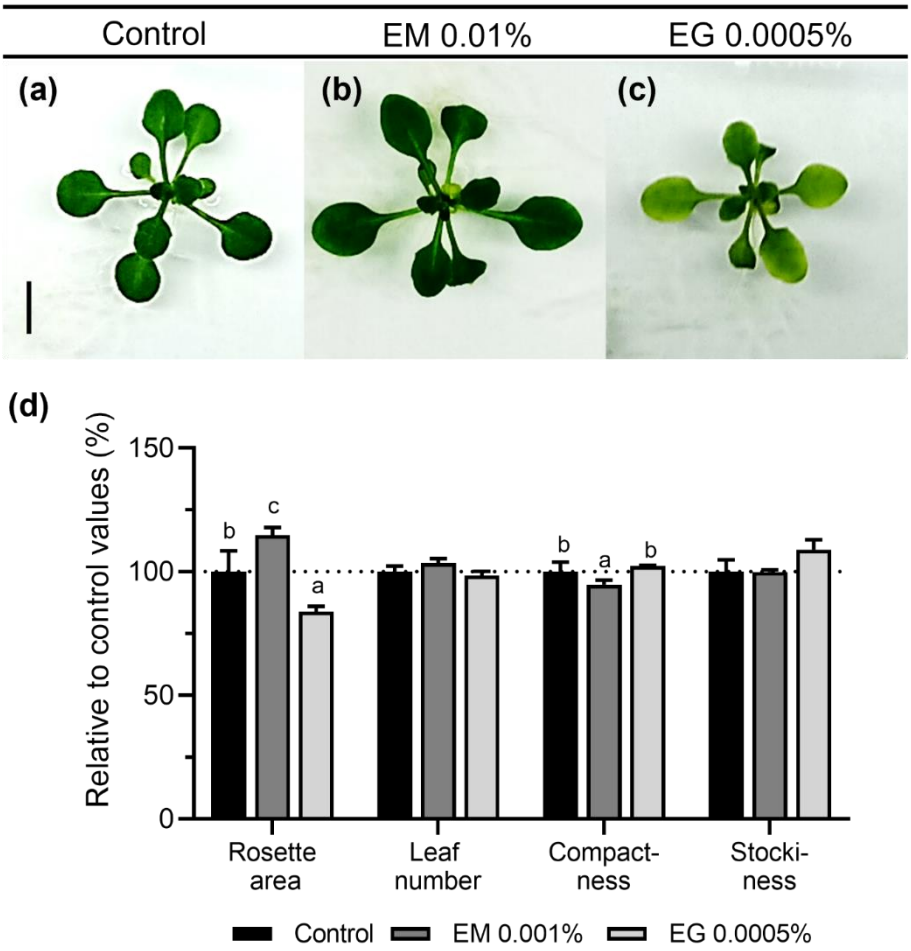

Figure 2.6. Rosette development of seedlings treated for 10 days with ½ MS (control), EM 0.01% v/v, and EG 0.0005% v/v. Seedlings of *A. thaliana* Col-0 grown for 7 days in ½ MS media were transferred to ½ MS supplemented with the corresponding treatment. **(a-c)** photos of representative phenotypes (scale bar= 5mm). **(d)** Relative to control values of Rosette projected area, Leaf number. **(e)** Rosette compactness and Stockiness **(f)**. Bars represent relative to control mean values and SEM of at least 3 independent experiments, with n~10 plants per assay. Mean significant differences for each group were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences. No letters indicated that there were no significant differences in that group.

To further analyze the impact of biostimulants in rosette development, we quantify if the changes in leaf area also impact dry and fresh weight. Thus, at concentrations of EG treatment ≤ 0.001%

v/v, there were no major differences between treatments in fresh or dry weight. However, there was a slight reduction in fresh and dry weight at concentrations ≤0.0005% v/v, and then at 0.001% v/v, recovered similar to control values. At higher concentrations (≥ 0.003% v/v), there was a negative effect on rosette growth as well as overall plant development (Fig. 2.6A and Fig. 2.1). With the EM biostimulant, no significant differences between treated plants and control ones were observed (Fig. 2.6B). However, a tendency to increase fresh and dry weight is observed on rosettes with EM treatment at concentrations below 0.02% v/v. Moreover, fresh weight peaked at 0.01% v/v with an increment of 13% compared to the control.

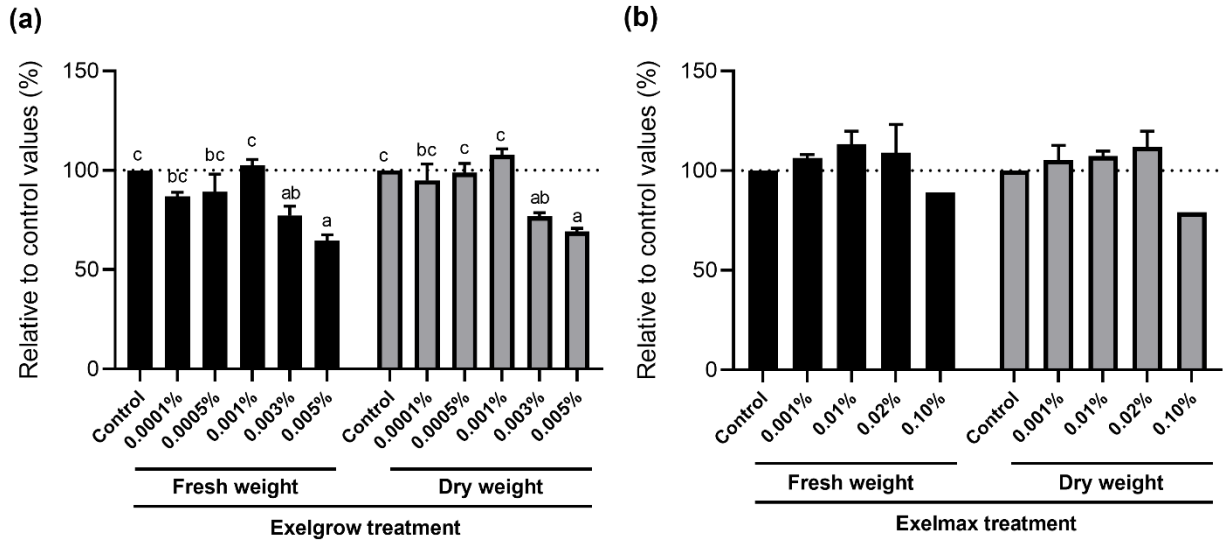

Figure 2.7. Fresh and dry weight of *A. thaliana* Col-0 rosettes grown at different concentrations of Exelgrow **(a)** and Exelmax **(b)** biostimulants (plants of the experiments presented in Figure 2.1). Values of treatment relative to control plants are means ± standard deviation of ≥10 plants per treatment and at least 3 biological replicates* (N = 3). Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences. No letters mean there are no significant differences. *Exception of EM 0.1% v/v, only 1 replicate.

2.4. Discussion

Ascophyllum nodosum-derived biostimulants have been widely used as a growth stimulant to improve yield and protect crops against different stresses. The composition of these products, and therefore the biostimulant activity, is highly susceptible to parameters such as the environmental conditions where the raw material was taken and the manufacturing process (Craigie et al., 2008; el Boukhari et al., 2020; Goñi et al., 2016; Goñi, Quille, & O'Connell, 2018; Wally et al., 2013); conditions that could be taken advantages of to obtain more than one product from the same raw material. This study characterized the phenotypic changes in Arabidopsis plants triggered by two ANE biostimulants derived from the same raw material but obtained with different manufacturing processes.

2.4.1. Standardization of biostimulants treatment *in vitro* culture conditions

The plant model organism *A. thaliana* is a small size plant with a short life cycle and a known and widely studied genome, and therefore, has excellent characteristics to develop a high-throughput system for the evaluation of the growth-promoting activity and to explore the interaction of the biostimulant with the biochemical pathways of the plant. As far as we know, no standardization for evaluating biostimulant treatments under *in vitro* culture conditions has been published. Moreover, when working *in vitro*, the biostimulant availability will be different compared to soil applications. Thus, in pots or the field, biostimulant applications will diffuse through the soil, while in agar plates, the seedlings have a constant interaction of the root system with the media, hence a permanent source of biostimulant. Consequently, the manufacturer's recommended use is not applicable, making it necessary to test various concentrations of each product to determine the appropriate dose of each biostimulant for the experimental conditions used in this work. With this approach, it was possible to study the activity of the biostimulants in a dose-dependent manner and focus on different phenotypes of interest. Both the application timing and the treatment period were considered in this standardization. For Arabidopsis, the adequate timing for biostimulants application was 7 days after sowing, and for most of the phenotypic analysis, it was necessary at least 7 days of biostimulant treatments. Additionally, we found that the range of activity of the biostimulants in this system was between 10^{-2}x and 1x the recommended

concentration by the manufacturer. Previous studies in Arabidopsis *in vitro* culture conditions have used similar treatment periods but differ in application timing. Wally (2013) transferred plants to treatment 4 days after sowing, and phenotypic parameters were evaluated at 3-, 5-, and 7- days after transfer to treatment. Similarly, Rayorath (2008a) evaluated root growth at the same periods as Wally (2013) but initiated the treatment in plants 5-days after sowing. The dose of application also varies; 0.01 and 0.1 g L^{-1} (Rayorath, Jithesh, et al., 2008), or 0.01% w/v (Wally et al., 2013).

2.4.2. The biostimulants had different effects on plant development

The findings of our study demonstrated the different biostimulant effects of both products on Arabidopsis plants. Previous research has shown that comparative phenotypic analysis using ANE obtained from various sources or extraction methods can have diverse growth-promoting impacts on plants (Goñi et al., 2016; Goñi, Quille, & O'Connell, 2018; Rayorath, Jithesh, et al., 2008).

Root growth improvement by ANE have been previously reported (Alam et al., 2014; N. Ali, Farrell, et al., 2016; O. Ali et al., 2022; Billard et al., 2014; Khan et al., 2012; Macdonald et al., 2014; Shukla & Prithiviraj, 2021; Wally et al., 2013). However, a detailed analysis of the impact of ANE biostimulants on root architecture, such as the one carried out in this work, has yet to be reported. In Arabidopsis, previous studies have shown contradictory effects of ANE on root development (Rayorath, Jithesh, et al., 2008; Wally et al., 2013), which is in accordance with the idea that extracts from different sources could have different biostimulant activity. We also analyzed the effect of the biostimulants on the primary root developed before and after the transfer to treatment. Moreover, the methodology applied allowed us to determine that: 1st, the biostimulants act in a dose-dependent manner, and therefore the concentration of application of the product will depend on the desirable effect on the plant; and 2nd, the biostimulants act differently in the PR section and LRs developed before and after the transfer to treatment.

Our results showed that while both Exelgrow and Exelmax induced changes in root architecture, they did so in distinct ways. Under this study's growth conditions, each product's most beneficial effects on root development were observed with EG 0.001% v/v and EM 0.01% v/v. In a simple view, EG 0.001% v/v-treated plants had characteristics similar to the control treatment. Notably,

plants had more developed lateral roots (longer lateral roots) and a significantly more developed root hair system on the primary root grown after transfer to treatment. EG could also have a role in the induction of lateral roots primordium, as EG-treated plants had a higher proportion of LRP in the early stages of development (I-II) at earlier periods of treatment (three days). EM 0.01% v/v treatment induced plants with a shorter primary root, accelerated lateral root development, a greater surface of lateral roots, and a significantly more developed root hair system, particularly on the primary root developed on treatment. The analysis on primordium development suggested that EM disrupted LRP initiation but enhanced the development of pre-existing LRPs, accelerating growth. With EM is also important to note that the biostimulant effect on lateral root development is focused on the upper part of the root, corresponding to the primary root formed previous to the exposure to the treatment. Moreover, LR development appears to be arrested in the newly developed PR. The root architecture induced by EM biostimulant could be a desirable outcome for crops grown in pots or with a drip irrigation system. The application of biostimulants EG and EM resulted in notable enhancements in the root hair system and a significant increase in the overall area covered by lateral roots, particularly with EM. These improvements could bring several benefits to the plants. The increased surface area of the root system, attributed to the growth of lateral roots and root hairs, amplified the absorptive capacity, thereby enhancing the uptake of nutrients and water in systems with limited inputs (Gilroy & Jones, 2000). Moreover, the development of a more robust network of lateral roots could enhance plant stability and anchorage (Bailey et al., 2002). Similarly, the elongation of root hairs, particularly in seedlings, contributed to improving anchorage and increasing water retention capabilities (Choi & Cho, 2019).

Interestingly, both EG and EM could maintain root dry weight similar to the control treatment, despite the negative effect observed in general root development at higher concentrations. The increased root weight relative to the root size could be related to the significant growth of the root hair system and the increment in LR number. Root hairs increase the root surface area and improve nutrient acquisition, which could also contribute to the increment in the root dry weight (Gilroy & Jones, 2000; Jungk, 2001). It has been previously reported that ANE improved nutrient uptake (N. Ali, Farrell, et al., 2016; Basak, 2008; Billard et al., 2014; Goñi et al., 2021). An analysis

of root nutrient content will be useful to better understand changes in root weight. Also, EM treatment increased root fresh but not dry weight independently of the concentration. This suggests that EM improved the water content of the root. An increment in water content could be associated with an increment in cell turgor or the hydraulic conductance of the cell (O. Ali et al., 2023). Cell conductance has a major role in the growth and elongation of root cells (Hsiao1 & Xu, 2000; Sarker et al., 2010). Therefore, the increment in the water content in the roots could be related to the capacity of EM of promoting lateral root growth and root hairs elongation. We suggest analyzing root cell growth dynamics in future studies.

Due to the limitations of the *in vitro* culture conditions used in this study for the shoot growth analysis, only parameters related to rosette development were quantified. Few studies have reported the effect of ANE biostimulants on Arabidopsis shoot development. ANE treatment has been found to increase rosette leaf number, plant height, and total biomass (Goñi et al., 2016; Rayorath, Jithesh, et al., 2008). However, we did not observe significant changes in these parameters with EG or EM treatment. Interestingly, there were significant differences in morphological parameters of rosette development, such as projected area, compactness, and stockiness. Compactness is the ratio between the projected rosette area and the convex hull area (i.e., the area inside the shortest line around a given object). Thus, compactness relates to leaf density and the surface covered by the rosette. Stockiness considers leaf shape and could help detect leaf serrations (Dhondt et al., 2014; Jansen et al., 2009). Changes in rosette compactness in EM-treated plants could indicate that the leaves had longer petioles. On the other hand, the EG biostimulant increment in the stockiness value could indicate that these plants had a more ovate leaf shape. Unfortunately, we did not perform more assays to deepen the rosette's morphology changes in response to the biostimulants treatment. Assays to study the cell cycle in rosette's leaf could be interesting to perform to better understand the changes in the projected area, especially with EM biostimulant.

2.4.3. Hormone-like effect of the biostimulants treatment

Root development is orchestrated by different hormones that have complex interactions to regulate the growth of the different tissues. The role of cytokinins (CK) and auxin (AUX) in the control of root architecture has been widely studied in Arabidopsis. In this study, we found that the phenotypic impact of EG and EM biostimulants were similar to CK and AUX treatment, respectively. Cytokinin treatment reduces primary root growth and inhibits lateral root initiation. Previous studies have shown that the inhibitory effect on primary root elongation is observed at high concentrations of CKs treatment (0.5 µM kinetin), while the reduction of LR density is observed at lower concentrations (≥ 0.1 µM kinetin) (Laplaze et al., 2007). CK acts on LR development by altering LRP initiation and by delaying or stopping the development of LRP (Laplaze et al., 2007). The EG biostimulant induces a similar effect on primary root growth and lateral root number and density (Fig. 2.1C). However, a different behavior was observed with LRP development (Fig. 2.4), since the effect was milder to the reporter by the CK treatment. It is possible that the EG 0.001% v/v could be more like to a lower concentration of CK treatment. However, we did not find studies that analyzed root development with lower concentrations of CK treatment.

Auxin treatment is well known to inhibit primary root growth and induce lateral root formation (Okumura et al., 2013). Detailed analysis of lateral root initiation and development has shown that auxin acts differently on the primary root formed before and after transfer to treatment and in a dose-dependent manner (Ivanchenko et al., 2010). On the pre-existing root, auxin (indole-3-acetic acid, IAA) induces LR initiation that increases when the concentration increases. On the newly developed PR, the promoting effect on LR initiation is observed only at low concentrations, when there is no inhibition on primary root growth (<12.5 nM IAA), and sharply decreased at higher concentrations. Auxins inhibit PR elongation, primarily by reducing cell length. At concentrations where IAA inhibited PR elongation, auxin treatment decreased the ability to induce LR initiation events (Ivanchenko et al., 2010). Similar phenomena were observed with EM treatment. At concentrations that EM inhibits PR elongation (> 0.01% v/v), LRs are mainly observed in the pre-existing PR; there was an increment in LR number in this part of the root, and strong inhibition of LR formation was observed in the newly developed PR (Figs. 2.1B, D and

Fig. 2.2). At the primordia development level, EM treatment reduced the distance between individual LRP and altered the typical organization of LRP through the PR (Figure 2.4 E-F) (de Smet, 2012). Similar phenotype it has been reported with exogenous AUX treatment in Arabidopsis roots (Geldner et al., 2004; Keicher et al., 2017).

The “hairy” phenotype induced by both EG and EM biostimulants in PR and LRs formed after transfer to treatment (Fig. 2.5), could also be associated with a phytohormone effect. However, the promotion of root hair elongation could be associated with several phytohormones; IAA, ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and cytokinin 6-benzylaminopurine (BA) had been reported that exhibited “hairy” phenotypes (S. Zhang et al., 2016). Brassinosteroid (BR) signaling, on the other hand, inhibits root hair formation, and mutants for BR receptor and biosynthesis genes also showed “hairy” phenotypes (Cheng et al., 2014).

EG and EM treatment induces an effect on roots similar to the application of CK and AUX, respectively. However, the phenotypes observed could be associated with other hormones as well. As is well known, plant development is orchestrated by an interaction network of several phytohormones. Due to the complex biostimulants composition, the products probably act holistically on plants, altering different pathways that induced the phenotypic outcome observed. To gain a comprehensive understanding of how EG and EM biostimulants exert their effects on plants, it is crucial to conduct additional genetic investigations focusing on cytokinin, auxin, and other phytohormone-related pathways. The forthcoming chapters will address these specific issues and provide insights into the underlying mechanisms of action.

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3. Chapter III
Effect of Exelgrow and Exelmax biostimulants on cytokinin and auxin signaling

3.1. Introduction

The mechanism of action of *Ascophyllum nodosum* extracts (ANE) biostimulants has been a topic of study for many years and to this day not completely elucidated. One of the proposed mechanisms has been associated with phytohormones due to the similarity of their effects on plant development with that of the phytohormones themselves (Khan et al., 2011). It has been reported that ANE treatment could trigger endogenous phytohormone accumulation in plants (Khan et al., 2011; Wally et al., 2013) and alter the regulation of hormone-related genes (Jithesh et al., 2018; Santaniello et al., 2017; Wally et al., 2013), ANE-biostimulants have been associated with inducing an auxin or cytokinin-like effect on plants. Moreover, most of the publications available regarding the molecular analysis of ANE effects on plants have been focused on the accumulation and/or gene-expression analysis related to these phytohormones on plants with the biostimulants treatment (De Saeger et al., 2019).

The most common approach to studying the hormone-like effect of ANE-biostimulants has been through bioassays based on hormone-responsive promoters, the expression analysis of specific genes for a pathway of interest, the use of mutants, and genome-wide expression analysis. Signaling and biosynthesis pathways of cytokinin (CK) and auxin (AUX) are preferred as the first approach to study the effect of the biostimulants on the endogenous CK and AUX pathways in roots (De Saeger et al., 2019).

On the root meristem, the balance between cell division and differentiation rate is crucial for meristem maintenance. CK and AUX interact antagonistically to control cell division and differentiation, and the crosstalk between these two hormones regulates root meristem development and primary root growth. On lateral roots, AUX promotes lateral root initiation and lateral primordium development (Su et al., 2011), and CK inhibits lateral root initiation that is required for the progression of lateral root development. Nevertheless, the coordinated action

of both AUX and CK is necessary for the development of a proper root architecture (Jing & Strader, 2019).

Cytokinin biosynthesis is modulated by ISOPENTENYL TRANSFERASE (IPT) and LONELY GUY (LOG) enzyme family. The first step is to convert ATP and dimethylallyl pyrophosphate (DMAPP) into iPRTP and iPRDP (isopentenyl-adenosine-5'-triphosphate and isopentenyl-adenosine-5'-diphosphate, respectively) by IPT enzymes. Subsequently, the CYTOCHROME P450 enzyme (e.g., CYP735A) catalyzes the formation of cytokinin ribotides to be finally converted to the active forms (tZ, iP, cZ, and DZ) by de LOG enzymes. Cytokinin signaling pathway is a multistep phosphorelay system similar to the bacterial two-component response system. Cytokinin binds to histidine kinase receptors (AHKs) localized at the plasma membrane, which auto-phosphorylates. The phosphate is then transferred to cytosolic histidine phosphor-transmitter proteins (AHPs). Then, AHPs are translocated to the nucleus and transfer the phosphate to type-A (negative) and -B (positive) response regulators (ARRs) and trigger cytokinin response (Kieber & Schaller, 2014).

The most abundant auxin is indole-3-acetic acid (IAA) and is primarily synthesized in a two-step process from the amino acid tryptophan, known as the tryptophan-dependent pathway. In the first step, tryptophan is converted to indole-3-pyruvate by the TRYPTOPHAN AMINOTRANSFERASE OF the ARABIDOPSIS (TAA) family of aminotransferases. Indole-3-pyruvate is then converted to IAA by the YUCCA family of flavin monooxygenases (Fig. 1.6a) (Schaller et al., 2015b). In the tryptophan-independent pathway, indole-3-glycerol phosphate (IGP) is converted to IAA through a process that remains mostly uncharacterized. In the tryptophan-dependent pathway, IGP is converted to Indole, the precursor of tryptophan, by the TRYPTOPHAN SYNTHASE α (TSA) in the chloroplast. It has been proposed that the first step of the tryptophan-independent pathway is the conversion of IGP to Indole by the enzyme INDOLE SYNTHASE (INS), a homologous of TSA, but in the cytosol (B. Wang et al., 2015; R. Zhang et al., 2008). IAA can also be obtained via the β-oxidation of IBA in the peroxisomes (Fig. 1.6a) (Schaller et al., 2015b). Auxin-inducible genes have Auxin Response Elements (AREs) in the promoters and are normally repressed by the interaction of members of three protein families: AUXIN RESPONSE FACTORS (ARF), Aux/IAA transcriptional repressor family, and TOPPLES (TPL). Dimers of ARFs are

bound to specific AREs in auxin-inducible genes. ARFs recruit Aux/IAA, which subsequently recruits TPLs corepressors, to prevent gene expression. In the presence of auxin, auxin brings together F-box proteins of the TRANSPORT INHIBITORRESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) family and the Aux/IAA transcriptional repressor family. The dimmer transfers activated ubiquitin from a ubiquitin-activating enzyme to the Aux/IAA, resulting in their degradation, and subsequent transcription activation of auxin-inducible genes (Leyser, 2018).

In Chapter I we showed that two commercial *A. nodosum* biostimulants, Exelgrow and Exelmax (ADAMA Ltd.), act differently in the primary root and lateral root growth of *A. thaliana.* Thus, Exelgrow and Exelmax induce a phenotype in roots similar to the application of cytokinin (CK) and auxin (AUX), respectively. To have a better understanding of the hormone-like effect on the root tissues of *Arabidopsis* by the biostimulants, we analyzed the hormonal response of *A. thaliana* plants to the treatment of the two commercial ANE-derived biostimulants *in vitro* culture conditions. First, we observed the tissue-specific response of CK and AUX in the root to the biostimulants with transgenic plant's genetic constructs with a reporter system, and then we analyzed the response to the biostimulants of mutant lines for key genes of the signaling and biosynthesis pathways of CK and AUX.

3.2. Materials and methods

3.2.1. Plant material and growth conditions

Transgenic lines of *A. thaliana* Col-0 with the p*TCSn::GFP/pDR5::VENUS-N7* and p*DR5::GUS* (Ulmasov et al., 1997) construct were used. The transgenic line of Arabidopsis *TCSn::GFP/DR5::VENUS-N7* had double markers for cytokinin and auxin. *TCSn* (Two Component signal Senser new) is a synthetic sensor of cytokinin that reflects the activity of type-B response regulators (Zurcher et al., 2013). DR5 is a synthetic auxin-responsive promoter, that contains several auxin response factors binding sites (Brunoud et al., 2012). In addition, mutant lines and their respective wild types (WT) were Col-8, *cre ahk2, cre ahk3,* Col-ipt, *ipt3/5/7,* WS, Col-0, and *tir1-1 afb2-1 afb3-1* were used.

Seeds were surfaced sterilized with 50% v/v NaClO (commercial chlorine, 4.9%) and 10% v / v Triton X-100 at 0.1%, for 10 min, and washed five times with sterile distilled water, 2 min each time. Sterile seeds were germinated in ½ MS medium salt (Murashige & Skoog, 1962) supplemented with 1% (w/v) sucrose, 0.05% MES sodium salts, 0.8% agar, pH 5.9. 7-days after sowing, seedlings with consistent growth were selected and transferred to square plates with ½ MS medium supplemented with the corresponding biostimulants treatment. Control plants were transferred to a new plate with only ½ MS medium. Plates were sealed with microporous tape to allow gas exchange. Plates were maintained vertically oriented in a growth chamber at 21±2°C with a day/night photoperiod of 16/8 h with a light intensity of 100 µmol $m^{-2}s^{-1}$. Mutant lines and wild-type plants were grown with and without biostimulants in the growth chamber for seven days.

3.2.2. Commercial biostimulants treatment

Several concentrations were previously tested to determine the concentration of biostimulants to be applied to *A. thaliana in-vitro* culture conditions, starting with the recommended concentration for field conditions (Chapter II). After an extensive phenotypic analysis, the following concentrations were selected for genetic analysis: Exelgrow 0.001% v/v, and Exelmax 0.01% v/v. The biostimulants do not need to be sterilized. The corresponding amount of biostimulants was mixed by inversion with the melted ½ MS media in a 50 ml falcon tube and then added to the plate.

3.2.3. GUS staining

For GUS (β-glucuronidase) staining, three days after transfer to treatment, transgenic Col-0 seedlings with *DR5::GUS* construct were put in staining buffer (~ 1ml per 20 seedlings of 10 days old), and kept in dark at 37°C until a stain was observed (~3h). For the staining buffer first was mixed 5 parts of Na-phosphate buffer (0.1M, pH 7.0) and 2 parts of Ferro-Ferricyanide buffer (5mM). Then, 1 mg/ml of X-Gluc dissolved in dimethylformamide (10 µl/mg) was added. Na-phosphate was prepared with 19.5% v/v Solution A (NaH_2PO_4 0.1 M), 30.5% v/v Solution B (Na_2HPO_4 0.1M), 0.1% Triton, and water. pH was adjusted to 7.0 with Solution A. Ferro-Ferricyanide buffer was prepared with 50 ml Na-phosphate buffer (pH 7.0), 0.08 g of K3Fe(CN)6,

and 0.105 g of K4Fe(CN)6. After the incubation time with the staining solution, plants were washed with Ethanol: acetic acid (3:1) three times; first with 70% ethanol, then 50%, and finally 20%. For root clearing, the roots were fixed in 70% Ethanol overnight. Then, roots were incubated in two solutions: first, a solution of 0.24 N HCl in 20% methanol at 62°C for 40 min, and second, a solution of 7% NaOH in 60% ethanol for 20 min at room temperature. Roots were hydrated in 40%, 20%, and 10% ethanol for 20 minutes for each step. Then roots were incubated in 50% glycerol (25% glycerol in 5% ethanol, final concentration) for 2 hr. Finally, plants were mounted in 25% glycerol. Images were taken with ZEISS Axioscope.A1 microscope. GUS signal quantification was carried out with ImageJ (1.53f51) software. For each treatment and genotype, eight seedlings per plate were analyzed in each assay, and the experiment was repeated three times (replicates). Data were expressed as relative values between treatment and control plants of the 24 plants per treatment ($Mean_{treatment}/Mean_{control}$*100).

3.2.4. Fluorescent lines microscopy

Three days after transfer to treatment, *TCSn::GFP/DR5::N7-VENUS* seedlings were directly mounted with distilled water. Images were taken with ZEISS LSM 880 confocal microscope. GFP was excited at 504 nm and VENUS at 531 nm. GFP and VENUS signal quantification was carried out with ImageJ (1.53f51) software. For each treatment and genotype, eight seedlings per plate were analyzed in each assay, and the experiment was repeated three times (replicates). Data were expressed as relative values between treatment and control plants of the 24 plants per treatment ($Mean_{treatment}/Mean_{control}$*100).

3.2.5. Phenotypic root analysis of mutant lines

Plates were scanned on the day of transfer (day 0) and at seven days of treatment (day 7). ImageJ (1.53f51) software was used for primary and lateral root quantification. Primary root length was measured on the day of transfer and on day seven of treatment. Then, primary root growth (PR) was determined as $length_{day7} - length_{day0}$ .The lateral root number was determined by visual quantification in photos. Lateral root density (LRD) was calculated as LR/PR $length_{day7}$. To

measure the length of lateral root (LR) and to be comparable, the length of all lateral roots was measured at the end of treatment (seven days). Then, total lateral root length (TLRL) was calculated as the plant's summation of all lateral roots. The TLRL of each treatment was obtained as the average TLRL of all the plants per treatment; 7-12 plants per treatment were analyzed. The assay was repeated two times (replicates). Data were expressed as mean values of all plants of the two replicates: for CK- and AUX-receptors mutants, n=15-20 per treatment, and for CK-biosynthesis mutants, n=15-24.

3.2.6. Statistical analysis

Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$). Number of plants and replicates are detailed in the description of each assay.

3.3. Results

3.3.1. EG and EM biostimulants had opposite effects on CK and AUX signaling on the root tip

Synthetic promoters have been used for many years to visualize in a tissue specific manner auxin and cytokinin signaling. Thus, synthetic promoters based on Aux/IAA-ARF (DR5, in case of auxin) and ARR (TCS, in case of cytokinin) signaling regulated the expression of reporter genes such as fluorescent proteins or β-glucuronidase (Ulmasov et al., 1997; Zurcher et al., 2013). Therefore, we used DR5::N7-VENUS and TCSn::GFP reporter lines to evaluate changes in auxin and cytokinin signaling in response to ANE biostimulants. As shown in Figure 3.1, auxin signaling is mainly observed in the vasculature, QC and columella cells. On the other hand, cytokinin signaling is more active in the lateral root cap and the proximal meristem, which correspond to a cell niche next to the QC (Fig 3.1 o).

Roots with EG biostimulant showed a clear upregulation of cytokinin signaling as observed by a higher intensity of GFP in the lateral root cap and the proximal meristem when compared to control (Fig 3.1 a, b). Moreover, EM does not seem to change cytokinin signaling. Surprisingly,

the VENUS signal in EM treatment was similar to control, contrary to what was expected in an auxin-like treatment. While EG treatment showed a minor down-regulation of auxin signaling (Fig. 3.1). To further confirm these observations, the GFP and VENUS intensity was quantified in the columella + lateral root cap and the proximal meristem. As shown in Figure 3.1 (p), EG treatment up-regulated cytokinin signaling while down-regulating auxin signaling. On the other hand, no changes were observed in auxin signaling after EM treatment. However, EM significantly down-regulates cytokinin signaling (Fig 3.1 p).

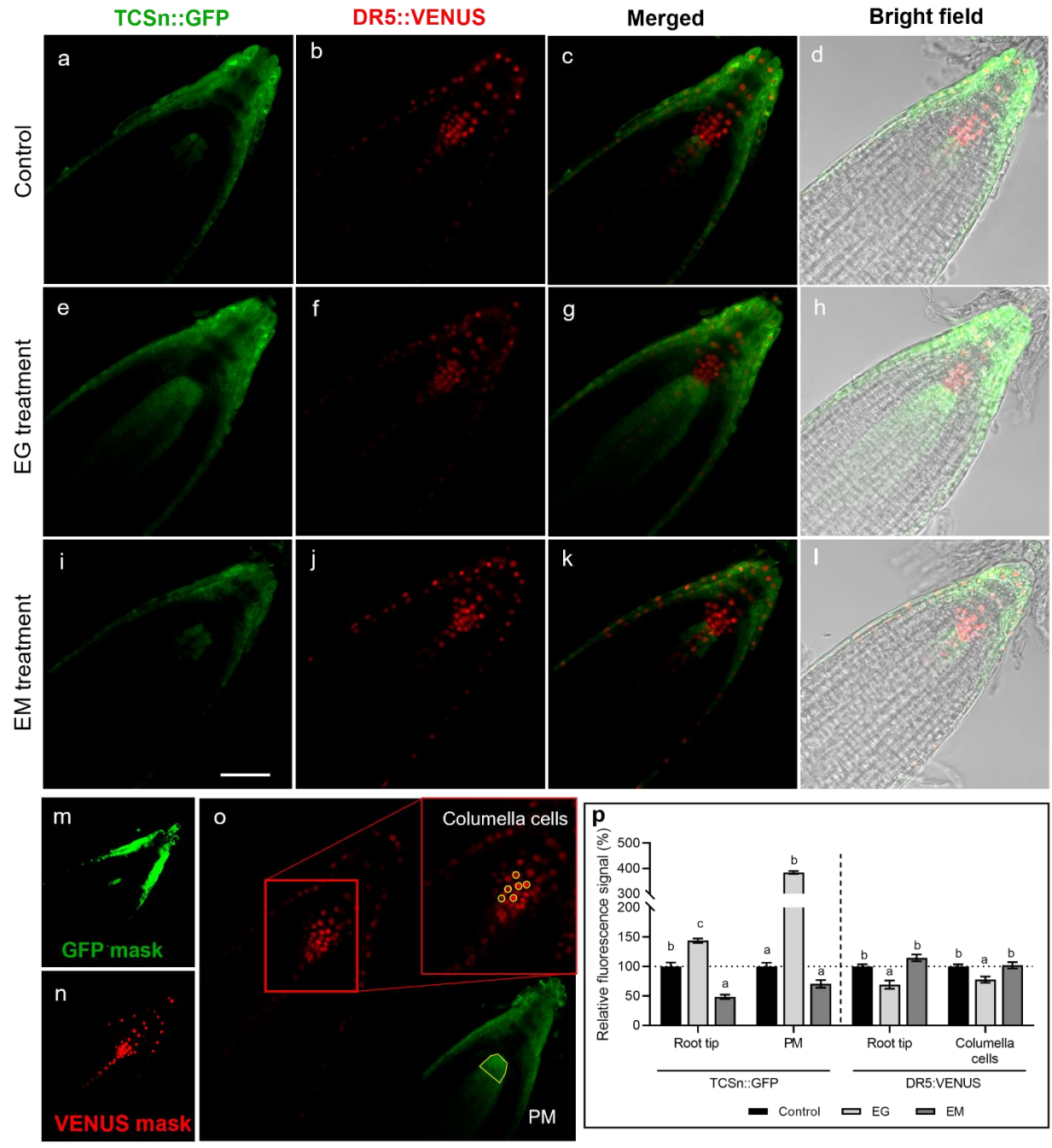

Figure 3.1. Auxin and cytokinin signal on primary root tip. (a-l), representative phenotypes of seedlings of *A. thaliana* with *TCSn::GFP/DR5::VENUS-N7* reporter system grown for seven days in ½ MS media and transferred to ½ MS supplemented with the corresponding treatment for three days. Auxin (red) and cytokinin (green) response gradients in seedlings treated with ½ MS (Control, **a-d**), EG 0.001% v/v **(e-h)**, and EM 0.01% v/v **(i-l)**. For GFP, excitation wavelength= 488 nm. For VENUS excitation wavelength= 514

nm. Scale bar=50 µm. GFP signal **(a,e,i)** was quantified on the root tip and in the proximal meristem area (PM) (**o,** yellow polygon). VENUS **(b,f,g)** was quantified in the root tip and the Columella cells (**o,** yellow circles). The total signal of the three brighter and the three less bright cells was measured. To select the pixels with a fluorescing signal on the root tip, a threshold was applied to every image; for GFP auto-threshold "otsu", and for VENUS [13,255]. The masks in **(m-n)** show the area of pixels selected by the threshold method. **(p),** fluorescence signal of GFP in the root tip and the PM, and of VENUS on the root tip and in Columella cells (sum of the 6 cells). Integrated density (IntDen) parameter was used for all the measurements. Bars represent the percentage relative to control treatment and SEM of the mean values of 16-20 plants per treatment, of three independent replicates. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$); different letters represent significant statistical differences.

To extend these results, we analyzed DR5 expression with the genetic construct *DR5::GUS*, where the promoter DR5 controls the expression of the bacterial β-D-glucuronidase (GUS) (Ulmasov et al., 1997). Three days after transfer to treatment, histochemical GUS staining was performed to quantify the GUS activity on the root (Fig. 3.2 A-C). In this case, to compare the GUS signal between treatments, the total GUS staining area on the root tip was calculated. A threshold was performed automatically at each image to select the pixels with GUS staining (Fig. 3.2 D). As expected, EM biostimulant significantly increased the GUS signal, and on EG-treated plants, the GUS signal was significantly lower than the control (Fig. 3.2 E). Also, the GUS signal on the mature primary root was stronger on EM-treated plants, just above the point of transfer to treatment (Figure 3.2 F-J). In contrast, no GUS stain was observed in this region either in control plants or after EG treatment.

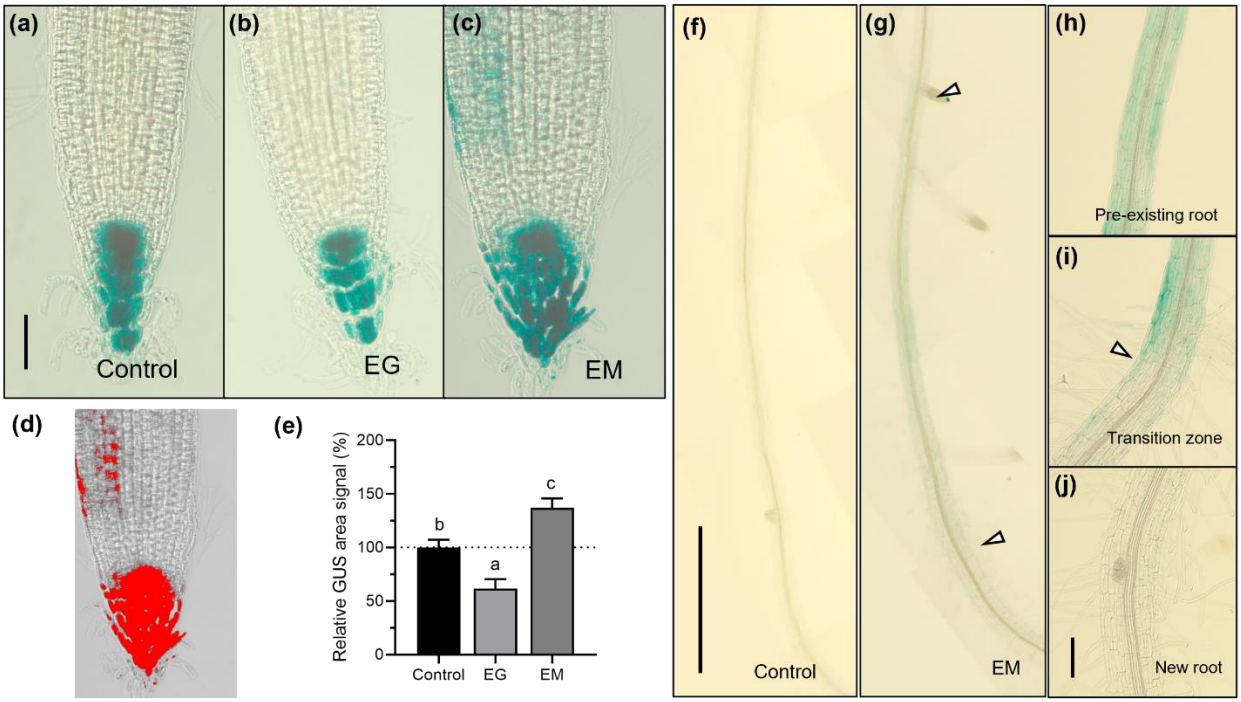

Figure 3.2. Auxin signal on the primary root. (a-c), representative phenotype of auxin response gradients visualized by GUS histochemical staining in seedlings of *A. thaliana* with *DR5::GUS* reporter system grown for seven days in ½ MS media and transferred to ½ MS supplemented with the corresponding treatment for three days; ½ MS (Control, a), EG 0.001% v/v **(b)** or EM 0.01% v/v **(c)**. Scale bar=50 µm. **(d)**, representative area measured. For pixels selection, the auto threshold "IsoData" on the red channel was applied. **(e)** total area of GUS stain. Bars represent the percentage relative to control treatment and SEM of the mean values of 16-18 plants per treatment, of three independent replicates; different letters represent significant statistical differences. **(f-g)**, Auxin signal on the mature primary root. The arrowhead indicates the zone with GUS signal just above the point of transfer to treatment (recognized by the excessive root hair development just before that point); scale bar=1000 µm. **(h-j)** Images show the transition from the previously formed root **(h)** to the portion formed after transfer to treatment **(j)**, Scale bar=100 µm. Lower auxin response in the primary root portion formed on EM treatment.

3.3.2. Role of CK-signaling and biosynthesis pathways on the effect of EG and EM

To determine whether CK signaling pathway is involved in the root architectural response to EG and EM biostimulants, we analyzed the effect on the double mutants for AHKs CK-receptors genes *cre ahk2* and *cre ahk3*, on root system architecture (Fig. 3.3). The EG biostimulant negatively affected PR growth, and LR number in WT and in the CK-receptors mutants but did not affect LR density. (Fig.3.3. A-C). In contrast, the EG biostimulant enhanced the TLRL in *cre ahk3* mutant, in comparison to control plants, but not in WT nor *cre ahk2* mutant. Regarding the distribution of LRs, the EG bioestimulant increased the proportion of roots <0.1 cm in *cre ahk2* and *cre ahk3*, and of 0.1-0.2 cm in *cre ahk3,* compared with the trend observed in WT. There was no effect on the proportion of longer roots in the mutant lines (>1.0 cm), following the same trend observed in WT (Fig. 3.3. E-F).

Previously we found that the EG biostimulant significantly increased root hair development in the PR developed after transfer to treatment. We qualitatively analyzed the effect of EG on the root hair development of the CK-receptor-related mutants. Interestingly, the phenotype of root hair enhancement on the primary root developed on treatment was completely lost in the *cre ahk2* and *cre ahk3* mutant lines with EG biostimulant (Fig. S3). The EM biostimulant slightly decreased PR growth on WT, but this difference was significant in the *cre akh3* double mutant. Furthermore, it increased LR number and density in the *cre ahk3* mutant, but not in the WT or *cre ahk2*, and TLRL in the WT and *cre ahk3* mutant. The EM biostimulant also increased the percentage of longer roots (>1.0 cm), a characteristic phenotype previously observed with both EM and EG (Chapter I). Interestingly, this phenotype was lost in both *cre ahk2* and *cre ahk3*. Increment in root hair development phenotype, unlike EG, was not lost in the mutant lines with EM treatment. However, the phenotype was weaker than in WT (Fig. S3); root hairs looked apparently shorter than in WT with EM.

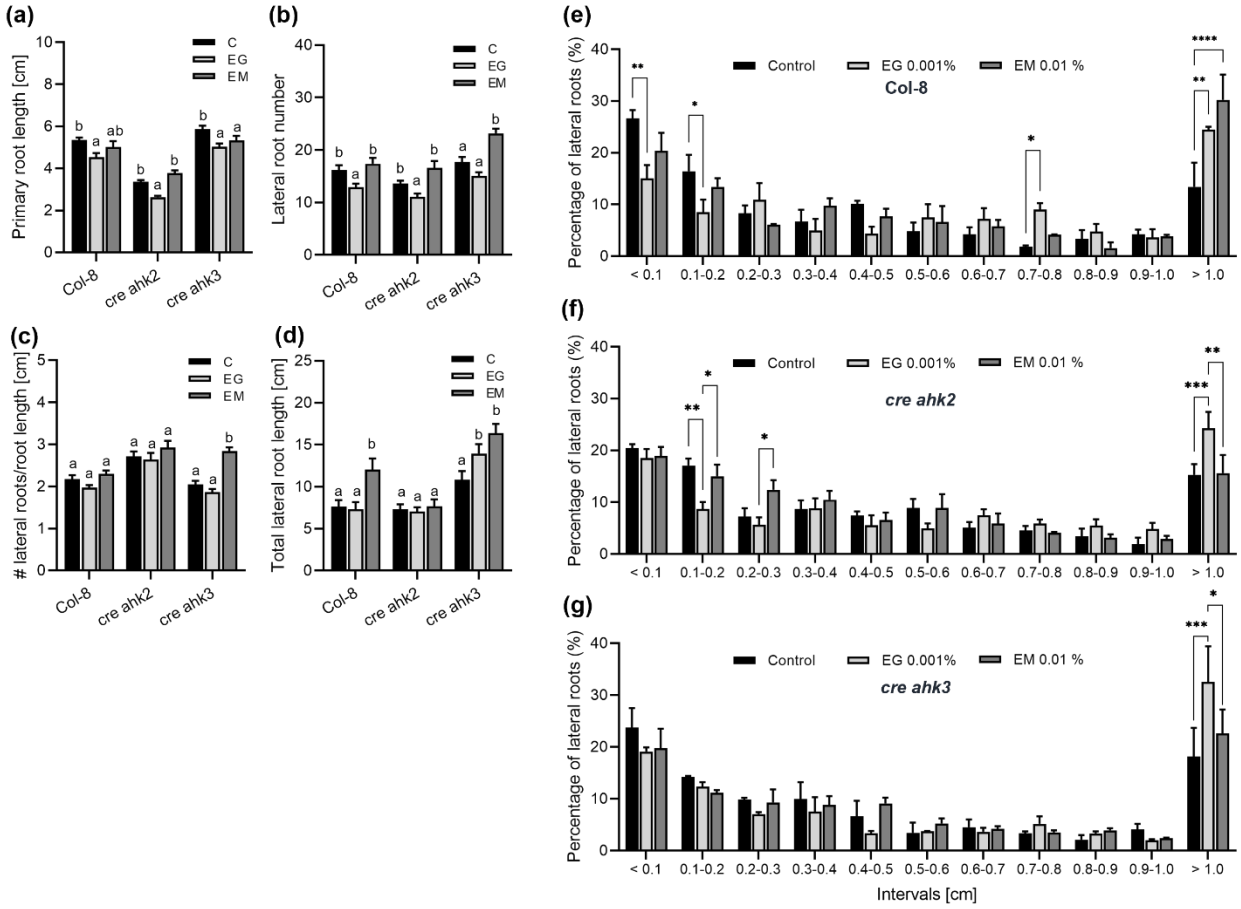

Figure 3.3. Effects of biostimulants EG and EM on the root system architecture of *A. thaliana* wild-type seedlings and CK-related signaling mutants. Arabidopsis Col-8 (WT), *cre ahk2,* and *cre ahk3* seedlings grown for seven days in ½ MS media were transferred to ½ MS supplemented with the corresponding treatment for seven days. **(a)**, primary root length, calculated as the difference in root length at day seven and day cero of treatment. **(b)**, lateral root number. **(c)**, lateral root density, lateral root number per cm of primary root. **(d)**, total lateral root length. Bars represent the mean values and standard error (SEM) of N~20 plants of two independent replicates. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$); different letters represent significant statistical differences. **(e-f)**, distribution of lateral root lengths of Col-8 **(e)**, *cre ahk2* **(f)**, and *cre ahk3* **(g)**. Results are presented as a percentage of the total lateral root number per treatment. Bars represent the mean values and (SEM) of two replicates (N = 2). Mean significant differences in each interval were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$; 0.03(*), 0.002(**), 0.0002(***), <0.0001(****).

To further understand the role of cytokinin in EG-EM mechanism in the regulation of root development, we analyzed the effect on the triple mutants for IPTs genes *ipt3/5/7* (Fig. 3.4). Unfortunately, after EM treatment only an impact in LR density was observed and there was no phenotype in lateral root length in WT. However, the *ipt3/5/7* mutant with the EG biostimulant had reduced PR growth, LR number and TLRL compared to control (Fig. 3.4 A-C). Nevertheless, the proportion of longer roots (>1 cm) was similar in WT and triple mutants (Fig 3.4 E-F). The EM biostimulant reduced PR growth in the mutant line (Fig. 3.4 A) and increased LR number and density in the WT, compared to control plants (Fig. 3.4 B-C); the trend in TLRL was similar in WT and mutant line (Fig. 3.4 D). Regarding the distribution of LRs, the EM biostimulant decreased the percentage of longer roots (<1.0 cm) in the WT, but the percentage was similar to control in the mutant line. However, the error was high though this are not conclusive results (Fig. 3.4 E-F).

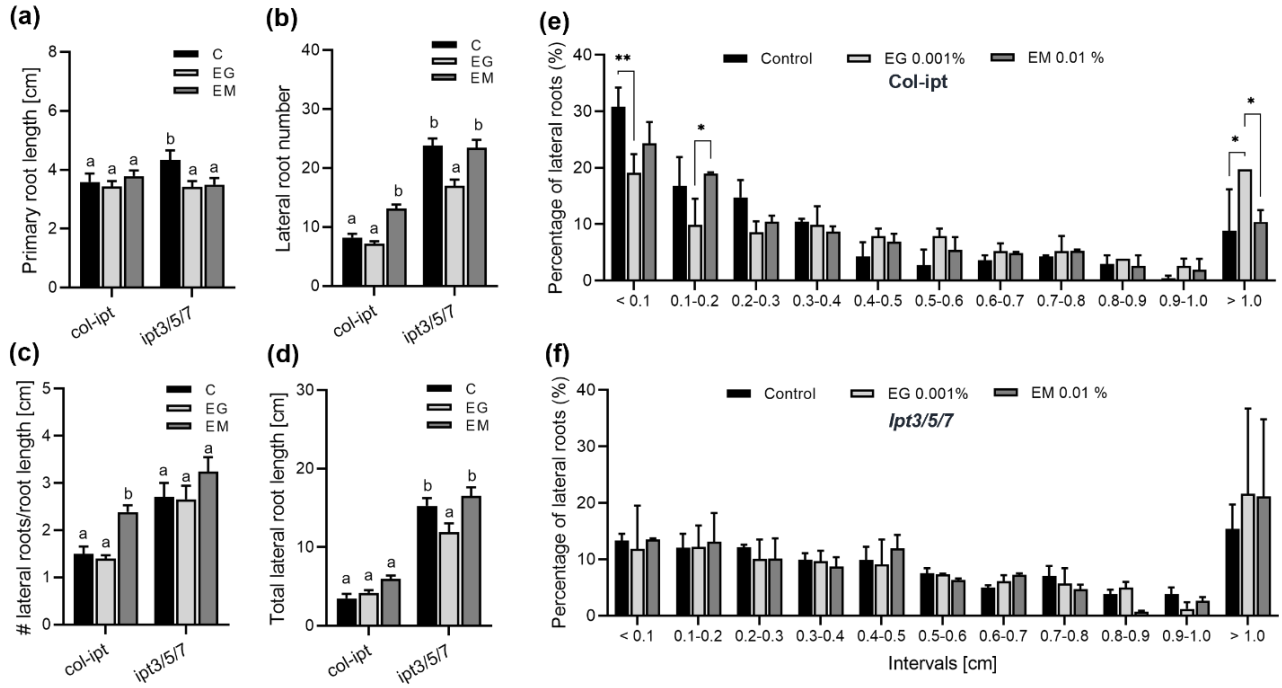

Figure 3.4. Effects of biostimulants EG and EM on the root system architecture of *A. thaliana* wild-type seedlings and CK-related biosynthesis mutants. Arabidopsis Col-ipt (WT), and *ipt3/5/7* seedlings grown for seven days in ½ MS media were transferred to ½ MS supplemented with the corresponding treatment for seven days. **(a)**, primary root length, calculated as the difference in root length at day seven and day cero of treatment. **(b)**, lateral root number. **(c)**, lateral root density, lateral root number per cm of primary

root. **(d)**, total lateral root length. Bars represent the mean values and standard error (SEM) of N=15-19 plants of two independent replicates. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$); different letters represent significant statistical differences. **(e-f)**, distribution of lateral root lengths of Col-ipt **(e)** and *ipt3/5/7* **(f)**. Results are presented as a percentage of the total lateral root number per treatment. Bars represent the mean values and (SEM) of two replicates (N = 2). Mean significant differences in each interval were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$; 0.03(*), 0.002(**), 0.0002(***), <0.0001(****).

3.3.3. Role of AUX-signaling pathway on the effect of EG and EM

Finally, we wanted to analyze whether the AUX signaling pathways were involved in the root architectural response to the biostimulants. For this purpose, we analyzed the effect of the triple mutants of members of the TIR1/AFB family of AUX-receptors genes, *tir1-1/afb2-1/afb3-1*, on root system architecture. The EG biostimulant did not induce any effect in PR, TLRL, and LR distribution on the mutant line, compared to the trend observed in the WT (Fig. 3.5). The only slight difference was observed in LR; the mutant line treated with the EG biostimulant had similar values than the control, but the WT showed lower LR compared to control, which also affected LR density (Fig. 3.5 B-C). Interestingly, the triple *tir1-1/afb2-1/afb3-1* mutant completely inhibits the positive impact of EM over LR number, density and TLRL, suggesting a key role of auxin perception in response to EM biostimulant (Fig. 3.5).

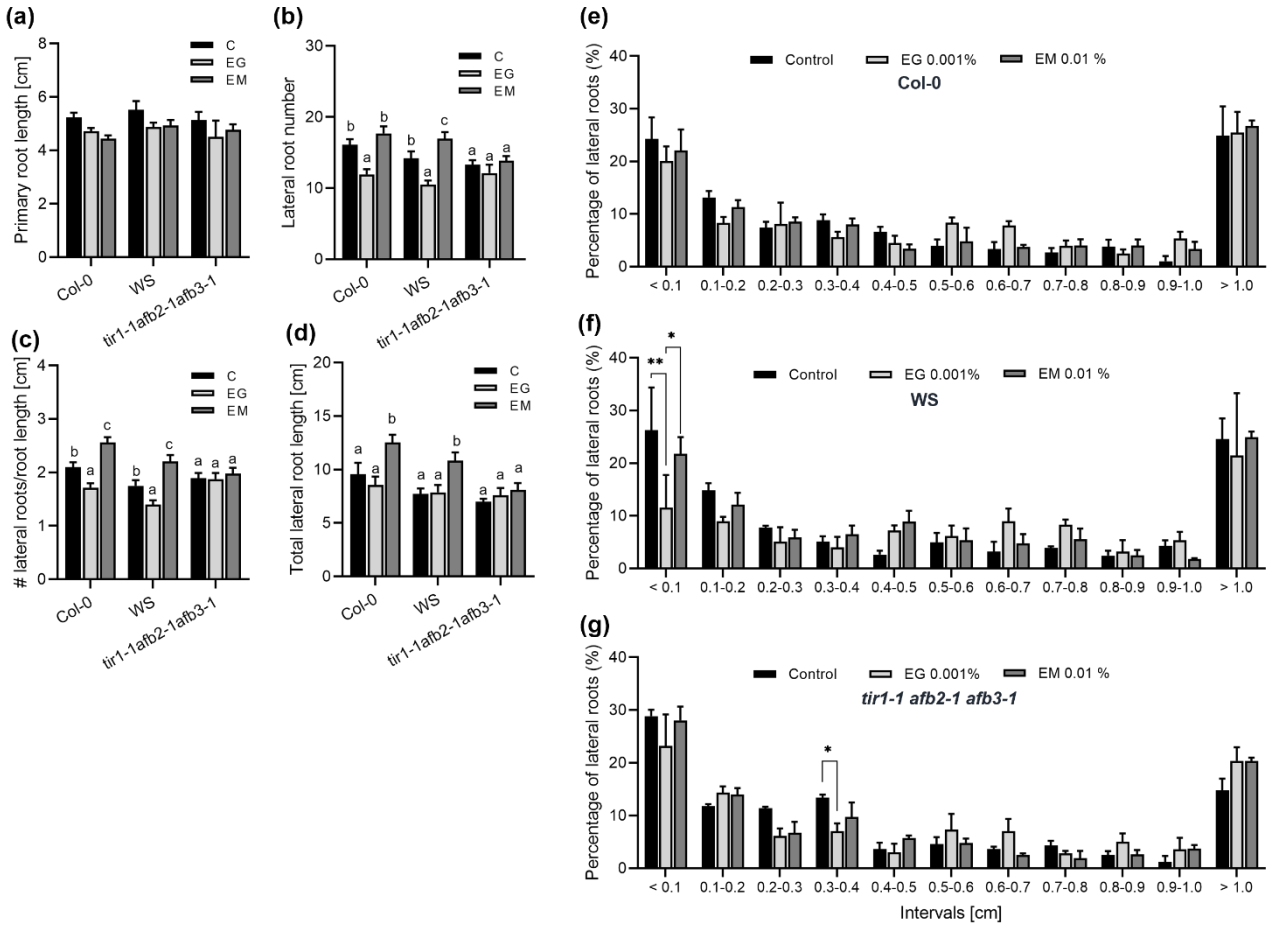

Figure 3.5. Effects of biostimulants EG and EM on the root system architecture of *A. thaliana* wild-type seedlings and AUX-related signaling mutants. Arabidopsis Col-0 (WT for *tir1-1)*, WS (WT for *afb2-1* and *abf3-1*), and *tir1-1 abf2-1 afb3-1* seedlings grown for seven days in ½ MS media were transferred to ½ MS supplemented with the corresponding treatment for seven days. **(a)**, primary root length, calculated as the difference in root length at day 7 and day 0 of treatment. **(b)**, lateral root number. **(c)**, lateral root density, lateral root number per cm of primary root. **(d)**, total lateral root length. Bars represent the mean values and standard error (SEM) of N=15-22 plants of three independent replicates. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$); different letters represent significant statistical differences. **(e-f)**, distribution of lateral root lengths of Col-0 **(e)**, WS **(f)**, and tir1-1afb2-1afb3-1 **(g)**. Results are presented as a percentage of the total lateral root number per treatment. Bars represent the mean values and (SEM) of three replicates (N = 3). Mean significant differences in each interval were determined with one-way ANOVA and Tukey's multiple comparisons test ($p<0.05$; 0.03(*), 0.002(**), 0.0002(***), <0.0001(****).

3.4. Discussion

The phytohormone-like effect of *A. nodosum* biostimulants has been reported before (De Saeger et al., 2019; Shukla et al., 2019). Previous analysis has been mainly focused on CK, AUX, and in a minor way, abscisic acid (ABA) pathways, due to the key role of these phytohormones on plant development and regulating growth under stress conditions (Kurepa & Smalle, 2022; Schaller et al., 2015b; Vishwakarma et al., 2017). We previously found that EG and EM had growth-promoting effects on *A. thaliana* Columbia 0 (Col-0) *in vitro.* EG 0.001% v/v-treated plants had no negative effect on PR, LR number, and rosette development. However, plants had more developed lateral roots (longer lateral roots) and a significantly more developed root hair system on primary root grown after transfer to treatment. On the other hand, EM 0.01% v/v-treated plants had shorter primary root, accelerated lateral root development, a greater surface of lateral roots, significantly more developed root hair system on the newly developed primary root, and larger rosettes (Chapter II). Here, we showed that EG and EM biostimulants modulate CK and AUX signaling pathways, each of them in a different manner.

The EG biostimulant promotes cytokinin signaling on the root tip when compared to control and EM-treated plants. Moreover, EG also showed a negative regulation of auxin signaling (Fig. 3.1 and 3.2). This opposite regulation was expected due to the antagonistic nature of these two hormones on the root meristem (Schaller et al., 2015b). TCSn promoter reflects the activity of type-B response regulators on the CK signaling pathway, which activates the transcription of CK-responsive genes (Zurcher et al., 2013). Therefore, the increased signaling of *TSCn::GFP* by the EG biostimulant could indicate that it contains compounds with CK-like activity or could be stimulating endogenous CK biosynthesis.

The analysis of the response of CK-receptor mutants to the EG biostimulant indicated that the perception of CK was not required for the effect of EG on root PR length and LR root development, suggesting that the biostimulants could be acting downstream of the CK-perception in the signaling pathway. Alternatively, is also possible that although cytokinin is positively regulated, LR development in response to EG could be regulated in a CK independent

manner. In fact, AHK3 appears to have a negative effect on the response of lateral root elongation to the EG biostimulant. Indeed, *cre ahk3* mutant showed higher TLRL compared to control, which was not observed in the WT. However, the distribution of LRs and the increase in the percentage of longer roots of plants treated with the EG biostimulant, compared to control, remained unaltered in the mutant lines compared to WT (Fig. 3.3).

Interestingly, the characteristic phenotype of root hair enhancement on the primary root developed was completely lost in the mutant *cre ahk2* and *cre ahk3* lines with EG biostimulant (Sup. Fig. S3), suggesting that the CK signaling pathway is required, at least, for the promotion of root hair development by the biostimulant. These results could also indicate that the EG biostimulant contained compounds with CK-like activity. In fact, it has been previously reported that cytokinin directly regulates root hair development through the modulation of ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4), that encodes a basic helix–loop–helix (bHLH) transcription factor (Takatsuka et al., 2023). Interestingly, this regulation is mediated by b-type ARRs such as ARR1 and ARR12.

The increased response to CK in the root tip could indicate that the EG biostimulant induces endogenous CK-biosynthesis. Thus, we also characterized the role of the CK-biosynthesis pathway on the effect of EG on root development. Interestingly, the mutant showed reduced root growth compared to control, an effect not observed in the WT (Fig. 3.4), suggesting that an active CK-biosynthesis pathway is required for EG, and therefore. Therefore, it is possible to speculate that EG stimulates CK-biosynthesis, however, more experiments are needed to confirm that. On the contrary, analysis with AUX-receptor gene mutants showed that the mutants were insensitive to EG biostimulant, suggesting that the canonical auxin signaling pathway is not required for EG (Fig. 3.5).

The EM biostimulant promotes auxin signaling on the root tip and in the mature primary root, in the zone just above the transfer point to treatment (Figure 2.5). A similar phenotype was observed in plants with IAA treatment, where the DR5::GUS signal was stronger in the PR portion formed previous to transfer to treatment (Ivanchenko et al., 2010). DR5 is a synthetic auxin-responsive promoter that contains AuxREs repeats (Ulmasov et al., 1997). Therefore, stronger

GUS signaling by the EM biostimulant indicated an increased AUX signaling in that tissue. However, the increased AUX-signaling on the root tip was not observed with the DR5::VENUS-N7 reporter gene system. As shown in Fig. 3.2, GUS staining does not seem to be stronger, the main difference is the area of expression. Thus, in response to EM, auxin signaling is not only high in QC and columella cells, but also in lateral root cap, where cytokinin signaling is more active. Since VENUS intensity was measured in columella cells, no difference was observed. The increment in auxin signaling in the lateral root cap could also explain the reduction in cytokinin signaling observed.

Analysis of mutants of AUX receptor genes suggested that EM biostimulant requires an active canonical auxin signaling pathway in the plant, at least for the stimulation of lateral development. This could indicate that the EM biostimulant contained compounds with AUX-like activity or could stimulate endogenous AUX biosynthesis (Fig. 3.5). However, the mutants for AUX-related biosynthesis genes could not be analyzed. Therefore, further experiments are needed to support this.

Unexpectedly, CK signaling pathways appear to have an important role in the EM effect on root development. The increment on TLRL, characteristic of the EM biostimulant, was lost in *cre ahk2* mutant; also, the phenotype of higher percentage of longer roots (>1.0 cm) was lost in both *cre ahk2* and *cre ahk3*, suggesting that EM stimulated lateral development through a CK-dependent pathway (Fig. 3.3). Moreover, root hairs of AUX-receptors mutants treated with EM biostimulant looked apparently shorter than in the WT, despite the incremented root hair development was not lost in the mutant line (Sup. Fig. S3). The results with CK-biosynthesis mutants with EM biostimulants, also suggest that an active CK-biosynthesis pathway is required for EM for the induction of LRs (Fig. 3.4).

Unfortunately, it was not possible to compare the biostimulant treatments with a specific concentration of CK, AUX, or other hormones, because the content of phytohormones in each product is unknown. However, previous studies have found that the concentration of phytohormones in *A. nodosum* extracts is relatively low and unlikely to induce a phytohormones-responsive phenotype (De Saeger et al., 2019). Therefore, other compounds found in the algae

could interact with the plant's phytohormones pathways, causing the observed hormone-like effect.

It should be noted that the assays with mutants were not developed under the ideal conditions, because: 1) we had some problems with contaminations and the growth of plants during that time; for that reason, we lost some plates, and few plants were analyzed from two repetitions of the assay; 2) the design of the assay was not optimal, because, for a proper and complete analysis with mutants, it should be tested the single mutants of each type (i.e., *cre, ahk3, tir1-1)*, as well as the double and triple mutants in each case; and 3) we did not have the mutants for AUX-biosynthesis-related genes available, therefore, we could not test the role of the AUX-biosynthesis pathway on the effect of the biostimulants on root development. Our results showed some insights into the role of the CK- and AUX-biosynthesis and signaling pathways in the root's architectural response to the biostimulants, however, the assay should be repeated with the corresponding modifications to corroborate the results obtained in this study.

Our data indicate that EG and EM interact with CK and AUX pathways differentially. The EG biostimulant induced CK signaling and repressed AUX signaling on the root meristem, and the EM biostimulant works in the opposite way by inducing AUX and repressing CK signaling. In addition, the biostimulants require CK and AUX-related pathways differentially for different aspects of root development. Despite the limitations on the mutants for CK and AUX-related biosynthesis and signaling pathways assay, these findings provide additional information about the mechanism of action of EG and EM biostimulants.

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4. Chapter IV
Transcriptomic changes induced by two commercial *A. nodosum* biostimulants on *A. thaliana* plants

4.1. Introduction

Ascophyllum nodosum extracts (ANE)-derived biostimulants are complex products with known and unknown components that contribute to their effects on plants. The known bioactive compounds include poly- and oligosaccharides that are not found in plants, including laminarin, mannitol, fucoidan and alginate; polyphenols such as phlorotannin; betaines; sterols; vitamins; amino-acids; macro- and micronutrients; and phytohormones, such as auxin, cytokinin, and abscisic acid (De Saeger et al., 2019). ANE are commonly used as a growth stimulant and to protect crops against biotic and abiotic stresses such as freezing, salinity, and drought (Oosten et al., 2017; Shukla et al., 2019). Despite little is known about the mode of action of these biostimulants, ANE are commonly known to have a phytohormone-like effect on plants. The biostimulant effect of ANE on plants has been associated with the phytohormones present in the algae. However, due to the relatively low concentrations of phytohormone in the biostimulant extract (Wally et al., 2013), other non-hormonal compounds within the algae may be responsible for the observed effects. It has been proposed that ANE could also contain unidentified compounds with "hormone-like effects". Molecules such as amino acids, polysaccharides, and organic acids may act as precursors or activators of endogenous plant hormones (De Saeger et al., 2019; Shukla et al., 2019; Yakhin et al., 2017). The complex composition and diverse bioactive components of ANE biostimulants make it challenging to fully understand their mode of action on plant growth processes.

Transcriptomic analysis is a powerful tool for studying gene expression in specific tissues and under different conditions. Few studies have investigated the transcriptomic response to ANE-induced plant growth promotion, and these studies have revealed significant changes in gene expression. However, there is limited overlap in the differential gene sets identified in these studies (De Saeger et al., 2019; Shukla et al., 2019; Yakhin et al., 2017). The bioactivity reported for ANE varies significantly, mainly because the composition of the extracts varies depending on

the manufacturing methodology (Craigie et al., 2008; Shukla et al., 2019). A recent study of the transcriptomic response to foliar application of an *A. nodosum* extract formulation (Stimplex) in tomato plants showed a large number of genes that were up-regulated (635) and down-regulated (456), including genes involved in the synthesis of key metabolites and activation of signal transduction pathways, related to the regulation of plant growth, stress response, and disease resistance. Interestingly, it was found that the biostimulant triggers defense and growth responses before any stress is applied or encountered (O. Ali et al., 2022). Moreover, it has been suggested that ANE biostimulants protect plants against several types of stress through a priming mechanism (De Saeger et al., 2019; Islam et al., 2021; Subramanian, Sangha, Gray, et al., 2011).

We previously found that Exelgrow (EG) and Exelmax (EM) act as root biostimulants, and they have different effects on root development in *A. thaliana* (Chapter II). We also found that both products interact with cytokinin (CK) and auxin (AUX) biosynthesis and signaling pathways (Chapter III). EG induced CK signaling and repressed AUX signaling on the root meristem, and EM works by inducing AUX and repressing CK signaling. Furthermore, different CK and AUX pathways were involved in various aspects of root development in response to EG and EM biostimulants. With the transcriptomic analysis, we expect to elucidate other hormonal pathways involved in the mechanism of action of EG and EM biostimulants on *A. thaliana* roots and identify the key biological processes modulated by these biostimulants.

4.2. Materials and methods

4.2.1. Plant material and growth conditions

Arabidopsis thaliana (Col-0) seeds were surface-sterilized with 50% v/v NaClO (commercial chlorine) and 10% v / v Triton X-100 at 0.1%, for 10 min, and washed five times with sterile distilled water, 2 min on each occasion. Sterile seeds were germinated in ½ MS medium salt (Murashige & Skoog, 1962) supplemented with 1% (w/v) sucrose, 0.05% MES sodium salts, 0.8% agar at pH 5.9. Seven days after sowing, seedlings with consistent growth were selected and transferred to square plates with ½ MS medium supplemented with the corresponding biostimulants treatment: Control, EM 0.01% v/v or EG 0.001% v/v. The corresponding amount of

biostimulant was mixed by inversion with the melted ½ MS media in a 50 ml falcon tube and then added to the plate. Control plants were transferred to a new plate with only ½ MS medium. Plates were sealed with microporous tape to allow gas exchange. Plates were maintained vertically oriented in a growth chamber at 21±2°C with a day/night photoperiod of 16/8 h with a light intensity of 100 µmol $m^{-2}s^{-1}$ during the germination and treatment period. The assay was repeated three times (replicates). Each replicate and treatment had 24 plants.

4.2.2. Experimental design and tissue collection

Previously we found that EG and EM biostimulants had different effects on the primary root (PR) developed before, and after, the biostimulant application. Hence, we decided to partition the root tissue into two segments: Root 1, denoting the PR developed before transfer to treatment, and Root 2, denoting the PR developed after transfer to treatment. To identify both parts of the root, the position of the root tip was marked on the plate the day of the transfer to treatment. On day seven of treatment, root tissues were collected and immediately frozen as follows: the primary root of all plants in the plate was cut at the point of transfer to treatment, using a clean scalpel, then all Root 2 tissue were collected and frozen, continuing with Root 1. Each tissue (Root 1 and Root 2) of the 24 plants per treatment of each replicate was frozen in the same tube (i.e., one replicate contains the root tissues of 24 plants).

4.2.3. RNA extraction and sequencing

Total RNA was extracted from Arabidopsis roots (25-50 mg) using PureLink mini kit (ThermoFisher) with RNAsolv reagent (Omega BIO-TEK) and On-column PureLink DNAse treatment (ThermoFisher). The manufacturer's protocol "Using TRIzol® Reagent with PureLink® RNAMini Kit" (Life Technologies, 2012) was followed, with an adaptation to be used with RNAsolv Reagent instead of TRIzol Reagent (Omega BIO-TEK, 2013). The isolated RNA was then quantified and sent to Novogene sequencing services (California, USA) for RNA sequencing. Sample integrity was assessed with Agilent 2100 bioanalyzer system (RIN>6.9). cDNA libraries for RNAseq analysis were prepared using non-stranded NEBNext Ultra II RNA Library Prep by Illumina kit, and sequencing was done with NovaSeq 6000 PE150, with approximately 20 million reads per sample (Novogene Co., Ltd.).

4.2.4. Bioinformatics analysis

Data quality of raw reads was done by Novogene and confirmed later with FastQC v0.11.9. Data was filtered by Phred > 25, minimum length of 50 bp, and N terminal adapters eliminated with Ktrim v1.4.1 (Sun, 2020). High-quality reads were mapped to *A. thaliana:* TAIR 10 reference genome with STAR v2.7.10b (Dobin et al., 2013), with default parameters and alphabetically ordered with Samtools v1.16.1 (Danecek et al., 2021). Araport11 was used for annotation reference. Raw counts were obtained with R package Rsubreads v2.12.0 (Liao et al., 2019). Genes with low counts (<5) in all biological replicates were discarded. Raw count file was separated by tissue in different data sets. Then, DESeq2 v1.38.1 (Love et al., 2014) was used to obtain differentially expressed genes (DEGs), using the mean of ratios (counts divided by sample-specific size factors determined by the median ratio of gene counts relative to geometric mean per gene) for normalization (https://hbctraining.github.io/DGE_workshop/lessons/02_DGE_count_normalization.html). The raw DEGs file was filtered based on $0.58 \leq \log_2$ (Fold change) ≤ -0.58, equivalent to a fold change threshold of 1.5, and an adjusted p-value < 0.1 (calculated by the Benjamin-Hochberg (BH) method). Comparisons were made between EG-control and EM-control for Root 1 and Root 2.

4.2.5. Visualization

Heatmaps were generated from the mean of the counts transformed by the variance stabilizer variable (VST) and plotted using the R package pheatmap v1.0.12. Principal component analysis was generated from the VST previously obtained in the heatmap, using the plotPCA function of DESeq2 and plotted using the R package ggplot2. Venn diagrams were created from lists of the differentially expressed genes for each comparison using the InteractiVenn web platform (http://www.interactivenn.net/).

4.2.6. Gene ontology

Gene ontology analysis was done using the online tool ShinyGOv0.77 (Xijin Ge et al., 2020). Terms were considered significantly enriched when the False Discovery Rate (FDR) ≤ 0.05 according to the nominal p-value from the hypergeometric test. Fold enrichment was calculated as the percentage of DEGs belonging to a pathway divided by the corresponding percentage in the background. The background corresponded to the subset of genes of the RNA-seq dataset with detectable expression. The top 20 enriched terms, ordered by fold enrichment, were used for figures. All results from the analysis are in Table S2, Table S3, and Table S4.

4.2.7. Network analysis

For the gene co-expression network, we followed the pipeline indicated by (Contreras-López et al., 2018). Briefly, counts were normalized and correlated using EBSeq (Leng et al., 2013) and Mnormt (Azzalini et al., 2022), respectively. Network attributes were calculated with Igraph (Csárdi & Nepusz, 2006) and visualized with Cytoscape (Shannon et al., 2003). Further clustering and gene ontology enrichment analysis was conducted using the ClusterMaker (Morris et al., 2011) and BinGO (Maere et al., 2005) plugins. Correlations were significant above 0.7 or below -0.7, with a p-value ≤ 0.01. To further assess possible transcription factor (TF)-targets interactions, we intersected the correlation matrix with a list of Arabidopsis TFs (from PlantTFDB, (Jin et al., 2017)) and a list of TF-target experimentally tested interactions through DAP-seq (O'Malley et al., 2016), allowing us to add an additional layer of evidence to the network analysis.

4.2.8. Phenotypic analysis of roots

The same plates used for RNA extraction with Col-0 seedlings were scanned the day of transfer (day 0) and at the 7th day of treatment. ImageJ (1.53f51) software was used for primary and lateral root quantification. Primary root length was measured on the transfer day and at the 7th day of treatment. Primary root growth (PR) was determined as $length_{day7} - length_{day0}$ .The lateral root number was determined by visual quantification in photos. LR density (LRD) was calculated as LR/PR_{day7}. To measure the length of lateral root (LR) and to be comparable, the length of all lateral roots was measured at the end of treatment (7th day). Then, Total Lateral

Root Length (TLRL) was calculated as the summation of all lateral roots of a plant. TLRL of each treatment was obtained as the average TLRL of all plants per treatment. Results correspond to mean values of three replicates (24 plants per condition and replicate). PR, LR, and LRD were reported as the percentage of control, calculated as (treatment/control*100).

4.3. Results

4.3.1. Phenotypic characterization of *A. thaliana* plants with EG and EM treatment

The phenotypic analysis of roots of the plants of *A. thaliana* used for the RNA-seq analysis showed similar results as the ones previously obtained in Chapter I with EG 0.001% v/v and EM 0.01% v/v (Fig. 4.1 A), indicating consistency of the biostimulant effect on roots of both products. EG biostimulants had no negative impact on PR growth, LR number, and density compared to control (Fig. 4.1 B). Also, EG-treated plants had increased TLRL and a greater percentage of longer roots (>1 cm) (Fig. 4.1 C-D). EM biostimulant reduced PR growth and slightly increased LR number, leading to a significant enhancement in LR density (Fig. 4.1 B); EM also increased TLRL to a major extent than EG (Fig. 4.1 C). In addition, EM-treated plants had a greater percentage of longer roots (Fig. 4.1 D).

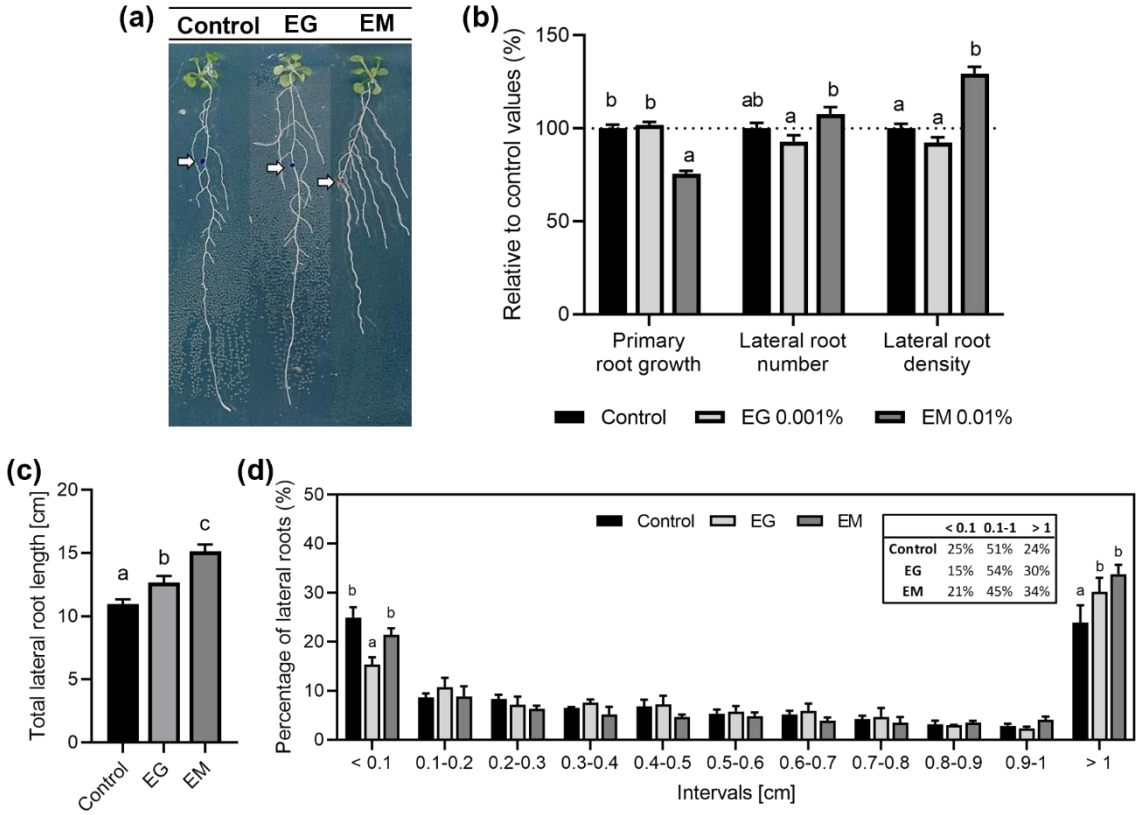
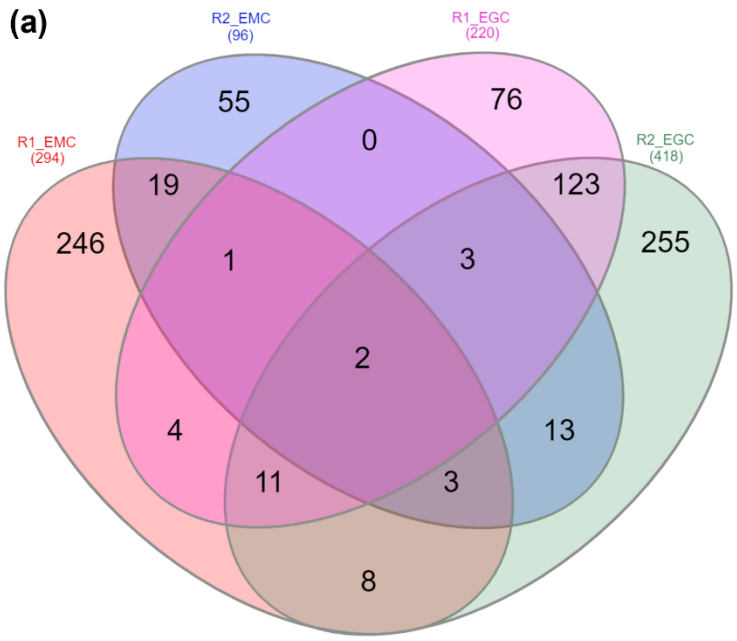


Figure 4.1. Phenotypic evaluation of root growth of *A. thaliana* with EG and EM biostimulants treatment. Seedlings of *A. thaliana* Col-0 ecotype grown for seven days in ½ MS media were transferred to ½ MS supplemented with the corresponding treatment, control (½ MS), EG 0.001% v/v, and EM 0.01% v/v. **(a)**, photos of representative plants of each treatment. The white arrow indicates the point of transfer to treatment. **(b),** primary root growth, lateral root number, and lateral root density. Results are presented as the percentage of control, calculated as (treatment/control*100)**. (c)**, total lateral root length. **(d)**, distribution of lateral root lengths. Results are expressed as the percentage of the total lateral root number per treatment. Bars represent mean values and standard error (SEM) of the 3 independent assays. Mean significant differences were determined with one-way ANOVA and Tukey's multiple comparisons tests ($p<0.05$); different letters represent significant statistical differences. No letters indicate no significant differences. In **(d),** the mean significant difference was calculated in each interval.

4.3.2. Summary of RNA-sequencing reads statistics and DEG report

More than 98% of the reads (~45 million reads) were retained after quality trimming, and the mapping rate to the reference genome was >95% for all samples (Sup. Table S1). A threshold of 1.5 fold change and adjusted p-value ≤ 0.1 was used for the differential expression analysis to determine differentially expressed genes between conditions. The principal components analysis showed a clear separation between Root 1 and Root 2 and between control and treated plants (Sup. Fig. S4). With EG biostimulant, 220 gene transcripts were DE (differentially expressed) in Root 1 and 418 in Root 2, compared to the control plants (Fig. 4.2). Among them, 189/31 were up-/down-regulated in Root 1 and 364/54 in Root 2 (Fig. 4.2A). 81 genes were specific to Root 1 and 279 genes to Root 2. With EM biostimulant, 294 gene transcripts were DE in Root 1 and 96 in Root 2, compared to control plants (Fig. 4.2). Among them, 143/151 were up-/down-regulated in Root 1, and 36/60 in Root 2 (Fig. 4.2B); 269 genes were specific to Root 1 and 71 genes to Root 2. The differences in DEGs between the EG and EM treatments were also visualized in the heat map (Sup. Fig. S5). Interestingly when we compared the DEGs of Root 1 and Root 2 of each biostimulant; in EG, they shared an important number of genes between both section of the root; EM, instead, showed a significantly greater differentiation in the transcriptomic changes between Root 1 and Root 2. This finding is consistent with the observed significant differences in the phenotype induced by the biostimulant across different parts of the root (Chapter II and Fig. 4.1).



(b)

EGvsC	Root 1	Root 2
Up	189	364
Down	31	54
Total	**220**	**418**

(c)

EMvsC	Root 1	Root 2
Up	143	36
Down	151	60
Total	**294**	**96**

Figure 4.2. Differentially expressed genes in Root 1 and Root 2 tissues. **(a)** Venn diagram of Root 1 and Root 2 DEGs by EG and EM biostimulants. Number of up and down-regulated genes by EG in each cluster is detailed in **(b).** Number of up and down-regulated genes by EM in each cluster is detailed in **(c).** Tissues cluster: EG vs control (EGvsC), and EM vs control (EMvsC). DEGs filtered by adjusted p-value < 0.1, and $0.58 \leq \log_2$ (Fold change) ≤ -0.58, equivalent to a fold change threshold of 1.5.

4.3.3. Gene ontology

Gene ontology (GO) enrichment analysis determined which functional pathways were significantly expressed in the EG- and EM-treated *A. thaliana* roots. We focus the analysis on the ontology category of biological processes. With EG biostimulant, the main enriched pathways in Root 1 were those associated with response to stress, such as systemic acquired resistance, salicylic acid (SA)-mediated signaling pathway, regulation of salicylic acid-mediated signaling pathway, response to biotic stimulus, and response to fungus, as well as those related to response to toxic substances and detoxification (Fig. 4.3a). In addition, genes related to polysaccharides metabolism, specifically to the chitin metabolic process, and terms related to the tricyclic triterpenoid metabolic process, specifically to thalianol metabolic process. In Root 2, EG

biostimulant enriched pathways like thalianol metabolic processes, response to salicylic acid and related pathways, response to oomycetes, defense response to bacterium, defense response to fungus, biological processes involved in interspecies interaction between organisms and response to external biotic stimulus, immune system response, and response to oxygen-containing compound (Fig. 4.3b). Many of the most enriched categories are related to each other and represent the same group of genes (Sup. Fig. S6). Hence, we analyzed the complete list of enriched GO terms and found two interesting categories: in Root 1, the regulation of jasmonic acid (JA) mediated signaling pathway and nitrile biosynthetic process (Sup. Table S2), and in Root 2, oxylipin biosynthetic process and hormone catabolic process (Sup. Table S3).

With EM biostimulant, the main GO terms were also primarily related to a stress response. In Root 1, the enriched pathways included the responses to oxidative stress and oxygen-containing compounds, the cellular response to toxic substances and related pathways; i.e., detoxification, reactive oxygen species metabolic processes, cellular oxidant detoxification and hydrogen peroxide catabolic processes, glycosinolate and glucosinolate biosynthetic processes, water and fluid transport, and response to water deprivation, phenylpropanoid metabolic process, and syncytium formation (Fig. 4.4a and Sup. Fig S7). Another enriched pathway found in the analysis was sulfate reduction (Sup. Table S4). In Root 2, EM biostimulant enriched pathways such as the cellular responses to copper ion, oxygen radicals, and related pathways; cellular detoxification and related pathways, polysaccharides catabolic processes, and chitin metabolic processes (Fig. 4.4b and Sup. Fig S7). Another enriched pathway found in the analysis was the camalexin biosynthetic process (Sup. Table S5).

Interestingly, when we analyzed the enriched pathways of the set of genes that were particular for each part of the root, with EM, there was a clear separation of metabolic processes for each tissue. Root 1's terms were related to sulfate reduction, syncytium formation, glycosinolate and glucosinolate biosynthetic processes, and water and fluid transport. Root 2's terms were related to camalexin metabolic and biosynthesis processes and chitin metabolic processes (Sup. Fig S8).

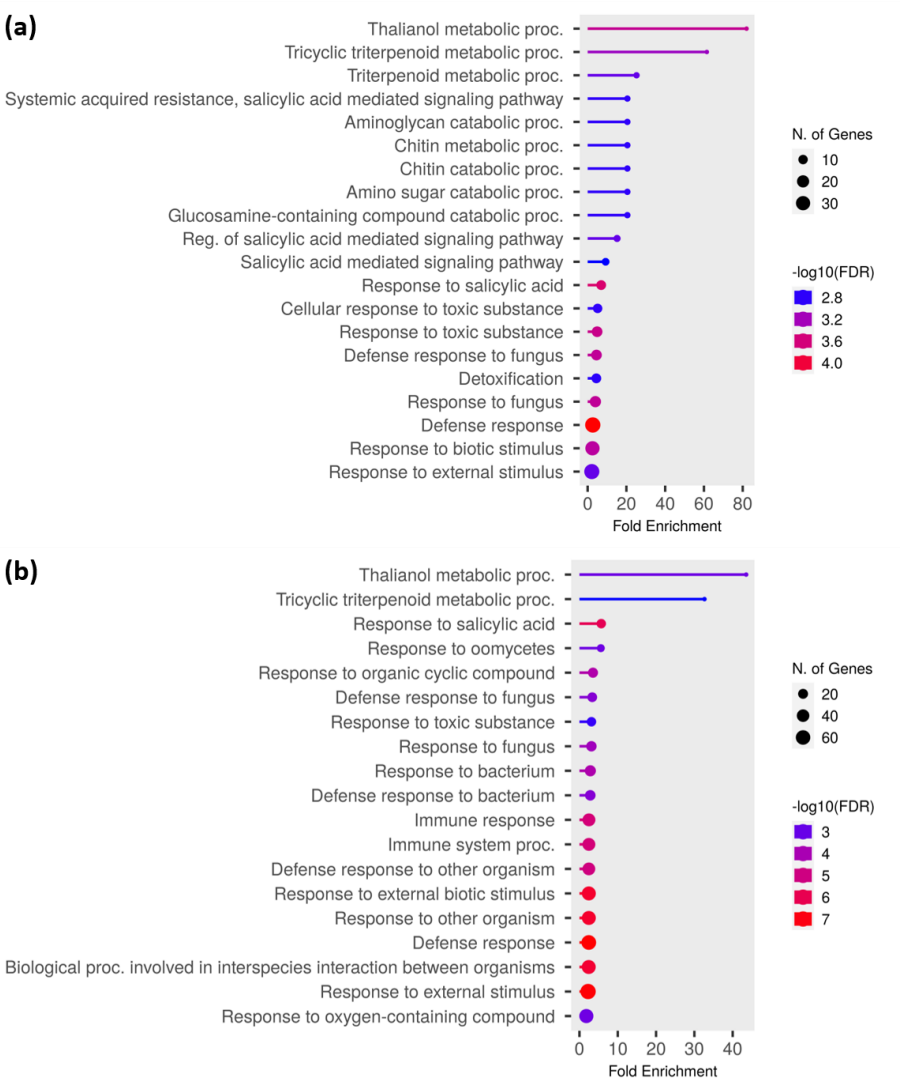


Figure 4.3. Gene ontology enrichment analysis of *A. thaliana* root tissues with EG biostimulant. The top 20 enriched factors for Biological Process in Root 1 **(a)**, and Root 2 **(b)** tissues, of DEGs of EG biostimulant compared with control. Analysis was done in ShinyGOv0.77.

The common DEGs between Root 1 and Root 2 were related to the response to oxidative stress and oxygen-containing compounds; the cellular response to toxic substances and the related pathways, and copper ion homeostasis. With EG, instead, no GO term was enriched with the set of DEGs of Root 1; in Root 2, the most enriched was oxylipin biosynthesis process, and the rest of the enriched biological processes appeared in the common set of DEGs of Root 1 and Root 2 (Sup. Fig S9).

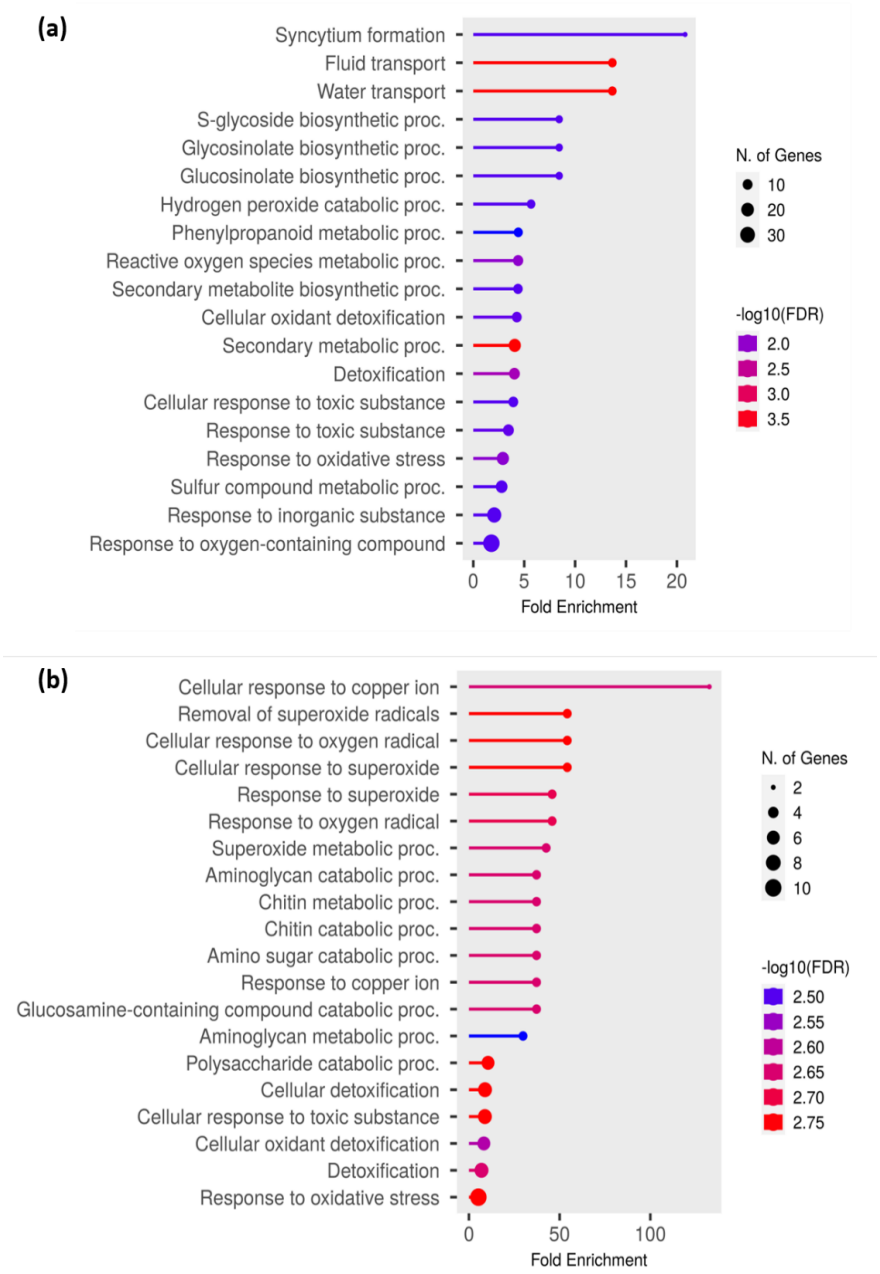


Figure 4.4. Gene ontology enrichment analysis of *A. thaliana* root tissues with EM biostimulant. The top 20 enriched factors for Biological Process in Root 1 **(a)**, and Root 2 **(b)** tissues of DEGs of EM biostimulant compared with control. Analysis was done in ShinyGOv0.77.

4.3.4. Central transcription factors in plant response to EG and EM biostimulants

We developed a network analysis to find central genes in the action mechanism of EG and EM biostimulants. In EG-treated plants, the transcription factors (TF) identified were Arabidopsis NAC domain containing protein 87 (ANAC087, AT5G18270) and ANAC004 (AT1G02230) in Root 1 (Sup. Fig. S10, and ANAC087 and ANAC004, along with WRKY DNA-binding protein 31 (WRKY31, AT4G22070) in Root 2 (Sup. Fig. S11), all of them up-regulated by the biostimulant. In EM-treated plants, only in Root 2 tissue was it possible to identify central genes. The analysis indicated the transcription factors MYB domain protein 56 (MYB56, AT5G17800) and asymmetric leaves 2-like 9 (ASL9, AT1G16530) as central in the response to EM, both of them down-regulated by the biostimulant (Sup. Fig. S12).

4.4. Discussion

We previously found that EG and EM act as root biostimulants, but they have different effects on root development (Chapter II) in *A. thaliana*. We also found that both products interact with cytokinin and auxin biosynthesis and signaling pathways (Chapter III). EG biostimulant induced CK signaling and repressed AUX signaling on the root meristem, and EM works in the opposite way by inducing AUX and repressing CK signaling. In addition, EG and EM biostimulants require different CK and AUX pathways for different aspects of root development. Surprisingly, the RNA-seq analysis by GO enrichment did not show enriched processes directly related to root growth and development or related to CK or AUX metabolism, as expected. Interestingly, the major transcriptomic changes observed in EG and EM-treated roots were related to defense response to biotic and abiotic stresses. Therefore, the changes in root development induced by EG and EM appears to be in concordance with the modulation of plant growth to prepare the plant for diverse biotic and abiotic stimulus. Recent studies where transcriptomic analysis was developed on tomato plants with ANE treatment (Stimplex) under no-stress conditions have shown similar results. The application of the biostimulant resulted in the activation of various complex processes that have the potential to enhance plant defense, growth, and productivity (O. Ali et al., 2022).

4.4.1. Exelgrow action mechanism

Previously we found that EG biostimulant enhanced root hair development (Chapter II). Analysis with mutants for key receptor genes of CK signaling pathways suggested that this pathway was required for the promotion of root hair development. However, the GO enrichment analysis did not show enriched processes directly related to CK or root hair development. Instead, we found that EG-biostimulant up-regulated several root hair-specific (RHS) genes, which was consistent with the hairy phenotype of these plants. In Root 1, *RHS 13,14,18,* and *19*, and *RHS 13* in Root 2 were upregulated. *RHS18* and *RSH19* have peroxidase activity and could be related to cell wall loosening and stiffening (Won et al., 2009), and the overexpression of *RSH19* has a positive effect on root hair growth, peroxidase activity, ROS production and impacts cell wall thickness (Marzol et al., 2022). Interestingly, *RSH13* and *RHS19* were found to be direct targets of the ANAC087 transcription factor identified in the network analysis.

In the context of cytokinin and auxin phytohormone pathways, the differential gene expressions observed in Root 1 and Root 2 shed light on their respective roles. In Root 1, the upregulation of *Arabidopsis response regulator 7* (*ARR7*) gene aligns with the elevated cytokinin signaling mentioned in Chapter III. Previous studies have reported that overexpression of type-A ARR genes, including *ARR7*, positively influences root development (Ren et al., 2009). Notably, *ARR7* overexpression has also been associated with enhanced freeze tolerance (Jeon et al., 2010; Shi et al., 2012). Furthermore, the upregulation of the *cytokinin oxidase 4* (*CKX4)* and *CKX5* in Root 2, and *CKX4* in Root 1, may be in response to the increased cytokinin levels induced by EG (Brenner et al., 2012). Auxin-responsive genes were found to be dysregulated by EG. Among them, the small auxin upregulated (SAUR) 76 (*SAUR 76)* was downregulated. The SAUR protein family plays a crucial role in regulating dynamic and adaptive growth, under the control of various hormones. Specifically, *SAUR76* influences meristematic activity, and its overexpression has a positive effect on root meristematic activity. Auxin and auxin-induced ethylene strongly upregulate *SAUR76* (Stortenbeker & Bemer, 2019). Interestingly, the downregulation of *SAUR76* by EG biostimulant correlates with reduced auxin signaling in roots, induced by the biostimulant (Chapter III). The thalianol metabolic process was also upregulated in response to EG biostimulant, specifically genes *thalianol synthase 1* (*THAS1), cytochrome p70545 (CYP705A5),*

and (*CYP708A2*). In *Arabidopsis*, the thalianol pathway is organized as an operon-like gene cluster (Field & Osbourn, 2008). Notably, these genes have been linked to auxin since thalianol can modulate the phytohormones auxin and jasmonate itself. Moreover, knockout and overexpression of these genes result in altered root development (Bai et al., 2021). Also, the cluster of thalianol-biogenesis genes could potentially be regulated by cytokinins (Fukudome & Koiwa, 2018). Interestingly, *THAS1* was found to be a direct target of the ANAC087 transcription factor identified in the network analysis, as well as *CYP705A5* and *CYP708A2* were found to be targets of ANAC004.

4.4.2. Exelgrow: priming for biotic stress defense

The major transcriptomic changes observed with EG biostimulant were related to defense response to biotic stresses. Here we identified several genes that could participate in activating and modulating the plant's defense mechanisms against various biotic stressors. Among these genes, an important number of the transcription factors identified in the GO enrichment analysis of EG-treated plants belong to the WRKY gene family, which plays crucial roles in plant biology, including responses to stress, regulation of hormone signaling pathways, and plant development (Bakshi & Oelmüller, 2014). Specifically, WRKY genes induced by EG biostimulant were associated with terms related to biotic stress, defense responses, and immune responses. In Root 1 we found *WRKY42, 51, 54, 58*, and *70,* and in Root 2 *WRKY18, 40, 31, 51, 54, 58*, and *70.* Among these, *WRKY70* has been identified as an activator of SA-induced genes and a repressor of JA-responsive genes (Li et al., 2004), and along with *WRKY51,* are potentially involved in basal resistance against *Pseudomonas syringae* (Hu et al., 2012). Additionally, WRKY70 and WRKY54 could also have a role in promoting brassinosteroids (BR)-mediated gene expression and plant growth (Chen et al., 2017). On the other hand, *WRKY18*, and *WRKY40* function as key nodes in Arabidopsis signaling networks, responding to various stimulus and potentially integrating multiple phytohormone signals, including SA, JA, and abscisic acid (ABA) (Bakshi & Oelmüller, 2014). Moreover, *WRKY18* and *WRKY40* have been associated with plant defense to biotic stress as positive regulators in effector-triggered immunity (Bakshi & Oelmüller, 2014; X. Xu et al., 2006).

EG biostimulant also upregulated the nitrile metabolic process, particularly the genes *NSP1* and *NSP3*, which encode for nitrile-specifier proteins (NPS). NPS and epithiospecifier proteins (EPS) are involved in the breakdown of glucosinolate, leading to the formation of nitriles, which are important for the chemical defense against herbivores and microbial pathogens in Brassicas (Burow et al., 2009; Chroston et al., 2022). Among these genes, *NSP1* and *NPS3* play an important role in the nitrile-forming activity in roots and are highly expressed (Wittstock et al., 2016). Interestingly, transgenic Arabidopsis plants overexpressing ESPs, resulting in increased nitrile accumulation, exhibited enhanced resistance to bacteria (*P. synringae pv.tomato* (PST) DC3000) and fungal (*Alternaria brassicicola)* pathogens (Miao & Zentgraf, 2007). Additionally, pre-treatment with soluble sugars has been found to reduce the spread of the bacterial pathogen PST in Arabidopsis. Moreover, exogenous application of 3-butene nitrile initiated an immune response, leading to increased production of SA and JA, alleviation of leaf lesions, and reduced pathogen growth when applied prior to infection with fungal and bacterial pathogens (Ting et al., 2020). The signaling of glucosinolate derivatives may also interact with sugar metabolism, potentially influencing root and plant growth (Gro Malinovsky et al., n.d.; Ting et al., 2020). Furthermore, the EG treatment upregulated the response to oomycetes, involving genes related to chitin metabolism, such as chitinases and *pathogen-related (PR)* genes, and SA-responsive genes such as *WRKY70* transcription factor (Besseau et al., 2012)*, downy mildew resistant 6 (DMR6)*, *DMR6-like oxygenase 1 (DLO1)* and *l-type lectin receptor kinase iv.1* (*LECRK41)* (Zeilmaker et al., 2015; K. Zhang et al., 2013); genes that are also involved in the defense response to fungus and bacterium. Interestingly, *DMR6* and *DLO1* were found to be direct targets of the ANAC087 transcription factor identified in the network analysis.

4.4.3. Role of salicylic acid and jasmonic acid pathways

EG treatment significantly influenced the expression of genes related to salicylic acid (SA) and jasmonic acid (JA) hormones signaling and defense responses in plants. The upregulation of *WRKY70, NPR3*, and *NPR4* suggests a complex regulatory network involved in SA-mediated signaling and plant immunity. *NPR3* and *NPR4* are SA co-receptors and negatively regulate SA-induced pathogen-related genes and, consequently, SA-induced plant immunity. Also, SA serves as an inhibitor of NPR3/NPR4 to release the repression of defense genes (Ding et al., 2018).

NPR3/NPR4 negatively regulates the expression of *WRKY70*, *WRKY51,* and *NAC004,* genes that are induced by SA (Ding et al., 2018). These genes were up-regulated in EG-treated plants, suggesting that the up-regulation of the negative regulator of SA, *NPR3,* and *NPR4*, could be in response to high SA levels. Reinforcing this idea, several genes involved in SA biosynthesis such as the isochorismate synthase 2 (ICS2) (Garcion et al., 2008), and SA metabolic process were up-regulated by the biostimulant (*DLO1, DMR6, UGT74F2)* (K. Zhang et al., 2013).

The category oxylipin biosynthetic process is enriched in Root 2. Oxylipins are lipid-derived compounds, many of which act as signals in the plant response to biotic and abiotic stress, including the phytohormone JA and related jasmonate metabolites (e.g. cis-(+)-12-oxo-phytodienoic acid (cis-OPDA), methyl jasmonate) (Dave & Graham, 2012). The upregulation of lipoxygenases (LOX) *LOX1* and *LOX6*, patatin-like protein 2 (*PLP2),* and *alpha dioxygenase 1(DOX1)* suggests an activation of JA biosynthesis and related defense pathways. LOX enzymes catalyzed the formation of OPDA, a precursor of JA, and are implicated in herbivore-induced JA burst, crosstalk between JA and SA, and the regulation of resistance to biotic stress (Wasternack & Song, 2017; Zhou et al., 2014). *PLP2* affects the balance between cell death and defense signaling; possible provide fatty acid precursors for the biosynthesis of specific oxylipins, and differentially affected resistance to different pathogens, by altering the expression of SA- and JA-dependent markers (La Camera et al., 2009). Alpha-DOXs enzymes are pathogen-inducible enzymes that catalyze the primary oxygenation of fatty acids into the group of 2(R)-hydroperoxide oxylipins. DOX1 and LOX1 promote plant defense by establishing systemic acquired resistance (SAR) in response to pathogens in a SA-dependent manner (Vicente et al., 2012). Also, *LOX1* was found to be a direct target of *WRKY31,* the transcription factor identified as central for the response to EG in the network analysis.

4.4.4. Exelmax action mechanism

We previously demonstrated that EM biostimulant induced auxin signaling (Chapter III). However, few genes directly related to auxin pathways were found in the transcriptomic analysis, and moreover, our findings indicated that brassinosteroids (BR) may had an important role in the action mechanism of EM biostimulant. Among the genes linked to auxin found in the RNAseq

analysis, the most relevant seems to be *indole-3-acetic acid inducible 5 (IAA5)*, down-regulated 5.6 folds. Interestingly, it has been shown that *IAA5* competes with the non-canonical *IAA IAA33*, which lacks the TIR1 binding domain. Auxin stabilized the IAA33 protein, which in turn prevented ARF10/16 degradations. Also, this mechanism does not result in the up-regulation of *IAA33* gene expression (Lv et al., 2020). Surprisingly, a member of the YUCCA (YUC) family of flavin monooxygenases that play a crucial role in auxin biosynthesis, *YUC6,* was down-regulated by EM. Interestingly, this gene had a dual role in Arabidopsis by participating in AUX biosynthesis, but also in improving drought tolerance independently of AUX production (Cha et al., 2015). *Asymmetric leaves 2-like 9 (ASL9*) belongs to the asymmetric leaves 2/lateral organ boundaries protein family, whose members function in diverse biological processes (Naito et al., 2007), and could be related to EM effect on LRs. *ASL9* is a direct target of *MYB56* transcription factor according to the network analysis and was highly down-regulated by EM biostimulant. In roots, *ASL9* is induced by CK (Naito et al., 2007), and the transcript accumulation inhibits lateral root formation. Both exogenous auxins, as well as a mutation in the cytokinin transcription factors type-B ARR, restores lateral root formation in *ASL9* overexpressor (Zuo et al., 2022).

EM biostimulant modulated the expression of genes associated with BR signaling. BR had an important role in the regulation of cell elongation through a mechanism that involves the action of several receptor-like kinases (RLKs). Among the RLKs induced by BR, the *HERCULES Receptor Kinase 1 (HERK1),* required for optimal cell elongation (Guo et al., 2009), was down-regulated by EM biostimulant. Its modulation is independent of BES1/BZR1, key transcription factors in the BR signaling pathway, and functions in an independent pathway to regulate genes implicated in the regulation of cell elongation by BR. Therefore, *HERK1* may be involved in communicating the BR signal to other pathways to control cell elongation. Additionally, *HERK1* appears to be induced by IAA (Guo et al., 2009). EM treatment also down-regulated the *GASHHHO1* (*GSO1)* expression, a RLK involved in the positive regulation of cell proliferation and the timing of cell division and differentiation in root apical meristem cells (Racolta et al., 2014). Mutants for *gso1* gene showed arrested primary root growth (Racolta et al., 2014). The down regulation of *HERK1, GSO1* and *CYCD3,* a cyclin implicated in cell cycle regulation induced by BR (Clark et al., 2021), could be related to the EM-negative effect on primary root growth. Up-regulation of two negative

regulators of BR signaling, CYP734A1 and *ATBS1-interacting factor 1 (AIF1)*, further supports the involvement of BR in the response to the biostimulant (Choudhary et al., 2012; H. Wang et al., 2009).

Interestingly, BR induces the expression of IAA5, and mutations in BR biosynthesis genes lead to down-regulation of *IAA5* (Nakamura et al., 2003). Moreover, low levels of BRs promote lateral root development (Wei & Li, 2016). Also, BR is a negative regulator of root hair formation; and the mutant for BR biosynthesis genes showed a phenotype with longer root hairs (Cheng et al., 2014; Vissenberg et al., 2020). The increased lateral root development and root hair formation on EM-treated roots could be attributed to low levels of BR and its crosstalk with auxin through IAA5, regulated by the biostimulant.

4.4.5. Exelmax priming for oxidative stress response and defense to nematodes

The major changes in gene expression in response to EM were related to oxidative stress response, detoxification, defense against nematodes, and water transport. Reactive Oxygen Species (ROS) are by-products of aerobic metabolism and are essential signaling molecules in regulating multiple plant developmental processes as well as in reinforcing plant tolerance to biotic and abiotic stimuli. Excessive ROS generation by environmental conditions may cause oxidative damage to biomolecules (Zandi & Schnug, 2022). Plants have a complex redox system, and in *A. thaliana,* this network involved two interconnected clusters: the first formed by Superoxide dismutase (SOD)-related, Thiol-redox, peroxidases, and other oxide-reductase; and the other formed entirely by class III peroxidases (Prx) (Oliveira et al., 2019). In Root 1 EM-treated plants, the hydrogen peroxide catabolic process is enriched, and several Class III peroxidases were found to be categorized in that GO term; two of them were up-regulated *Prx21* and *Prx56*, and five were down-regulated *Prx15, 49, 52, 53* and *69*. Studies in cold stress tolerance and susceptible population of *A. thaliana* have found that, in response to cold stress, class III peroxidases were differentially expressed in the cold-tolerant population. *Prx 52*, and *Prx 53* were exclusively down-regulated in the cold-tolerant population, and *Prx15* was down-regulated in Col-0 and in the cold-tolerant population under cold stress. *Prx 56*, was exclusively up-regulated by the cold-tolerant population, and Prx49 was down-regulated in both (Eljebbawi et al., 2022).

Superoxide dismutase, a metalloenzyme, is actively present in the defense against oxidative damage from ROS. In Arabidopsis there are three main isoforms of these enzymes: Cu/Zn-SOD, Fe-SOD, and Mn-SOD (Zandi & Schnug, 2022). In roots with EM-biostimulants, two of the genes that encode for these enzymes are up-regulated: *Cu/Zn-SOD 1 (CDS1)*, and the chaperone of CSD1, *CCS*; and one, *Fe-SOD 1* (*FSD1)* is down-regulated. Pattern expression of these genes differs according to the stress stimulus. *CSD1* appears to be up-regulated under cold stress in *Arabidopsis* Col-0*,* and *FDS1*, on the other hand, is down-regulated under the same stress conditions (Filiz et al., 2019).

Syncytium is a multinuclear cell that is commonly induced by nematodes in *Arabidopsis* roots, which functions as a feeding site for nematodes (Szakasits et al., 2009). Syncytium formation GO term is enriched in Root 1 by EM biostimulant, and the genes related to this biological process were down-regulated by EM, suggesting that the biostimulant could induce tolerance to nematodes parasites. The genes found in this term were 3 *α-expansins (EXPA), EXPA1, 4* and *15*. Moreover, two pectate lyase-like (PLL) genes, *PLL18* and *PLL19,* were down-regulated by EM. Studies have shown that both genes are up-regulated by cyst and root-knot nematodes in their feeding site, and the loss of function of these genes particularly affects the formation of syncytia and, therefore, the development of cyst nematodes (Wieczorek et al., 2014). It has been also suggested that BR induced systemic resistance against root-knot nematode *Meloidogyne incognita* by a BR-dependent regulation of ROS accumulation in tomato roots (Song et al., 2018). Moreover, consistent with BRs major function in promoting cell elongation, BR also regulates the expression of various *EXPA* and *PLL* genes (Guo et al., 2009).

The enrichment of genes involved in glucosinolate biosynthesis indicates that EM biostimulant may influence plant defense against generalist herbivores and pathogens. Several genes involved in both aliphatic and indolic glucosinolate metabolic pathways were downregulated by EM in Root 1, including *indole glucosinolate o-methyltransferase 5* (*IGMT5)* (Yi et al., 2015). Interestingly, disruption of *IGMT5* increases resistance against the root-knot nematodes (Pfalz et al., 2016). Furthermore, camalexin biosynthetic processes were enriched in Root 2 tissue of plants with EM biostimulant. Camalexin is the major phytoalexin found in *Arabidopsis* and has been reported that play defensive functions against several pathogens (Lemarié et al., 2015). The

gene *GH3.5*, up-regulated by EM, has been implicated in the biosynthesis of camalexin precursor dihydrocamalexic acid (M. Y. Wang et al., 2012). Interestingly, *GH3.5* also conjugates IAA and SA to modulate auxin and pathogen response pathways, mediating the crosstalk between these hormones (Westfall et al., 2016).

Previously, we found that EM apparently increased the water content of roots (Chapter II). The RNAseq analysis showed that several aquaporins from the type of plasma membrane intrinsic proteins (PIP) and tonoplast intrinsic proteins (TIP) were up-regulated by EM in root, such as: *PIP1B, PIP2B, PIP1-1, PIP2-1, TIP2-1,* and *TIP2-3*, suggesting that EM biostimulant enhances water transport and osmotic regulation in plant roots. Aquaporins work as water channels and facilitators of the transport of small solutes and gases, having an important role in several physiological contexts, e.g., water transport in root cells, tissue expansion, nitrogen, and micronutrient acquisition. Aquaporin's homeostasis changes in response to variable environmental signals. Re-localization of aquaporins regulates turgor and intracellular water movement under water-, salt- and nutrient- stresses, and they are rapidly induced under drought stress (Maure et al., 2008), and cold stress (Rahman et al., 2020). Moreover, ROS-induced damage dominates the aquaporins response and determines the performance of the plant under stress conditions (Maure et al., 2008). Interestingly, aquaporins also seem to be involved in plant-pathogen interaction and appear to have a role in the proper osmotic regulation of the giant cells formed in response to nematode infection (Maure et al., 2008). It has also been suggested that BR may control aquaporin activities in *A. thaliana* on normal growth conditions (Kapilan et al., 2018; Morillon et al., 2001).

Our study highlights the comprehensive impact of EG biostimulant on hormone signaling and defense-related gene expression, shedding light on the complex mechanisms underlying plant responses to the biostimulant and their potential applications in enhancing plant defense and stress tolerance. EM biostimulant elicits complex molecular responses in plants, involving the regulation of oxidative stress, defense against nematodes and pathogens, and water transport. These insights contribute to our understanding of the mechanisms underlying the beneficial effects of EG and EM biostimulants and their potential applications in agriculture and stress

management. Further research is needed to validate these results in field conditions and to explore the practical implications for sustainable agriculture.

4.5. References

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5. General discussion and prospects

5.1. General discussion

Biostimulants act in a dose-dependent manner, therefore the concentration of application of the product will depend on the desirable effect on the plant. Also, the biostimulants act differently in the PR section, and LRs developed before and after the transfer to treatment. Under this study's growth conditions, each product's most beneficial effects on root development were observed with EG 0.001% v/v and EM 0.01% v/v. We could summarize the biostimulant effect of EG and EM in Arabidopsis at the concentrations we recommended for *in vitro* culture conditions as the following: EG-treated plants had characteristics similar to the control treatment, there is no negative effect on the general root and rosette development, and notably, plants had more developed lateral roots (longer lateral roots) and a significantly more developed root hair system on the primary root grown after transfer to treatment. EG could also have a role in the induction of lateral roots primordium. EM biostimulant repressed the primary growth, accelerated lateral root development, increased the surface of lateral roots, and induced a significantly more developed root hair system and rosettes with a greater surface. The analysis on primordium development suggested that EM disrupted LRP initiation but enhanced the development of pre-existing LRPs, accelerating growth. Also, the biostimulant effect on lateral root development is focused on the upper part of the root, corresponding to the primary root formed previous to the exposure to the treatment. This could be a desirable outcome for crops grown in pots or with a drip irrigation system. The increment in root surface area by the biostimulants amplified the absorptive capacity, which could potentially enhance the nutrient and water uptake in limited systems (Gilroy & Jones, 2000).

The up-regulation of several SA-responsive genes (e.g. *NPR3, NPR4, WRKY54, WRKY70, ALD1, LURP1, DMR6*) and the up-regulation of the *ICS2* gene, involved in the SA-biosynthesis pathway (Lefevere et al., 2020), by EG-treatment, suggested that the biostimulants act by inducing SA-biosynthesis and signaling pathways. In EG-treated plants, the *ethylene forming enzyme (EFE)* gene, related to ethylene (ET) biosynthesis, was up-regulated, as well as the biosynthesis of JA precursors. In general SA-responsive pathways regulates the defense against biotrophic pathogens, while JA- and ET- responsive pathways regulate the response to necrotrophic

pathogens, herbivory, and wounding (Stroud et al., 2022). SA and JA had antagonistic interactions against the different pathogens, and crosstalk between SA and JA pathways requires the involvement of transcriptional reprogramming by transcription factors. For example*, WRKY70* negatively regulates JA-mediated defenses and positively regulates SA-responsive genes in response to pathogens (Shim et al., 2013). Taking it all together, these results suggest that EG interplay with SA, JA, and ET pathways, and the interplay between SA, JA, and ET regulates the plant's response to different biotic stresses. This finding underscores the significant impact of the EG biostimulant in activating and modulating the plant's defense mechanisms against various biotic stressors.

We found that the brassinosteroids pathway could have an important role in the mechanism of action of EM in *Arabidopsis.* Brassinosteroids regulate many growths and developmental process, such as cell division and elongation in the meristem, senescence, vascular development, reproduction, root and shoot growth, and response to biotic and abiotic stresses (Manghwar et al., 2022). Also, the BR mechanism to induce stress tolerance appears to be modulated by the production of ROS. BR-mediated ROS signals modulate redox homeostasis and detoxification, leading to the activation of transcription factors that control transcription of BR-regulated and stress-responsive genes to enhance tolerance to biotic and abiotic stresses through the accumulation of protective proteins (Ahammed et al., 2020), consistently with the high EM-modulation of oxidative stress-related genes. Additionally, several genes that respond to BR are dysregulated by EM, such as *WRKY70, HERK1, CYCD3;1,* and *SODs*. These results suggest that EM interplays with BR for the regulation of ROS signals and defense against nematodes and pathogens.

At early stages of EG/EM treatment (three days of treatment), CK/AUX signaling was induced in the root tissue (Chapter III). However, no major CK/AUX responses were observed in the RNAseq analysis at seven days of EG/EM treatment, and several SA/BR-regulated genes were expressed instead. BR and AUX are involved synergistically or antagonistically in multiple plant developmental processes such as hypocotyl elongation, root development, regulation of root meristem, tropism, etc., and crosstalk between these hormones plays an important role in the regulation of these processes (Tian et al., 2018; Xuan & Beeckman, 2021). Similarly, SA interplays

with CK, AUX, and other hormones either synergistically or antagonistically for the control of plant growth and adaptation under biotic stress (Singh et al., 2022).

In conclusion, EG and EM biostimulants exhibit biostimulant activity in the roots of *Arabidopsis thaliana*, although with distinct effects. Furthermore, EG and EM interact differently with the CK and AUX pathways. During the early stages of biostimulant treatment (three days), EG promotes CK signaling and suppresses AUX signaling in the root meristem. In contrast, EM stimulates AUX signaling and inhibits CK signaling. As the biostimulant treatment progresses (seven days), the transcriptomic analysis revealed insights into the action mechanism of the biostimulants: EG interacts with the SA, JA, and ET pathways, which collectively regulate the plant's response to various biotic stresses. On the other hand, EM interacts with the BR pathway to regulate ROS signals and defense against nematodes and pathogens. We proposed that EM indirectly regulates AUX through BR, while EG indirectly regulates CK through SA to regulate plant growth and response to stress. Additionally, EG-induced priming in *A. thaliana* plants may enhance tolerance to biotic stress, whereas EM-induced priming may enhance tolerance to stress and nematode infection, by the regulation of ROS production. Finally, we identified key transcription factors in the modulation of plant growth and tolerance to stress by the biostimulants: ANAC087 (AT5G18270), ANAC004 (AT1G02230), and ATWRKY31 (AT4G22070) in EG; and the transcription factors MYB56 (AT5G17800), and ASL9 (AT1G16530) as central in the response to EM, that directly interacted with several key genes identified in the transcriptomic analysis.

5.2. Prospects

The present thesis studied the phenotypic, hormonal response, and transcriptomic effect of two commercial biostimulants derived from *A. nodosum* algae. The results demonstrated the difference between both products and insights into the mechanism of action of each product on plants. However, some results were inconclusive, and further studies are required. It is proposed that future work should be focused on:

- A phenotypic characterization of the effect on the aerial part of the plants, including flower and fruit development. It will be also interesting to evaluate the impact on cell cycle in leaves and fruits due to the positive effect observed on rosette area by EM biostimulant.

- The assay with mutants for the CK and AUX-related pathways should be repeated with all the missing lines (single mutants and for genes related to the AUX biosynthesis pathway).

- Test the potential priming mechanism of both biostimulants against the different stress proposed in this research. Because both products induced genes related to a stress defense mechanism, the product could be applied a week before the induction of the stress in the plants. It is expected that plants with the treatment had a better response to the stress than untreated plants.

- Validate these results in other species of agricultural interest and explore the practical implications for sustainable agriculture. This analysis should be done in field conditions. The growth biostimulant effect of these products, as well as their potential to enhance plant stress resistance, make them suitable for application in crops to complement and reduce the use of agrochemicals in farming.

5.3. References

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SUPPLEMENTARY MATERIAL

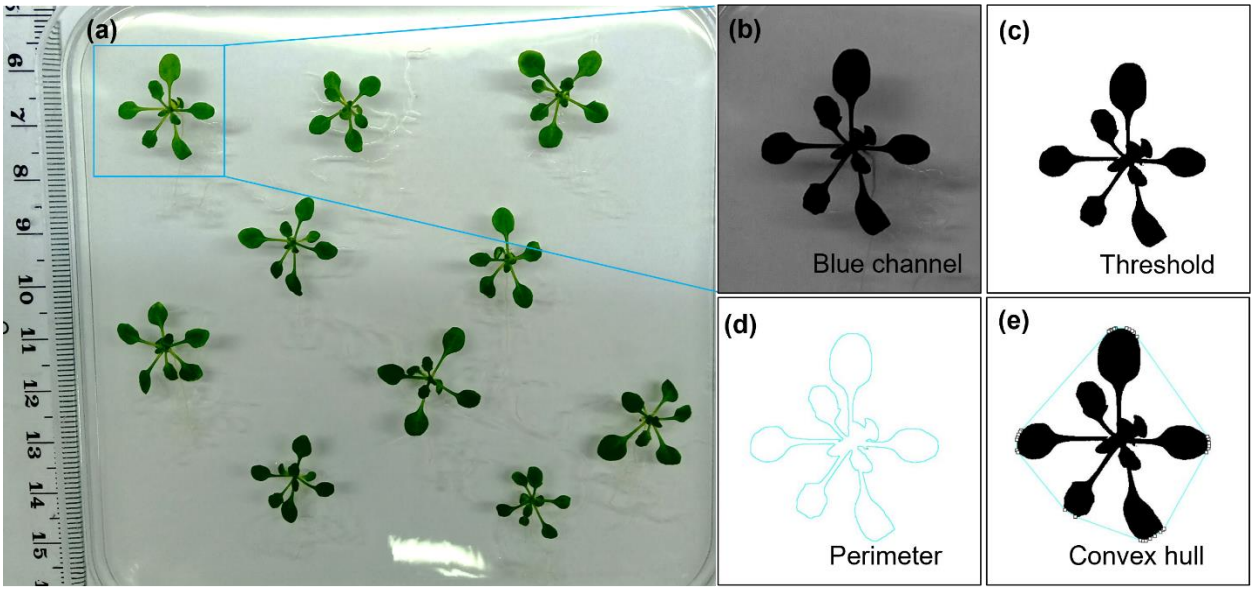

Figure S1. Rosette parameters measurement. **(a)** Photo of the plate that was used for the analysis. The photos were taken next to a ruler to set the image's scale. The images were analyzed with ImageJ 1.53f51 software. First, "Split channels" was applied, and the blue was selected to further process **(b)**. To measure the Projected area, a threshold was applied to select the rosette's pixels and eliminate the background **(c)**. Then, the rosette's area and perimeter were measured. The "Wand tool" was used to select each rosette **(d)**. To measure the convex hull area, the "convex hull" was selected (**e,** cyan line), and then a Mask was created for each convex hull to measure the area.

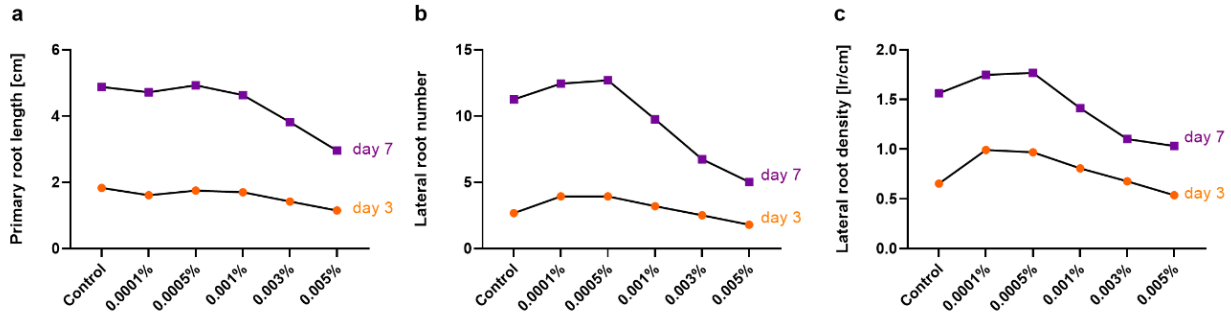

Figure S2. Comparative results of root assay at different treatments period. Seedlings of *A. thaliana* Col-0 3 and 7 days after treatment with Exelgrow at different concentrations. **(a)** primary root length, **(b)** Lateral root number, and **(c)** lateral root density. Primary root length represents the delta between day 3 or 7 and day 0 of treatment. Lateral root density is calculated as lateral root number/ primary root length (at day 3 or 7 of treatment). Points represent mean values of at least 3 biological replicates of n≥10 for each condition.

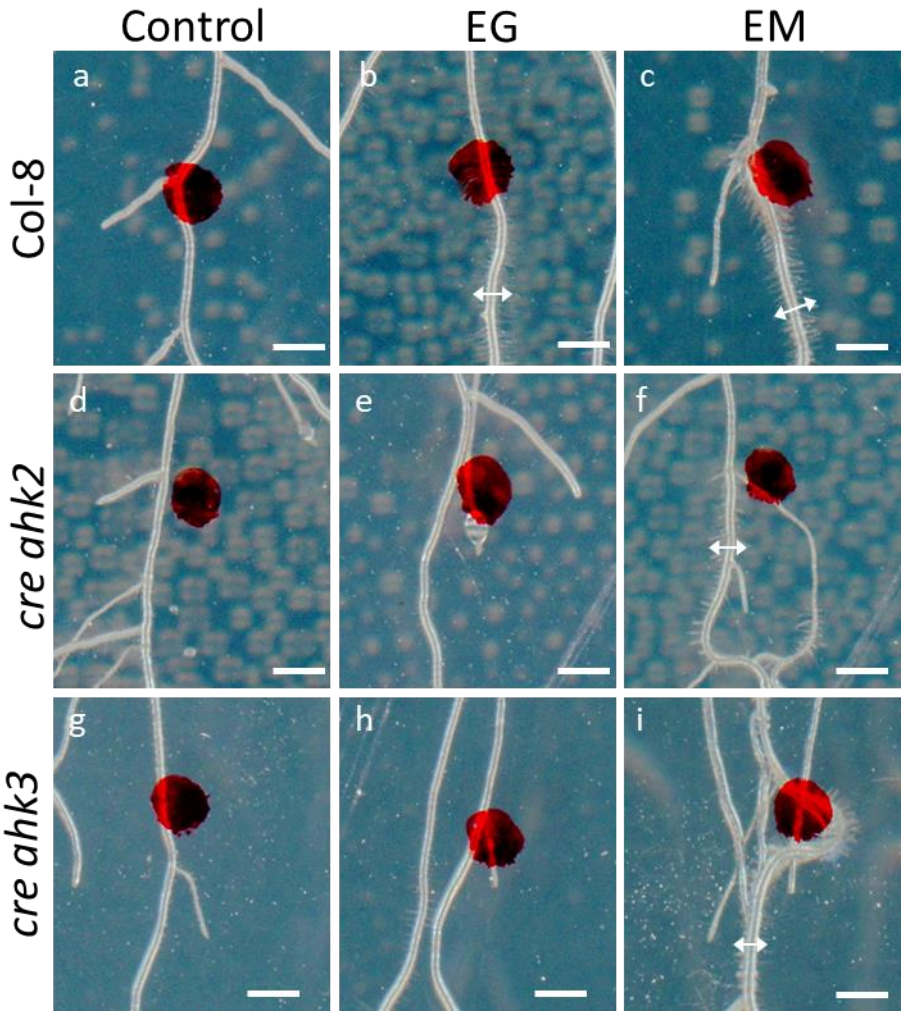

Figure S3. Root hair development on transgenic lines of *A. thaliana* for CK receptor genes. Photos showed representative phenotypes of plants of Col-8 (WT) **(a-c)**, double mutant line *cre ahk2* **(d-f)**, and double mutant line *cre ahk3* **(g-i)**, with control (1/2 MS), Exelgrow (EG), and Exelmax (EM) tretament. Scale bar=1mm. The red dot indicated the position of the root at the time of transfer to treatment, the "zone of transfer to treatment". White arrows indicate width of root hairs. No arrows indicate that there is no root hair development or at least is not detectable at eye view.

Table S1: Quality information of the transcriptomic analysis

Sample ID	Description	Raw reads R1+R2	Post trimming	Q25 (%)	Unique align (%)	Multi loci align (%)	Total align (%)
CB1	Control_Root1_R1	41.748.092	41.051.906	98,33	96,99	2,50	99,49
CB2	Control_Root1_R2	46.367.618	45.455.078	98,03	96,81	2,78	99,59
CB3	Control_Root1_R3	49.678.604	48.819.022	98,27	96,59	3,01	99,60
EGB1	EG_Root1_R1	44.406.398	43.600.750	98,19	97,33	2,26	99,59
EGB2	EG_Root1_R2	43.003.606	42.252.430	98,25	97,33	2,23	99,56
EGB3	EG_Root1_R3	41.345.416	40.646.602	98,31	97,39	2,18	99,57
EMB1	EM_Root1_R1	39.997.064	39.330.790	98,33	97,83	1,75	99,58
EMB2	EM_Root1_R2	40.559.794	39.862.568	98,28	97,06	2,57	99,63
EMB3	EM_Root1_R3	45.840.776	45.070.826	98,32	97,50	0,33	97,83
CC1	Control_Root2_R1	49.201.804	48.408.424	98,39	97,19	2,43	99,62
CC2	Control_Root2_R2	45.192.216	44.417.214	98,29	97,02	2,62	99,64
CC3	Control_Root2_R3	47.170.968	46.367.602	98,30	97,66	1,92	99,58
EGC1	EG_Root2_R1	58.109.762	57.109.928	98,28	97,41	2,20	99,61
EGC2	EG_Root2_R2	45.806.120	44.981.438	98,20	96,98	2,62	99,60
EGC3	EG_Root2_R3	46.519.236	45.722.516	98,29	97,49	2,03	99,52
EMC1	EM_Root2_R1	45.244.722	44.435.622	98,21	96,81	2,82	99,63
EMC2	EM_Root2_R2	51.740.872	50.783.818	98,15	96,77	2,79	99,56
EMC3	EM_Root2_R3	43.755.706	42.919.396	98,09	97,54	2,07	99,61

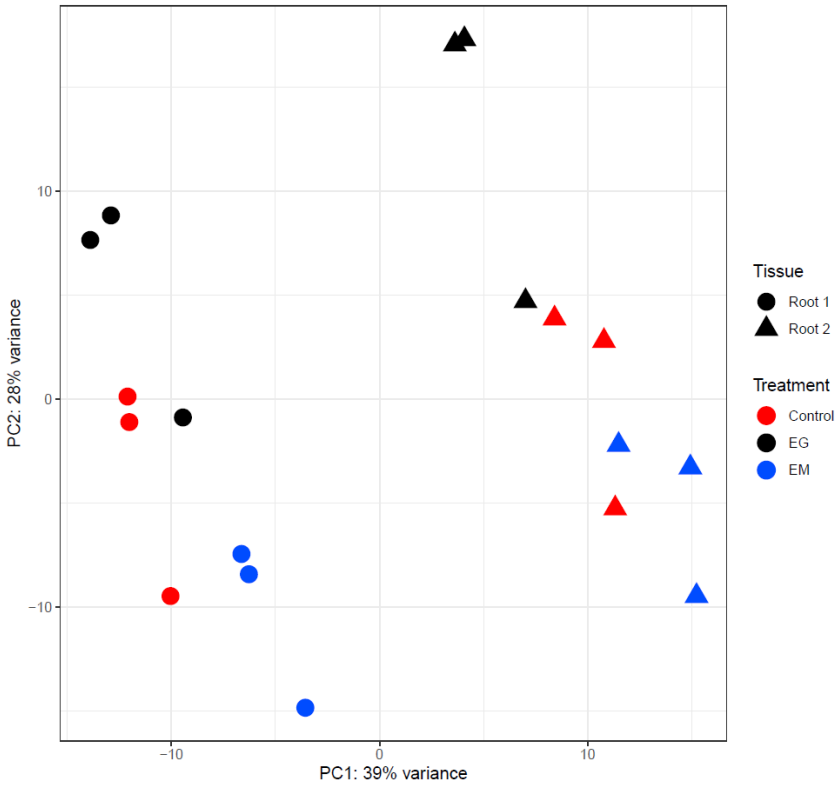

Figure S4: Principal components analysis. Tissues Root 1 and Root 2 with control (1/2 MS), Exelgrow 0.001% v/v (EG), and Exelmax 0.01% v/v (EM) treatments.

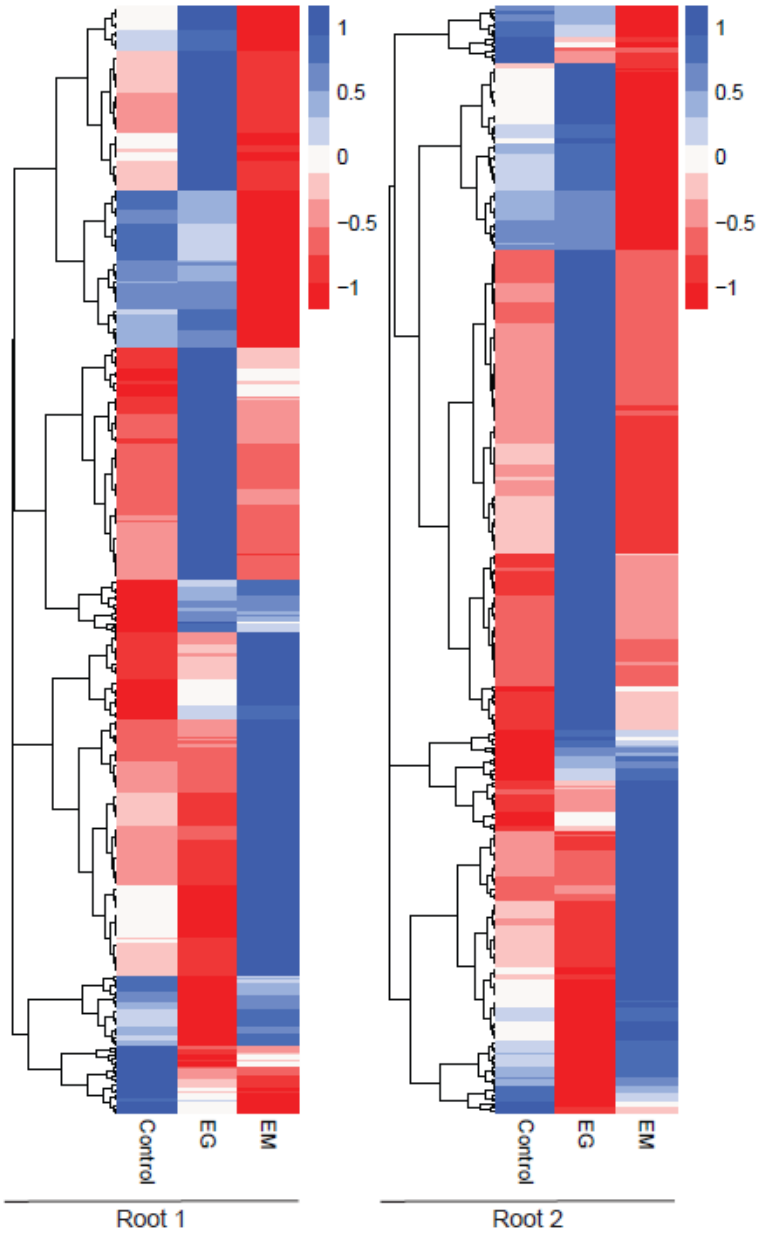

Figure S5: Heat map of RNAseq analysis of Root 1 and Root 2 tissue of plants with control (1/2 MS), Exelgrow 0.001% v/v (EG), and Exelmax 0.01% v/v (EM).

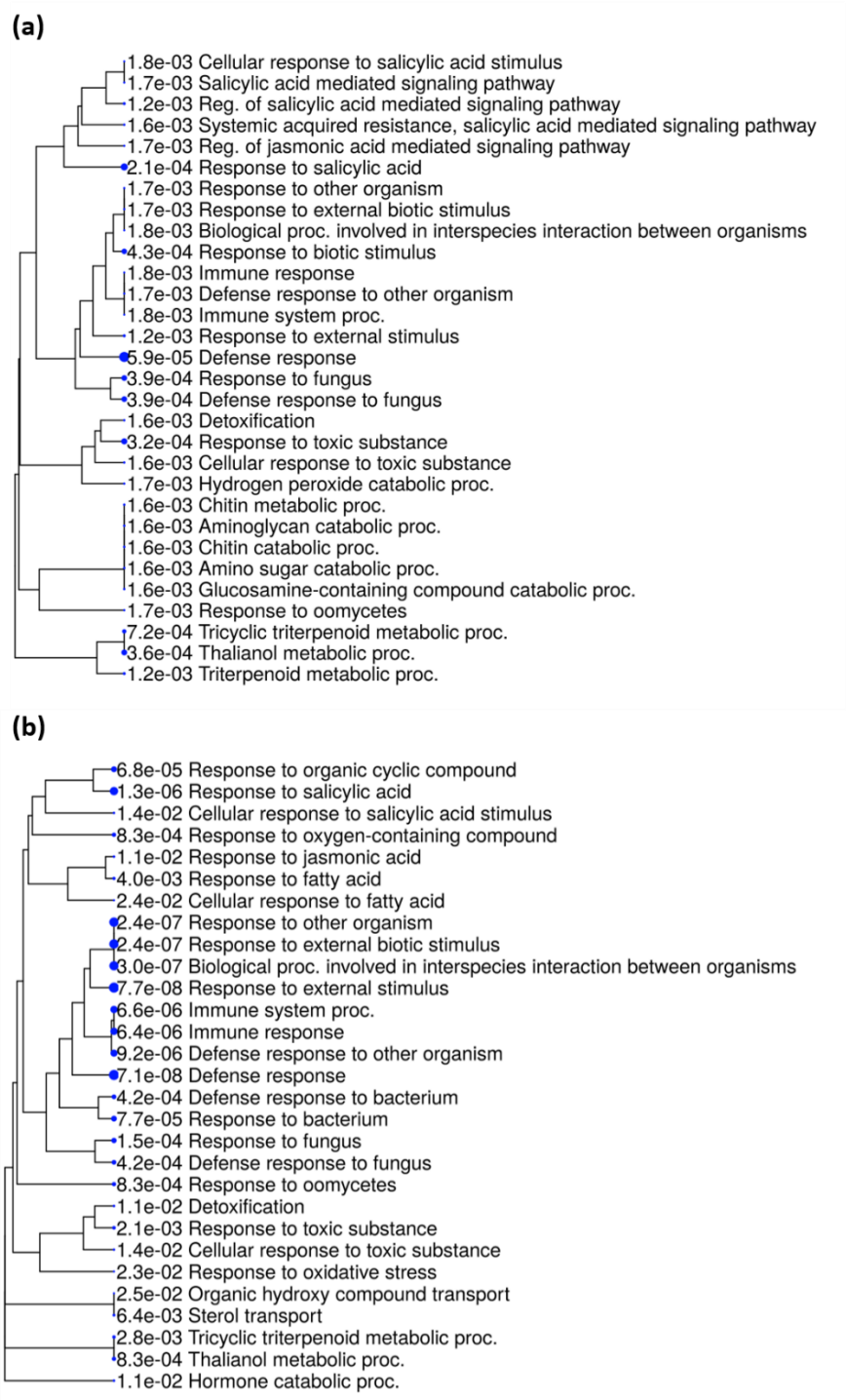

Figure S6: A hierarchical clustering tree summarizing the correlation among significant pathways listed in the GO enrichment of EG compared to control analysis, in Root 1 **(a)**, and Root 2 **(b)**. Pathways with many shared genes are clustered together. The larger dots indicate more significant P-values.

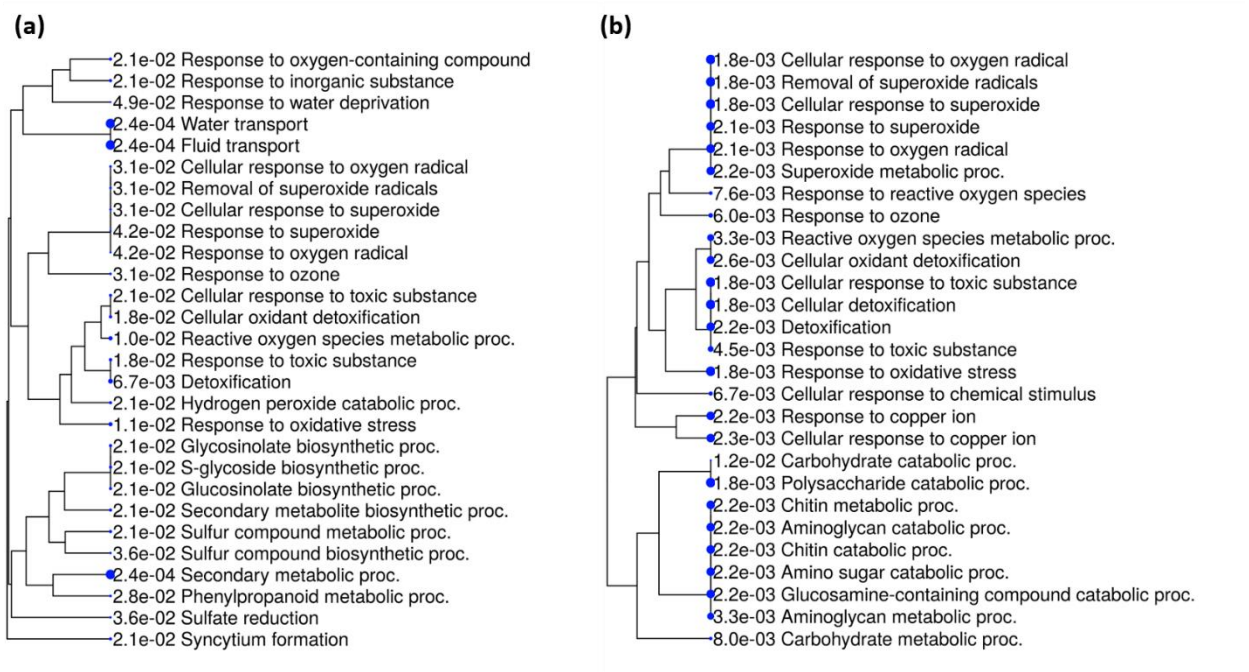

Figure S7: A hierarchical clustering tree summarizing the correlation among significant pathways listed in the GO enrichment of EM compared to control analysis, in Root 1 **(a)**, and Root 2 **(b)**. Pathways with many shared genes are clustered together. The larger dots indicate more significant P-values.

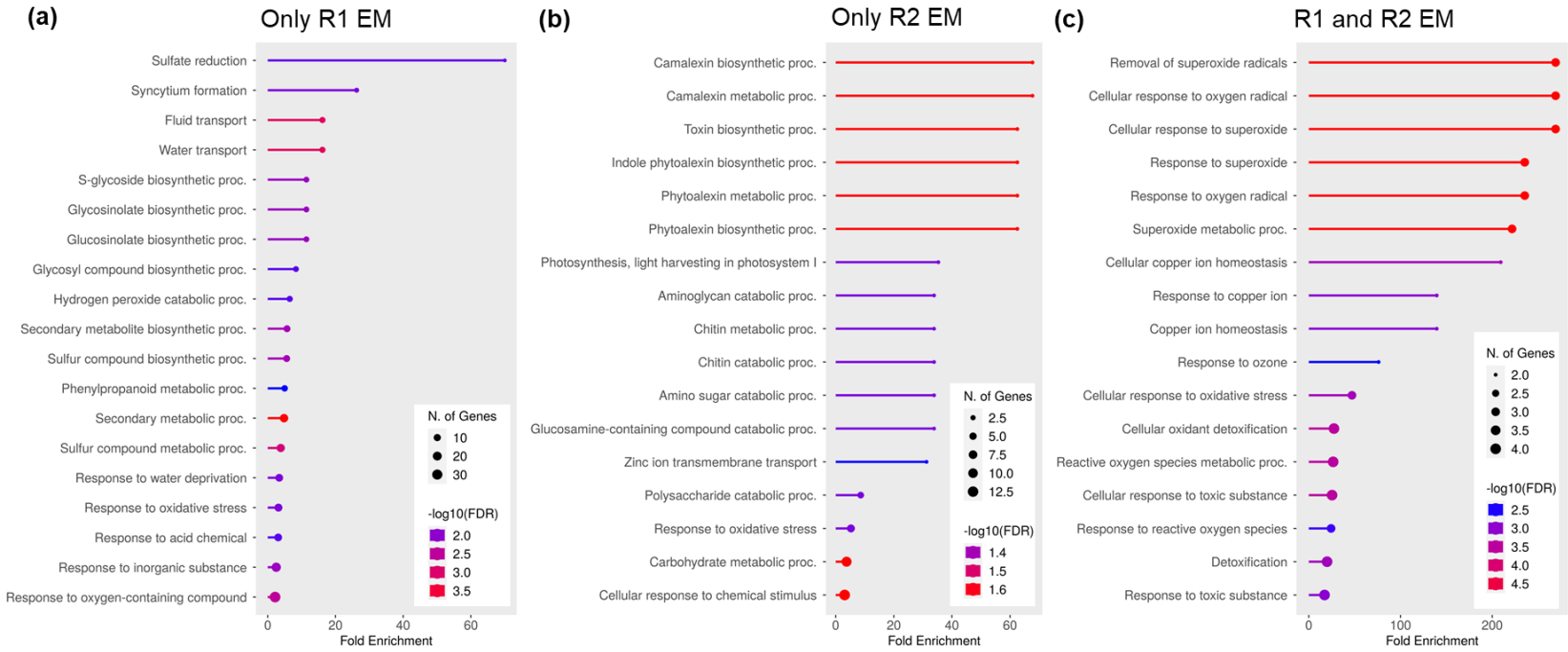

Figure S8. Gene ontology enrichment analysis of *A. thaliana* root tissues with EM biostimulant. The top 20 enriched factors for Biological Process for the DEGs found only in Root 1 **(a)**, and Root 2 **(b)** tissues, and common for both tissues (**c**). Analysis was done in ShinyGOv0.77

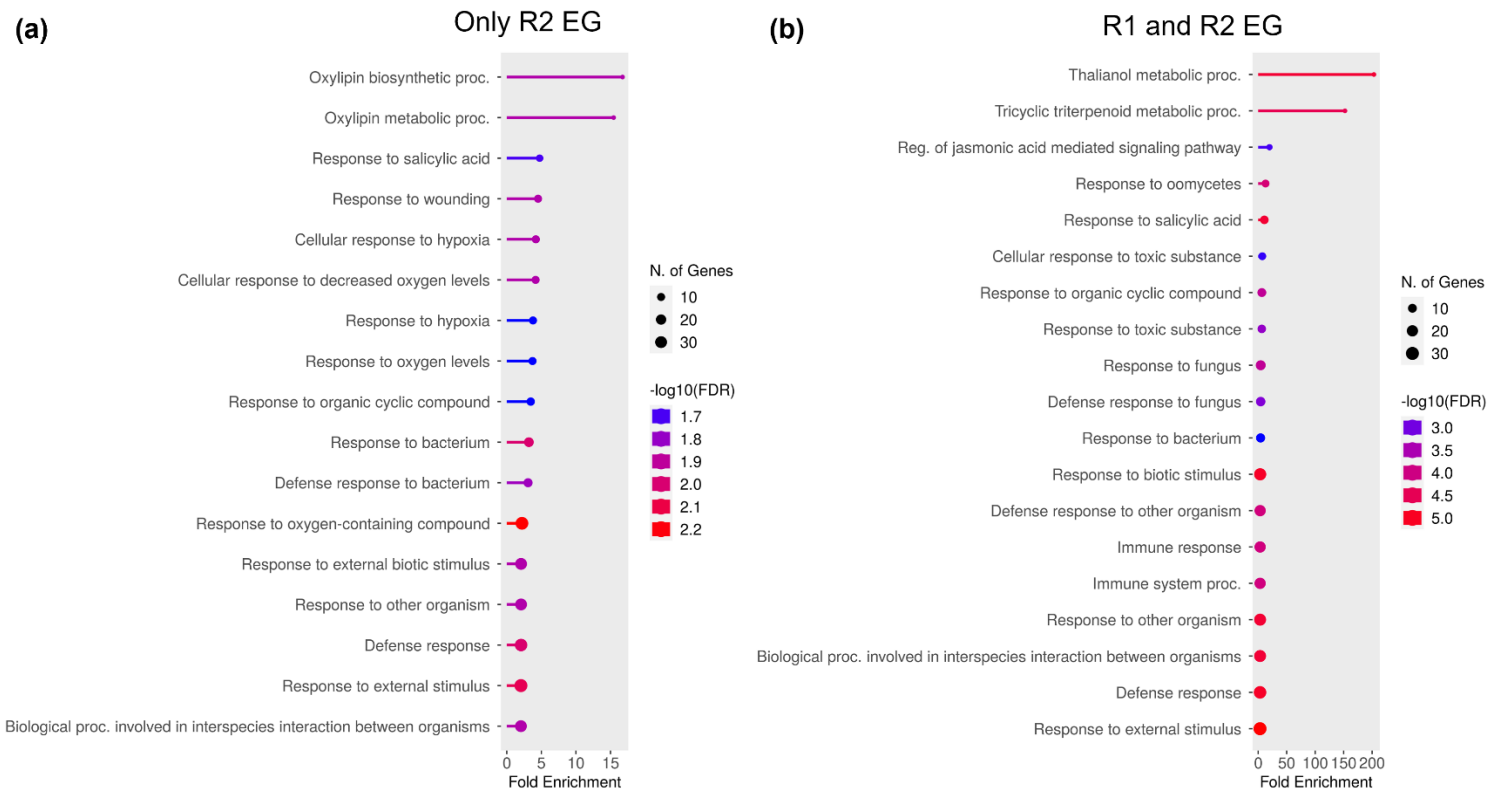

Figure S9. Gene ontology enrichment analysis of *A. thaliana* root tissues with EG biostimulant. The top 20 enriched factors for Biological Process for the DEGs found only in Root 2 **(a)**, and common for both tissues (**b**). Analysis was done in ShinyGOv0.77

Table S2. List of significantly enriched ontologies of Root 1 with EG biostimulant

Enrichment FDR	Genes in list	Pathway Genes	Fold Enrichment	Functional categories
3.62E-04	3	3	81.99	Thalianol metabolic process
7.18E-04	3	4	61.49	Tricyclic triterpenoid metabolic process
8.92E-03	2	4	54.66	Salicylic acid catabolic process
2.34E-02	2	7	32.79	Nitrile metabolic process
2.34E-02	2	7	32.79	Nitrile biosynthetic process
2.34E-02	2	6	32.79	Floral organ senescence
3.06E-02	2	8	27.33	Cellular response to iron ion starvation
3.06E-02	2	7	27.33	Phenol-containing compound catabolic process
1.23E-03	4	19	25.23	Triterpenoid metabolic process
3.79E-02	2	8	23.42	Hormone catabolic process
3.79E-02	2	14	23.42	Oligopeptide transmembrane transport
1.57E-03	4	24	20.50	Aminoglycan catabolic process
1.57E-03	4	24	20.50	Chitin metabolic process
1.57E-03	4	24	20.50	Chitin catabolic process
1.57E-03	4	24	20.50	Amino sugar catabolic process
1.57E-03	4	24	20.50	Glucosamine-containing compound catabolic process
1.57E-03	4	18	20.50	Systemic acquired resistance, salicylic acid mediated signaling pathway
2.13E-03	4	28	16.40	Aminoglycan metabolic process
2.53E-03	4	32	15.62	Glucosamine-containing compound metabolic process
1.23E-03	5	30	15.18	Regulation of salicylic acid mediated signaling pathway
1.71E-03	5	41	12.06	Regulation of jasmonic acid mediated signaling pathway
3.04E-02	3	27	11.71	Brassinosteroid homeostasis
3.79E-02	3	31	10.25	Phytosteroid biosynthetic process
3.79E-02	3	31	10.25	Brassinosteroid biosynthetic process

3.79E-02	3	31	10.25	Steroid hormone biosynthetic process
1.38E-02	4	49	9.65	Amino sugar metabolic process
1.69E-03	6	66	9.28	Salicylic acid mediated signaling pathway
4.75E-02	3	33	9.11	Cell wall macromolecule catabolic process
4.75E-02	3	35	9.11	Phytosteroid metabolic process
4.75E-02	3	35	9.11	Brassinosteroid metabolic process
4.75E-02	3	38	9.11	Oligopeptide transport
1.82E-03	6	73	8.63	Cellular response to salicylic acid stimulus
2.13E-03	6	96	8.20	Carbohydrate derivative catabolic process
2.85E-02	4	52	7.63	Salicylic acid metabolic process
1.69E-03	7	97	7.45	Hydrogen peroxide catabolic process
1.70E-03	7	110	7.36	Response to oomycetes
2.11E-04	11	169	6.99	Response to salicylic acid
1.55E-02	5	84	6.72	Systemic acquired resistance
4.22E-02	4	69	6.43	Phenol-containing compound metabolic process
7.36E-03	6	98	6.39	Jasmonic acid mediated signaling pathway
4.39E-02	4	67	6.31	Iron ion homeostasis
9.20E-03	6	103	6.07	Cellular response to jasmonic acid stimulus
4.05E-03	7	120	5.92	Hydrogen peroxide metabolic process
1.16E-02	6	107	5.79	Cellular response to fatty acid
1.57E-03	10	198	5.16	Cellular response to toxic substance
2.02E-03	9	183	5.05	Cellular oxidant detoxification
3.24E-04	14	292	4.88	Response to toxic substance
1.27E-02	7	143	4.78	Leaf senescence
3.18E-03	9	197	4.67	Cellular detoxification
1.48E-02	7	149	4.63	Plant organ senescence
3.86E-04	14	562	4.55	Defense response to fungus
1.59E-03	11	252	4.49	Detoxification
2.54E-02	7	168	4.13	Aging
3.86E-04	16	650	4.00	Response to fungus
2.05E-03	12	319	3.81	Response to organic cyclic compound

3.82E-02	7	189	3.68	Reactive oxygen species metabolic process
3.06E-02	10	371	2.97	Secondary metabolic process
6.35E-03	14	465	2.97	Response to oxidative stress
5.85E-05	36	1700	2.65	Defense response
3.16E-02	12	457	2.62	Defense response to bacterium
1.71E-03	23	1193	2.51	Defense response to other organism
4.34E-04	30	1482	2.49	Response to biotic stimulus
1.82E-03	23	1205	2.47	Immune response
1.82E-03	23	1209	2.46	Immune system process
3.79E-02	13	534	2.44	Response to bacterium
1.71E-03	27	1457	2.28	Response to external biotic stimulus
1.71E-03	27	1457	2.28	Response to other organism
1.82E-03	27	1473	2.25	Biological process involved in interspecies interaction between organisms
1.23E-03	35	1914	2.14	Response to external stimulus
4.39E-02	22	1318	1.87	Transmembrane transport
2.24E-02	30	1692	1.79	Response to oxygen-containing compound
2.90E-02	29	1716	1.77	Cellular response to chemical stimulus

Table S3. List of significantly enriched ontologies of Root 2 with EG biostimulant

Enrichment FDR	Genes in list	Pathway Genes	Fold Enrichment	Functional categories
8.29E-04	3	3	43.52	Thalianol metabolic process
2.81E-03	3	4	32.64	Tricyclic triterpenoid metabolic process
4.29E-02	2	4	29.02	Salicylic acid catabolic process
1.12E-02	3	8	21.76	Hormone catabolic process
6.42E-03	5	24	9.89	Sterol transport
2.48E-02	4	24	9.67	Oxylipin biosynthetic process
2.59E-02	4	26	9.16	Oxylipin metabolic process
2.48E-02	5	41	6.80	Organic hydroxy compound transport
1.28E-06	17	169	5.69	Response to salicylic acid
8.29E-04	10	110	5.58	Response to oomycetes
1.38E-02	7	73	5.34	Cellular response to salicylic acid stimulus
3.59E-02	6	66	5.02	Salicylic acid mediated signaling pathway
4.88E-02	6	84	4.58	Systemic acquired resistance
2.40E-02	8	107	4.25	Cellular response to fatty acid
4.29E-02	7	98	4.12	Jasmonic acid mediated signaling pathway
3.96E-03	13	200	3.60	Response to fatty acid
6.78E-05	21	319	3.53	Response to organic cyclic compound
1.12E-02	12	196	3.41	Response to jasmonic acid
3.89E-02	9	159	3.41	Hormone metabolic process
4.19E-04	19	562	3.33	Defense response to fungus
1.35E-02	12	198	3.31	Cellular response to toxic substance
4.60E-02	9	143	3.29	Leaf senescence
3.89E-02	10	168	3.15	Aging
2.14E-03	17	292	3.14	Response to toxic substance
1.46E-04	23	650	3.12	Response to fungus
3.21E-02	11	197	3.05	Cellular detoxification
1.12E-02	14	252	3.03	Detoxification
4.88E-02	10	183	3.00	Cellular oxidant detoxification

2.48E-02	13	239	2.86	Cellular response to hypoxia
7.65E-05	28	534	2.84	Response to bacterium
2.48E-02	13	241	2.83	Cellular response to decreased oxygen levels
2.48E-02	13	241	2.83	Cellular response to oxygen levels
4.23E-04	24	457	2.82	Defense response to bacterium
4.71E-02	13	265	2.57	Response to hypoxia
2.48E-02	16	371	2.53	Secondary metabolic process
6.41E-06	43	1205	2.48	Immune response
6.62E-06	43	1209	2.46	Immune system process
9.17E-06	42	1193	2.46	Defense response to other organism
2.38E-07	54	1457	2.45	Response to external biotic stimulus
2.38E-07	54	1457	2.45	Response to other organism
2.38E-07	55	1482	2.45	Response to biotic stimulus
7.06E-08	62	1700	2.44	Defense response
2.97E-07	54	1473	2.42	Biological process involved in interspecies interaction between organisms
2.26E-02	20	465	2.29	Response to oxidative stress
7.65E-08	69	1914	2.25	Response to external stimulus
8.29E-04	56	1692	1.80	Response to oxygen-containing compound
2.48E-02	49	1716	1.60	Cellular response to chemical stimulus

Table S4. List of significantly enriched ontologies of Root 1 with EM biostimulant

Enrichment FDR	Genes in list	Pathway Genes	Fold Enrichment	Functional categories
1.45E-02	2	3	64.63	Sulfate reduction
4.40E-02	2	6	32.31	Floral organ senescence
1.11E-02	3	12	24.24	Syncytium formation
1.45E-02	3	14	20.77	Removal of superoxide radicals
1.45E-02	3	14	20.77	Cellular response to oxygen radical
1.45E-02	3	14	20.77	Cellular response to superoxide
1.99E-02	3	16	18.18	Response to superoxide
1.99E-02	3	16	18.18	Response to oxygen radical
5.33E-05	7	39	17.40	Fluid transport
5.33E-05	7	39	17.40	Water transport
2.33E-02	3	17	17.11	Superoxide metabolic process
2.69E-02	3	18	16.16	Response to copper ion
4.36E-02	3	22	13.22	Sulfate assimilation
1.45E-02	4	33	11.75	Response to ozone
6.21E-03	5	46	10.54	S-glycoside biosynthetic process
6.21E-03	5	46	10.54	Glycosinolate biosynthetic process
6.21E-03	5	46	10.54	Glucosinolate biosynthetic process
4.52E-02	4	49	7.91	Anthocyanin-containing compound metabolic process
1.87E-02	5	63	7.69	Glycosyl compound biosynthetic process
5.15E-03	7	97	7.00	Hydrogen peroxide catabolic process
4.15E-02	5	78	6.21	Lignin metabolic process
1.21E-02	7	120	5.65	Hydrogen peroxide metabolic process
7.00E-04	11	189	5.64	Reactive oxygen species metabolic process
2.38E-03	10	183	5.30	Cellular oxidant detoxification
8.15E-03	8	147	5.28	Phenylpropanoid metabolic process
5.13E-03	9	166	5.26	Secondary metabolite biosynthetic process
5.15E-03	9	169	5.16	Sulfur compound biosynthetic process
4.85E-04	13	252	5.00	Detoxification

3.95E-03	10	197	4.92	Cellular detoxification
3.95E-03	10	198	4.90	Cellular response to toxic substance
4.36E-02	6	119	4.89	S-glycoside metabolic process
4.36E-02	6	119	4.89	Glycosinolate metabolic process
4.36E-02	6	119	4.89	Glucosinolate metabolic process
5.33E-05	18	371	4.70	Secondary metabolic process
3.89E-02	7	155	4.38	Response to reactive oxygen species
1.61E-03	13	292	4.32	Response to toxic substance
4.85E-04	18	465	3.75	Response to oxidative stress
1.70E-03	16	437	3.55	Sulfur compound metabolic process
5.87E-03	14	402	3.38	Response to water deprivation
6.21E-03	14	411	3.30	Response to water
1.11E-02	14	442	3.07	Response to acid chemical
4.85E-04	27	962	2.72	Response to inorganic substance
4.85E-04	39	1692	2.23	Response to oxygen-containing compound

Table S5. List of significantly enriched ontologies of Root 2 with EM biostimulant

Enrichment FDR	Genes in list	Pathway Genes	Fold Enrichment	Functional categories
2.28E-03	2	3	132.50	Cellular response to copper ion
1.76E-03	3	14	54.21	Removal of superoxide radicals
1.76E-03	3	14	54.21	Cellular response to oxygen radical
1.76E-03	3	14	54.21	Cellular response to superoxide
2.07E-03	3	16	45.87	Response to superoxide
2.07E-03	3	16	45.87	Response to oxygen radical
2.24E-03	3	17	42.59	Superoxide metabolic process
2.24E-03	3	24	37.27	Aminoglycan catabolic process
2.24E-03	3	24	37.27	Chitin metabolic process
2.24E-03	3	24	37.27	Chitin catabolic process
2.24E-03	3	24	37.27	Amino sugar catabolic process
2.24E-03	3	18	37.27	Response to copper ion
2.24E-03	3	24	37.27	Glucosamine-containing compound catabolic process
2.29E-02	2	12	36.14	Cellular copper ion homeostasis
2.58E-02	2	12	33.13	Camalexin biosynthetic process
2.58E-02	2	12	33.13	Camalexin metabolic process
2.65E-02	2	13	30.58	Toxin biosynthetic process
2.65E-02	2	13	30.58	Indole phytoalexin biosynthetic process
2.65E-02	2	13	30.58	Indole phytoalexin metabolic process
2.65E-02	2	13	30.58	Phytoalexin metabolic process
2.65E-02	2	13	30.58	Phytoalexin biosynthetic process
3.29E-03	3	28	29.81	Aminoglycan metabolic process
3.29E-03	3	32	29.81	Glucosamine-containing compound metabolic process
3.66E-02	2	18	24.84	Phenylpropanoid catabolic process
3.66E-02	2	18	24.84	Lignin catabolic process
5.98E-03	3	33	23.85	Response to ozone
3.82E-02	2	18	23.38	Copper ion homeostasis
4.16E-02	2	20	22.08	Cellular response to metal ion

8.03E-03	3	35	20.56	Cellular response to reactive oxygen species
4.83E-02	2	23	19.88	Photosynthesis, light harvesting in photosystem I
4.83E-02	2	26	19.88	Zinc ion transmembrane transport
1.14E-02	3	49	18.07	Amino sugar metabolic process
1.76E-03	6	189	10.55	Polysaccharide catabolic process
3.70E-02	3	84	10.46	Systemic acquired resistance
3.82E-02	3	96	10.11	Carbohydrate derivative catabolic process
1.76E-03	7	197	8.86	Cellular detoxification
1.76E-03	7	198	8.81	Cellular response to toxic substance
2.20E-02	4	104	8.55	Response to endoplasmic reticulum stress
2.63E-03	6	183	8.22	Cellular oxidant detoxification
7.65E-03	5	155	8.21	Response to reactive oxygen species
3.29E-03	6	189	7.79	Reactive oxygen species metabolic process
2.24E-03	7	252	6.92	Detoxification
3.82E-02	4	140	6.63	Response to light intensity
4.48E-03	7	292	5.90	Response to toxic substance
1.16E-02	6	298	5.73	Carbohydrate catabolic process
1.76E-03	10	465	5.23	Response to oxidative stress
3.24E-02	7	494	3.83	Polysaccharide metabolic process
3.34E-02	9	741	3.09	Cellular response to oxygen-containing compound
8.03E-03	13	1196	2.97	Carbohydrate metabolic process
6.70E-03	17	1716	2.54	Cellular response to chemical stimulus
4.55E-02	15	1850	2.13	Catabolic process

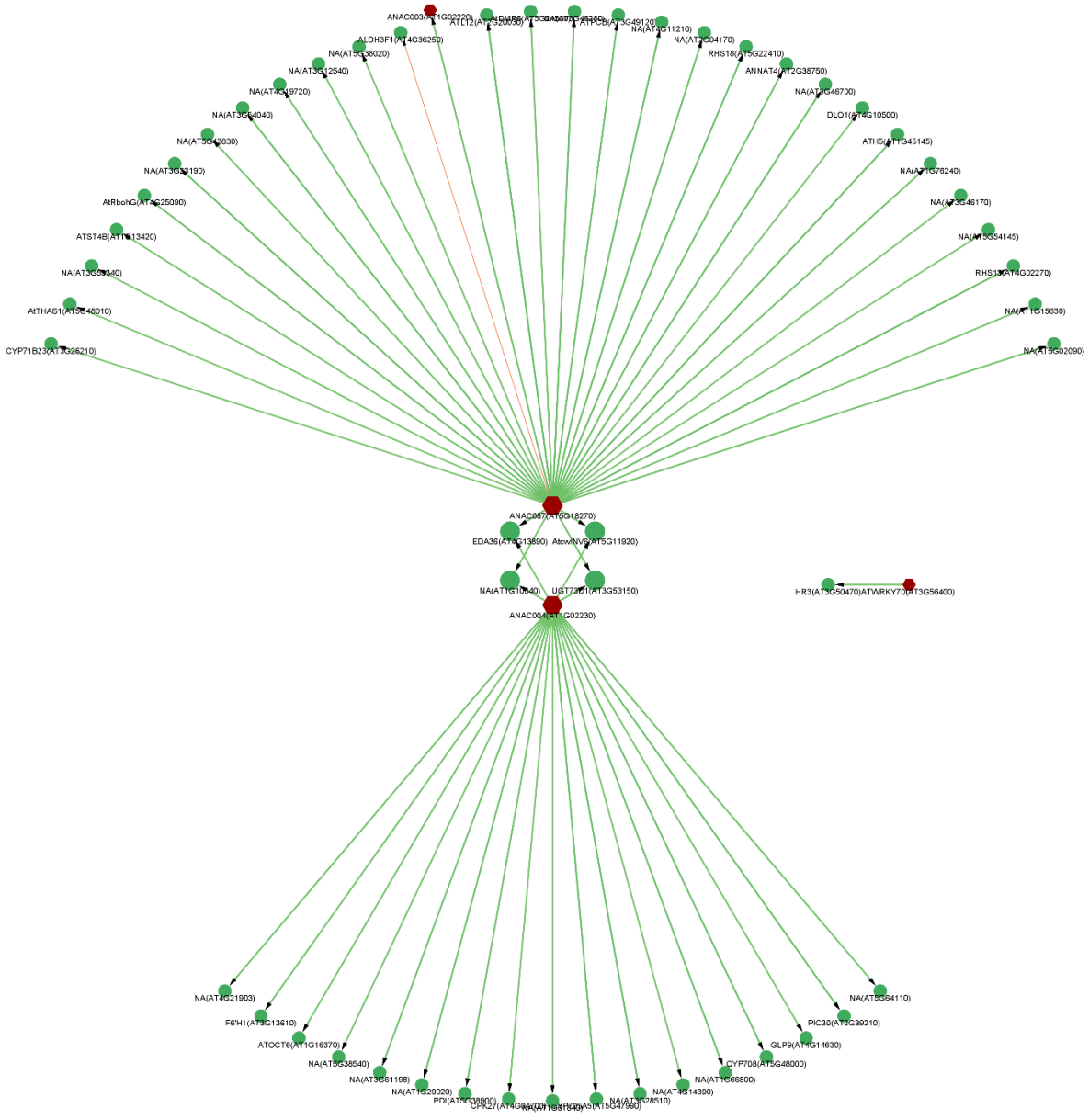

Figure S8: Network analysis of DEGs of Root1 tissue with EG biostimulant. Transcription factors are in red hexagons. Other genes are in green circles. Green arrow indicates positive interaction, and red arrow negative interaction.

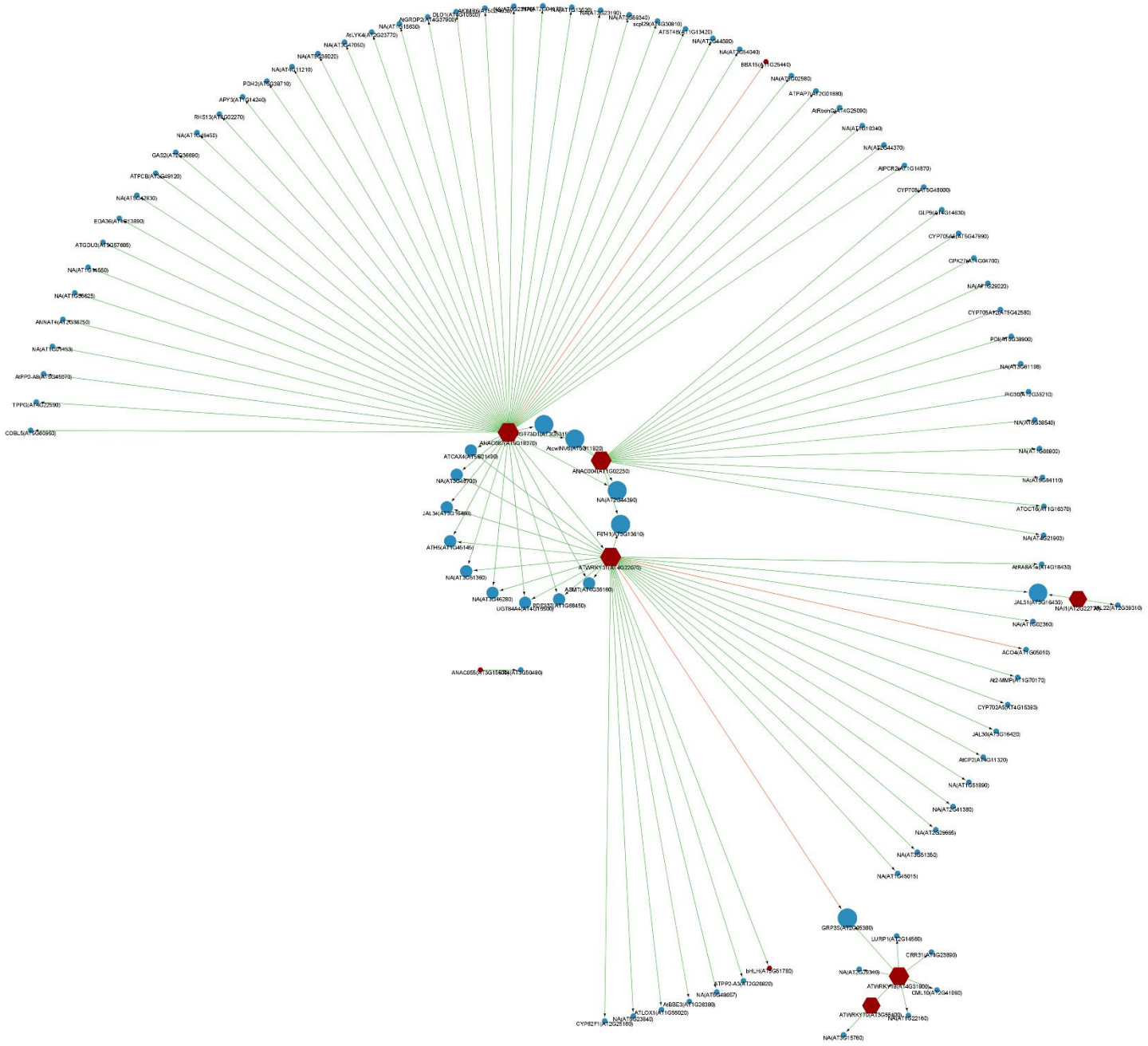

Figure S9: Network analysis of DEGs of Root2 tissue with EG biostimulant. Transcription factors are in red hexagons. Other genes are in blue circles. Green arrow indicates positive interaction, and red arrow negative interaction

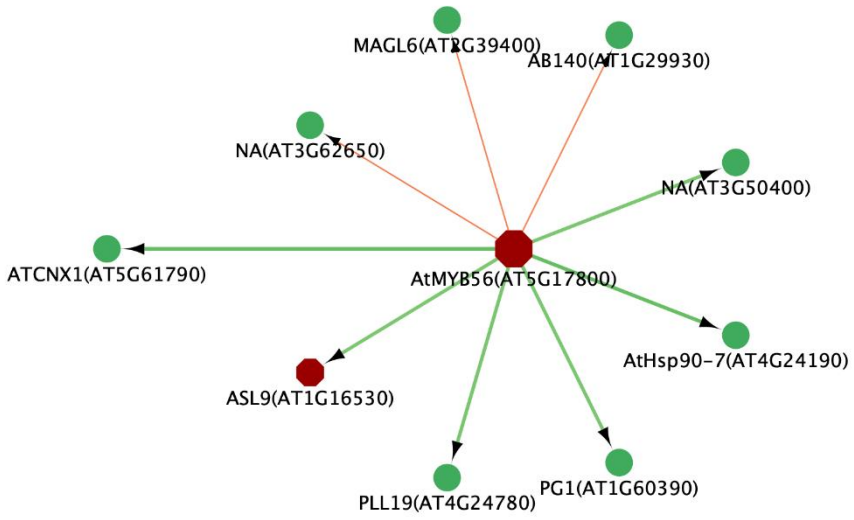

Figure S10: Network analysis of DEGs of Root2 tissue with EM biostimulant. Transcription factors are in red octagons. Other genes are in green circles. Green arrow indicates positive interaction, and red arrow negative interaction.