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# OMICS APPROACH TO DISSECT COMPLEX MORPHO-PHYSIOLOGICAL AND MOLECULAR RESPONSES TO NUTRITIONAL AND BIOSTIMULANT STIMULI IN PLANTS

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*“You only live once”*

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## GENERAL ABSTRACT

This thesis was focused on the application of omics technology to understand the effect of nutritional and biostimulant stimuli in sugar beet and tomato. The application of such technologies in the agricultural research field is of great help to the rapid validation of more sustainable inputs in agriculture to preserve production and food security, ensuring environmental and economical sustainability along the process, and lessening the negative impact of agricultural intensification based on chemicals, started in the middle of the last century. Sugar beet is important since the production of white sugar has made it one of the world's most important crops: it currently provides around 20% of the sugar requirement worldwide. Tomato instead, is one of the most important vegetables cultivated in the world, also well known for its beneficial properties. These traits make them suitable as model crop, for the evaluation of novel sustainable inputs with omics tools. The **first part** of the thesis presents a study on sugar beet leaf endophytes composition in response to variation of the availability of sulfur, a determinant nutrient for the growth and later sugar yield and purity in sugar beet taproot. A metabarcoding of the leaf bacterial 16S rRNA gene was carried out to determine leaf endophytic community structure and the results showed a fluctuation of the endophytes population in response to sulfur availability. These results demonstrate how important are nutrients but also how the plant microbiome can play a fundamental role as an indicator of the plant's status. In the **second part,** a comparison was done between a chemical Ca-based fertilizer and a novel Ca-base fertilizer. The study included morphological and molecular evaluation of the effects of these two substances applied to tomato cv. Microtom. Results showed an increased tomato fruit yield for Ca-based biostimulant, as well as an improved gene expression for nutrient uptake related genes, correlated to an increment in the leaf ionome content. Taken together, these results showed also the beneficial biostimulant activity of the novel Ca-based biostimulant. In

the **third part**, an intense study was done on an *Ascophyllum nodosum* extract (ANE), a promising biostimulant, through the transcriptome analysis in plants grown in controlled condition and in open field. Three dosages were tested, and the effect was monitored after 24, 48 and 72 hours after treatment. Through transcriptome, it was highlighted the role of ANE in triggering molecular responses linked to the plant hormonal metabolism of ABA and auxin synthesis, which resulted in significative increment in morphological traits for plants grown under controlled conditions, and in a significative increment in the sugar beet taproot yield and sugar content. These results demonstrate the biostimulant activity of ANE and promote transcriptomics as a valuable tool to dissect the complex metabolic response to different stimuli, in cultivated plants.

## RIASSUNTO GENERALE

Questa tesi di dottorato è incentrata sull'applicazione delle tecnologie omiche per comprendere l'effetto degli stimoli nutrizionali e biostimolanti nella barbabietola da zucchero e nel pomodoro. L'applicazione di tali tecnologie nel campo della ricerca in agricoltura è di grande aiuto per la rapida adozione di input più sostenibili in agricoltura, al fine di preservare la produzione agricola e la sicurezza alimentare, garantendo la sostenibilità ambientale ed economica nel processo produttivo agricolo e riducendo l'impatto negativo dell'intensificazione agricola basata sui prodotti chimici, iniziata a metà del secolo scorso. La barbabietola da zucchero è fondamentale, infatti fornisce circa il 20% del fabbisogno mondiale di zucchero. Il pomodoro è uno degli ortaggi più importanti coltivati nel mondo, noto per le sue proprietà benefiche. Queste caratteristiche rendono tali piante adatte a diventare colture modello, per la valutazione di nuovi input sostenibili, con strumenti omici. La **prima parte** della tesi presenta un studio sulla variazione della composizione della popolazione di endofiti fogliari di barbabietola da zucchero in risposta alla variazione della disponibilità di zolfo, un nutriente fondamentale per la crescita della pianta e successivamente per la resa e la purezza dello zucchero nel fittone della barbabietola. È stato eseguito il metabarcoding del gene 16S rRNA batterico estratto da foglie, al fine di determinare la struttura della comunità endofitica fogliare. I risultati hanno mostrato una fluttuazione della popolazione di endofiti in risposta alla disponibilità di zolfo. Questi risultati mostrano quanto sia importante lo stato nutrizionale della pianta, ma anche come il microbioma fogliare possa giocare un ruolo fondamentale come indicatore biologico dello stato nutrizionale della pianta. Nella **seconda parte**, è stato effettuato un confronto tra un fertilizzante chimico a base di calcio e un nuovo biostimolante a base di calcio. Lo studio ha visto la valutazione morfologica e molecolare degli effetti di queste due sostanze

applicate al pomodoro cv. Microtom. I risultati hanno mostrato un aumento della resa nelle piante di pomodoro trattate con il biostimolante, nonché una migliore espressione genica di alcuni geni correlati all'assorbimento dei nutrienti, legata anche ad un aumento del contenuto di nutrienti a livello fogliare. Presi insieme, questi risultati hanno mostrato anche l'attività biostimolante del nuovo prodotto a base di Ca. Nella **terza parte** è stato condotto un intenso studio su un estratto di *Ascophyllum nodosum* (ANE), usato come biostimolante, attraverso l'analisi del trascrittoma in piante coltivate in condizioni controllate e in pieno campo. Sono stati testati tre dosaggi e l'effetto è stato monitorato a 24, 48 e 72 ore dopo il trattamento. Attraverso il trascrittoma è stato evidenziato il ruolo di ANE nell'innescare risposte molecolari legate al metabolismo ormonale vegetale relativo alla percezione di ABA e alla sintesi dell'auxina. Questo ha determinato un significativo incremento dei caratteri morfologici nelle piante coltivate in condizioni controllate, nonché un significativo incremento della resa sia del fittone della barbabietola da zucchero resa che del suo contenuto zuccherino. Questi risultati dimostrano l'attività biostimolante dell'ANE e promuovono la trascrittomica come un prezioso strumento per studiare meglio la complessa risposta metabolica a diversi stimoli, nelle piante coltivate.

## GENERAL AIMS

The general aim of the thesis is to present study cases of omics technologies application in agriculture, to improve the sustainability of the agricultural production sector. The specific aims are:

I. To study the endophytes microbial population changes in response to abiotic stressors, such as nutrient availability (contribute n. 1).

II. To investigate the effect on ionome and gene expression of a novel biostimulant product and characterize the mode of action of the new product on tomato (contribute n. 2).

III. To address the mode of action of *Ascophyllum nodosum* extract with biostimulant effect on sugar beet through the complete transcriptome analysis of the untreated and treated plants with the RNA-seq approach (contribute n. 3).

## INTRODUCTION

We are living in ages of big challenges for the world agricultural system. The world population is expected to rise to 10 billion in 2055 (https://countrymeters.info/cn/World) with agriculture must ensure enough food supply to feed the growing population.
Nonetheless, agriculture is facing the impact of climate changes which are threatening food security by creating a more unpredictable environment and exposing crops to severe biotic and abiotic stressors, with a reduction in yield quantity and quality as a result.
Since 1880, world temperature has risen globally by more than one degree (https://earthobservatory.nasa.gov/world-of-change/global-temperatures) and the warming rate is increasing very rapidly in the last decades, with the consequences of very extreme climatic events happening all over the world. This increases the urgency and the need for more adaptable crops, to face the rapid environmental changes.
Modern agriculture, which began after the II World War, and was initiated by N. Bourlaug around 1950, has adopted all the available breeding technologies and synthetic products to increment crop yields. As a result, in the last years, we observed an intensification of the agricultural production system which led to a loss of biodiversity and land and marine ecosystem pauperization. In recent years, public concerns around the "environmental question" have risen, making people pay more attention to the sustainability of the food production process and the agricultural impact on the environment in general. The demand for more sustainability in the agricultural sector increased, and agriculture had to change its models, to adequate the request for more environmentally friendly systems, adopting new and more innovative production ways, which also considered the unpredictable impacts of climate change and the world population rapidly increasing.
In these days we are moving our steps into this complex situation which makes the agricultural decision-making process even more difficult than in the past years. Sustainability in agriculture nowadays refers not only to environmental sustainability but also to social and economic sustainability. We need new technologies which can help us in the process of decision-making, fastening the acquisition and the improvement of modern crops, and enhancing their environmental and economical suitability.
Since the dawning era of agriculture, around ten thousand years ago, humankind has used technology and technological improvement to ameliorate and maintain, over the years, the quality, and the quantity of crop production. Throughout the century lots of interesting cutting-edge techniques came up, but the space to clearly summarize them here is not enough.
Coming to the most recent advancement in agriculture, the ages of the Green revolution are the most remembered and the ones with a direct impact on our present.

The industrialization of agriculture from those years to the present time has created the condition for the development of new varieties, new production systems, and new technical inputs, which resulted in the intensification of agriculture and the improvement of the quality and quantity of crop products. But all of this with a cost, from the environmental and social point of view.
The research and the technology development now need to be addressed towards solutions able to conciliate all the needs that the present society requires with all the factors involved in agricultural production.
Many are the solutions and for the purpose of this thesis, the focus will be only on the most relevant among them, the ones that are presented in this work.
In the last decades, a series of new tools came out as promising technologies to help to cope with all the present and oncoming challenges. They are defined as "Omics" technologies, which are high-throughput technologies that give a holistic view of complex biological systems, such as cultivated plants.
The interest in omics technologies in the context of agriculture scientific research, rapidly increased in these 20 years: for example, in the last two years more than 600 papers related to the application of omics technologies in agriculture were published and the rate incremented exponentially, as compared to the beginning of 2000 (Source: Scopus, Elsevier).
Omics technologies include a series of multi-disciplinary tools such as genomics, transcriptomics, proteomics, metabolomics, phenomics, metagenomics, and ionomics.
Nowadays, omics-approach is a well-established method to study the molecular basis of crop development through modifications in DNA, transcript levels, but also proteins, metabolites, and mineral nutrient contents variation against a backdrop of environmental and physiological stress responses.
Nonetheless, the advent of Next Generation sequencing (NGS) solutions has created the ideal condition for the omics-approach studies, thanks to the capacity of NGS solutions to generate rapidly high throughput significant data for genomes, metagenomes, transcriptomes, proteomes, metabolomes, phenomes, and ionomes.
One interesting application of omics technology in the research field of agriculture is the study of the plant-microbiome interaction and its responses to different environmental conditions. For the first time in history, we have new insights into this long-lasting relationship between plants and their microbiome in the phyllosphere, rhizosphere, and endosphere. It is possible now to characterize all the endophytes and the epiphytes, and their response to any external stimuli.
The term "microbiome" refers to any microbial genome associated with the relative plant microbiota which are exerting important functions for plant growth and health.
With the metagenome analysis today, it is not only possible to characterize the entire microbiome of the plant, but also to understand its importance for plant

development and its changes during plant growth as well as in response to any abiotic or biotic factors.
By studying the functionality of plant-microbiome interactions and factors involved in the microbial community shaping it is possible to reach a better understanding of the plant as a meta-organism and how plants can benefit from their microbial partners.
Furthermore, microorganisms have shown the potential to be applied as biofertilizers or biopesticides, and this have increased the interest among the researchers to investigate deeper their possible function or integrative function as alternatives to chemical products in sustainable agricultural practices.
Another interesting application of an omic-technology relevant for this thesis in the plant ionomics.
Plant ionome is defined as the mineral nutrient content of the entire plant or a portion of it, which represents the inorganic fraction of the cellular system examined.
Ionomics, which is the study of the plant ionome, requires a high- throughput technologies and, thanks to the integration with other genetic tools, it can give us much information on the functional status of a plant, which is directly linked to the plant responses versus any different environmental conditions.
This is a powerful acquisition, since plant ionome depends on many factors, such as the genetic variety, any imposed treatments to the plants as well as favourable or adverse environmental conditions.
By combining this tool with all the other omics tools, the researchers can understand how plant nutrient acquisition is regulated, which can lead to the development of a new sustainable system to enhance the quality of the crop yields, as well as the nutritional quality of the final products.
Both the two omics-technologies presented here can be combined with another omic-tecnology, the transcriptomics analysis.
Transcriptomics focus on the study of the transcriptome, the totality of the RNA messengers of a determinate tissue or cell, and the transcriptome sequencing technique, the RNA-Seq, is a high-throughput technique that can generate a high amount of data.
The direct application of transcriptomics is the study of the gene's expression pattern over a certain time, from hours to days, in response to any abiotic or biotic stimuli in plants.
The transcriptome of individuals is complex and dynamic, but the recent advances in the RNA-seq area have allowed research to dissect the variability and obtain more precise data, which often is linked to gene products, that is an indicator of important biological traits and an indicator of the specific biological pathways where it is involved.
Thanks to this omic technology it is possible to identify many candidate genes of interest, that are useful indicators of stress resistance, or of a particular biological characteristic, or to understand how environmental stimuli can affect crops.

Interestingly, the RNA-seq application allowed researchers to study the effects of the application on crops of certain substances, such as biostimulants.
Biostimulants are natural substances of different origins that, applied to crops, can enhance their tolerance to abiotic stresses, nutrient acquisition, and the quality of the final products.
In the new EU regulation 1009/2019 biostimulants are divided into two macro-categories: microbial biostimulants and non-microbial biostimulants. The first category includes all the biostimulants made by one type of microbial species or a consortium of bacteria. In the non-microbial biostimulant are included: seaweed extract, plants or animal extracts, humic substances, inorganic compounds, protein hydrolisates, chitosan, and other innovative substances.
In recent years, biostimulants are gaining more and more interest because of their beneficial effects on plants and their limited or absent environmental impact, helping farmers to pursue more sustainability in crop production. They have also a direct or indirect positive effect on crop yields, which makes their use more interesting, and one of the cutting-edge solutions now available on the market that can help to achieve more environmental, but also social sustainability in the agricultural process in developed and undeveloped countries.
One of the main targets of omics technologies is the deep study of the mode of action of the active substances contained inside the biostimulants, to improve our knowledge of how they work as well as how to use them in more proficient ways to maximize their effects with the minimum effort necessary to do it.
In summary, the need for agriculture to adapt to the growing population and the rapid climate change has posed the urge for improving crop yields and crop stress resilience. The research field can help to cope with these challenges and the use of omics technologies can fasten the adoption of more suitable solutions, such as the diffusion of new technical inputs like biostimulants.

**CONTRIBUTE 1**

# Endophytic Microbiome Responses to Sulfur Availability in *Beta vulgaris* (L.)

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**Abstract**

Sulfur is an essential plant macronutrient, and its adequate supply allows an efficient root storage and sugar extractability in sugar beets (*Beta vulgaris* L.). In this study, we investigated the effect of changes in sulfur availability on the endophytic community structure of sugar beets. Plants were hydroponically grown in a complete nutrient solution (S-supplied), a nutrient solution without $MgSO_4$ (S-deprived), and a nutrient solution without $MgSO_4$ for six days and resupplied with 100 µM $MgSO_4$ for 48 h (S-resupplied). The sulfur status was monitored by inductively coupled plasma ICP–OES, and combustion analysis together with the evaluation of microRNA395 as a biomarker for sulfate status. Metabarcoding of the bacterial 16S rRNA gene was carried out in order to determine leaf endophytic community structure. The Shannon diversity index significantly differed ($p < 0.05$) between sulfate-supplied and sulfate-deprived seedlings. Validation by Real-Time PCR showed a significant increase ($p < 0.05$) of *Burkholderia* spp. in sulfate-deprived plants as compared to sulfate- supplied ones. The study sheds new light on the effects of nutrient deficiency on the microbiome of sugar beet plants.

**Keywords:** Sulfur; sugar beet; endophytic community; Next Generation Sequencing (NGS)

## 1. Introduction

Abiotic stresses, such as a nutrient deficiency, can shape the presence and number of plant growth-promoting bacteria recruited from the environment. In sugar beets (*Beta vulgaris* L.), sulfur deficiency affects the sugar yield and technological quality of roots (*Burba, 1996*). Sulfur is an essential plant nutrient, taken in by roots and metabolized in leaves. It is required to synthesize key amino acids such as methionine and

cysteine, which are needed to produce functional and structural proteins (*Hoffmann et al., 2004)*. In sugar beets, low S concentration leads to the accumulation of alfa-amino N, resulting in less root storage capacity and lower sugar extractability. Studies have revealed that, in S-deficient plant tissues, non-S containing amino acids such as glutamine and asparagine accumulate, in addition to non-protein compounds such as amides (*Thomas et al., 2000)*. Moreover, sulfate plays an important role in crop health and stress tolerance. As an example, glutathione (one of the forms of organic S) can act as a messenger, carrying information on fungal attacks (*Bloem et al., 2005; Hernàndez et al., 2017)*.

Research on plant microbiomes has been attracting huge interest and investments to clarify the role of plant-associated microorganisms. The plant microbiome is an essential indicator of a plant's physiological condition, and the effect of biotic and abiotic stresses

can alter this fragile balance and lead to pathological conditions (*Brader et al. 2017*). Bacteria, fungi, and viruses, which constitute the plant microbiome, can interact with each other and with the plant itself in ways that may result in stress-coping adaptation, enhancing nutrient uptake, plant growth, and morphogenesis (*Singh et al., 2019)*. In this scenario, bacteria communicate with plants through the release of phytohormones, small molecules, and volatile substances. Plants, on their side, release a variety of molecules such as vitamins, organic acids, and sugars used by bacteria as nutrients or signals (*Compant et al., 2019)*. The composition of bacterial communities differs among plant species and tissues (*Jones et al., 2019)*. Also, studies have revealed that microbiome changes are detectable according to plant developmental stage, seasonality, nutritional status, and the presence of stress (*Berendsen et al., 2018*). The plant's aerial part (or the phyllosphere) is an extreme habitat directly

exposed to many abiotic stresses including UV, low water and nutrient availability, temperature, salinity, and pressure (*Thapa et al., 2018*). This environment selects only microorganisms able to grow and survive under all environmental conditions and to acquire specific adaptation mechanisms. Therefore, differences in microbiome composition can be detectable on a leaf's surface (Lindow and Brandl, 2003). The epiphytic and endophytic microbial communities of the phyllosphere play a crucial role in processes related to plant development such as the biological control of diseases and the synthesis of phytohormones such as auxin, cytokinin, and gibberellin, which affect growth through modulating endogenous hormone levels in association with their plant-derived counterparts (Vorholt, 2012; Bulgarelli et al., 2013).

Generally, endophytic bacteria are non-pathogenic organisms and do not cause visible damage or morphological changes in their hosts. Several studies have highlighted the effectiveness of endophytic bacteria in protecting plants from abiotic stresses such as drought, salinity, and low temperature and in mediating plant defense response to pathogenic attacks (Liu et al., 2017). To describe the plant's microbiome, the advent of sequencing technologies has revolutionized the method of detecting microbial species, also expanding it to those species that are non-culturable and hence unsuitable to be studied with traditional methods (Guttmann et al., 2014). The 16S rRNA gene, composed of nine hypervariable regions interspaced with highly conserved elements, is commonly sequenced to reveal the specific taxonomy of the bacterial species upon database alignments (Bukin et al., 2019).

In this study, the effect on plant endophytic community structure, following changes in sulfur availability, was investigated. Leaf sulfur status was monitored by inductively coupled plasma ICP–OES, and combustion

analysis together with an evaluation of microRNA395 as a biomarker for sulfate status (Jones-Rhoades and Bartel, 2004). Then, metabarcoding of bacterial 16S rRNA gene was carried out to determine leaf endophytic community structure in response to changes in sulfate availability. From the metagenomic sequencing information, we designed specific primers to validate and quantify the presence of the detected bacteria with Real-Time PCR. The specific aim was to detect plant-growth-promoting and/or specific physiological condition-indicator bacteria in sugar beet seedlings following a sulfur deficiency condition.

## 2. Results

To verify the effectiveness of nutritional treatments, the sulfur status of S-deprived and S-resupplied plants were firstly evaluated with the optical emission spectroscopy with inductively coupled plasma (ICP–OES). The sulfur content was also monitored by combustion analysis, and results were significantly correlated ($p < 0.01$) with them obtained by ICP–OES. The sulfate content of plants grown at 100 µM sulfate was significantly higher ($p < 0.01$) than a plant grown in a Hoagland solution without $MgSO_4$, as represented in Figure 1.

At the molecular level, we proceeded to test the expression of the marker miR395. The presence of this marker was reduced in plants grown with high S availability as shown in Figure 2.

A total of 2,395,918 sequences were obtained for 18 samples and, among these, 1,845,486 were considered valid. The average length of the obtained sequences was 241 nucleotides, with a median and mode of, respectively, 260 and 216 nucleotides. At 97% identity cut-off, 149 OTUs, classified into 34 families, 41 genera, and 74 species, were detected using the Greengenes database version 13.5 (public database available

at https://greengenes.secondgenome.com/?prefix=downloads/greengenes_database/) and the curated MicroSEQ® 16S Reference Library v2013.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) on the Torrent Suite Software 5.12 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Plant bacterial diversity significantly differed among treatments with a *p*-value of 0.024 calculated using the Shannon index (Figure 3).

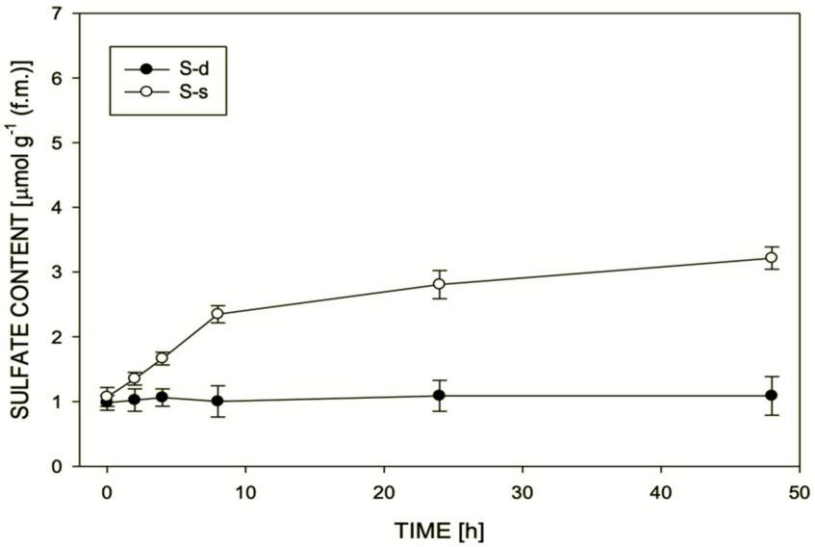


**Figure 1.** Sulfate content of S-deprived (S-d) and S-supplied (S-s) plants were obtained after 2, 4, 8, 24, and 48 h of treatment. The graph shows the means of replicates (12 observations each time) and error bars represent the standard error of the means.

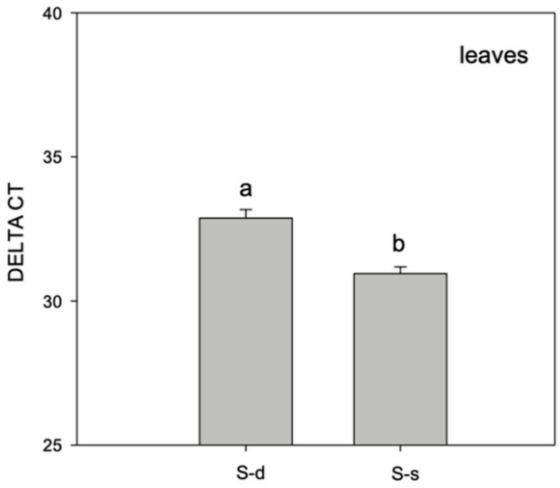


**Figure 2.** Bar plot showing the expression of miR395 in S-deprived (S-d) and S-supplied (S-s) plants for 48 h. Means of replicates and standard error is reported. The experiment has been performed three times to validate the results.

A Bray–Curtis distance genus-level PCoA analysis was plotted to visualize clustering of samples (Figure 4). The first two principal coordinate axes accounted for 33% and 19% of the variation. Particularly, the S-supplied plants can be clearly distinguished from the S-deprived and S-resupplied plants that clustered independently.

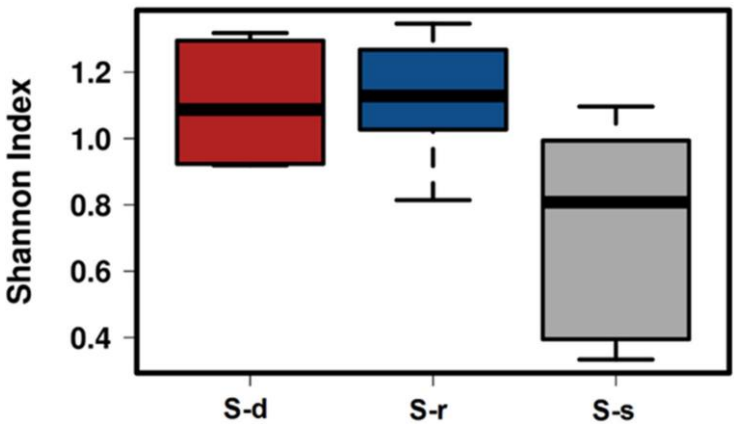


**Figure 3.** Boxplots of Shannon Index reflecting the diversity of bacterial microbiomes in S-deprived (S-d), S-resupplied (S-r), and S-supplied (S-s) plants. Boxes represent the interquartile range between the first and third quartile. The horizontal line inside the box defines the median (ANOVA; $\alpha < 0.05$).

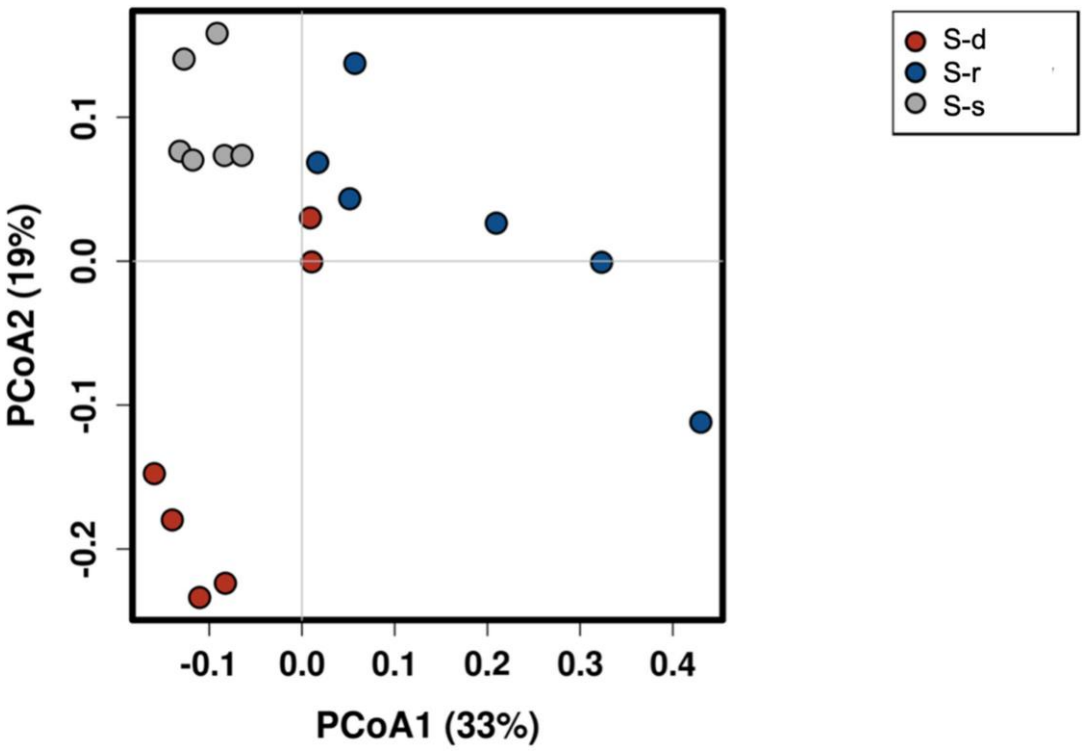


**Figure 4.** Principal Coordinates Analysis (PCoA) plots of Bray-Curtis distances between S-deprived (S-d), S-resupplied (S-r), and S-supplied (S-s) plants with the percentage variance explained by the principal coordinates.

The bacterial community composition in the leaves was represented by pie charts, based on the relative bacterial genera abundance (Figure 5).

*Pseudomonas* was the most abundant genus across the three treatments reaching 39% and 38%, respectively, for S-deprived and S-resupplied samples and 52% for S-supplied plants. In addition to *Pseudomonas*, five other genera were found in all treatments (Figure 6). These were: *Stenotrophomonas*, *Sphingomonas*, *Pantoea*, *Paenibacillus*, and

*Massilia*. Among the other bacteria, four were found only on S-deprived plants: *Pedobacter*, *Enterobacter*, *Duganella*, and *Burkholderia*. The genera *Oxalicibacterium*, *Microbacterium*, *Acinetobacter* were found only on S-resupplied plants (Figure 6). The only genus found on S-supplied plants was *Xanthomonas* (Figure 6).

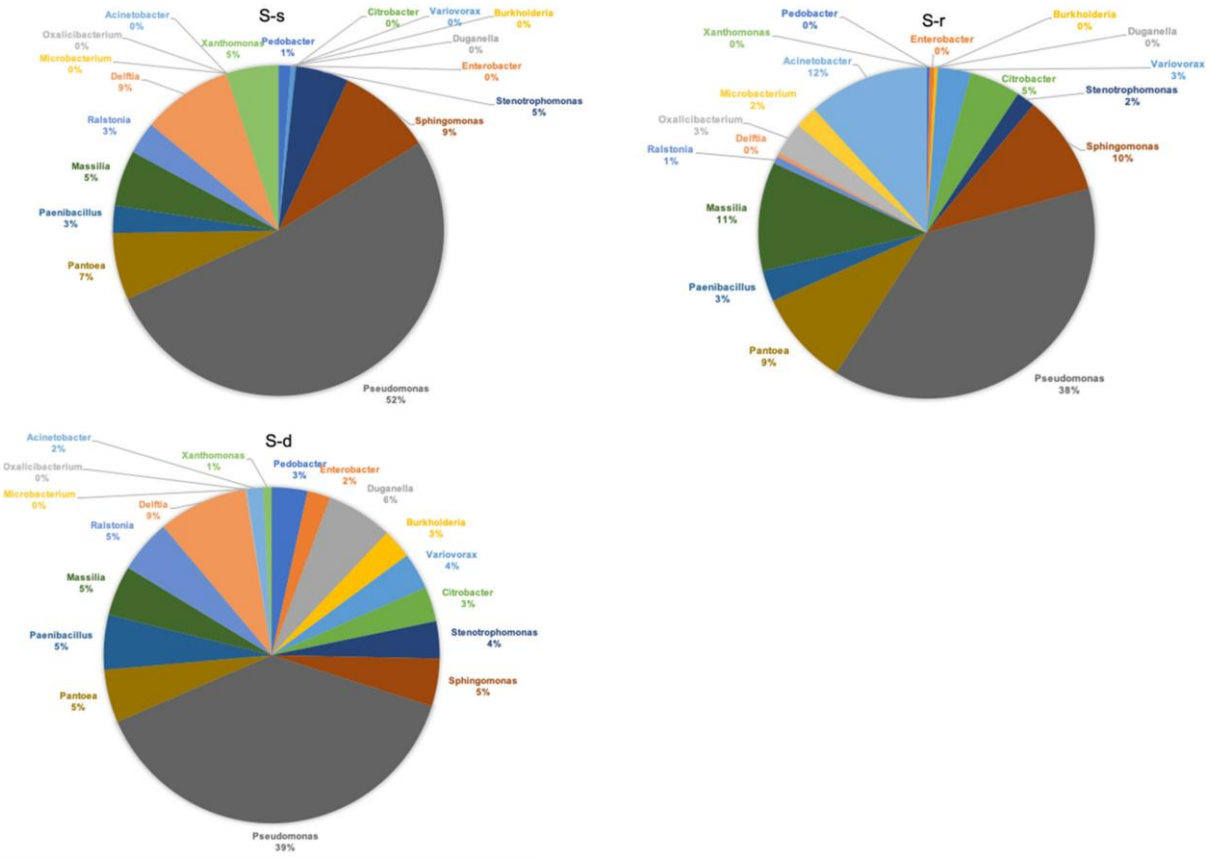


**Figure 5.** Pie charts showing bacteria genera abundance expressed as a percentage over the total detected in S-supplied (S-s), S-resupplied (S-r), and S-deprived (S-d) plants.

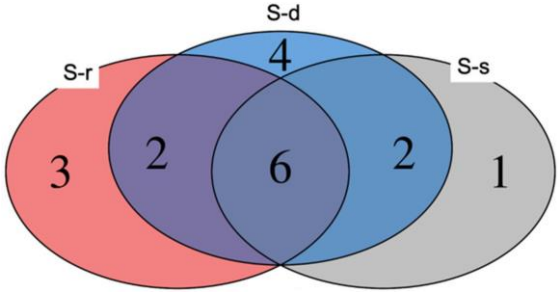


**Figure 6.** Venn diagram showing the number of shared and exclusive OTUs. Areas 1, 3, and 4 represent OTUs found exclusively in S-supplied (S-s), S-resupplied (S-r,), and S-deprived (S-d) plants, respectively. Overlapping area 6 represents the OTUs in common between all treatments.

Subsequently, we validated by qPCR the sequencing results using specific primer pairs to amplify and quantify the presence of bacteria. The qPCR technique was performed on the stored DNA not used for library preparation and coming from plant samples of the first, second and third growing cycle. The results obtained confirmed the presence of *Pseudomonas* genus in all treatments, with no significant difference among them ($p$ = 0.5767) (Figure 7). The most significant differences across treatments were found for *Burkholderia*, which showed an average Ct of 25.9 on S-deprived samples, 28.1 on S-resupplied samples, and 29.4 on S-supplied samples (Figure 7).

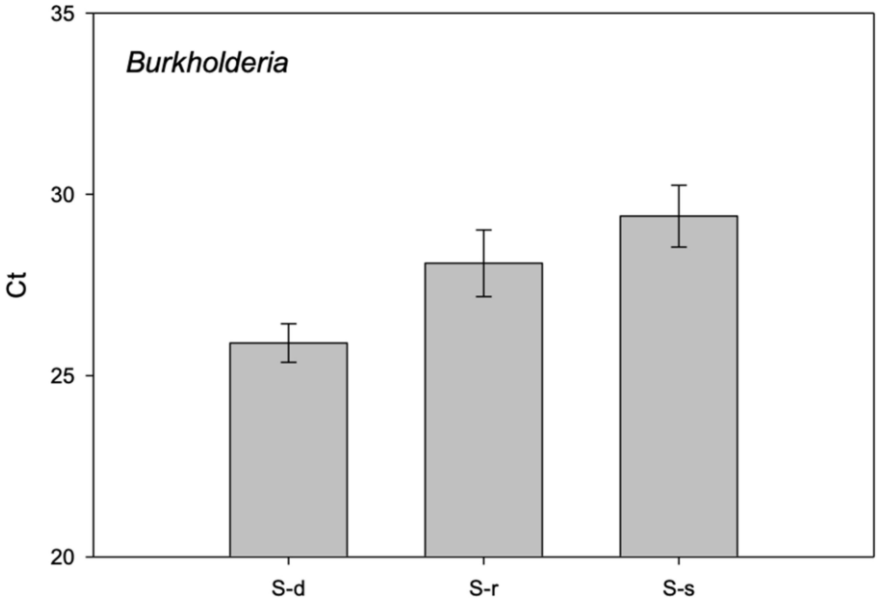


**Figure 7.** Relative abundance by qPCR of *Burkholderia* genera in S-deprived (S-d), S-supplied (S-s), and S-resupplied (S-r) plants. Threshold cycle means and corresponding standard errors are reported (the lower the values, the higher the number of target gene copies within plant tissues). Vertical bars indicate standard errors.

The other bacteria that were only found on S-deprived, S-resupplied, and S-supplied plants, were not confirmed by Real-Time PCR analysis on the other two growing cycles, since the differences between groups were not significant (data not shown).

## 3. Discussion

Environmental conditions and biotic and abiotic stresses can shape a plant's micro- biome. In our study, germination took place in a growth chamber with distilled water- moistened filter paper, and seedlings were then transferred and grown in a hydroponic solution. These conditions not

only allow the presence and detection of seed and plant endophytes but also bacteria naturally present in the water and surrounding environment, which can be considered as contaminants hardly avoidable during DNA isolation steps. However, we focused our attention on the microorganisms in common between treatments as constituents of the seed microbiome inherited by vertical transmission, and on the microorganisms that are specifically present in each treatment to identify those most related to overcoming nutritional stress. Since sulfur metabolism is known to take place in the leaf, we described the microbiome in the outer and inner parts of this tissue, the phyllosphere and endosphere, respectively.

To assess the effective plant sulfur nutritional status, we adopted two different techniques. The optical emission spectroscopy with inductively coupled plasma (ICP–OES) has widely been used to monitor the overall nutritional status of plant tissue, detect micronutrient quantity, and evaluate the presence of heavy metals contamination (Hansen et al., 2013). In our study, this technique was employed as a first step to assess the real sulfur presence or absence inside leaves. Moreover, at the molecular level, the Real-Time quantitative RT–PCR quantified the expression of a specific microRNA called miR395. This marker has been previously adopted to analyze the sulfate starvation in *Beta vulgaris*, *Arabidopsis*, and sorghum (Stevanato et al., 2015; Jagadeeswaran et al., 2014; Zhang et al., 2011). Here, the shortage of sulfate was connected to an increased expression of miR395, following the same trend observed in the previous paper. Once we detected the effective sulfur nutritional status, we proceed to analyze its relationship with leaf endophytes.

Plant bacteria diversity analysis with Shannon index revealed a significant bacteria diversity of sulfur-supplied plants compared to the sulfur-deprived and sulfur-resupplied plants. Specifically, 48 h of treatments with

$MgSO_4$ seems to be not enough to restore the presence of bacteria in stressed plants. The same result has also been confirmed by PCoA, where S-deprived and S-resupplied plants clustered together. However, this speculation should be confirmed by a more dedicated experiment, since until now there is no evidence in the available literature.

In our study, the most abundant detected endophyte was *Pseudomonas*. This bacterium is commonly reported in seeds of very different plant species, and usually present in the phyllosphere, with a very important ecological function. *Pseudomonas* is known as a plant- growth-promoting bacterium capable of secreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase to enhance plant growth. As a constituent of the phyllosphere, *Pseudomonas* is reported to limit the loss of water and damage due to UV radiation (Stone et al., 2018). Moreover, a high diversity of *Pseudomonas* species consortia is known to be positively correlated with the accumulation of plant biomass and the assimilation of nutrients (Goswani et al., 2013). Among the constituents of the core microbiome, we detected *Sphingomonas*, *Pantoea*, *Paenibacillus*, *Massilia*, and *Stenotrophomonas*. *Sphingomonas* is a gram-negative bacterium with an essential role in environmental remediation, capable of accumulating intracellular Zn2+ and reducing the uptake of Cd2+ by plant roots. This genus can bind the heavy metal and enhance the expression of cysteine-rich metallothionein proteins (Asaf et al., 2020). *Sphingomonas* also have an important role in counteracting biotic and abiotic stresses, such as the mitigation of salinity stress (Khan et al., 2017). A similar role was reported by Grover et al. (Ryan et al., 2009) for *Pantoea* and *Paenibacillus* as PGPB providing tolerance to different abiotic stress environments in tomato, pepper, canola, bean, and lettuce. Moreover, the genus *Stenotrophomonas* has been reported to be highly adaptable to nutrient-limited environments colonizing and surviving on the

plant surface. Also, this genus contributes to enhancing growth conditions for bacteria by increasing water permeability (Coenye and Vandamme, 2003).

Analysing the microbiome composition of S-deprived, S-resupplied, and S-supplied plants, we revealed the presence of very distinct genera for each treatment. These genera were validated with Real-Time PCR on the same sample used for sequencing analysis and on plants from additional growing cycles. The presence of *Pseudomonas* was confirmed on all plants among replicates without any significant difference between treatments. *Pedobacter*, *Enterobacter*, *Duganella*, *Microbacterium*, *Oxalicibacter*, *Acinetobacter*, and *Xanthomonas* showed a variable presence, but not significant, in all samples and among replicates. However, the Real-Time PCR results confirmed a significant difference of *Burkholderia* among the three treatments. Specifically, the *Burkholderia* genus was abundant in S-deprived plants. This genus includes versatile bacteria that survive in many ecological niches, from soil to animals and humans (Compant et al., 2008). The type of interaction with the plant host can be pathogenic, symbiotic, or both. *Burkholderia* species are mainly known as the most potent plant-growth- promoting rhizobacteria, with colonization reported for at least 30 plant species (Grover et al., 2011). The molecular mechanisms underlying the interaction between these microorganisms and the host plant are still unknown. However, *Burkholderia* species are recognized for the benefits they bring to agriculture. Among these, the most important are nitrogen fixation, IAA and siderophore production, inorganic phosphate solubilization, and pathogen inhibition (Suárez-Moreno et al., 2012; Castro-Gonzales et al., 2011). This genus is also capable of epiphytic and endophytic colonization of grapevine tissues and organs protecting the plants against heat as well as chilling stress (Ait Bakra et al., 2006). Similarly,

*Burkholderia* increased drought stress resilience of maize (Naveed et al., 2014), induced metabolic changes in *Arabidopsis* under salt tolerance (Pinedo et al., 2015) and improved the growth, physiology, and antioxidant activity of *Brassica napus* L. in chromium-contaminated soil (Nafees et al., 2018).

This evidence let us speculate that a plant under abiotic stress could foster the growth of specific microorganisms in the background of its hosted taxa, particularly boosting those that can protect it and leading to their relative abundance shifts in comparison to others that would not be as efficient in helping to cope with that particular stress factor. This could therefore have happened to our plants subjected to the nutritional stress of sulfur deficiency. Having this result been repeatedly confirmed in three growing cycles, it stands as a confidence–worth observation.

In conclusion, the evidence gathered from our results suggests that: (1) seed-borne bacteria constitute a consistent presence in sugar beet, featuring a large contingent of various taxa; and (2) within the 'inner spore bank' offered by these, the plant would respond to nutritional stress as early as at seedling stage by inducing and allowing taxon- specific endophytic growth (this selective enhancement can supposedly be exerted by increased synthesis of defined chemical compounds). Such a strategy is already known to shape microbial communities in the outer rhizosphere. The observed patterns indicate that this mechanism is operative also in the inner tissues and that it preferentially fosters the bacteria that could be needed to respond to a given stress, upshifting them immediately after seed germination. The further investigation focused on the dynamics of endophytic plant growth-promoting bacteria offer several promising developments for future trends in improved agriculture and horticulture.

# 4. Material and Methods

## *4.1. Plant Material*

The seeds of sugar beet (*Beta vulgaris* (L.)) used in this study belong to the “Shannon” variety provided by Lion Seeds Ltd. (Maldon, UK). This genotype is a hybrid obtained crossing a multigerm pollinator resistant to rhizomania with a susceptible male-sterile monogerm line.

## *4.2. Growing Conditions*

The sugar beet seeds were surface sterilized by dipping them in an ethanol solution (76%) for 5 min. The treatment was repeated three times alternating it with a rinse with distilled water. Seeds were then placed on Petri dishes with distilled water-moistened filter paper and left to germinate for 48 h at a temperature of 25 ◦C in the dark. Germinated seeds were then transferred into three 500 mL glass pots with Hoagland solution (Arnon and Hoagland, 1940). Thirty seedlings were maintained in a pot for 8 days with a full Hoagland nutrient solution containing $MgSO_4$ corresponding to 100 µM sulfate (S-supplied plants). In parallel, other 30 seedlings were maintained for 8 days in a Hoagland solution without $MgSO_4$ (S-deprived plants). Further 30 seedlings were initially maintained for 6 days in a Hoagland solution without $MgSO_4$ and then transferred into another pot with a full Hoagland nutrient solution containing 100 µM sulfate (S-resupplied plants) and kept in that condition for 48 h. The experiment was repeated three times for validation aims.

## *4.3. Ionomic Analysis*

Sulfate content was determined using inductively coupled plasma optical emission spectrometry (ICP–OES, Spectro, Kleve, Germany). Firstly, fresh leaves were ground to powder in liquid nitrogen. Then, sulfate was extracted in 20 cm3 of millipore water by incubation at 70 ◦C for 30 min and centrifuged at 20,000× *g* for 30 min. The supernatant was filtered using a 0.45-µm pore size filter and analyzed both by ICP–OES instrument (Spectro Analytical Instruments, Kleve, Germany), and combustion analysis (Elementar vario MACRO CNS, Elementar Analysensysteme GmbH, Hanau, Germany).

### *4.4. RNA Extraction*

Total RNA was extracted from 100 mg of the fresh leaf using the Eurogold TriFastTM kit (EuroClone, Milan, Italy), following the manufacturer's instructions. RNA quantification was performed with Qubit RNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### *4.5. Real-Time Quantitative RT–PCR*

The reverse transcription was achieved using QuantiMir kit (System Biosciences, Mountain View, CA, USA), according to the manufacturer's instructions, starting from 1 µg of total RNA. Quant Studio 12K Flex Real-Time PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to detect the expression of miR395 with the following reaction mixture: 5 µL of Power Syber Green PCR master mix (Life Technologies, Carlsbad, CA, USA), 0.1 µL of forward primer, 0.1 µL of reverse primer, 1.4 µL of nuclease-free water, and 1 µL of each sample. The PCR program was set up with an initial denaturation at 95 ◦C for 10 min followed by 50 cycles at 95 ◦C for 15 s and 60 ◦C for 1 min. The primer sequence used to detect miR395 was obtained from (Stevanato et al., 2015; Axtell and Bartel, 2005). The threshold method was used to analyze miR395 expression

and the ΔCt values, calculated based on Hajizadeh et al. (2011), involved the use of 18S rRNA as an internal standard.

### *4.6. DNA Extraction*

After 8 days since the transfer of germinated seeds into Hoagland solutions, fresh leaves were collected from each pot and processed to extract DNA. For each leaf sample, 50 mg was homogenized with 300 µL of RLT buffer in two ml Eppendorf tubes using a Tissue Lyser (Qiagen, Hilden, Germany) for 5 min at 30 hertz. The tubes were centrifuged at 6000 rpm for 3 min, and the supernatant obtained was processed using Biosprint 96 (Qiagen, Hilden, Germany) to extract DNA. The automatic extraction involved the use of six 96 well S-Block plates set up as follows. One plate filled with 300 µL of sample supernatant, 200 µL of isopropanol, and 20 µL of MagAttract magnetic beads (Qiagen, Hilden, Germany). A second plate with 500 µL of buffer RLT. The third and fourth plates with 500 µL of 96% ethanol. The fifth plate with 500 µL of 0.02% Tween solution and the last plate filled with 100 µL of nuclease-free water. After the extraction process, DNA was quantified with a Qubit fluorimeter (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the Qubit™ DNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### *4.7. Metabarcoding of Bacterial 16S rRNA Gene Sequencing and Data Analysis*

DNA sequencing was performed with the Ion GeneStudio S5 instrument using a 16S Ion Metagenomics Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions. The kit provides two separate primer tubes to amplify the regions V2, V4, V8, and V3, V6–7, V9, respectively. The amplification program of the 16S hypervariable regions involved an initial step of 10 min at 95 ◦C followed

by 25 cycles at 95 ◦C for 30 s, 58 ◦C for 30 s, 72 ◦C for 20 s and a hold at the stage at 72 ◦C for 7 min. Pooled amplicons were used for library preparation with an Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Unique barcodes were ligated to each library using an Ion Xpress Barcode Adapter (Thermo Fisher Scientific). A 6-cycle PCR reaction was performed to amplify libraries at 58 ◦C for 15 s and 70 ◦C for 1 min, then at 4 ◦C for up to one hour. PCR reactions were performed in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Twenty-five pM of the pooled libraries were used to prepare template-positive Ion Sphere Particles (ISPs) with One-Touch 2 and One-Touch ES system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and subsequently sequenced with Ion GeneStudio S5 on an Ion 520 chip (Ion 520 Chip Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Sequencing data were analyzed with Torrent Suite software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Ion Reporter cloud software (version 5.16, Thermo Fisher Scientific Inc., Waltham, MA, USA) was adopted to process 16S metagenomic data. A BaseCaller module filtered out the low-quality sequences, together with barcode assignment, adaptor trimming at 3*t* end, and base calling. OTU clustering was done against the curated Green-

genes database v.13.8 and curated MicroSEQ 16S Reference Library v2013.1, using a read abundance threshold of 10 and 97% sequence similarity. Shannon index was calculated for the three groups to compare the bacterial diversity and PCoA based on the bacterial abundances was also used to determine the overall similarity between groups using the Calypso tool (Zakrzewski et al., 2017).

### *4.8. Primer Design and Real-Time PCR*

The 16S DNA sequences of the bacteria, which characterize the three treatments, were used to design forward and reverse primers using the Primer Express V3.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primer sequences are reported in Table 1.

**Table 1.** Forward and reverse primer sequences of bacteria species characterizing the three treatments designed by Primer Express V3.0 software.

| **Name** | **Primer Forward 5*t*,3*t*** | **Primer Reverse 5*t*,3*t*** |
|---|---|---|
| *Pseudomonas* | GCGCGTAGGTGGCTTGATAA | GGATGCAGTTCCCAGGTTGA |
| *Pedobacter* | CTGGTCCGGTGTCTCAGTAC | GGGGATCTGAGAGGATGACC |
| *Enterobacter* | CATGGGAGTGGGTTGCAAAA | TCACAAAAGTGGTAAGCGCC |
| *Duganella* | GCTACTGATCGATGCCTTGG | GCGGCCGATATCTGATTAGC |
| *Burkholderia* | CCTCTGCCATACTCTAGCCC | ATGTGAAATCCCCGGGCTTA |
| *Microbacterium* | GGTACCGTCACTTTCGCTTC | GTGAGGGATGACGGCCTT |
| *Oxalicibacterium* | GCGCAACCCTTGTCATTAGT | TGTCACCGGCAGTCTCATTA |
| *Acinetobacter* | GCGAGGAGGAGGCTACTTTA | CGGTGCTTATTCTGCGAGTA |
| *Xanthomonas* | AAGGTGGGGATGACGTCAAG | TGTGTAGCCCTGGTCGTAAG |

A QuantStudio 12K Flex unit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to perform Real-Time PCR with the following mix: 5 µL of Sybr Green Real-Time PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 0.1 µL of forward primer, 0.1 µL of reverse primer, and 1.4 µL of nuclease-free water. One µL of the sample was analyzed for each well. The cycling program consisted of 10 min of pre-incubation at 95 ◦C, 50 cycles of 15 s at 95 ◦C, and 1 min at 60 ◦C.

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## References

Ait Bakra, E., Nowak, J., Clement, C., 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. Appl. Environ. Microbiol 72, 7246–7252.

Arnon, D.I., Hoagland, D.R., 1940. Crop production in artificial culture solution and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Sci 50, 463–483.

Asaf, S., Numan, M., Khan, A.L., Al-Harrasi, A., 2020. Sphingomonas: From diversity and genomics to functional role in environmental remediation and plant growth. Crit. Rev. Biotechnol 40, 138–152.

Axtell, M.J., Bartel, D.P., 2005. Antiquity of microRNAs and their targets in land plants. Plant Cell 17, 1658–1673.

Berendsen, R.L., Vismans, G., Yu, K., Song, Y., Jonge, R., Burgman, W.P., Burmølle, M., Herschend, J., Bakker, P.A.H.M., Pieterse, C.M.J., 2018. Disease-induced assemblage of a plant-beneficial bacterial consortium. ISME J 12, 1496–1507.

Bloem, E., Haneklaus, S., Schnug, E., 2005. Significance of sulfur compounds in the protection of plants against pests and diseases. J. Plant Nutr 28, 763–784.

Brader, G., Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L.-J., Sessitsch, A., 2017. Ecology and genomic insights into plant- pathogenic and plant-nonpathogenic endophytes. Annu. Rev. Phytopathol 55, 61–83.

Bukin, Y.S., Galachyants, Y.P., Morozov, I.V., Bukin, S.V., Zakharenko, A.S., Zemskaya, T.I., 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci. Data 6, 190007.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E.V.L., Schulze-Lefert, P., 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol 64, 807–838.

Burba, M., 1996. Der schädliche stickstoff als kriterium der rübenqualität. Zuckerind 121, 165–173.

Castro-Gonzalez, R., Martinez-Aguilar, L., Ramirez-Trujillo, A., Santos, P., Caballero-Mellado, J., 2011. High diversity of culturable Burkholderia species associated with sugarcane. Plant Soil 345, 155–169.

Coenye, T., Vandamme, P., 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol 5, 719–729.

Compant, S., Nowak, J., Coenye, T., Clément, C., Ait Barka, E., 2008. Diversity and occurrence of Burkholderia spp. in the natural environment. FEMS Microbiol. Rev 32, 607–626.

Compant, S., Samad, A., Faist, H., Sessitsch, A., 2019. A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. J. Adv. Res 19, 29–37.

Goswami, D., Vaghela, H., Parmar, S., Dhandhukia, P., Thakker, J.N., 2013. Plant growth promoting potentials of Pseudomonas spp. strain OG isolated from marine water. J. Plant Interact 8, 281–290.

Grover, M., Ali, S.Z., Sandhya, V., Rasul, A., Venkateswarlu, B., 2011. Role of microorganisms in adaptation of agriculture crops to abiotic stresses. World J. Microb. Biot 27, 1231–1240.

Guttman, D., McHardy, A.C., Schulze-Lefert, P., 2014. Microbial genome enabled insights into plant–microorganism interactions. Nat. Rev. Genet 15, 797–813.

Hajizadeh, H., Razavi, K., Mostofi, Y., Musavi, A., Cacco, G., Zamani, Z., Stevanato, P., 2011. Identification and characterization of genes differentially displayed in Rosa hybrida petals during flower senescence. Sci. Hort 128, 320–324.

Hansen, T.H., Bang, T.C., Laursen, K.H., Pedas, P., Husted, S., Schjørring, J.K., 2013. Multielement plant tissue analysis using ICP spectrometry, in: Plant Mineral Nutrients. Humana Press, Totowa, NJ, USA, pp. 121–141.

Hernández, J.A., Barba-Espín, G., Diaz-Vivancos, P., 2017. Glutathione-mediated biotic stress tolerance in plants, in: Glutathione in Plant Growth, Development, and Stress Tolerance. Springer, Cham, Switzerland, pp. 309–329.

Hoffmann, C., Stockfisch, N., Koch, H.-J., 2004. Influence of sulfur supply on yield and quality of sugar beet (Beta vulgaris L.)—determination of a threshold value. Eur. J. Agron 21, 69–80.

Jagadeeswaran, G., Li, Y.F., Sunkar, R., 2014. Redox signaling mediates the expression of a sulfate-deprivation-inducible microRNA395 in Arabidopsis. Plant J 77, 85–96.

Jones, P., Garcia, B., Furches, A., Tuskan, G., Jacobson, D., 2019. Plant host-associated mechanisms for microbial selection. Front. Plant Sci 10, 862.

Jones-Rhoades, M.W., Bartel, D.P., 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell 14, 787–799.

Khan, A.L., Waqas, M., Asaf, S., Kamran, M., Shahzad, R., Bilal, S., Aaqil Khan, M., Kang, S.-M., Kim, Y.-H.,

Yun, B.-W., 2017. Plant growth-promoting endophyte Sphingomonas sp. LK11 alleviates salinity stress in Solanum pimpinellifolium. Environ. Exp. Bot 133, 58–69.

Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microb 69, 1875–1883.

Liu, H., Carvalhais, L.C., Crawford, M., Singh, E., Dennis, P.G., Pieterse, C.M., Schenk, P.M., 2017. Inner plant values: Diversity, colonization and benefits from endophytic bacteria. Front. Microbiol 8, 2552.

Nafees, M., Ali, S., Naveed, M., Rizwan, M., 2018. Efficiency of biogas slurry and Burkholderia phytofirmans PsJN to improve growth, physiology, and antioxidant activity of Brassica napus L. in chromium-contaminated soil. Environ. Sci. Pollut. Res 25, 6387–6397.

Naveed, M., Mitter, B., Reichenauer, T.G., Wieczorek, K., Sessitsch, A., 2014. Increased drought stress resilience of maize through endophytic colonization by Burkholderia phytofirmans PsJN and Enterobacter sp. FD17. Environ. Exp. Bot 97, 30–39.

Pinedo, I., Ledger, T., Greve, M., Poupin, M.J., 2015. Burkholderia phytofirmans PsJN induces long-term metabolic and transcriptional changes involved in Arabidopsis thaliana salt tolerance. Front. Plant Sci 6, 466.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., Lelie, D., Dow, J.M., 2009. The versatility and adaptation of bacteria from the genus Stenotrophomonas. Nat. Rev. Microbiol 7, 514–525.

Singh, D., Raina, T.K., Kumar, A., Singh, J., Prasad, R., n.d. Plant microbiome: A reservoir of novel genes and metabolites. Plant Gene.

Stevanato, P., Fedito, P., Trebbi, D., Cagnin, M., Saccomani, M., Cacco, G., 2015. Effect of sulfate availability on root traits and microRNA395 expression in sugar beet. Biol. Plant 59, 491–496.

Stone, B.W., Weingarten, E.A., Jackson, C.R., 2018. The role of the phyllosphere microbiome in plant health and function. Ann. Plant Rev. Online 1, 533–556.

Suárez-Moreno, Z., Caballero-Mellado, J., Coutinho, B.G., Mendonça-Previato, L., James, E.K., Venturi, V., 2012. Common features of environmental and potentially beneficial plant-associated Burkholderia. Microb. Ecol 63, 249–266.

Thapa, S., Prasanna, R., 2018. Prospecting the characteristics and significance of the phyllosphere microbiome. Ann. Microbiol 68,. Thomas, S.G., Bilsborrow, P.E., Hocking, T.J., Bennett, J., 2000. Effect of sulphur deficiency on the growth and metabolism of sugar beet (Beta vulgaris cv druid. J. Sci. Food Agric 80, 2057–2062.

Vorholt, J.A., 2012. Microbial life in the phyllosphere. Nat. Rev. Microbiol 10, 828–840.

Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M.J., Berger, B., Krause, L.C., 2017. A user-friendly web-server for mining and visualizing microbiome-environment interactions. Bioinformatics 33, 782–783.

Zhang, L., Zheng, Y., Jagadeeswaran, G., Li, Y., Gowdu, K., Sunkar, R., 2011. Identification and temporal expression analysis of conserved and novel microRNAs in Sorghum. Genomics 98, 460–468.

## CONTRIBUTE 2

# Molecular and ionomic responses of Solanum lycopersycum L. (cv. Micro-Tom) plants treated with a novel calcium-based plant biostimulant

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**Abstract**

In this study, we investigated the leaf treatment effects of a novel trace elements calcium-based fluid mixture with a supposed biostimulant action on *Solanum lycopersicon* L. (cv. Micro-Tom. Seedlings were grown on standard peat substrate and treated with two different products: a calcium-based fluid mixture and a common calcium fertilizer, $CaCl_2$. Both treatments were compared to an untreated control. We first investigated the effects of treatments on fruit yield and dry matter production in greenhouse-grown tomato. These effects were assessed in leaves by gene expression profiling of 60 genes involved in different biological pathways and functional categories, and by ionomic analysis. Leaf treatment on tomato with the calcium-based fluid mixture allowed the highest fruit yield per plant (6.17 fruits $plant^{-1}$) and above-ground dry matter (13.99 g $plant^{-1}$) to be obtained. Also, 4 genes related to the nutrient transporter category, *NCX*, *NRAMP3*, *SI BOR2*, *CHLM*, were upregulated in plants treated with the novel product. *CRK*, a gene related to the calcium-dependent protein kinases (*CDPK*), was upregulated in plants treated with the novel product whereas *SODCC.1*, a gene related to the superoxide dismutase family, was downregulated in the same plants. A substantial reduction of elemental contents was observed for $CaCl_2$ treated plants, while the novel Ca-based mixture increased the leaf mineral content of Zn (+61%) and Mn (+65%). These results highlighted the biostimulant activity of the novel product resulting in the changes in fruit yield and dry matter production, gene expression and ionome profiles of tomato leaves.

Keywords: tomato, biostimulant, leaf treatment, gene expression, leaf ionome

## 1. Introduction

To ensure adequate yields and quality, and provide food security, new sustainable and efficient solutions are needed to meet the requirements of a rapidly growing human world population (*Evenson and Gollin, 2003; Godfray et al., 2010; Smith, 2015; Fróna et al., 2019*). In this way, the use of bioeffectors, known as biostimulants, helps in various ways to enhance the crop yield, exerting a beneficial action at different levels in plants (*Bulgari et al., 2015; Van Oosten et al., 2017)*. Plant biostimulants are substances or microorganisms applied to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of nutrients content (*Du Jardin 2015*). The key mechanisms targeted by the biostimulants are strongly related to the nature of the biostimulant itself. Due to the complexity of the chemical composition and the simultaneous action of two or more compounds, a complete mode of action characterization is still lacking (*Bulgari et al.,2015; Brown and Saa, 2015; Yakhin et al., 2017; Van Oosten et al., 2017).* Among crops, tomato is one that benefits from the use of biostimulants. To study plant response to biostimulants, the variety *Solanum lycopersicon* cv. *Micro-Tom* is frequently adopted due to its peculiar botanical traits and the availability of a public and completely sequenced genome (*Mueller et al., 2005*). Recent studies have demonstrated that tomato transcriptome and ionome

can be strongly modified by leaf treatment with fertilizers or other substances, such as biostimulants (*Caruso et al., 2019; Della Lucia et al. 2022)*. Particularly, several studies have reported that calcium is a crucial nutritional macro element for tomato growth and development and has a still not well-characterized repertoire of metabolic pathways modulated by biostimulants in tomato.

Within this context, the aim of this study was to assess the leaf treatment effects of a novel trace elements calcium-based fluid mixture with a supposed biostimulant action on *Solanum lycopersicon* L. (cv. Micro-Tom). We first investigated the effects of treatments on fruit yield and dry matter production in greenhouse-grown tomato. These effects were then assessed in leaves by gene expression profiling of 60 genes coding for microelements transporters, calcium signal pathway, and enzymes involved in abiotic stress tolerance, and by ionomic analysis.

## 2. Materials and methods

### *2.1 Plant materials and growth conditions*

Seeds of tomato (*Solanum lycopersicon* L., cv. Micro-Tom) were provided by Sipcam Oxon S.p.a. and grown in 13 cm diameter pots filled with standard peat substrate. A mineral-based slow-release fertilizer (nitrophoska© type) with nitrogen, phosphorus and potassium concentration of 12%, 12% and 17%, respectively; the fertilizer also has an additional 2% of MgO, 24% of sulfur, 0.02% of boron and 0.10% of

zinc and was applied to the substrate, at a rate of about 15-20 g per pot. Each pot was irrigated with around 200-300 mL of water, every two days. Water in excess was resupplied until the complete absorption by the peat substrate. Pots were maintained for 50 days in a climatic chamber at 25/20 °C and a 16/8 light/dark photoperiod. There were two batches, one for the ionome analysis and one for the molecular analysis.

### *2.2 Treatments application and tissue collection*

Plants were divided into three sets: one control and two sets of treated plants. Three different treatments were applied to the tomato plants as a foliar spray, at a volume of 10 mL per plant. This quantity corresponds to the novel Ca-based product volume usually sprayed as treatment in tomato crops. The untreated control was supplied with an equal volume of water. The other two treatments applied were: a calcium-chloride solution with a concentration of 10.050 gr $L^{-1}$, and a novel calcium-based mixture with a concentration of 5 mL $L^{-1}$. The concentrations of the two products used for the experiments are the ones that are agronomically effective, by improving tomato agronomic traits, as reported in the product technical indications. The novel Ca-based product was provided by Sipcam-Oxon S.p.a. and its chemical composition is reported in Tab. 1. Each solution was diluted in ultra-pure water and the pH was assessed as around 7.

Treatment application occurred at the flowering stage classified by the code BBCH 65 in the official BBCH scale classification method, corresponding to the first open flower of the fifth inflorescence. The BBCH scale is defined as a system for uniform coding of phenologically similar growth stages, with a two-digit code that precisely identifies all phenological stages for the majority of plant species. For each code, morphological traits are used, and a brief description of each stage is given, from seed germination to harvesting (*Meier et al., 2009*).

The fruit yield per plant and dry matter (DM) were evaluated to study the growth and production promotion in the treated plants. The sampling for this evaluation occurred 100 days after plant establishment in the growth chamber. For the fruit yield per plant, fruit samples were harvested at BBCH 89, when they have the typical fully ripe colour. To measure the above-ground dry matter of plants at harvest time (leaf, stem, and fruit), samples were oven-dried at 50 °C for at least five days. The dried sample was then determined using a digital scale.

For the gene expression analysis, three plants per treatment were chosen and sampled 48 hours after treatment application. The timing was chosen according to previous experiments in which the best timing after treatment to observe changes in gene expression was assessed. There were three biological replications for each plant and a total of 27 samples were collected. Each sample was formed by three leaf disks collected per single plant. The second, third or fourth unfolded leaf on the main shoot was selected, using all of them alternatively. Samples were immediately stored at -80 °C until RNA extraction. For the ionomic analysis, 6 plants

per treatment were selected and sampled before leaf treatment application and after 7 days. Each plant sample consisted of 3 g of fresh tomato leaves, choosing the second, third, or fourth unfolded leaf on the main shoot. Leaves were chosen in accordance with our previous experiment where it was observed that the optimal accumulation of mineral elements occurs between the second and fourth leaf, for our considered tomato phenological stage. The leaves were stored at -20 °C, ready for the ionomic analysis.

### *2.3 Selection of candidate genes and PCR primer design*

A panel of 60 genes (encoding proteins) involved in different biological pathways and functional categories, such as calcium and other nutrients transport and metabolism, hormonal metabolism, and stress responses were selected and cDNA annotation sequences were provided by the MiBASE database (http://www.pgb.kazusa.or.jp/mibase/) (*Aoki et al., 2010*). The gene-specific primers were designed using an online PCR Primer Design Tool offered by Eurofins (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design).

### *2.4 RNA isolation and Real-time RT PCR*

Total RNA was extracted and purified using an automatized procedure with the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) in a BioSprint 96 workstation (Qiagen) following the manufacturer's instructions. The molarities of RLT and RPW buffers were increased to achieve higher extracted RNA yields. The quality of the RNA extraction was assessed via quantification, using a Qubit RNA HS Assay Kit in a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The total extracted

RNA was stored at -80 °C for the next analysis. One-step reverse transcription-quantitative PCR (RT-qPCR) in real-time was run in triplicate on a QuantStudio 12K Flex Real-Time PCR System (Life Technologies, USA) in a reaction volume of 10 µL containing 9 µL of qPCR BIO Sybrgreen 1-Step Mix (Resnova – PCR Biosystem) and 4 ng of template RNA, using the following thermocycler program: 10 minutes at 45 °C and 2 minutes at 95 °C for the holding stages, followed by 40 cycles at 95 °C for 5 seconds and 25 seconds at 60 °C. The process also includes a step for amplicons dissociation, recorded by the PCR machine as melting curves graphs. The melting stage was recorded after 40 cycles and starts by heating the amplicons for 15 seconds at 95 °C, 60 °C for 1 minute, and 95 °C for 15 seconds. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$. Ubiquitin (UBI) and Actin (ACT), two classical tomato housekeeping genes with stable expression levels reported in the literature (*Løvdal and Lillo, 2009*), were used for the normalization of expression levels in this experiment.

### *2.6 Ionome analysis*

The element concentration in the leaves treated with the two different products and the untreated ones was determined by ionomic analysis. Sampling of the leaves occurred before and seven days after the treatment application Leaf samples were digested with concentrated $HNO_3$ in a microwave system. The element concentration was determined by inductively coupled plasma ICP-OES. Elements were quantified using certified multi-element standards. The content of Al, As, B, Ba, Ca, Cd, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, S, Si, Sr, Ti, and Zn were evaluated

through the ICP-OES optical system. Contents were considered in *mg kg$^{-1}$* of dry matter.

### *2.5 Statistical Analysis*

The analysis of variance (ANOVA one-way) was performed to determine any statistical differences between treated and untreated samples, for the evaluated variables in the gene expression analysis. A p-value < 0.05 was considered for each analysis. Treatment means were separated by the Duncan test while variance homogeneity was evaluated using Levene's test. Principal components analysis (PCA) of ionome variation between untreated and treated samples was also performed. Variables were presented in graphs as the mean and standard error of the mean (standard error). All statistical analyses were carried out using Statistica software v. 13.4 (TIBCO Software, USA).

## 3. Results

### 3.1 Growth and yield traits

The results indicated that the application of the novel Ca-based mixture had significant effects on tomato plants dry mass and fruit yield. The maximum number of fruits yield per plant (6.17 fruits plant$^{-1}$) was

observed, which was statistically greater than $CaCl_2$ and the untreated plants (Fig. 1).

Plants treated by the novel Ca-based mixture also produced the significantly highest above-ground dry matter (13.99 g $plant^{-1}$). Instead, no significant difference was recorded for plant DM between the $CaCl_2$ application and untreated plants (Fig. 1).

### *3.2 Gene expression analysis*

Sixty genes reported being involved in several different plant metabolic pathways were selected among all the EST gene sequences reported in the MiBASE – Microtom database. Genes category includes calcium and other nutrients metabolism (Mg, Cu, Zn, B), hormonal metabolism, oxidative stress triggered metabolism, and water stress triggered metabolism. All 60 primers pairs were tested for gene expression in leaf samples. Only gene primers pairs with a single peak in the melting curve analysis performed by the machine were selected. A single peak confirms the specificity of PCR amplification and a PCR product of the expected size, as described by Czechowski et al. (2005).

Only twenty primer pairs out of sixty amplified single PCR products with a single peak resulted during the melting curve analysis. All the selected primers included genes with between 20 and 29 Ct. The twenty genes were divided into 2 groups: one with all the genes related to the nutrient transporter and the other one related to the oxidative and water stress triggered metabolism (Tab. 2).

Genes expressions were tested by the Duncan post hoc test and only 6 genes out of twenty were selected. These genes showed a statistically significant difference in terms of change in the relative expression as compared to the untreated plants at a *p-value* < 0.05. Genes with no differences between treated and untreated samples were discarded because of the absence of any putative effects from the treatment, meaning that the gene expression of the analyzed genes was not affected. The result of the Duncan test is reported in Tab. 3. Duncan tests reveal differences in some genes belonging to both categories.

Genes *CRK* and *SODcc.1*, related to the stress signaling metabolism, significantly changed their expression. (Fig. 2). In particular, gene *CRK*, a Ca-dependent protein-kinase of tomato were upregulated two-fold in samples treated with the Ca-based mixture, as compared to the untreated control. Gene *SODcc.1* instead, was downregulated eight-fold in samples treated with the Ca-based mixture, and upregulated in samples treated with $CaCl_2$, but not significantly. Genes *NCX*, *NRAM3*, *SI_BOR2*, and *CHLM*, related to nutrient transporters, were upregulated two-fold in samples treated with the Ca-based mixture, but only *CHLM* and *NRMAP3* showed a difference with the Duncan test (Fig. 3).

### *3.3 Ionome analysis*

The leaf element composition of the treated and untreated leaves was analyzed before and seven days after treatment. The comparison of the

ionome profile revealed significant differences in the elemental composition, seven days after treatment. ANOVA test results are reported in Tab. 4. All three main variables, treatment, time, and element are highly statistically significant. Means of the content profile and increment or decrement for the considered element, before and seven days after the treatment are shown in Tab. 5, 6, and 7. Calcium content didn't change significantly seven days after treatment: only in the sample treated with $CaCl_2$, the calcium content showed an increment of 2%, from 31737.78 *mg* $kg^{-1}$ to 32325.79 *mg* $kg^{-1}$. This means that neither treatment affected the calcium ionome in leaves. The other plant macro and microelements contents were different. Based on the results of gene expression, some microelements related to the targeted transporter were studied in-depth: B, Mg, Fe, Na, and Zn. Zinc is one of the key elements that showed an increment of 60% after seven days (from 48.99 to 78.93 *mg* $kg^{-1}$), in samples treated with the Ca-based mixture, while both untreated control and $CaCl_2$ treated plants decreased their zinc content by about 50% (from 62.66 to 29.40 *mg* $kg^{-1}$) and 40% (from 65.81 to 37.23 *mg* $kg^{-1}$) respectively. Boron and magnesium content increased by 8% (from 60.61 to 65.84 *mg* $kg^{-1}$) and 7% (from 9479.24 to 10154.01 *mg* $kg^{-1}$) respectively in plants treated with the Ca-based mixture, while a decrement of 10% (from 84.03 to 75.44 *mg* $kg^{-1}$) was observed for boron and an increment of 13% (from 10192.13 to 11588.39 *mg* $kg^{-1}$) for magnesium, both in plants treated with $CaCl_2$. For iron, there were no percentage differences in the Ca-based mixture treatment, but a reduction of 24% (from 124.55 to 94.40 *mg* $kg^{-1}$) and 20 % (from 159.34 to 128.58 *mg* $kg^{-1}$) was observed in untreated control and $CaCl_2$ treatment respectively.

A decrease of around 37% in sodium content was observed in all the experimental conditions: from 331.44 to 211.62 *mg* $kg^{-1}$ for the untreated samples; from 430.50 to 257.55 for the Ca-based mixture treated

samples; and from 329.19 to 206.26 *mg* $kg^{-1}$ for the $CaCl_2$ treated samples. Despite gene expression analysis results not showing changes in the expression of manganese metabolism-related genes, manganese content was 65% higher after seven days in the Ca-based mixture treatment, from 234.94 to 388.15 *mg* $kg^{-1}$, but the content decreased by 5% in the other two conditions: from 324.60 to 307.21 *mg* $kg^{-1}$ for the untreated plants; and from 331.65 to 315.74 *mg* $kg^{-1}$ for the $CaCl_2$ treatment. Moreover, manganese is the second component of the novel Ca-based mixture, with a concentration of 1.5%.

PCA analysis was performed to evaluate the relationship between treatment and ionome content in treated and untreated samples. PCA analysis revealed separate clustering for the Ca-based mixture treatment, while the others were clustering together (Fig. 4). Factor 1 and factor 2 of the first PCA graph (Fig. 5a) related to data collected before the treatment explained 62.52% and 11.65% of the total variation, respectively. Factor 1 and factor 2 of the second PCA graph (Fig.5b) related to data collected seven days after the treatment explained 58.30 % and 13.90% of the total variation, respectively.

## 4. Discussion

In this study, we studied the effects of a novel Ca-based product on tomato plants. A $CaCl_2$ mineral fertilizer and an untreated control were included for comparison. The treatment with the Ca-based product

showed the highest growth and production promotion in tomato plants, as compared to the other two treatments. This is in line with other authors who found similar results by applying biostimulants or biostimulant-like substances of different and complex nature with an organic component like the Ca-based mixture (*Rouphael et al., 2018; Canellas et al., 2019; Jindo et al., 2020; Canellas et al., 2020*). De Hita et al. (2019) reported the effect of the application of humic substances for root and leaf treatments. Root treatments showed more consistent effects compared to leaf treatments, which have a transient effect in increasing plant growth and require several applications during the plant cycle. But despite these findings, our observations on leaf treatment highlight an important effect that resulted in improved plant growth and yield, with only one treatment during the plant life cycle. The effect of treatments on root and shoot development could be explained by the changes we observed in gene expression and in the ionome of tomato leaves. Interestingly, the gene related to the calcium CDPK-related protein kinase was notably upregulated in plants treated with the calcium-based mixture. *CDPKs* are a large and partially characterized family of proteins in plants. They are calcium sensor proteins and play an important role as a mediator of responses to endogenous and environmental stimuli (*Cheng et al.,2002*). The protein structure is also conserved in tomato *CDPKs*, with four characterized domains in the *N*-terminal domain, Ser/Thr kinase domain, autoinhibitory junction domain, and calmodulin-like domain. The *CDPKs* converts the variation of cytosolic [$Ca^{2+}$] into biochemical and genetic products through a phosphorylation process, like membrane solute transporters (including the $Ca^{2+}$-ATPase, AtACA2), ion and water channels, *NADPH* oxidases, enzymes involved in carbon and nitrogen metabolism, cytoskeletal proteins, proteases, and DNA-binding proteins, control of the cell cycle, phytohormone signal transduction, light-regulated

gene expression, gravitropism, thigmotropism, nodulation, cold acclimation, salinity tolerance, drought tolerance, and responses to pathogens (*White and Broadley, 2003*). Neither the treatments with the Ca-based mixture nor the one with $CaCl_2$ significantly affected the concentration of calcium in the leaves, with no significant differences between treated and untreated plants. This is in line with several authors who underline the role of calcium as an elicitor and also the mediator of the signals to activate all the correlated downstream transcriptional processes, like the active regulation of different ion and micronutrient membrane transporters, with a *CDPKs* complex activation, resulting in the overexpression of the related genes (*Sanders et al., 2002; White and Broadley, 2003; Dodd et al., 2010*). The presence of a polysaccharides mixture in the novel Ca-based mixture could be also a possible explanation for the observed cross-talk between the different metabolic pathways that regulate the growth and development of plants. Polysaccharides are also known as elicitors and this could have an influence on the expression of signaling-related genes, as it has already been reported that carbohydrates and sugars-related compounds can activate PAMP/DAMP responses in plants, which also involves genes related to the CDPKs family, like the one we studied (*Moghaddam and Van den Ende, 2012; Trouvelot et al., 2014).* Moreover, oligosaccharides are reported to be effective in enhancing plant tolerance to biotic and abiotic stresses (*Ibrahim and Abdellatif, 2016; Davidsson et al., 2017; Chaliha et al., 2018; Zang et al., 2019; Narula et al, 2020).*

The *SOD* gene, a Cu/Zn superoxide dismutase (Cu/Zn SOD) was downregulated in plants treated with the calcium-based mixture. Superoxide dismutases instead are a large category of metal-containing enzymes with the important function of catalyzing the dismutation of reduced oxygen species (ROS), which are toxic to plants. Because of this,

they play a fundamental role during abiotic and biotic stress events. *SODs* are highly regulated in the plant by gene expression and their activities are also strongly influenced by environmental stimuli (*Bowler et al, 1994*). In the case of tomatoes, genomic *SODs* gene family classification was studied by Feng et al. (2016). In their study, they revealed the presence of nine genes related to different metal ion-dependent *SODs*, with many *cis-elements in* the promoter sequences, that respond to different stresses. This correlates *SODs* tomato genes to stress responses to drought, low-temperature, defense stresses, anaerobic induction, fungal elicitors, SA, MeJA, GA, IAA, and ethylene. Interestingly*, SlSOD1*, mentioned in the study by Feng et al. (2016), is the same gene sequence used in the current experiment and is overexpressed during salinity stress. Other studies revealed that the *SODs* enzymes are activated under stress events also in other crops, and they work more as stress fighters than stress indicators. Despite the large literature about $Ca^{2+}$ signaling related stress defensive responses, our novel Ca-based mixture product instead acts in an intriguingly different way, enhancing general plant signaling and improving plant nutrients acquisition instead of the plant defence system. We noticed that gene *NRAMP3*, zinc transporter, and CHLM, magnesium transporter, are upregulated in the treatment. In Rai et al. (2021) the authors reviewed many studies in which it was reported that NRAMPs genes family are directly involved in the iron homeostasis in plants through a complex crosstalk between other micronutrients, such as Zn. Nonetheless, we found an increased level of zinc content in tomatoes treated with the novel Ca-based mixture and no interesting variances in the iron content after seven days, but it was notably decreased in the other two treatments. This supports our hypothesis of a putative biostimulant action in accordance with other previous results showing the ability of biostimulants to improve nutrient uptake with an overexpression of the related nutrient transporters genes.

In particular, this improvement includes modifications of the root absorption area and modulation of the plant cell membrane activities related to nutrient acquisition (*White and Broadley, 2003; Zandonadi et al., 2016; Zanin et al., 2019; Nardi et al., 2021; González-Morales et al., 2021)*.

## 5. Conclusion

In this study, the comparison between treatment with a novel Ca-based mixture with supposed biostimulant activity and calcium-chloride solution showed interesting results with a gene expression/ionome approach. While the calcium-chloride solution did not exert a great impact at the genome level in the leaf and seems to be slightly detrimental to the general leaf ionome, the treatment with the novel substance importantly influenced these two components. These results confirm the initial hypothesis of a supposed biostimulant activity of the novel substance. We have demonstrated, according to the definition of biostimulant substance provided by Du Jardin (2015), that: 1) the novel Ca-based mixture can enhance nutrient use efficiency by increasing the expression of genes related to the nutrient transport metabolism; 2) the novel Ca-based product is a complex mixture of macro and microelements fundamental for plant nutrition without acting like a chemical fertilizer; 3) The novel Ca-based mixture enhances general plant signaling. Given the first two main characteristics extrapolated from our results, the novel product is confirmed as a substance with biostimulant activity, and the way it was characterized is appropriate to understanding more about the mode of action.

## 6. Acknowledgments

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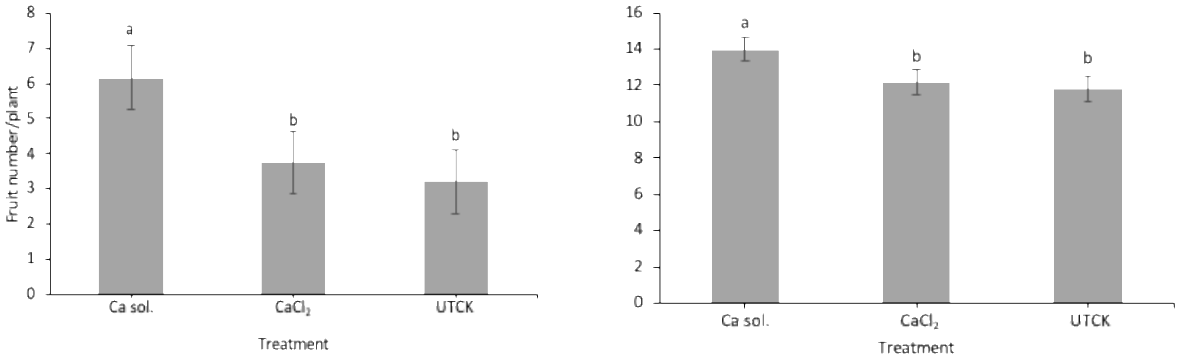


**Fig. 1** Fruit number plant$^{-1}$ and total aboveground DM plots for the three treatments. Means and error bars for each treatment are shown. Post-hoc Duncan's test at *p<0.05* was performed to discriminate means between treatments

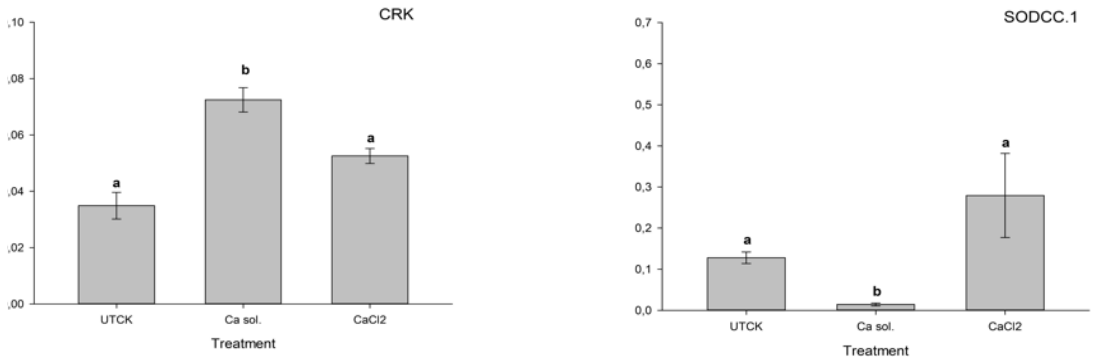


**Fig. 2** Relative expression of genes encoding for stress-related proteins. Means and error bars for each treatment are shown. Post-hoc Duncan's test at *p<0.05* was performed to discriminate means between treatments

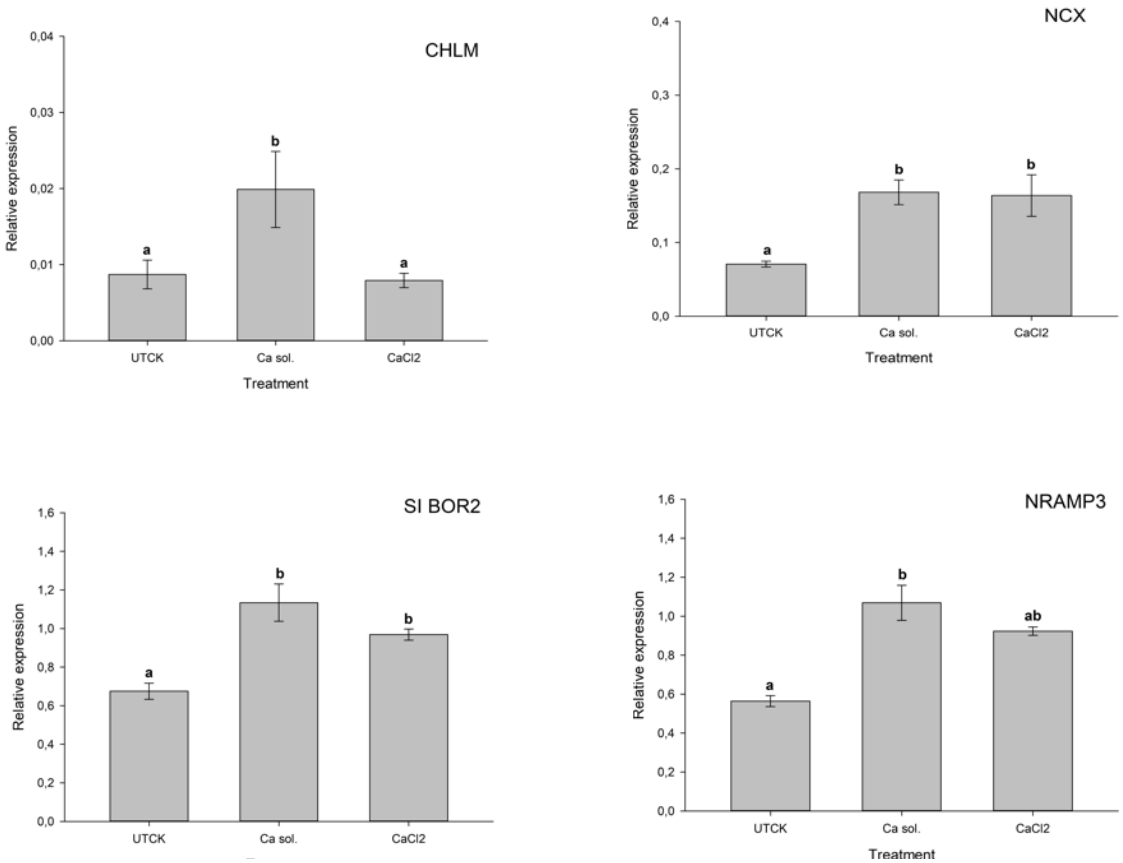


**Fig. 3** Relative expression of genes encoding for nutrient transporter. Means and error bars for each treatment are shown. Post-hoc Duncan's test at *p<0.05* was performed to discriminate means between treatments

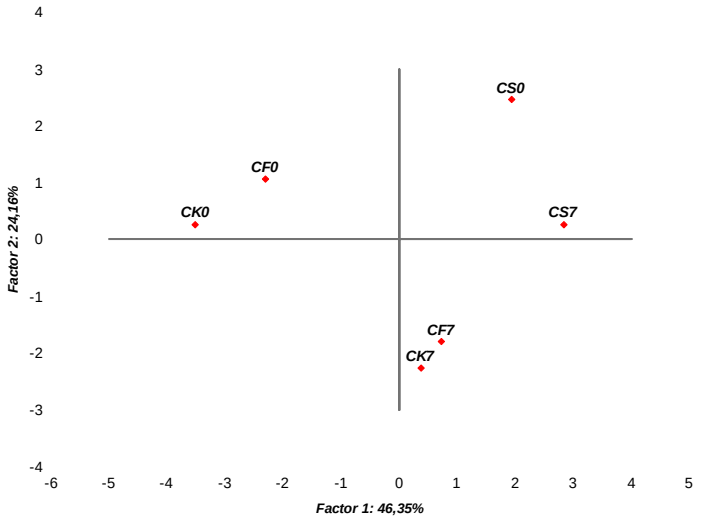


**Fig. 4** Classification of the clusters based on leaf ionome content as a function of the treatment. "CK" = Untreated control; "CF" = $CaCl_2$ treatment; "CS" = Ca-based solution product treatment. "0" = before treatment; "7" = seven days after treatment

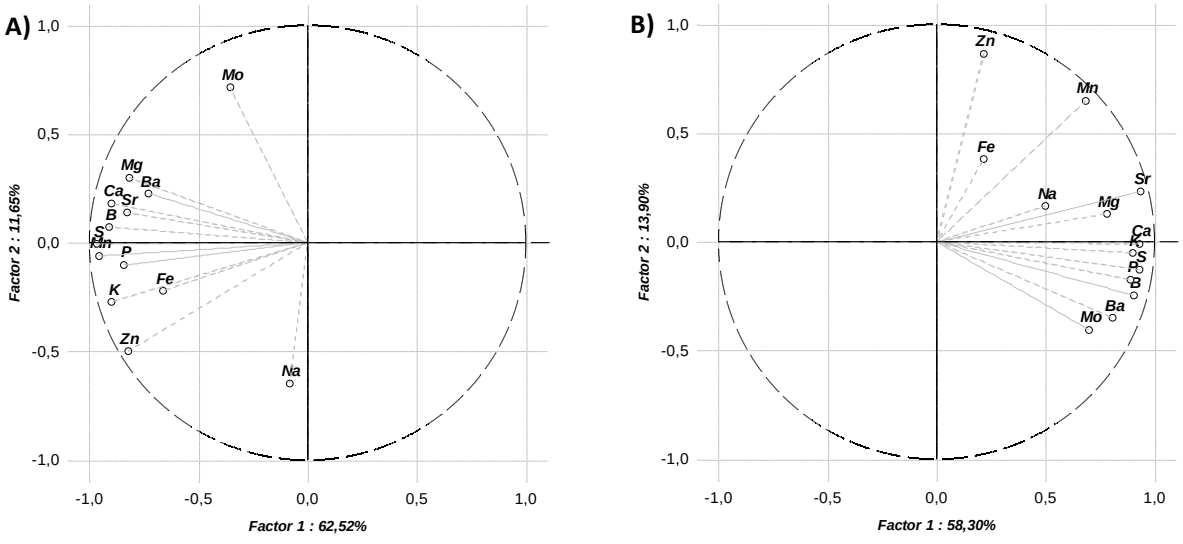


**Fig. 5** PCA analysis before the treatment (A) and seven days after (B). These graphs show the correlation between variables, and closeness in the graphs indicates correlation strength

**Tab. 1** Composition of the novel Ca-based solution product enriched with an inorganic complexing agent provided by Sipcam Italia S.p.a.

| | |
|---|---|
| CaO sol. in water (% w/w) | **5** |
| Mn sol. In water (% w/w) | **1.5** |
| Zn sol. In water (% w/w) | **0.5** |
| polysaccharides mixture (% w/w) | **20** |
| Electrical conductance (mS/cm) | **28.8** |
| pH | **4.25** |
| Density (kg/l) | **1.407** |

**Tab. 2** List of genes analyzed, showing statistical significance with ANOVAand *p<0,05.* All sequences are freely available at http://www.pgb.kazusa.or.jp/mibase/clone_uni_name.htmL

| Category | Function | Literaure name | EST clone sequence name |
|---|---|---|---|
| ***Stress related metabolism*** | CDPK-related protein kinase | CRK | *LEFL2013N24* |
| | SODCC.1 superoxide dismutase [Cu-Zn] 1 | SODCC.1 | *FC11BA12* |
| ***Nutrient transporters*** | probable boron transporter 2 | Si BOR2 | *LEFL1003CF07* |
| | generic Fe and Zn vacuolar efflux transporter | NRAMP3 | *LEFL1039BB08* |
| | Sodium/calcium exchanger NCL | NCX | *LEFL1011AD10* |
| | magnesium protoporphyrin IX methyltransferase, chloroplastic | CHLM | *LEFL1031AD01* |

**Tab. 3** Duncan's Multiple range test (*p<0,05*) results on relative expression of analyzed genes. Differences are calculated as compared to the untreated control

| | NCX | NRAMP3 | SI_Bor2 | CHLM | SODcc.1 | CRK |
|---|---|---|---|---|---|---|
| **Untreated control** | - | - | - | - | - | - |
| **Ca solution** | * | * | * | * | *n.s.* | ** |
| **$CaCl_2$** | * | * | * | *n.s.* | *n.s.* | * |

**Tab. 4** Analysis of Variance for *p<0,05,* for the ionome content. Means are mg/kg dm. The statistically significant factors and interactions are highlighted

| | *SS* | *D.o.f.* | *MS* | *F* | *p* | |
|---|---|---|---|---|---|---|
| ***Intercept*** | 3.231048E+10 | 1 | 3.231048E+10 | 3632.543 | 0.000000 | |
| ***Treatment*** | 1.546550E+08 | 2 | 7.732749E+07 | 8.694 | 0.000202 | ** |
| ***Time*** | 1.459378E+08 | 1 | 1.459378E+08 | 16.407 | 0.000062 | ** |
| ***Element*** | 7.422705E+10 | 12 | 6.185588E+09 | 695.422 | 0.000000 | ** |
| ***Treatment*Time*** | 3.841534E+07 | 2 | 1.920767E+07 | 2.159 | 0.116767 | *n.s.* |
| ***Treatment*Element*** | 3.833803E+08 | 24 | 1.597418E+07 | 1.796 | 0.012841 | * |
| ***Time*Element*** | 8.046658E+08 | 12 | 6.705549E+07 | 7.539 | 0.000000 | ** |
| ***Treatment*Time*Element*** | 1.836289E+08 | 24 | 7.651205E+06 | 0.860 | 0.657598 | *n.s.* |
| ***Error*** | 3.47E+09 | 390 | 8.89E+06 | | | |

**Tab. 5** Means (mg/Kg dm) and percentage of variations for the analyzed elements in UTCK samples. Percentages are the increment or decrement after seven days for the considered elements

| | *UTCK - 0 hours* | *UTCK - 7 days* | *% variation* |
|---|---|---|---|
| ***B*** | 84.552 | 85.957 | 1.66 |
| ***Ba*** | 2.112 | 1.591 | -24.67 |
| ***Ca*** | 33046.202 | 31378.811 | -5.05 |
| ***Fe*** | 124.550 | 94.399 | -24.21 |
| ***K*** | 45467.204 | 33323.328 | -26.71 |
| ***Mg*** | 10564.873 | 10674.459 | 1.04 |
| ***Mn*** | 324.603 | 307.209 | -5.36 |
| ***Mo*** | 29.140 | 31.238 | 7.20 |
| ***Na*** | 331.439 | 211.620 | -36.15 |
| ***P*** | 8820.634 | 7067.112 | -19.88 |
| ***S*** | 26684.005 | 21727.695 | -18.57 |
| ***Sr*** | 32.933 | 28.161 | -14.49 |
| ***Zn*** | 62.665 | 29.396 | -53.09 |

**Tab. 6** Means (mg/Kg dm) and percentages of variations for the analyzed elements in Ca-based solution product treated samples. Percentages are the increment or decrement after seven days for the considered elements

| | *Ca solution - 0 hours* | *Ca solution - 7 days* | *% variation* |
|---|---|---|---|
| ***B*** | 60.612 | 65.839 | 8.62 |
| ***Ba*** | 1.768 | 0.947 | -46.43 |
| ***Ca*** | 28673.219 | 28646.059 | -0.09 |
| ***Fe*** | 107.566 | 108.290 | 0.67 |
| ***K*** | 35425.961 | 32008.752 | -9.65 |
| ***Mg*** | 9479.239 | 10154.013 | 7.12 |
| ***Mn*** | 234.933 | 388.144 | 65.21 |
| ***Mo*** | 17.915 | 20.351 | 13.60 |
| ***Na*** | 430.497 | 257.547 | -40.17 |
| ***P*** | 6234.708 | 5880.697 | -5.68 |
| ***S*** | 19003.177 | 18079.315 | -4.86 |
| ***Sr*** | 29.282 | 28.766 | -1.76 |
| ***Zn*** | 48.992 | 78.926 | 61.10 |

**Tab. 7** Means (mg/Kg dm) and percentages of variations for the analyzed elements in $CaCl_2$ treated samples. Percentages are the increment or decrement after seven days for the considered elements

| | ***$CaCl_2$ - 0 hours*** | ***$CaCl_2$ - 7 days*** | ***% variation*** |
|---|---|---|---|
| ***B*** | 84.036 | 75.447 | -10.22 |
| ***Ba*** | 2.051 | 1.751 | -14.62 |
| ***Ca*** | 31737.784 | 32325.792 | 1.85 |
| ***Fe*** | 159.340 | 128.585 | -19.30 |
| ***K*** | 44437.169 | 32294.276 | -27.33 |
| ***Mg*** | 10192.131 | 11588.387 | 13.70 |
| ***Mn*** | 331.648 | 315.745 | -4.80 |
| ***Mo*** | 19.748 | 17.714 | -10.30 |
| ***Na*** | 329.187 | 206.264 | -37.34 |
| ***P*** | 8073.711 | 6403.117 | -20.69 |
| ***S*** | 25043.394 | 18170.229 | -27.45 |
| ***Sr*** | 30.618 | 29.478 | -3.72 |
| ***Zn*** | 65.807 | 37.233 | -43.42 |

## References


Aoki, K., Yano, K., Suzuki, A., Kawamura, S., Sakurai, N., Suda, K., Kurabayashi, A., Suzuki, T., Tsugane, T., Watanabe, M., Ooga, K., Torii, M., Narita, T., Shin-i, T., Kohara, Y., Yamamoto, N., Takahashi, H., Watanabe, Y., Egusa, M., Kodama, M., Ichinose, Y., Kikuchi, M., Fukushima, S., Okabe, A., Arie, T., Sato, Y., Yazawa, K., Satoh, S., Omura, T., Ezura, H., Shibata, D., 2010. Large-scale analysis of full-length cDNAs from the tomato (Solanum lycopersicum) cultivar Micro-Tom, a reference system for the Solanaceae genomics. BMC Genomics 11, 210. https://doi.org/10.1186/1471-2164-11-210

Bolouri Moghaddam, M.R., Van den Ende, W., 2012. Sugars and plant innate immunity. Journal of Experimental Botany 63, 3989–3998. https://doi.org/10.1093/jxb/ers129

Bowler, C., Camp, W.V., Montagu, M.V., Inzé, D., Asada, P.K., 1994. Superoxide Dismutase in Plants. Critical Reviews in Plant Sciences 13, 199–218. https://doi.org/10.1080/07352689409701914

Brown, P., Saa, S., 2015. Biostimulants in agriculture. Front. Plant Sci. 6. https://doi.org/10.3389/fpls.2015.00671

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., Ferrante, A., 2015. Biostimulants and crop responses: a review. Biological Agriculture & Horticulture 31, 1–17. https://doi.org/10.1080/01448765.2014.964649

Canellas, L.P., Canellas, N.O.A., da S. Irineu, L.E.S., Olivares, F.L., Piccolo, A., 2020. Plant chemical priming by humic acids. Chemical and

Biological Technologies in Agriculture 7, 12. https://doi.org/10.1186/s40538-020-00178-4

Canellas, L.P., Canellas, N.O.A., Soares, T.S., Olivares, F.L., 2019. Humic Acids Interfere with Nutrient Sensing in Plants Owing to the Differential Expression of TOR. J Plant Growth Regul 38, 216–224. https://doi.org/10.1007/s00344-018-9835-6

Caruso, G., De Pascale, S., Cozzolino, E., Cuciniello, A., Cenvinzo, V., Bonini, P., Colla, G., Rouphael, Y., 2019. Yield and Nutritional Quality of Vesuvian Piennolo Tomato PDO as Affected by Farming System and Biostimulant Application. Agronomy 9, 505. https://doi.org/10.3390/agronomy9090505

Chaliha, C., Rugen, M.D., Field, R.A., Kalita, E., 2018. Glycans as Modulators of Plant Defense Against Filamentous Pathogens. Front. Plant Sci. https://doi.org/10.3389/fpls.2018.00928

Cheng, S.-H., Willmann, M.R., Chen, H.-C., Sheen, J., 2002. Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family. Plant Physiology 129, 469–485. https://doi.org/10.1104/pp.005645

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W.-R., 2005. Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. Plant Physiology 139, 5–17. https://doi.org/10.1104/pp.105.063743

Davidsson, P., Broberg, M., Kariola, T., Sipari, N., Pirhonen, M., Palva, E.T., 2017. Short oligogalacturonides induce pathogen resistance-

associated gene expression in Arabidopsis thaliana. BMC Plant Biology 17, 19. https://doi.org/10.1186/s12870-016-0959-1

De Hita, D., Fuentes, M., García, A.C., Olaetxea, M., Baigorri, R., Zamarreño, A.M., Berbara, R., Garcia-Mina, J.M., 2019. Humic substances: a valuable agronomic tool for improving crop adaptation to saline water irrigation. Water Supply 19, 1735–1740. https://doi.org/10.2166/ws.2019.047

Della Lucia, M.C., Baghdadi, A., Mangione, F., Borella, M., Zegada-Lizarazu, W., Ravi, S., Deb, S., Broccanello, C., Concheri, G., Monti, A., Stevanato, P., Nardi, S., 2022. Transcriptional and Physiological Analyses to Assess the Effects of a Novel Biostimulant in Tomato. Frontiers in Plant Science 12. https://doi.org/10.3389/fpls.2021.781993

Dodd, A.N., Kudla, J., Sanders, D., 2010. The Language of Calcium Signaling. Annu. Rev. Plant Biol. 61, 593–620. https://doi.org/10.1146/annurev-arplant-070109-104628

du Jardin, P., 2015. Plant biostimulants: Definition, concept, main categories and regulation. Scientia Horticulturae, Biostimulants in Horticulture 196, 3–14. https://doi.org/10.1016/j.scienta.2015.09.021

Evenson, R.E., Gollin, D., 2003. Assessing the Impact of the Green Revolution, 1960 to 2000. Science 300, 758–762. https://doi.org/10.1126/science.1078710

Feng, K., Yu, J., Cheng, Y., Ruan, M., Wang, R., Ye, Q., Zhou, G., Li, Z., Yao, Z., Yang, Y., Zheng, Q., Wan, H., 2016. The SOD Gene Family in Tomato: Identification, Phylogenetic Relationships, and Expression Patterns. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.01279

Fróna, D., Szenderák, J., Harangi-Rákos, M., 2019. The Challenge of Feeding the World. Sustainability 11, 5816. https://doi.org/10.3390/su11205816

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C., 2010. Food Security: The Challenge of Feeding 9 Billion People. Science 327, 812–818. https://doi.org/10.1126/science.1185383

González-Morales, S., Solís-Gaona, S., Valdés-Caballero, M.V., Juárez-Maldonado, A., Loredo-Treviño, A., Benavides-Mendoza, A., 2021. Transcriptomics of Biostimulation of Plants Under Abiotic Stress. Front. Genet. https://doi.org/10.3389/fgene.2021.583888

Ibrahim, H.A., Abdellatif, Y.M.R., 2016. Effect of maltose and trehalose on growth, yield and some biochemical components of wheat plant under water stress. Annals of Agricultural Sciences 61, 267–274. https://doi.org/10.1016/j.aoas.2016.05.002

Ilyas, M., Nisar, M., Khan, N., Hazrat, A., Khan, A.H., Hayat, K., Fahad, S., Khan, A., Ullah, A., 2020. Drought Tolerance Strategies in Plants: A Mechanistic Approach. J Plant Growth Regul. https://doi.org/10.1007/s00344-020-10174-5

Jindo, K., Olivares, F.L., Malcher, D.J. da P., Sánchez-Monedero, M.A., Kempenaar, C., Canellas, L.P., 2020. From Lab to Field: Role of Humic Substances Under Open-Field and Greenhouse Conditions as Biostimulant and Biocontrol Agent. Front. Plant Sci. https://doi.org/10.3389/fpls.2020.00426

Løvdal, T., Lillo, C., 2009. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Analytical Biochemistry 387, 238–242. https://doi.org/10.1016/j.ab.2009.01.024

Meier, U., Bleiholder, H., Buhr, L., Feller, C., Hack, H., Heß, M., Lancashire, P.D., Schnock, U., Stauß, R., Boom, T. van den, Weber, E., Zwerger, P., 2009. The BBCH system to coding the phenological growth stages of plants – history and publications –. Journal für Kulturpflanzen 61, 41–52. https://doi.org/10.5073/JfK.2009.02.01

Mueller, L.A., Solow, T.H., Taylor, N., Skwarecki, B., Buels, R., Binns, J., Lin, C., Wright, M.H., Ahrens, R., Wang, Y., Herbst, E.V., Keyder, E.R., Menda, N., Zamir, D., Tanksley, S.D., 2005. The SOL Genomics Network. A Comparative Resource for Solanaceae Biology and Beyond. Plant Physiology 138, 1310–1317. https://doi.org/10.1104/pp.105.060707

Nardi, S., Schiavon, M., Francioso, O., 2021. Chemical Structure and Biological Activity of Humic Substances Define Their Role as Plant Growth Promoters. Molecules 26, 2256. https://doi.org/10.3390/molecules26082256

Narula, K., Elagamey, E., Abdellatef, M.A.E., Sinha, A., Ghosh, S., Chakraborty, N., Chakraborty, S., 2020. Chitosan-triggered immunity to Fusarium in chickpea is associated with changes in the plant extracellular matrix architecture, stomatal closure and remodeling of the plant metabolome and proteome. The Plant Journal 103, 561–583. https://doi.org/10.1111/tpj.14750

Rai, S., Singh, P.K., Mankotia, S., Swain, J., Satbhai, S.B., 2021. Iron homeostasis in plants and its crosstalk with copper, zinc, and manganese. Plant Stress 1, 100008. https://doi.org/10.1016/j.stress.2021.100008

Rouphael, Y., Giordano, M., Cardarelli, M., Cozzolino, E., Mori, M., Kyriacou, M.C., Bonini, P., Colla, G., 2018. Plant- and Seaweed-Based Extracts Increase Yield but Differentially Modulate Nutritional Quality of Greenhouse Spinach through Biostimulant Action. Agronomy 8, 126. https://doi.org/10.3390/agronomy8070126

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F., 2002. Calcium at the Crossroads of Signaling. The Plant Cell 14, S401–S417. https://doi.org/10.1105/tpc.002899

Smith, P., 2015. Malthus is still wrong: we can feed a world of 9–10 billion, but only by reducing food demand. Proceedings of the Nutrition Society 74, 187–190. https://doi.org/10.1017/S0029665114001517

Van Oosten, M.J., Pepe, O., De Pascale, S., Silletti, S., Maggio, A., 2017. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. Chemical and Biological Technologies in Agriculture 4, 5. https://doi.org/10.1186/s40538-017-0089-5

White, P.J., Broadley, M.R., 2003. Calcium in Plants. Ann Bot 92, 487–511. https://doi.org/10.1093/aob/mcg164

Yakhin, O.I., Lubyanov, A.A., Yakhin, I.A., Brown, P.H., 2017. Biostimulants in Plant Science: A Global Perspective. Front. Plant Sci. https://doi.org/10.3389/fpls.2016.02049

Zandonadi, D.B., Santos, M.P., Caixeta, L.S., Marinho, E.B., Peres, L.E.P., Façanha, A.R., 2016. Plant proton pumps as markers of biostimulant action. Sci. agric. (Piracicaba, Braz.) 73, 24–28. https://doi.org/10.1590/0103-9016-2015-0076

Zang, H., Xie, S., Zhu, B., Yang, X., Gu, C., Hu, B., Gao, T., Chen, Y., Gao, X., 2019. Mannan oligosaccharides trigger multiple defence responses in rice and tobacco as a novel danger-associated molecular pattern. Molecular Plant Pathology 20, 1067–1079. https://doi.org/10.1111/mpp.12811

Zanin, L., Tomasi, N., Cesco, S., Varanini, Z., Pinton, R., 2019. Humic Substances Contribute to Plant Iron Nutrition Acting as Chelators and Biostimulants. Front. Plant Sci. https://doi.org/10.3389/fpls.2019.00675

## CONTRIBUTE 3

# Transcriptome analysis reveals a key role of *Ascophyllum nodosum* extract treatment on the hormone homeostasis regulation in sugar beet

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## Abstract

The molecular and phenotypic effects of Ascophyllum nodosum extract (ANE) were assessed on sugar beet. Sugar beet samples were grown both in open field and hydroponics. Root morphology together with sugar yield and quality traits were also analysed in plants grown both in open field and hydroponics. The molecular effects of ANE on open-field grown plants were studied by RNA-seq and validated by quantitative real-time PCR analysis. RNA-seq revealed about 1.600 differential expressed genes DEGs between the ANE-treated and non-treated plants. A p-value < 0.05 and a log2-fold change cutoff of 1 were used as significant criteria to select the DEGs. Cluster analysis on Gene ontology (GO) revealed different categories of biological processes with up-regulated genes and down regulated genes. Among then, categories related to the hormones pathways were selected, and eight DEGs involved in auxin and ABA homeostasis were then validated using qPCR. The phenotypic characterization indicated that ANE treatment led to a significant increase ($p<0.01$) in total root length and length of fine roots of plants grown under hydroponics conditions. The sugar yield of plants grown under field conditions was higher ($p<0.01$) in treated field plots as compared to the control. Our study uncovered relevant and positive effects of the ANE treatments in the regulation of hormone homeostasis and key traits related to sugar beet development and yield.

**Keywords:** sugar beet, root traits, RNA-seq, qPCR, molecular pathways, sugar yield, quality traits

## 1. Introduction

Environmental concerns about climate change and food security influence modern agriculture desire for environmentally friendly ways to sustain crop productivity and lessen its reliance on chemical fertilizers (Xu and Geelen, 2018). Plant biostimulants (PBs) have evolved into unique and sustainable agricultural inputs during the last few decades (Del Buono, 2021). Their impact on plants is mediated by various mechanisms, including the ability to create hormone-like activity and the stimulation of plant-soil microbe interaction (Nardi et al., 2017; Van Oosten et al., 2017; Hellequin et al., 2020; Della Lucia et al., 2021). Seaweed extracts are the major class of plant biostimulants, and for this reason, they are subjected to the study of multidimensional plant response. Seaweeds are macroscopic species, high in polyunsaturated fatty acids (PUFAs), polysaccharides, enzymes, and bioactive peptides, that are found in coastal and aquatic habitats (Courtois, 2008; de Jesus et al., 2013; Ahmadi et al., 2015; Shukla et al., 2018; Okolie et al., 2018).

Treatments with seaweed extracts can impact the transcription levels of plant genes encoding the endogenous synthesis of growth hormones such as cytokinin, auxin (IAA), gibberellic acid (GA), and abscisic acid (ABA) (Ali et al., 2019; Ali et al. 2021; Vijayanand et al., 2014; Craigie, 2011; Chouliaras et al., 2009; Kaladharan and Sridhar, 1999; Wally et al., 2013). IAA influences practically every stage of plant growth and development such as plant cell division and differentiation, translating in lateral root initiation and elongation (Zhang et al., 2022; Perrot-Rechenmann, 2010). ABA modifies plant physiological functions by

triggering the expression of genes related to the response to environmental stimuli (Zhang et al., 2022; Yamaguchi-Shinozaki and Shinozaki, 2006; Quach et al., 2014). In particular, ABA is substantially associated with drought stress (Zhang et al., 2022; Zhang et al., 2006). IAA signaling pathways components are necessary for a comprehensive ABA response in terms of growth changes (Zhang et al., 2022; Emenecker and Strader, 2020; Bouzroud et al., 2018), suggesting that ABA may be capable of integrating IAA signals to modify plant responses to environmental stresses.

The already demonstrated impact of ANE on plant hormone activities, has in consequence multifaceted effects on plant development and yield. But the mechanism that regulated all these plant responses is not well understood yet and need careful assessment in crops.

Sugar beet (*Beta vulgaris* L.) is a root crop that grows mostly in temperate areas and accounts for approximately 20% of global yearly sugar output (Biancardi et al., 2010). The use of PBs during critical stages could hasten root growth allowing for rapid soil colonization and, consequently, water and nutrient uptake. Barone et al. (2017) identified extracts from microalgae extracts such as *Scenedesmus quadricauda* and *Chlorella vulgaris* to be potential biostimulants in the initial growth stages of sugar beet cultivation through an enhanced nitrogen uptake and root development.

The use of multiple omics techniques combining high-throughput transcriptomics and phenomics was the most effective approach to characterize the PBs effects and their mode of action on important crops such as corn, soybean, and tomato (Della Lucia et al., 2021; Briglia et al., 2019). Particularly, the RNA-seq approach is used not only to clarify the mode of action of PBs but it might become a successful strategy to

develop new products (Franzoni et al., 2022). In this work, we aimed to assess the effects of *Ascophyllum nodosum* extract (ANE) on sugar beet. Molecular effects of ANE were verified by RNA-seq and quantitative real-time PCR analysis. Root morphology together with sugar yield and quality traits were also analysed to better elucidate the treatment effects.

## 2. MATERIALS AND METHODS

### 2.1. Chemical characterization of the ANE extract

The ANE extract used was provided by Sipcam SpA (Italy). Dry weight (DW) was measured by placing samples of the ANE extract in a drying oven until a standard weight at 105°C was reached. Samples were cooled for 2 h inside a closed bell jar, then the dry matter obtained was again weighted. The ANE extract ash content was estimated by incineration of the sample in a muffle furnace at 550°C to constant mass and was expressed as % with respect to DW. Carbohydrate content was assessed according to Moxley and Zhang (2007) by ion exchange chromatography using a Dionex DX500 system equipped with CarboPac PA20 using an isocratic elution of 20 mM NaOH. The sensor (pulsed amperometric-EDet1) used the Gold Standard PAD waveform with an AgCl reference electrode which included the following electrode potentials set as waveform A; E1: +0.1 V for 400 ms. E2: −2.0 V for 1 ms. E3: +0.6 V for 1 ms. E4: −0.1 V for 6 ms. Samples were prepared to treat 100 mg of the dried ANE with 3 mL of 72% $H_2SO_4$ (w/w) at 30°C for 20 min then diluted with 84 ml of distilled water and 4% $H_2SO_4$ (w/w) and finally autoclaved at 121°C for 20 min (Puglisi et al. 2018). The total carbohydrate content was expressed as g/kg of the dry weight (DW) of the extract. Lipids were extracted from 250 mg of freeze-dried samples using dichloromethane

following the method described by Folch et al. (1957). Total lipids content was expressed as g $kg^{-1}$ of the dry weight of the extract. Total protein content was quantified according to the Bradford (1976) method using BSA as a standard curve and was expressed as mg protein g $kg^{-1}$ of DW of extract. Total phenols were measured according to Arnaldos et al. (2001). Briefly, soluble phenolic acids were extracted with 3 mL pure methanol (1:10 w/v). The extracts were maintained in an ice bath for 30 min and then centrifuged at 5000 ×g for 30 min at 4 °C. The supernatants were stored at −20 °C until analysis.

### 2.2. Plant material

A diploid sugar beet hybrid (Cv. Beniamina, KWS, Einbeck, Germany) resistant to Cercospora leaf spot and rhizomania was used.

### 2.3. Hydroponic experiment

Seeds have been sterilized for 5 minutes in 76% ethanol and rinsed in distilled water three times. Seeds were put on wet filter paper and incubated in a growth chamber at 25°C for 48 hours. Germinated seeds were transplanted after germination into 500 ml glass pots with Hoagland solution (Arnon and Hoagland, 1940). After eight days, plants were transferred into box with multiple ANE dilutions (Control; 1:500; 1:1000; 1:2000; 15000; 1:10000). Leaf samples were collected 48 hours after ANE application. The experiment was performed in triplicate.

Root morphological traits were evaluated on 11-day-old seedlings using a scanner-based image processing method (WINRHIZO Pro. Regent Instruments. QC. Canada). To improve contrast, root systems were stained for 15 minutes with 0.1% (w/w) toluidine blue (Sigma-Aldrich. Montréal. QC). In a Plexiglas tray, the root systems were put in 3 mm of

water and lateral roots were distributed to reduce root overlap. The tray was scanned (STD-1600 EPSON) at a resolution of 1200 dpi. Total root length and fine root length were measured using the WINRHIZO software.

### 2.4. Field experiment

The effect of ANE treatment at molecular and yield levels was evaluated between March and August 2020 and 2021 in San Martino di Venezze. Rovigo. Italy (45°06'12.9"N. 11°53'52.5"E). The experimental design comprised four randomized blocks. Each randomized block was split into four sub-plots of 2.7 x 10 m. The sugar beet plant density in each sub-plot was 10 plants/$m^2$. An additional control-pot was added to the experiment. Plants were treated with foliar sprays of ANE solution at a rate of 1000, 500 and 250 ml $ha^{-1}$ (dilution 1:250; 1:500; 1:1000 respectively). The foliar biostimulant treatment was applied at BBCH32 and BBCH40. Leaf samples were collected 24, 48, and 72 hours after treatment. Then, samples were immediately transferred to dry ice and subsequently stored at −80°C for transcriptome and gene expression analysis.

#### 2.4.1. RNA sequencing

Samples treated at BBCH32 with the three dilutions of ANE doses were harvested 24 h, 48 h and 72 h after treatment for RNA-Seq analysis together with controls. Sampling was performed using a pool of two leaf disks from four plants from each experimental condition. RNA sequencing protocol was performed fully in-house and has also been described in Della Lucia et al. (2021): mRNA was extracted using the Dynabeads mRNA Direct Micro Kit (Thermo Fisher Scientific, Carlsbad, CA), then quantified by an Agilent 4150 TapeStation system (Agilent Technologies, USA). Sequencing libraries were prepared using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific). Libraries were quantified through D1000

screen Tape (Agilent Tapestation 1500), normalized to get a molar concentration of 100pM, pooled, and sequenced using three Ion 540™ Chips on the Ion Torrent S5 System (Thermo Fisher Scientific). In all the steps, manufacturer's instructions were followed.

### 2.4.2. Sequencing data and differential gene expression analysis

Low-quality reads were removed from the raw RNA-seq data with a phred-like Q value>20. Screened reads were mapped to the referenced sugar beet genome (publicly accessible from NCBI, GenBank accession GCA 000188115.3) by using Bowtie2 (v2.4.2) (Langmead and Salzberg. 2012). Samtools (v1.11) (Li et al. 2009) was used to examine the mapped files, and raw read values for all annotated genes were determined using bedtools multiBamCov (Quinlan and Hall. 2010). Non informative data were removed by filtering genes with a total expression level minor than 20 reads. To execute the inferential analysis and identify differentially expressed genes (DEGs) among the different experiments, the DESeq2 R package (v.1.30.0) (Love et al. 2014) was used. A p-value < 0.05 and a log2-fold change ≥ 1.0 were used as significant criteria to select the DEGs. Genes with log2-fold change > 1 were considered up-regulated, whereas genes with log2-fold change < 1 were considered down-regulated. DEGs were then functionally classified by Biological Process according with Gene Ontology (GO) enrichment analysis using the web-based toolkit ShinyGO v0.75 (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) at an FDR threshold of 0.05, and lollipop plots and tree hierarchical clustering of GO terms were generated on the same online platform.

### 2.4.3. Pots experiment

Sugar beet plants were grown in pots containing a substrate for the subsequent validation of the RNA-seq results. Briefly, sugar beet seedlings were grown in 13 cm diameter pots filled with standard peat substrate, pH 7. A mineral-based slow-release fertilizer (nitrophoska© type) was applied prior to seedling germination to the substrate at a rate of 20 g per pot. Fertilizer chemical composition included nitrogen, phosphorus, and potassium at a concentration of 12%, 12% and 17%, respectively; the fertilizer also had an additional 2% of MgO, 24% of sulfur, 0.02% of boron and 0.10% of zinc. Each pot was irrigated with around 250 ml of water, every two days. Water in excess was resupplied until the complete absorption by the peat substrate. Pots were maintained for 50 days in a climatic chamber at 25/20 °C and a 16/8 light/dark photoperiod. At BBCH 32 plants were treated with foliar sprays of ANE solution at a rate of 1000-, 500- and 250-ml $ha^{-1}$ (dilution 1:250; 1:500; 1:1000 respectively). Leaf samples were collected before treatments and 24 and 48 after treatment. Sampling was performed by taking two leaf disks per plants from each experimental condition. A total of four biological replicates were collected from treated and untreated plants Then, samples were immediately stored at −80°C for the validation analysis.

### 2.4.4. Validation of selected DEGs by RT-qPRC

Total RNA for validation was extracted using the RNeasy plant mini kit (Qiagen) following the manufacturer instructions. Table 1 lists the primer sequences and gene annotations used for validation. Primer Express V3.0 from Thermo Fisher Scientific was used to build primers using mRNA sequences of referenced sugar beet genome. Real-time quantitative reverse transcription PCR (RT-qPCR) amplification and

detection were performed using a Quant Studio 12K Flex Real-Time PCR (Thermo Fisher Scientific) and the Quantitect SYBR Green RT-PCR kit (Qiagen) using the one-step protocol. 5 µl of Syber Green + 0.5 µl of retrotranscriptase + 0.5 µl of both forward and reverse primers + 2.5 µl of nuclease-free water + and 1 µl of RNA were included in the 10 µl reaction mix. Each sample were run in triplicate. The resulting threshold cycle (Ct) values were standardized against the mean transcript levels of three housekeeping genes (GAPDH, Actin, and UBI) using the ΔΔCt method, where Ct is calculated as the difference between the target gene's Ct and the control gene's Ct (Livak et al. 2001; Schmittgen and Livak. 2008). Then, to assess the level of the fold, the level of expression before the treatment was use as control, for the treated and untreated conditions, in the different timing.

### 2.4.5. Yield analysis

ANE was tested for its effects on sugar beet yield traits such as root yield, sugar yield, and processing quality-related parameters. After BBCH 49 harvestable size of the beetroot-topped sugar beets from each subplot were gathered and assessed to determine the average of root weight yield, sugar yield, and processing quality-related parameters as impacted by ANE treatment. A total of 100 sugar beet roots were collected from each subplot. Roots of each plant were cleaned before being sawed into 1 kg of micronized tissues (brei) using a specialized saw (AMA-KWS. AMA Werk GmbH. Alfeld. Germany). Approximately 70 g of homogenized brei samples were promptly frozen at -40°C. Sugar content and major non-sugars were determined using an automatic brei-mixer following cold digestion of the brei in lead acetate 0.75% (w/w) solution (Schneider, 1979), (Venema Automation b.v. Groningen, The Netherlands). A Thorn-Bendix 243 polarimeter (Bendix Corp, Nottingham, UK) was utilized to

quantify the sugar content. Finally, a flame photometer was employed to determine the K and Na contents (Model IL 754. Instrumentation Laboratory S.p.A., Milan, Italy). The colorimetric analysis (PM2K; Carl Zeiss GmbH, Oberkochen, Germany) method proposed by Kubadinow and Wieninger (1972) and Stevanato et al.2010 was employed to measure the -amino N. The purity was calculated by Wieninger and Kubadinow (1971) and Stevanato et al. (2010) as the proportion of sugar from the roots that the manufacturer could extract and was quantified at 405/492 nm using plate reader Uniplan AIFR-01 (CJSC Picon. Russia).

## 3. Results

### 3.1. Chemical characterization of the ANE extract

The chemical composition of the ANE biostimulant assessed in this study varied significantly. The results from Tab. 2 show the constituents of ANE biostimulants was analysed by determining the amounts of certain major components evaluated by total DW such as dry matter ash, total carbohydrates. And polyphenols. As expected, the most abundant elements are carbohydrates and phenolic compounds which account for up to 60% and 15% of all matter.

### 3.2. Root morphological analysis of plants grown in hydroponics

To evaluate if the ANE treatment on sugar beet leaves has an impact on the root system, root morphological characteristics (total root elongation and length of fine roots) were measured. Morphological changes were observed after 48h treatment (Fig.1). Each data point is the mean of data values from forty seedlings per each of the 5 ANE dilutions, plus the control. The treatment significantly improved plant growth, root size, and morphology of sugar beet plants (Tab. 3). ANE treatment, regardless the dilution, showed notable stimulatory activity in root elongation: significant ($p<0.05$) variations were measured from the plants treated with different concentrations observed and showing a positive effect on root traits compared with untreated. Conversely, in comparative analysis with the control, lower dilution of extracts (B, the dilution of 1:500 in Fig. 1) showed the least efficiency in promoting root development. Significant improved root morphological traits are observed in dose 1:5000 and with the dilution of 1:10000. In general, plants that were supplemented with ANE had a larger root surface than untreated plants and longer thin roots than controls.

### 3.3. Transcriptomic analysis – field plants

RNA-seq profiling and gene expression analysis has been performed to understand the molecular reaction of leaf treated with ANE on sugar beet under field conditions at growth stages BBCH 32. Leaf samples were collected at three sampling time points (24, 48, and 72 hours after treatment), from plants treated with the three ANE dilutions and the control plants. After removing low-quality adaptor and barcode sequences, 90.82, 88.51 and 91.29 total million raw reads were obtained from the transcriptome libraries of leaf treated with different dilutions of

ANE (1:250, 1:500 and 1:1000) each treatment has two-timed points (after 24h and 48h) at BBCH32, respectively. More than 97.75% exceptional reads (clean reads) were clustered and utilized for downstream assessment. The alignment outcome projected that 68.21%-71.03% of clean reads credibly mapped with respective to specified genome. 57.51 (63.3%), 55.30 (63.82%), and 57.95 (64.79%) million reads from cDNA libraries were contrarily mapped on the referenced genome. After setting up the mapped reads ensued in the recognition of a total of 15.507 genes in all the samples that were used for further analysis effect induced by ANE leaf treatment in *Beta vulgaris* L. transcriptome.

### 3.3.1. Identification of DEGs response to ANE treatment

DEGs were analyzed for the three different ANE dilutions (1:250; 1:500; 1:1000) in respect to the untreated samples, at three time points (24, 48 and 72 hours after the treatment), at BBCH32. Most DEGs have been found after 24h and 48h compared to the DEGs identified at 72h. The comparison between various times of application and control plants revealed a total of 6064 DEGs sorted into 2625 downregulated and 3439 upregulated genes. A total of 4038 significant DEGs ($p < 0.05$) were found 24h after the treatment, irrespectively to the dilution used. Among them, 2649 were up-regulated genes and 1389 were downregulated genes. A total of 2026 significant DEGs ($p < 0.05$) were found 48h after the treatment, irrespectively to the dilution used. Among them, 790 were up-regulated genes and 427 were downregulated genes. Results for 72 are not reported since the DEGs analysis did not shown any significant differentially expressed genes.

By looking at DEGs with $p < 0.05$, the dilution 1:500 was the one stimulating the higher number of up-regulated genes at 24 hours after the

treatment (1108 DEGs) but at 48 hours after treatment the best dilution was 1:250 (317 DEGs). Nonetheless the dilution 1:1000 was the one with the highest number of down-regulated DEGs in both the timing (620 DEGs at 24 h and 481 DEGs at 48 h). This dilution was also the one with the less up-regulated DEGs in both the timing (666 DEGs at 24 h and 182 at 48 h after treatment). These results indicate the dilution 1:1000 as the less performative among the dilution triade. A graphic view of these results is provided by the Volcano plots in fig. 3 where it is possible to appreciate the different distribution of up-regulated and down-regulated DEG for all the treatments in the two different timing. In the Volcano plots, DEGs at $p < 0.05$ are shown, but also DEGs fund after adjusted $p < 0.1$. Based on the number of DEGs with adj $p < 0.1$ a Eulero-Venn diagram analysis was performed.

Looking at the common DEGs, the Eulero-Venn diagrams in fig. 2a and fig. 2b, highlight few numbers of genes in common between different dilutions in the two-sampling timing. The most interesting results are genes in common between the three dilutions: at 24 hours after treatment there were only 20 for up-regulated DEGs and 3 for the down-regulated; at 48 hours after treatment there were zero up-regulated DEGs and 65 down-regulated DEGs. Taken together with the number of DEGs, these results underline a dose-dependent effect of ANE on gene expression, but it is not able to clearly provide time-dependent effect of the ANE treatment.

### 3.3.2. Gene ontology (GO) analyses of DEGs

Differentially regulated genes after 24h and 48h treatments were sorted based on their functional category and a GO enrichment analysis was performed through the web-based tool ShinyGO v0.75.

The complete list of GO terms allied to DEGs is provided in fig. 4 and fig. 5., where it is shown the significantly enriched GO terms correlating to the biological processes of up-regulated and down-regulated DEGs in response to ANE after 24h and 48h (FDR < 0.05).

A cluster analysis on gene lists from the GO analysis was also performed to highlight specific pathways with significant homogeneity in their DEGs composition. From the GO analysis the gene lists related to hormonal pathways were clustering together and “Cellular response to auxin stimulus” was the category with the highest fold enrichment value. All the pathways related to hormones in general were also clustering together. Based on this, candidate genes related to the hormonal pathways were selected from these genes lists for the upregulated genes. The downregulated candidate genes were selected from the GO category with the highest number of gene DE, which is “Organonitrogen compound metabolic process”. The complete list of the candidate genes for validation is reported in table 5. From this table, eight genes were selected based on available literature of their functions, for the next step of validation.

### 3.3.3. **Gene expression analysis – validation**

To validate the RNA-Seq results, the expression calibration of eight DEGs with phytohormonal activity was evaluated using qRT-PCR. Eight genes were selected involved in auxin and ABA hormonal homeostasis, as well as receptors-like kinases (RLKs) genes. The selected genes are named *ARF19. NH23. YUCCA6. PYL4. MYB30. PP2C62. HAIKU2. And HSPRO2.* Real-time RT-qPCR has been conducted on five plants per treatment at BBCH32 phenological stages at various times of application (before treatment, after 24 h and after 48 h). When fold change was < 2, gene was considered down-regulated while when fold-change was > 2,

genes were considered up-regulated. A value of 2 is commonly used in study related to analysis of DEGs, as a fold change cut-off. According to the overview of GO and qRT-PCR evaluation, the selected eight genes were principally showing a stimulatory response after ANE biostimulant. Interestingly, the relevant hormone-related genes are differentially expressed. Among the selected genes 3 genes, *PYL4, MYB30 and PP2C62* encode ABA-related genes. *MYB30* along with *PYL4* were showing always a fold change < 2, meaning that they were always down-regulated. While *PP2C* was always up-regulated in treated plants, but down-regulated in the untreated control (fig 6).

Three genes *ARF19. NH23* and *YUCCA6* was selected in response to Auxins stimulation. These targeted genes were continuously upregulated in both sampling times, which correlated with the positive effect on auxin biosynthesis. In the untreated control instead, the level of the fold change was always < 2, indicating that the three genes were down regulated in absence of a treatment, over time (fig. 7)

In general, treated plants showed higher-level gene expression related to auxins compared to control.

Two genes are recorded for their membrane receptor activity *HAIKU2 and HSPRO2.* And gene expression changes in membrane receptor activity induced by the application of ANE are presented (fig. 6). Promising effects were observed in both the leaf-treated sample with ANE and the control on different sampling times. Treated plants showed higher values for the *HSPRO2* and lower values for *HAIKU2* among the different dosages but when compared with the untreated both the genes were overexpressed. Overall comparative expression levels of the eight identified genes indicated distinct levels of expression between ANE treated and untreated plants.

## Yield analysis

The effects of the interaction between foliar application of ANE biostimulant on the sugar beet in open field conditions were measured through the evaluation of the yield and other quality parameters on sugar beet taproot. An ANOVA was used to elucidate differences between the treatments with a 0.05 p-value threshold and evaluated traits are presented in Table 3. Plants treated with A. nodosum extract produced higher values of root yield content (77.20, 76.80 and 76.10 t/ha) and sugar yield (12.70, 12.00 and 11.90 t/ha). We found no relevance effect of treatment on the aspect of sugar purity like K. Na. and α-amino-N constituents in the sugar beet root juice in treated plants compared to untreated. Interestingly evident that improved root morphological traits in hydroponics conditions with the improved root yield and final sugar content in the open field experiment, were significantly improved in response to the foliar treatment of ANE extract in both growing conditions, regardless of ANE dosages.

## Discussion

Plant biostimulants are a new group of agricultural products that serve to boost crop quality and yield while guarding against biotic and abiotic challenges (Rajput et al., 2019). Effects vary depending on application and dose and are also affected by various agronomical and environmental factors (Di Mola et al., 2019). The ability to predict plant response to biostimulants is necessary, in the outgrowth of sustainable agriculture.

The impact of ANE on plant growth is multidimensional and leads to an overall increase in production. Previous research has shown that

seaweed extracts are neither precisely antibacterial nor abundant in nutrients. They, instead, operate ambiguously on plant metabolism by generating signalling cascades that begin reactions that lead to biotic and abiotic stress mitigation together with an increased growth and performance as a result (Baipal et al., 2019; Nair et al., 2012). Due to the vast range of bioactive components found in seaweeds. there is only little knowledge of the intricate mechanisms by which seaweed extracts affect plant development processes (Ramkissoon et al., 2019; Jayaraj et al., 2009). In this study, an ANE was used to evaluate their effects on sugar beet plants under controlled and field conditions.

We focused on the plant's transcriptome because biostimulants have a broad spectrum of activity that encompasses numerous metabolic pathways in plants and biological processes, involved mainly in hormone homeostasis. Then, the detailed characterization of the expression levels on selected genes gene in the three distinct doses by qRT-PCR correlates with the time of application was done.

Out of eight genes, three genes are related to ABA-related function: PYL4 (abscisic acid receptor), MYB30 (Transcription factors) and PP2C 62 (Phytohormones synthesis). MYB30 is responsible for some regulatory roles in root development with ABAs signalling. But it is down streamed along with PYL4 genes which function as an ABA receptor. And *PP2C62,* which is a probable protein phosphatase gene, was continuously upregulated in both sampling times.

It is known that PYL4 act as an ABA receptor, it is also reported by Dittrich.et al..2019 that PYL4/5 (abscisic acid receptor) is necessary for a CO2-induced guard-cell response and regulating the stomatal functioning in Arabidopsis.

Lackman et al,.(2011) exposed that overexpression of NtPYL4 led to a reprogramming of the cellular metabolism that reduced alkaloid accumulation and gave ABA sensitivity for synthesis of alkaloids in tobacco hairy roots.

MYB30 is noted as a transcription factor acting as an ABA-responsive factor. In our study downregulation of MYB30 shows a significant correlation with ABA signalling. Satomi et.al. 2018 tested MYB30 with plant hormones and reported that MYB30 influences root growth regulation under ABAs signalling (Sakaoka et al., 2018). Also, an effective regulator of the hypersensitive response (HR) programmed cell death linked to pathogen resistance in plants, and BR signalling has been identified as the R2R3-MYB transcription factor MYB30 (Zheng et al., 2012). MYB30 is involved in enhancing HR and BR signalling by instantly interacting with BES1 and improving its activity (Li et al., 2009). Furthermore, MYB30 influences HR and disease resistance by regulating salicylic acid (SA) status and the expression of SA-related genes (Raffaele et al., 2006). According to our findings, ABA signalling is also impacted by MYB30.

PP2C is a well-known as a negative regulator of ABA synthesis and its function and role in ABA signalling is reported by several authors in literature (Qui et al., 2022; Ali et al., 2020; Jung et al., 2020; Umezawa et al., 2010; Meyer et al., 1994).

The PYR/PYLs, PP2C along with SnRK2s and ABF ABA receptors, form together the core network of ABA signal control (Zhu, 2016). The treatment with the ANE analysed in this work can modulate these three genes, and from the results, the major response is the ABA signalling interruption, due to the up regulation of PP2C, prior to the dosage, but with a more longer lasting effect when the dose is high. The higher dose

last more, probably because, in practical terms, more ANE is distributed on the plant, reducing any loss of product due to evaporation or any other environmental factor that can enhance product losses. In other term, a higher dosage, in open field can be more efficient for these reasons. The inhibition of the ABA signalling by the way, trigger a non-stress status response, which can result in higher crop performance, and a general healthy status.

Another three genes encoding for the Auxin metabolism: ARF19 (Phytohormones transporter), NH23 (Defensive genes), and YUCCA6 (Phytohormones synthesis). We found that the ARF19 gene actively functions in the auxin metabolism. A similar correlation with Li et al., (2016) that demonstrated the recent biochemical and genetic study of the auxin response factor (ARF). Through proteins involved in DNA binding, transcriptional activation, or repression, ARF genes control many plants' developmental phases, and ARFs function as a crosstalk centre between the auxin and ethylene, playing also an important part in ethylene regulations in A. thaliana (Li et al., 2006).

We also found that the up regulated phytohormone synthesis gene YUCCA6 belongs to the YUCCA family; a "TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA)/YUCCA (YUC)" route and is almost significant and well distinguish auxin production pathway in plants [61].

Plant growth has been observed to be greatly aided by indole acetic acid (IAA) and numerous cytokinins (Cao et al., 2019. IAA engages in a comprehensive growth activity including cell division, vascular expansion, root development, and apical dominance. Additionally, ABA can prevent IAA-mediated lateral root primordia in peanut by reducing AUX-dependent auxin transport *Arachis hypogaea* (Guo et al., 2017).

According to Zhao et al. (2015) ABA regulates IAA homeostasis on rice through modulating IAA-related gene expression in *Oryza sativa*, revealing the connectivity between the IAA and ABA signalling pathways.

All auxin-related genes reported in this study well correlated with previous research works and are highly responsible for auxin metabolism. The enhanced root phenotype correlates with auxin stimulatory activity by the ANE treatment indicating that auxin can promote cell division in the root pericycle, essential for the beginning and elongation of adventitious roots (Da-Xi et al., 2003). So, their up regulation can explain the morphological results observed in this study, which directly influenced also the final yield discovered in the field.

In this study, two genes were indicating RKLs related genes: HAIKU2 (receptor-like protein kinase) and HSPRO2 (Phytohormones transporters). HAIKU2, a broad group of genes with functions in signal transduction mechanisms in plant growth and metabolism, encodes a leucine-rich repeat receptor kinase. Extracellular transmembrane and kinase domains are all present in receptor kinases (Luo et al., 2005). HSPRO2 was previously reported by Cai et al., (1997) as a Leucine Rich Repeat (LRR) protein homologue of the resistance-granting sugar beet $HS^1PRO^1$ to *Heterodera schachtii*, a sugar beet cyst nematode, and engages in interactions with the protein kinase SNF1 (Gissot et al., 2006). HSPRO2 elevation is effect of Salicylic Acid (SA) and restrained by jasmonic acid/Ethylene (JA/ET) (Bose et al., 2018). Our study suggests the HSPRO2 is also linked to ABA signalling when treated with ANE. Our growing results emphasize that carbohydrates present in seaweed as major components in triggering plant biostimulant activity, as also reported in (Carmodi et al., 2020).

These findings elucidated by an activation of the plant signalling system in response to the application of ANE leaf treatment as compared to untreated plants with the help of molecular analysis. The increased plant growth paraments observed in the present study included root morphology, since transcription of genes responsible for these variables was significantly influenced in treated plants compared to control. Auxin play a fundamental role in this.

Auxin signalling is improved during lateral root development when some ARF genes, that are brassinosteroid-insensitive, are phosphorylated (Li et al., 2016). It was determined that root morphological features such as root elongation rate, total root length, and the number of root tips under hydroponics had increased significantly.

These findings are especially intriguing for sugar beet since roots influence plant development and water-nutrient uptake, which improves final sugar content (Stevanato et al., 2010).

In terms of agronomic yield traits of sugar beet, we studied plant biomass and sugar content leaf application of the ANE-based biostimulant. Our results show that treated plants have relatively high sugar yield when compared with untreated plants. We found no discernible variations in the impurity level between treated and control plants concerning the connection among ANE treatments. This is in contrast to Rahimi et al. (2020) whose research revealed a significant drop in Na, K, α-amino N. Therefore, hormonal homeostasis-induced improvements in root morphological traits are directly reflected in final yield without a significant impact on the quality.

A visual description of the gene expression induction and repression after the ANE is reported in fig.

For the several reasons described before, the increment of yield and sugar content in this work correlated with the imposed treatment with ANE, but this cannot explant the inefficacy on the quality parameter.

## Conclusion

The genes investigated in this work reflect several metabolic processes that are closely tied to the development of the sugar beet root morphology. Additionally, treated root morphological analysis from controlled condition evident ANE stimulatory activity led to better root morphological features and correlates with remarkable yield attributes in open field sugar beet plants. A pathway for cytokinin and GA was not investigated in this current work. Further study should investigate this trend also in other crops, to search shared or unique responses to ANE, and search a common ground for ANE mode of action.

**Table1.** Gene name and primer sequences of validated genes with qPCR. The eight DEGs selected for validation are listed, they are divided according to the hormonal cluster they belong to. Each gene description is also provided. Primer forward (PF) and reverse (PR) are given for each gene.

| Gene Cluster | Gene name | Gene description | Primer sequence (5'-3') |
|---|---|---|---|
| **ABA-related genes** | *PYL4* | Abscisic acid receptor | PF: TGAAACCCTCGTTAGCTCATGA<br>PR: TGGAGATGGGCAGCAGAGA |
| | *MYB30* | Transcription factor MYB30-like | PF: GCGCGGCCCTTGAAA<br>PR: ACCCCTGAACAAGCCTCTGA |
| | *PP2C62* | Probable protein phosphatase 2C 62 | PF: AATTCGGAGATGCAGGTGAAA<br>PR: TCTCTCTCCAATTCTGCTTCATTTT |
| **IAA-related genes** | *YUCCA6* | Indole-3-pyruvate monooxygenase | PF: GGAGGCGGCAGTGACAAC<br>PR: GTCGCCACCACCAACCA |
| | *ARF19* | Auxin response factor 19 | PF: ACTTTACCTGGCTCCACAGCTT<br>PR: TCCTAGTTGACGGGATAGATCAGAA |
| | *NH23* | Nudix hydrolase 23, chloroplastic | PF: CCGTTTTAGACCGTTCCGAAT<br>PR: GAAGAAGAGGAAGCACTTAAATTTGAG |
| **RKLs-related genes** | *HAIKU2* | Receptor-like protein kinase | PF: TGCAACAACGCTGGAAATGT<br>PR: GGGAGCGTGCCAGATAAGC |
| | *HSPRO2* | Nematode resistance protein-like HSPRO2 | PF: TTCAATCAACACCAAATCTCACAA<br>PR: GTGAAATTACCGGCGTTGAAG |

**Table 2.** Composition of the of the ANE biostimulant. Each value is expressed based on the total dry matter of the ANE extract.

| Characteristic | Quantity |
|---|---|
| Dry matter (g $l^{-1}$) | 90.39 ± 2.3 |
| Ash (%) | 29.58 ± 0.9 |
| Carbohydrate (g $kg^{-1}$) | 387.70 ± 12.1 |
| Lipids (g $kg^{-1}$) | 2.50 ± 0.1 |
| Protein (g $kg^{-1}$) | 37.80 ± 0.8 |
| Total phenolic compounds (g $kg^{-1}$) | 101.20 ± 1.8 |

**Table 3.** Mean values of root yield. sugar yield. and processing quality-related traits in ANE-treated and untreated sugar beet grown in San Martino di Venezze. Rovigo. Italy (45°06’21.9’’N; 11°53’40.1’’E). Results are expressed as the mean of four randomized replicates with 60 plants each. ANOVA was used to evaluate differences between the treatments with a 0.05 p-value threshold. Mean values followed by asterisk differ significantly from untreated samples.

| Treatment | Root yield (t $ha^{-1}$) | Sugar yield (t $ha^{-1}$) | Potassium (meq % °S) | Sodium (meq % °S) | Alfa-N (meq % °S) | Sugar purity (%) |
|---|---|---|---|---|---|---|
| Control | 75.6* | 11.4 | 26.12 | 5.56 | 5.71 | 92.2 |
| 1:250 | 76.1 | 12.7 * | 25.37 | 6.31 | 5.92 | 92.4 |
| 1:500 | 77.2* | 11.9 * | 27.17 | 5.42 | 6.15 | 91.9 |
| 1:1000 | 76.8 | 12.0 * | 25.14 | 5.23 | 5.54 | 92.3 |

**Table 4.** Table showing the number of DEG genes (up and down) at each timing and dilution

| Sampling time after treatment | Dilution | Downregulated | Upregulated |
|---|---|---|---|
| 24 hours | 1:250 | 471 | 875 |
| | 1:500 | 298 | 1108 |
| | 1:1000 | 620 | 666 |
| 48 hours | 1:250 | 354 | 317 |
| | 1:500 | 401 | 291 |
| | 1:1000 | 481 | 182 |

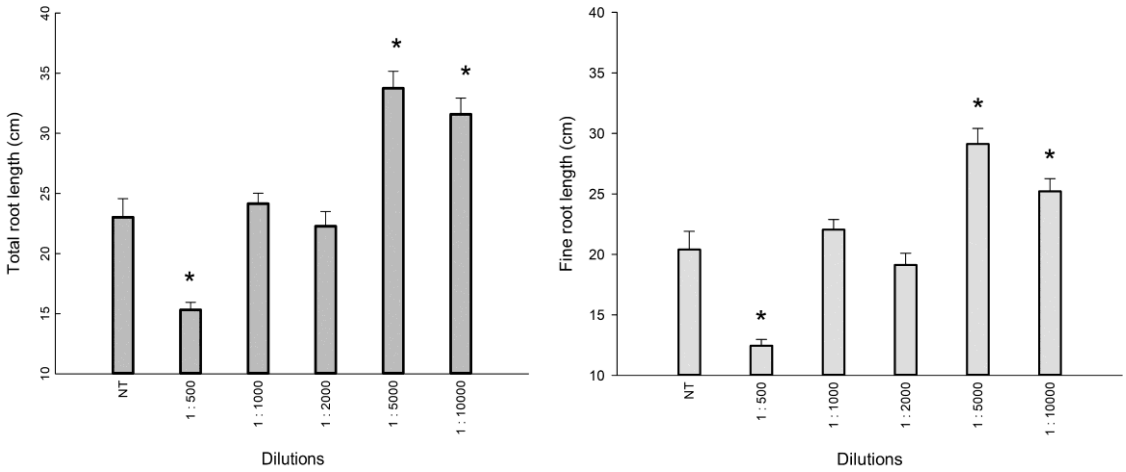
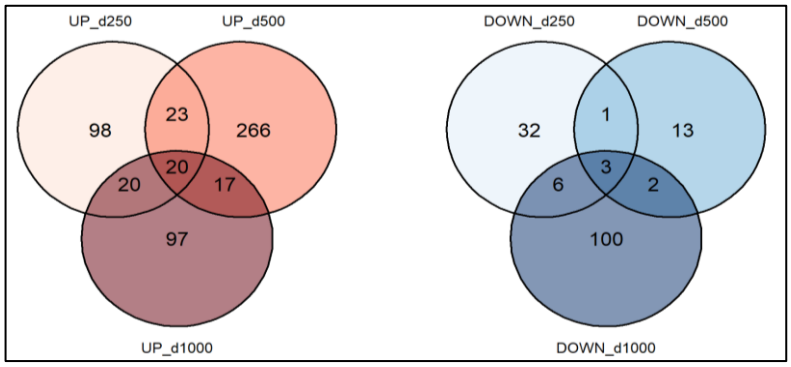



**Figure 1**. Bar plots of total root length (cm) and fine root length (cm) of seedlings grown in hydroponic conditions. "*" means dilutions statistically significative at $p < 0.05$, after Duncan's post hoc test

a.




b.

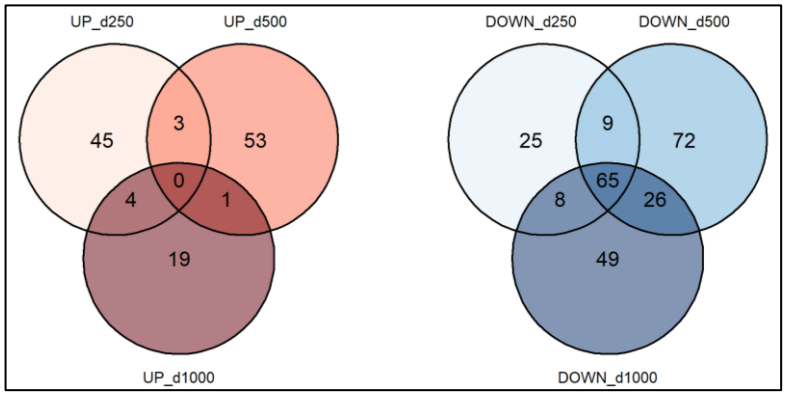



**Figure 2**. Number of DEGs specific and unique for each dilution**.** DEGs at the three dilutions in respect to the control, 24 hours after treatment (a) and 48 hours after treatment (b)

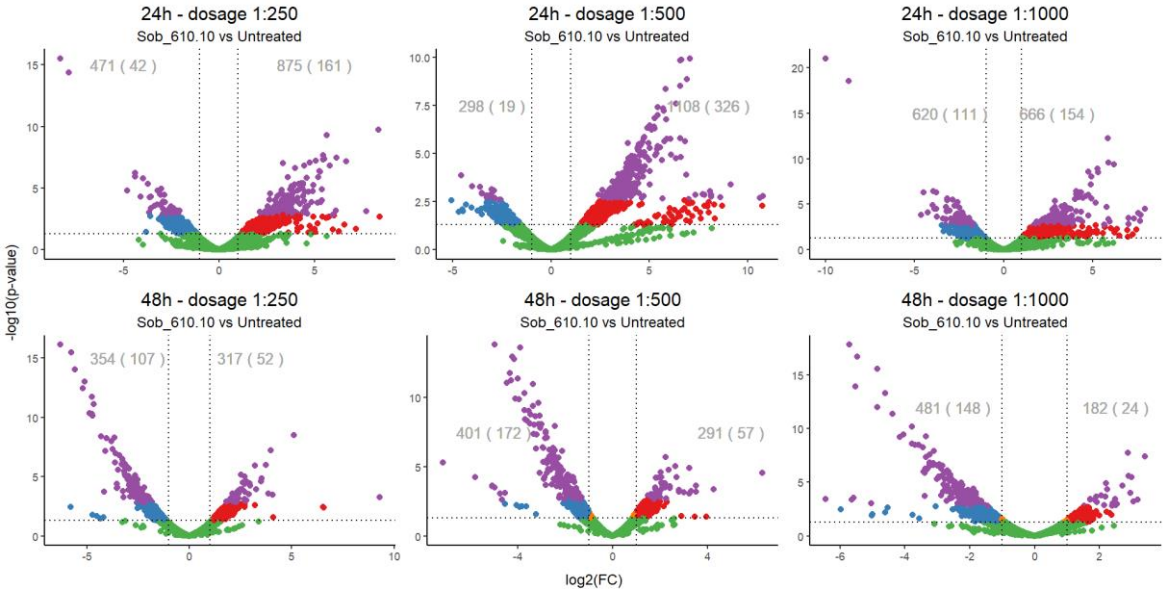



**Figure 3**. Volcano plots showing DEG genes (p-value<0.05) at 24h and 48 h after treatment. Per each dilution. Green and orange dots are genes not DE for the p-value or the FC; Red dots are up-regulated genes (p-value<0.05); Blue dots are down-regulated genes (p-value < 0.05); Purple dots are genes DE due to the adjusted p-value (p-adj<0.1). Sob 610.10 is the code name of the ANE used in this work.

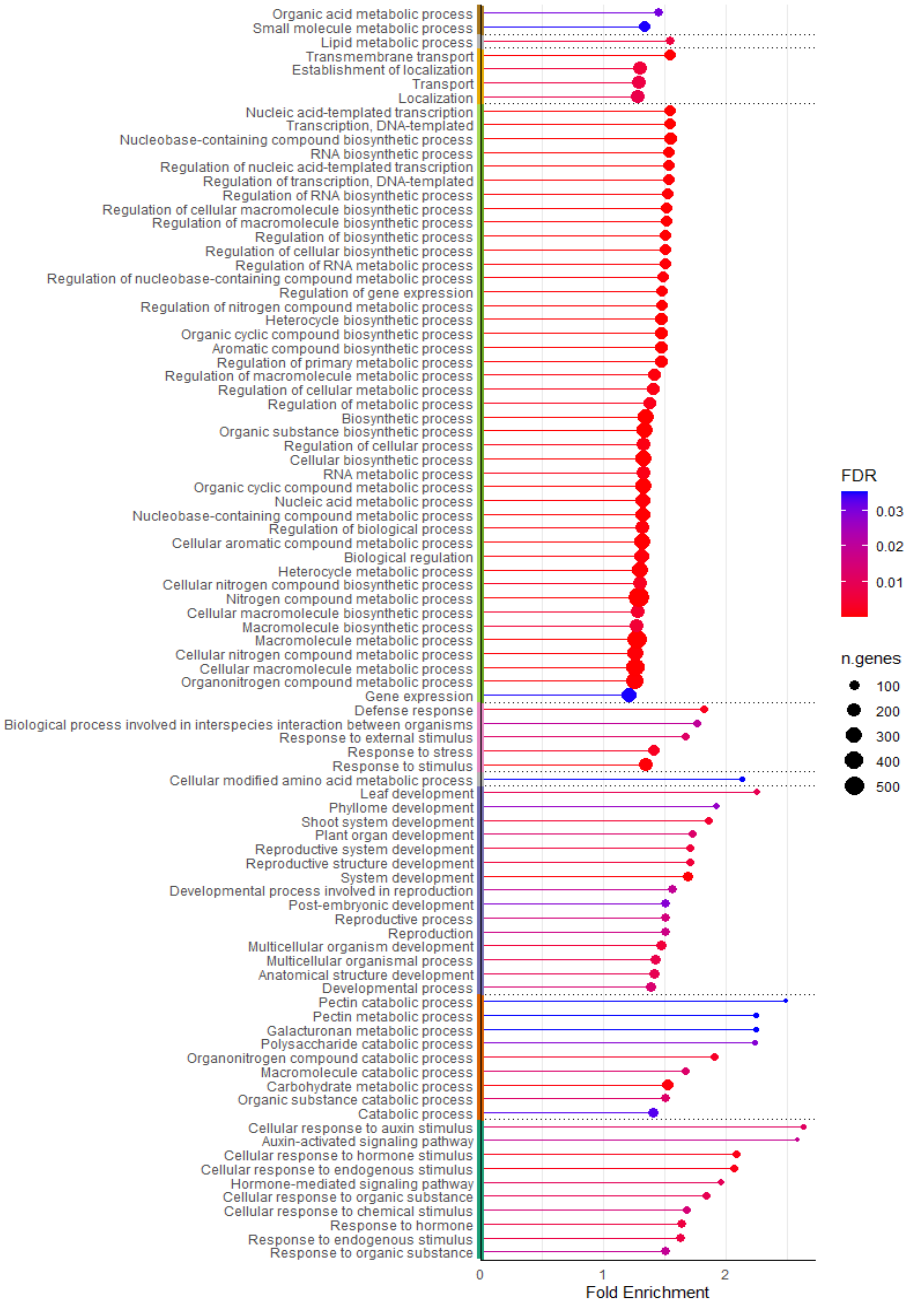



**Figure 4.** Enrichment chart showing fold enrichment analysis of GO biological processes for up-regulated genes after ANE treatment. The cluster analysis based on similarity is also reported and each homogeneous group is separated by a dashed line. In each cluster, pathways are in descending order.

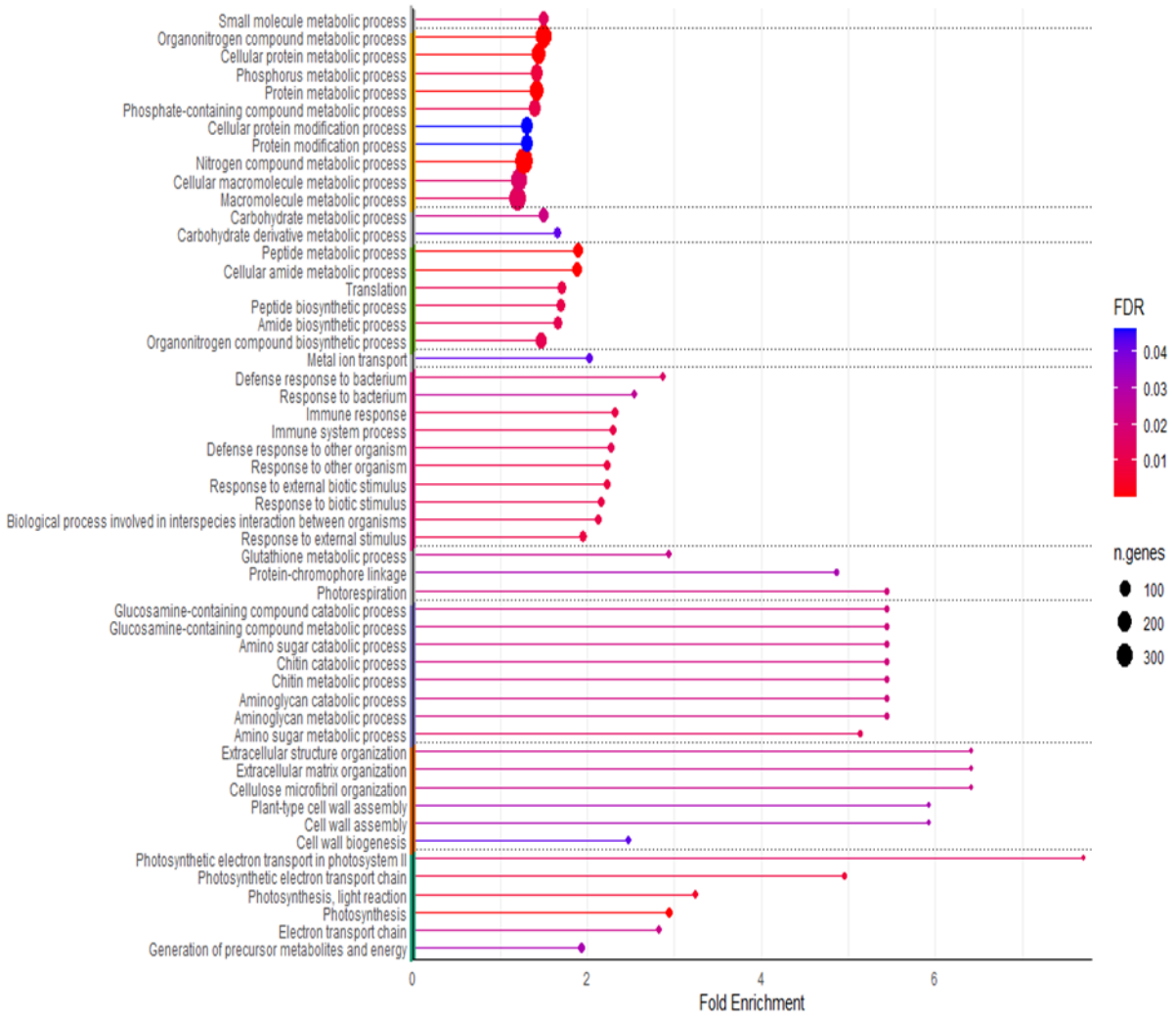



**Figure 5**. Enrichment chart showing fold enrichment analysis of GO biological processes for down-regulated genes after ANE treatment. The cluster analysis based on similarity is also reported and each homogeneous group is separated by a dashed line. In each cluster, pathways are in descending order.

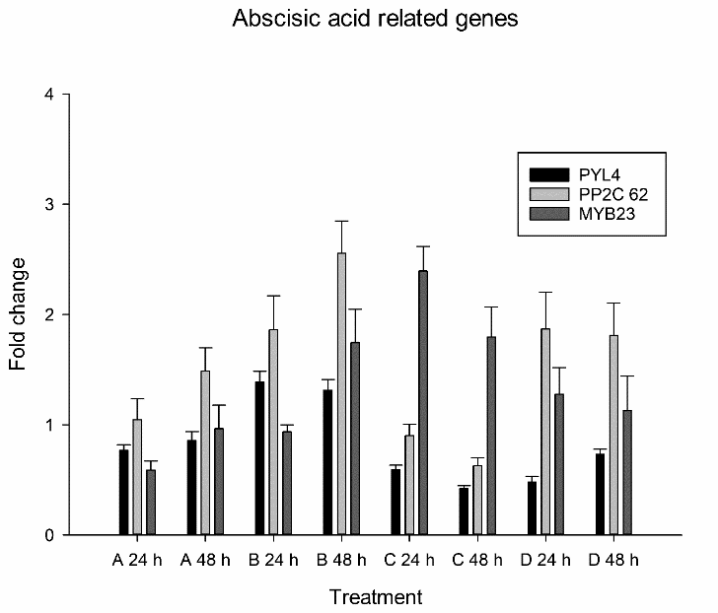



**Figure 6**. Barplot of the fold change for gene expression analysis of the ABA-related genes 24 and 48 hours after treatment. Each barplot shows the mean of 8 replicates with the standard error of the mean. (A: Utck; B: 1:250; C: 1:500; D: 1:1000).

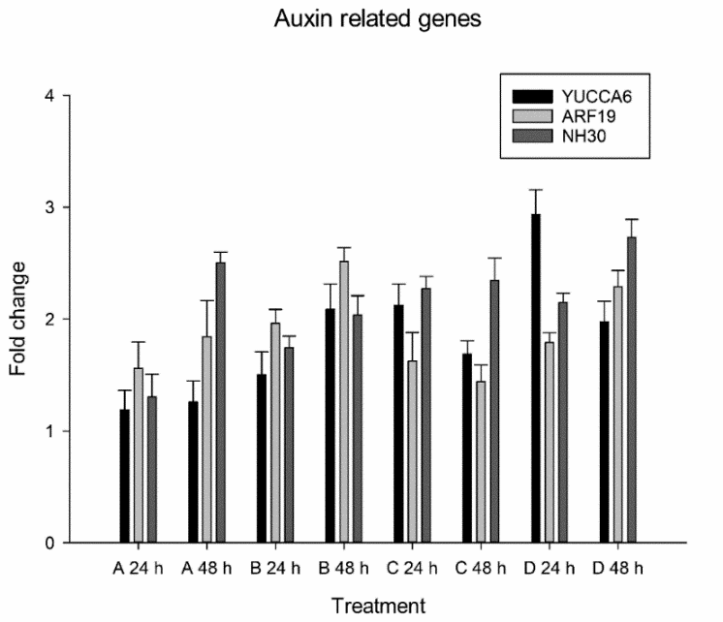



**Figure 7**. Barplot of the fold change for gene expression analysis of the auxin-related genes 24 and 48 hours after treatment. Each barplot shows the mean of 8 replicates with the standard error of the mean. (A: Utck; B: 1:250; C: 1:500; D: 1:1000).

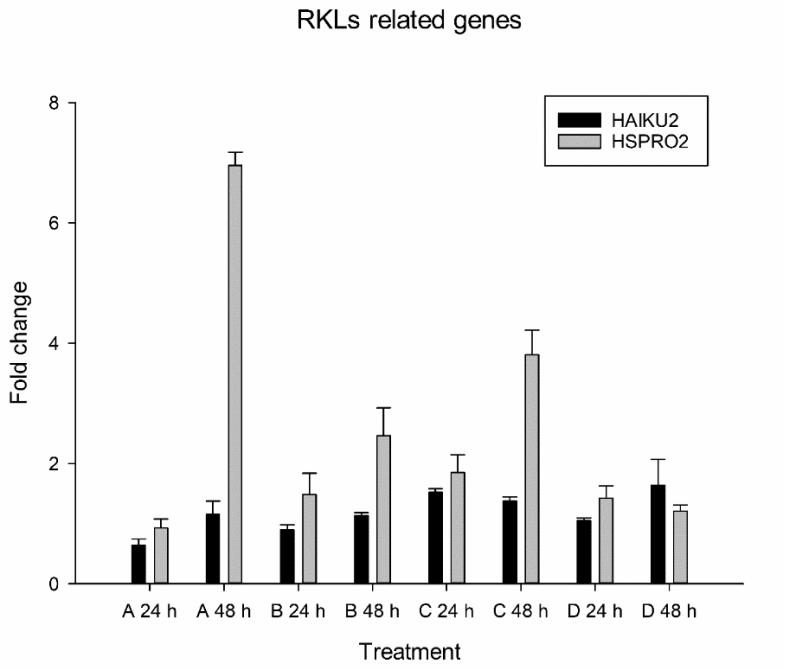



**Figure 8**. Barplot of the fold change for gene expression analysis of the RLKs-related genes 24 and 48 hours after treatment. Each barplot shows the mean of 8 replicates with the standard error of the mean. A: Utck; B: 1:250; C: 1:500; D: 1:1000.

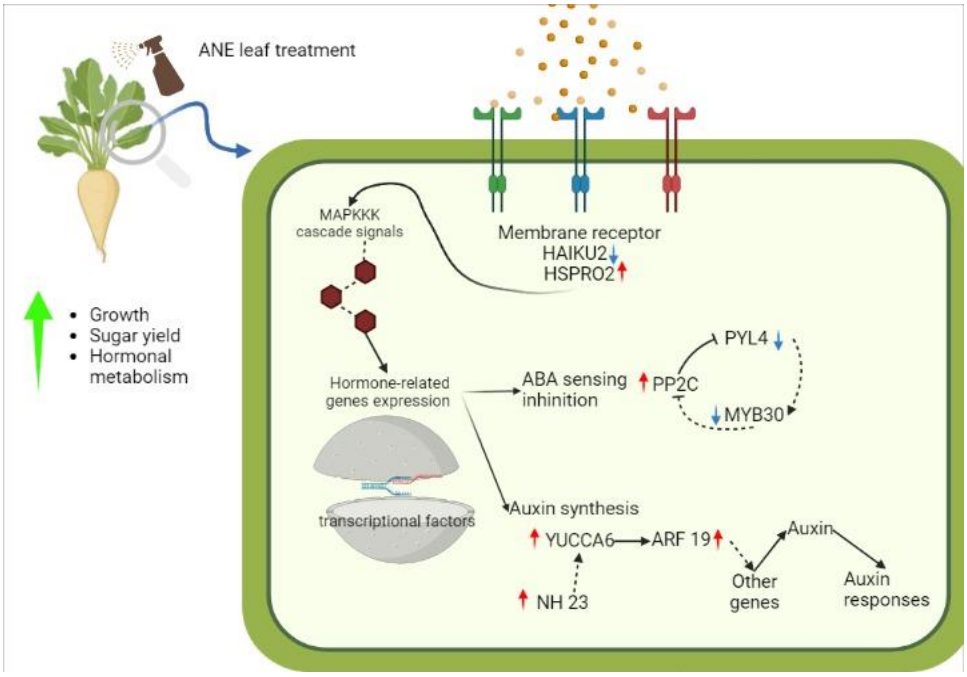



**Figure 9.** ANE mode of action on sugar beet, based on the effect of the biostimulant on genes related to ABA and auxin.

## References

1. Xu, L. and Geelen, D. Developing Biostimulants From Agro-Food and Industrial By-Products. 2018 *Front. Plant Sci.* 9:1567. doi: 10.3389/fpls.2018.01567

2. Del Buono. D. Can biostimulants be used to mitigate the effect of anthropogenic climate change on agriculture? It is time to respond. Science of the Total Environment 2021. 751. 141763.

3. Nardi. S.; Pizzeghello. D.; Schiavon. M.; Ertani. A. Plant biostimulants: physiological responses induced by protein hydrolyzed- based products and humic substances in plant metabolism. Scientia Agricola 2016. 73. 18–23.

4. Van Oosten. M.J.; Pepe. O.; De Pascale. S.; Silletti. S.; Maggio. A. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. Chemical and Biological Technologies in Agriculture 2017. 4. 1–12.

5. Hellequin. E.; Monard. C.; Chorin. M.; Daburon. V.; Klarzynski. O.; Binet. F.; et al. Responses of active soil microorganisms facing to a soil biostimulant input compared to plant legacy effects. Scientific Reports 2020. 10. 1–15.

6. Della Lucia. M.C.; Bertoldo. G.; Broccanello. C.; Maretto. L.; Ravi. S.; Marinello. F.; Sartori. L.; Marsilio. G.; Baglieri. A.; Romano. A.; et al. Novel effects of leonardite-based applications on sugar beet. Frontiers in Plant Science 2021. 12. 646025.

7. Courtois. J. Oligosaccharides from land plants and algae: production and applications in therapeutics and biotechnology. Current Opinion in Microbiology 2009. 12. 261–273.

8. de Jesus Raposo. M.F.; de Morais. R.M.S.C.; de Morais. A.M.M.B. Health applications of bioactive compounds from marine microalgae. Life sciences 2013. 93. 479–486.

9. Ahmadi. A.; Zorofchian Moghadamtousi. S.; Abubakar. S.; Zandi. K. Antiviral potential of algae polysaccharides isolated from marine sources: a review. BioMed research international 2015. 2015.

10. Shukla. P.S.; Borza. T.; Critchley. A.T.; Hiltz. D.; Norrie. J.; Prithiviraj. B. Ascophyllum nodosum extract mitigates salinity stress in Arabidopsis thaliana by modulating the expression of miRNA involved in stress tolerance and nutrient acquisition. PLoS One 2018. 13. e0206221.

11. Okolie. C.L.; Mason. B.; Critchley. A.T. Seaweeds as a source of proteins for use in pharmaceuticals and high-value applications. Novel Proteins for Food. Pharmaceuticals. and Agriculture: Sources. Applications. and Advances 2018. 217.

12. Ali. O.; Ramsubhag. A.; Jayaraman. J. Biostimulatory activities of Ascophyllum nodosum extract in tomato and sweet pepper crops in a tropical environment. PLoS One 2019. 14. e0216710.

13. Ali. O.; Ramsubhag. A.; Jayaraman. J. Phytoelicitor activity of Sargassum vulgare and Acanthophora spicifera extracts and their prospects for use in vegetable crops for sustainable crop production. Journal of Applied Phycology 2021. 33. 639–651.

14. Vijayanand. N.; Ramya. S.S.; Rathinavel. S. Potential of liquid extracts of Sargassum wightii on growth. biochemical and yield parameters of cluster bean plant. Asian Pacific Journal of Reproduction 2014. 3. 150–155.

15. Craigie. J.S. Seaweed extract stimuli in plant science and agriculture. Journal of applied phycology 2011. 23. 371–393.

16. Chouliaras. V.; Tasioula. M.; Chatzissavvidis. C.; Therios. I.; Tsabolatidou. E. The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity. fruit maturation. leaf nutritional status and oil quality of the olive (Olea europaea L.) cultivar Koroneiki. Journal of the Science of Food and Agriculture 2009. 89. 984–988.

17. Kaladharan. P.; Sridhar. N. Cytokinins from marine green alga. Caulerpa racemosa (Kuetz) Taylor. Fishery Technology 1999. 597 36. 87–89.

18. Wally. O.S.; Critchley. A.T.; Hiltz. D.; Craigie. J.S.; Han. X.; Zaharia. L.I.; Abrams. S.R.; Prithiviraj. B. Regulation of phytohormone biosynthesis and accumulation in Arabidopsis following treatment with commercial extract from the marine macroalga Ascophyllum nodosum. Journal of plant growth regulation 2013. 32. 324–339.

19. Zhang. Q.; Yuan. W.; Wang. Q.; Cao. Y.; Xu. F.; Dodd. I.C.; Xu. W. ABA regulation of root growth during soil drying and recovery can involve auxin response. Plant. Cell & Environment 2022. 45. 871–883.

20. Perrot-Rechenmann. C. Cellular responses to auxin: division versus expansion. Cold Spring Harbor perspectives in biology 2010. 2. a001446.

21. Zhang. J.; Jia. W.; Yang. J.; Ismail. A.M. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Research 2006. 97. 111–119.

22. Yamaguchi-Shinozaki. K.; Shinozaki. K.; et al. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annual review of plant biology 2006. 57. 781–803.

23. Quach. T.N.; Tran. L.S.P.; Valliyodan. B.; Nguyen. H.T.; Kumar. R.; Neelakandan. A.K.; Guttikonda. S.K.; Sharp. R.E.; Nguyen. H.T. Functional analysis of water stress-responsive soybean GmNAC003 and GmNAC004 transcription factors in lateral root development in Arabidopsis. PLoS One 2014. 9. e84886.

24. Emenecker. R.J.; Strader. L.C. Auxin-abscisic acid interactions in plant growth and development. Biomolecules 2020. 10. 281.

25. Bouzroud. S.; Gouiaa. S.; Hu. N.; Bernadac. A.; Mila. I.; Bendaou. N.; Smouni. A.; Bouzayen. M.; Zouine. M. Auxin response factors (ARFs) are potential mediators of auxin action in tomato response to biotic and abiotic stress (Solanum lycopersicum). PloS one 2018. 13. e0193517.

26. Biancardi. E.; McGrath. J.M.; Panella. L.W.; Lewellen. R.T.; Stevanato. P. Sugar beet. In Root and tuber crops; Springer. 2010; pp. 617 173–219.

27. Barone. V.; Baglieri. A.; Stevanato. P.; Broccanello. C.; Bertoldo. G.; Bertaggia. M.; Cagnin. M.; Pizzeghello. D.; Moliterni. V.; Mandolino. G.; et al. Root morphological and molecular responses induced by microalgae extracts in sugar beet (Beta vulgaris L.). Journal of Applied Phycology 2018. 30. 1061–1071.

28. Della. M.L.; Baghdadi. A.; Mangione. F.; Borella. M.; Zegada-Lizarazu. W.; Ravi. S.; Deb. S.; Broccanello. C.; Concheri. G.; Monti.A.; et al. Transcriptional and Physiological Analyses to Assess the Effects of a Novel Biostimulant in Tomato. Frontiers in plant science 2021. 12.

29. Briglia. N.; Petrozza. A.; Hoeberichts. F.A.; Verhoef. N.; Povero. G. Investigating the impact of biostimulants on the row crops corn and soybean using high-efficiency phenotyping and next generation sequencing. Agronomy 2019. 9. 761.

30. Franzoni. G.; Cocetta. G.; Prinsi. B.; Ferrante. A.; Espen. L. Biostimulants on Crops: Their Impact under Abiotic Stress Conditions. 627 Horticulturae 2022. 8. 189.

31. Moxley. G.; Zhang. Y.H.P. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. Energy & Fuels 2007. 21. 3684–3688.

32. Folch. J.; Lees. M.; Sloane Stanley. G.H.; et al. A simple method for the isolation and purification of total lipids from animal tissues. J biol Chem 1957. 226. 497–509.

33. Bradford. M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 1976. 72. 248–254.

34. Chatris. J.; Quintela. J.; Folch. J.; Planas. E.; Arnaldos. J.; Casal. J. Experimental study of burning rate in hydrocarbon pool fires. Combustion and flame 2001. 126. 1373–1383.

35. Langmead. B.; Salzberg. S.L. Fast gapped-read alignment with Bowtie 2. Nature methods 2012. 9. 357–359.

36. Quinlan. A.R.; Hall. I.M. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 2010. 26. 841–842.

37. Love. M.I.; Huber. W.; Anders. S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology 2014. 15. 1–21.

38. Barone. V.; Bertoldo. G.; Magro. F.; Broccanello. C.; Puglisi. I.; Baglieri. A.; Cagnin. M.; Concheri. G.; Squartini. A.; Pizzeghello. D.; et al. Molecular and morphological changes induced by leonardite-based biostimulant in Beta vulgaris L. Plants 2019. 8. 181.

39. Ertani. A.; Schiavon. M.; Nardi. S. Transcriptome-wide identification of differentially expressed genes in Solanum lycopersicon L. in response to an alfalfa-protein hydrolysate using microarrays. Frontiers in plant science 2017. 8. 1159.

40. Livak. K.J.; Schmittgen. T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2- Delta Delta CT method. methods 2001. 25. 402–408.

41. Arnon. D.; Hoagland. D.; et al. Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Science 1940. 50. 463–485.

42. Schneider. K.W. F.: Sugar Analysis. ICUMSA Methods. Official and Tentative Methods Recommended by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA). herausgeg. vom. Starch-Stärke 1980. 32. 325–6.

43. Kubadinow. N.; Wieninger. L. Analyses of alphaamino nitrogen in sugar beets and in processing juices. Zucker 1972. 25. 43–47.

44. Stevanato. P.; Zavalloni. C.; Marchetti. R.; Bertaggia. M.; Saccomani. M.; McGrath. J.M.; Panella. L.W.; Biancardi. E. Relationship between subsoil nitrogen availability and sugarbeet processing quality. Agronomy journal 2010. 102. 17–22.

45. Rajput. R.S.; Ram. R.M.; Vaishnav. A.; Singh. H.B. Microbe-based novel biostimulants for sustainable crop production. In Microbial diversity in ecosystem sustainability and biotechnological applications; Springer. 2019; pp. 109–144.

46. Di Mola. I.; Ottaiano. L.; Cozzolino. E.; Senatore. M.; Giordano. M.; El-Nakhel. C.; Sacco. A.; Rouphael. Y.; Colla. G.; Mori. M. Plant-based biostimulants influence the agronomical. physiological. and qualitative responses of baby rocket leaves under diverse nitrogen conditions. Plants 2019. 8. 522.

47. Bajpai. S.; Shukla. P.S.; Asiedu. S.; Pruski. K.; Prithiviraj. B. A biostimulant preparation of brown seaweed Ascophyllum nodosum suppresses powdery mildew of strawberry. The plant pathology journal 2019. 35. 406.

48. Nair. P.; Kandasamy. S.; Zhang. J.; Ji. X.; Kirby. C.; Benkel. B.; Hodges. M.D.; Critchley. A.T.; Hiltz. D.; Prithiviraj. B. Transcriptional and metabolomic analysis of Ascophyllum nodosum mediated freezing tolerance in Arabidopsis thaliana. BMC genomics 2012. 13. 1–23.

49. Jayaraj. J.; Rahman. M.; Wan. A.; Punja. Z. Enhanced resistance to foliar fungal pathogens in carrot by application of elicitors. Annals of Applied Biology 2009. 155. 71–80.

50. Ramkissoon. A.; Ramsubhag. A.; Jayaraman. J. Phytoelicitor activity of three Caribbean seaweed species on suppression of 666 pathogenic infections in tomato plants. Journal of Applied Phycology 2017. 29. 3235–3244.

51. Dittrich. M.; Mueller. H.M.; Bauer. H.; Peirats-Llobet. M.; Rodriguez. P.L.; Geilfus. C.M.; Carpentier. S.C.; Al Rasheid. K.A.; Kollist.H.; Merilo. E.; et al. The role of Arabidopsis ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration. Nature Plants 2019. 5. 1002–1011.

52. Lackman. P.; González-Guzmán. M.; Tilleman. S.; Carqueijeiro. I.; Pérez. A.C.; Moses. T.; Seo. M.; Kanno. Y.; Häkkinen. S.T.; Van Montagu. M.C.; et al. Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. Proceedings of the National Academy of Sciences 2011. 108. 5891–5896.

53. Sakaoka. S.; Mabuchi. K.; Morikami. A.; Tsukagoshi. H. MYB30 regulates root cell elongation under abscisic acid signaling. 674 Communicative & Integrative Biology 2018. 11. e1526604.

54. Zheng. Y.; Schumaker. K.S.; Guo. Y. Sumoylation of transcription factor MYB30 by the small ubiquitin-like modifier E3 ligase SIZ1 mediates abscisic acid response in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 2012. 109. 12822–12827.

55. Li. L.; Yu. X.; Thompson. A.; Guo. M.; Yoshida. S.; Asami. T.; Chory. J.; Yin. Y. Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. The Plant Journal 2009. 58. 275–286.

56. Raffaele. S.; Rivas. S.; Roby. D. An essential role for salicylic acid in AtMYB30-mediated control of the hypersensitive cell death program in Arabidopsis. FEBS letters 2006. 580. 3498–3504.

57. Li. D.; Li. Y.; Qian. J.; Liu. X.; Xu. H.; Zhang. G.; Ren. J.; Wang. L.; Zhang. L.; Yu. H. Comparative transcriptome analysis revealed candidate genes potentially related to desiccation sensitivity of recalcitrant Quercus variabilis seeds. Frontiers in plant science 2021.12.

58. Zhu. J.K. Abiotic stress signaling and responses in plants. Cell 2016. 167. 313–324.

59. Li. S.B.; Xie. Z.Z.; Hu. C.G.; Zhang. J.Z. A review of auxin response factors (ARFs) in plants. Frontiers in plant science 2016. 7. 47.

60. Li. M.; Qin. C.; Welti. R.; Wang. X. Double knockouts of phospholipases D ζ 1 and D ζ 2 in Arabidopsis affect root elongation during phosphate-limited growth but do not affect root hair patterning. Plant physiology 2006. 140. 761–770.

61. Cao. X.; Yang. H.; Shang. C.; Ma. S.; Liu. L.; Cheng. J. The roles of auxin biosynthesis YUCCA gene family in plants. International 689 journal of molecular sciences 2019. 20. 6343.

62. Ugarte. R.A.; Sharp. G.; Moore. B. Changes in the brown seaweed Ascophyllum nodosum (L.) Le Jol. Plant morphology and biomass produced by cutter rake harvests in southern New Brunswick. Canada. In Proceedings of the Eighteenth International Seaweed Symposium. Springer. 2006. pp. 125–133.

63. Luo. M.; Dennis. E.S.; Berger. F.; Peacock. W.J.; Chaudhury. A. MINISEED3 (MINI3). a WRKY family gene. and HAIKU2 (IKU2). a

leucine-rich repeat (LRR) KINASE gene. are regulators of seed size in Arabidopsis. Proceedings of the National Academy of Sciences 2005. 102. 17531–17536. 696

64. Cai. D.; Kleine. M.; Kifle. S.; Harloff. H.J.; Sandal. N.N.; Marcker. K.A.; Klein-Lankhorst. R.M.; Salentijn. E.M.; Lange. W.; Stiekema. W.J.; et al. Positional cloning of a gene for nematode resistance in sugar beet. Science 1997. 275. 832–834.

65. Gissot. L.; Polge. C.; Jossier. M.; Girin. T.; Bouly. J.P.; Kreis. M.; Thomas. M. AKINbg Contributes to SnRK1 Heterotrimeric Complexes and Interacts with Two Proteins Implicated in Plant Pathogen Resistance through Its KIS/GBD Sequence1.

66. Bose. S.; Gangopadhyay. G.; Sikdar. S.R. RiHSPRO2. a nematode resistance protein-like homolog from a wild crucifer Rorippa indica (L.) Hiern. is a promising candidate to control mustard aphid Lipaphis erysimi. Arthropod-Plant Interactions 2018. 702 12. 701–714.

67. Guo. D.; Liang. J.; Qiao. Y.; Yan. Y.; Li. L.; Dai. Y. Involvement of G1-to-S transition and AhAUX-dependent auxin transport in abscisic acid-induced inhibition of lateral root primodia initiation in Arachis hypogaea L. Journal of plant physiology 2012. 705 169. 1102–1111.

68. Zhao. F.Y.; Cai. F.X.; Gao. H.J.; Zhang. S.Y.; Wang. K.; Liu. T.; Wang. X. ABA plays essential roles in regulating root growth by interacting with auxin and MAPK signaling pathways and cell-cycle machinery in rice seedlings. Plant growth regulation 2015. 708 75. 535–547.

69. Carmody. N.; Goñi. O.; Łangowski. Ł.; O'Connell. S. Ascophyllum nodosum extract biostimulant processing and its impact on enhancing heat stress tolerance during tomato fruit set. Frontiers in plant science 2020. 11. 807.

70. Da-Xi. Z.; Ke. Y.; Zhi-Hong. X.; Hong-Wei. X. Effect of polar auxin transport on rice root development. Journal of Integrative Plant 712 Biology 2003. 45. 1421.

71. Stevanato. P.; Trebbi. D.; Saccomani. M. Root traits and yield in sugar beet: identification of AFLP markers associated with root 714 elongation rate. Euphytica 2010. 173. 289–298.

72. Rahimi. A.; Kiralan. M.; Ahmadi. F. Effect of Humic Acid Application on Quantitative Parameters of Sugar Beet (Beta vulgaris L.) 716 Cv. Shirin. Alexandria Science Exchange Journal 2020. 41. 85–91.

73. Jung, C.; Nguyen, N.H.; Cheong, J.-J. Transcriptional Regulation of Protein Phosphatase 2C Genes to Modulate Abscisic Acid Signaling. Int. J. Mol. Sci. 2020, 21, 9517

74. Qiu, J.; Ni, L.; Xia, X.; Chen, S.; Zhang, Y.; Lang, M.; Li, M.; Liu, B.; Pan, Y.; Li, J.; Zhang, X. Genome-Wide Analysis of the Protein Phosphatase 2C Genes in Tomato. Genes 2022, *13*, 604

75. Ali, A.; Pardo, J.M.; and Yun, D.-J. (2020) Desensitization of ABA-Signaling: The Swing From Activation to Degradation. Front. Plant Sci. 11:379

76. Meyer, K..; Leube, M.P.; Grill, E. (1994). A Protein Phosphatase 2C Involved in ABA Signal Transduction in Arabidopsis thaliana. Science 264, 1452–1455.

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