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Characterization of plant physiological and molecular responses to biostimulant applications

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GENERAL ABSTRACT

In the last decades, agriculture has been seeking novel strategies to decrease its pressure on ecosystems and the environment and adjust production levels and quality of food to meet the demand of a growing world population. Among the available approaches and technologies, plant biostimulants are promising solutions to improve agricultural practices in this direction. Biostimulants are substances or microorganisms that, irrespective of their nutrient content, can improve plant nutrient use efficiency, plant tolerance to abiotic stresses, and yields and quality traits of harvested products.

This thesis aims to contribute to the development of the understanding of biostimulation processes in plants. The approach concerned the evaluation of the effects of different biostimulant substances in two important crops: sugar beet (*Beta vulgaris* L.) and tomato (*Solanum lycorpesicum* L.). The molecular and physiological responses were mainly assessed through the analysis of the plant transcriptome, the leaf microbiome composition, and selected morpho-physiological parameters and yield traits.

The first contribution included in the thesis presents a study focused on the evaluation of the effects of leonardite applications on sugar beet. Specifically, to assess the impact of leonardite application on the composition of the leaf microbial communities, a comparative analysis of the leaf microbiome of plants grown in laboratory-controlled hydroponic conditions and plants grown in the field was carried out. Moreover, the treatment-induced modulation of expression of key genes related to hormonal and signaling metabolism in leaves and the impact on beet yield traits were also studied. A significant increase in sugar yield was observed in treated plants. Even more, plants treated with leonardite compared with untreated ones had a significant increase in the abundance of Oxalicibacterium spp., a beneficial endophyte bacterial genus, and higher expression levels of *LAX2* gene, encoding an auxin transport protein.

In the second contribution, the effectiveness of a novel calcium-based biostimulant in reducing the effects of drought stress on tomato plants was evaluated. The adopted approach consisted of transcriptomic analysis combined with physiological profiling. We observed an increased photosynthetic rate, chlorophyll content, and fruit dry matter yield of biostimulant-treated plants under water deficiency compared to standard calcium-based fertilizer.

Finally, the third contribution presents a methodological approach for characterizing the effects of a biostimulant product on a model crop. In detail, phenomics and global transcriptomics were used for dissecting responses to an *Ascophyllum nodosum* extract on tomato plants cultivated in the laboratory, greenhouse, and open field conditions. The gene functional categories most significantly modulated by the treatment were those related to stimulus-response and

photosynthesis. Indeed, the seaweed extract promoted tomato net photosynthesis, stomatal conductance, and fruit yield across all culture conditions.

Overall, this thesis is providing a contribution to the current knowledge on the plant responses to three selected plant biostimulants: one derived from leonardite and rich in humic acids, a calcium-and-polysaccharides-based formulation, and an *Ascophyllum nodosum* extract. Data presented are both collected from plants grown in laboratory conditions and from field-cultivated crops. Characterizing biostimulant effects on plants and disentangling their modes of action are critical ongoing challenges that will benefit from the joint effort of scientific community and agriculture stakeholders.

RIASSUNTO GENERALE

Negli ultimi decenni, il settore agricolo sta ponendo attenzione alla ricerca di strategie innovative per alleviare l'impatto negativo esercitato dalle avversità ambientali sugli ecosistemi agricoli e per adeguare la quantità e la qualità delle produzioni alla popolazione mondiale in continua crescita. Fra i mezzi tecnici oggi a disposizione, i prodotti biostimolanti sono fra quelli più promettenti al fine di mitigare gli effetti negativi dei suddetti fattori di stress. I biostimolanti sono sostanze e/o microorganismi che, indipendentemente dal loro contenuto nutrizionale, hanno la capacità di accrescere nelle piante l'efficienza d'uso dei nutrienti, la tolleranza agli stress abiotici, le rese e alcuni parametri qualitativi dei prodotti raccolti.

Il presente lavoro di tesi contribuisce alla comprensione di alcuni di questi processi di biostimolazione in piante coltivate. L'approccio metodologico impiegato ha volto l'attenzione alla valutazione degli effetti di diversi prodotti biostimolanti in due importanti specie vegetali ad ampia diffusione: la barbabietola da zucchero (*Beta vulgaris* L.) e il pomodoro (*Solanum lycorpesicum* L.). Sono state studiate le risposte molecolari e fisiologiche attraverso l'analisi del trascrittoma della pianta, lo studio della composizione del microbioma fogliare e la valutazione di parametri morfo-fisiologici e di resa delle colture.

Il primo contributo incluso nella tesi presenta uno studio che ha avuto come obbiettivo la valutazione degli effetti dell'applicazione di un biostimolante a base di leonardite su piante di barbabietola da zucchero. In particolare, è stata eseguita un'analisi comparativa del microbioma fogliare di piante coltivate in idroponica in laboratorio ed in pieno campo con lo scopo di comprendere l'impatto del trattamento a base di leonardite sulla composizione delle comunità microbiche associate alla foglia. Inoltre, sono stati analizzati l'espressione di alcuni geni coinvolti nel metabolismo ormonale e nella segnalazione cellulare e l'effetto del trattamento sulle rese produttive e su alcuni parametri della qualità estrattiva della barbabietola. Un aumento significativo nella resa di saccarosio è stato registrato nelle piante trattate. Nelle stesse, è stata inoltre riscontrata una maggior abbondanza di batteri del genere *Oxalicibacter*, descritti come endofiti che promuovono la crescita della pianta. Infine, il gene *LAX2* che codifica per un trasportatore delle auxine, è risultato sovra-espresso nelle piante trattate.

Nel secondo contributo, è stata studiata l'efficacia di un formulato biostimolante a base di calcio nel ridurre gli impatti della carenza idrica in pomodoro. Combinando l'analisi del trascritto di piante trattate con una caratterizzazione fisiologica, sono stati osservati (i) un aumento del tasso di fotosintesi netta, (ii) del contenuto di clorofilla e (iii) del peso secco dei frutti nelle piante sottoposte a stress idrico e trattate con il biostimolante rispetto a quelle trattate con un fertilizzante standard.

Infine, il terzo contributo presenta un approccio metodologico per la caratterizzazione degli effetti di un biostimolante in pomodoro. Nello specifico, tecniche di fenomica e trascrittomica sono state impiegate per esaminare le risposte prodotte dal trattamento con un estratto dell'alga *Ascophyllum nodosum* in piante di pomodoro coltivate in laboratorio, in serra e in pieno campo. Le categorie di geni modulate in modo più significativo dal trattamento sono state quelle implicate nella risposta agli stimoli ambientali e nella fotosintesi. In accordo con questo risultato, nelle piante trattate è stato registrato (i) un più alto tasso di fotosintesi netta, (ii) una maggiore conduttanza stomatica e, infine, (iii) una resa produttiva più alta in tutte e tre le condizioni di allevamento testate.

In conclusione, questa tesi fornisce un contributo alle conoscenze nell'ambito dello studio delle risposte a tre diversi biostimolanti. I dati presentati sono stati raccolti in cella climatica, serra e pieno campo. La caratterizzazione degli effetti dei biostimolanti in piante coltivate con lo studio approfondito delle loro modalità d'azione sono risultati importanti della presente ricerca per il miglioramento della sostenibilità del settore agricolo e per il suo adattamento alle avversità ambientali.

GENERAL INTRODUCTION

BACKGROUND AND JUSTIFICATION

Nearly half of the habitable land surface on Earth is used for agriculture (Ellis et al., 2010) and its role is crucial to address the global environmental and climate challenges (FAO, 2020).

Globally, agriculture is a major contributor to climate change, being responsible for 14% of greenhouse gas emissions (Poore and Nemecek, 2018) and it is the main cause of soil degradation, biodiversity loss, and freshwater use and pollution through phosphorous and nitrogen leaching (IPBES, 2019). At the same time, climate change is having severe impacts on agricultural production and the food system. The sensitivity of agricultural systems to the effects of climate change is diverse and uneven, impacting more in developing countries and intensifying food insecurity for vulnerable people (FAO, 2020). Moreover, while the increased concentration of atmospheric CO_2 is expected to have a fertilizing effect on many crops, the increased photosynthetic efficiency may come with side effects like the loss of nutritional value of certain crops (Leisner, 2020). The uncertainty in climate trends and the increasing frequency of extreme weather events are projected to cause fluctuations in agricultural yields, fisheries, and food availability (FAO, 2020).

Introducing regenerative methods of agricultural production is considered one of the main actions to redesign the global food system to achieve food security and dietary health while guaranteeing environmental and ecological sustainability and social justice (Rockström et al. 2020). Sustainably managed, agriculture can shift from being a cause of climate change and an environmental threat to becoming a solution to the ecosystems restoration challenge and improving water, soil, and human health.

To reduce its ecological footprint, agriculture is asked to increase its resource efficiency, which means producing “more with less” without increasing land use, while reducing greenhouse gas emissions and encouraging adaptation to climate change (Gan et al., 2011). The proposed approaches are several and carry different degrees of radicalism which, according to Sumberg (2022), can be categorized in two main simplified branches. One recommends innovation and technology applied to agriculture in a framework of “sustainable intensification” to increase the efficiency of processes. The other wishes for a shift towards alternative agricultures for example “natural farming”, organic farming, permaculture, or agroforestry, inspired by the principles of ecology and system thinking. Combined approaches are also applicable and should be selected and possibly adapted to the geographical, cultural, and socio-economic specific situation.

Overall, the goal of strengthening the resilience of agri-food systems can benefit from shifting towards models of low-input agriculture. As part of this trend, the European Union’s *Farm to*

Fork Strategy released in 2020, has set binding level targets to reduce by 50% the use of chemical pesticides by 2030. Also, the use of fertilizers is expected to decrease by at least 20% by 2030 as a consequence of practices that prevent nutrient losses and deterioration of soil fertility (EC, 2020). Moreover, in the current market situation of increased energy and fossil fuels costs it is not economically sustainable for farmers to heavily rely on inorganic fertilizers and other off-farm inputs for crop nutrition.

BIOSTIMULANTS

The application/use of plant biostimulants seems to be a promising solution that tackles both the need to reduce environmental polluting inputs and the need to face climate change-induced extreme weather conditions scenarios. Abiotic and biotic stresses, together with poor water and nutrient use efficiency are among those factors impacting the reduction of the yield potential of crops. Other than water, mineral fertilizers, and pesticides, plants can be supplemented with other organic or inorganic substances that if applied to plants can promote their growth and development, and their capacity to face abiotic or biotic stresses. Such substances are plant biostimulants. The interest in plant biostimulants in the last decades has been growing at a global level.

The biostimulant market reached up to USD 3 billion in 2021 globally, and it is projected to reach more than USD 5.1 billion by 2027. The European market share is around 45%, while both North America and Asia have around the 20%, and Latin America has 15% (Corsi et al., 2022).

Providing a thorough and agreed-upon definition of biostimulants is still a matter of debate. The dynamic evolution of this field is constantly asking for updates in the conceptualization of this category of products. According to Yakhin and co-authors (2017), the concept of biogenic stimulants is firstly described by Filatov in 1944 as the influence on metabolic and energetic processes in animals and exerted by biological compounds produced by various organisms in stressful conditions. Moving across gradual efforts in conceptualizing this class of materials, the current definition that seems to meet a broad consensus defines biostimulants as substances or microorganisms, or mixtures of such ingredients, that are applied to plants to promote nutrient efficiency, yields, and crop quality, and the plant capacity to face biotic and abiotic stresses, without providing direct nutritional supply as fertilizers or being classified as pesticides (Calvo et al., 2014, du Jardin, 2015). This definition settled the basis for the one included in the European Union (EU) Fertilizing Products Regulation (2019/1009), in which biostimulants are considered as a discrete class of fertilizers and defined by their claimed agronomic effects: "*plant biostimulant means a product stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency; (b) tolerance to*

abiotic stress; (c) quality traits; (d) availability of confined nutrients in soil or rhizosphere". In these cited definitions there is no such reference to the nature of the substances used or to the mechanisms that cause such stimulation in plants. This is due, in part, to the complexity of the composition of many biostimulant products that can contain different compounds or can be derived by specific extraction processes from a biological substrate. Also, the combination of different substances may be crucial in determining the biological activity of certain products, so that, defining the features of each constituent may not lead to the understanding of the product function and properties (Yakhin et al., 2017).

Several mechanisms have been described for different plant biostimulants. Brown and Saa (2015) try to provide a general one hypothesizing that the positive effects observed on crop yields and growth can be ultimately traced back to their ability to reduce the plant stress response metabolism through the interaction with the signaling pathway of the plant. In this way, the plant organism reduces the amount of energy employed for stress response, increasing the allocation of assimilates to its growth and development metabolisms.

Main categories of plant biostimulants

The main categories of biostimulants on the bases of their composition are humic acids, seaweed, plant extracts, protein hydrolysates, beneficial microorganisms, and other organic and inorganic materials.

Humic substances (HS)

Humic substances (HS) are the main components of soil organic matter, and they originate from the decay and transformation of plant and microbial residues (Stevenson, 1994). These organic compounds are ubiquitously found in soils, sediments, and natural bodies and are related to pivotal processes in soil conservation, soil fertility, and plant-soil interactions (Piccolo, 2002). They constitute the stable fraction of soil organic matter, not being easily decomposed by the soil microbial community because of their strong interaction with the soil mineral components and because of their complex and heterogenous chemical structure (Trevisan et al., 2010). HS have recently been described as relatively low-molecular size compounds behaving as supra-molecular structures stabilized by weak bonds (H-bonds, van der Waals, π-π, ion-dipole) (Šmejkalová & Piccolo, 2008). It has been shown that HS can be dissociated into low and high-molecular sizes by microbial activity and by the organic acids released by plants as root exudates and that the chemical structure of HS impacts their biological activity (Nardi et al., 2000). There is an intricate interplay in the rhizosphere occurring between the pant, the organic matter, and the microorganisms, which needs to be considered when studying biostimulants based on HS.

HS have an indirect effect on plant growth deriving from their positive effect on soil fertility which is exerted mainly through an improved nutrients availability and soil structure, increased soil ion exchange and water retention capacities, and reduced toxicity by heavy metals and aluminum (Chen et al., 2004; Elkins & Nelson, 2002, Janos et al., 2009, Nardi et al., 2002). HS are also known to have a direct biological activity on plant physiology and metabolism. This activity depends on the dose, the molecular weight, the source material of the humic fraction, the method of application to plants, the plant species, and the growing conditions (da Silva et al., 2021; Nardi et al., 2021). HS have been shown to stimulate root nutrition through diverse mechanisms. They modify root system morphology promoting the differentiation of lateral roots and root hairs. This activity is described as associated with the hormone-like properties of HS and the consequent influence that they are playing on signaling pathways (Zandonadi et al., 2010). Indeed, HS retain in their structure indole-3-acetic acid (IAA) and other molecules originating from plants or microorganisms in the soil, responsible for their auxin-like activity. Also, HS affect the expression of genes encoding nutrient transporters and plasma membrane H^+-ATPases (Quaggiotti et al., 2004). The release of protons outside the root cell plasma membranes, not only creates an electrochemical potential that favors the uptake of nutrients, but also leads to the loosening of cell walls and ultimately cell elongation, thus promoting root growth (Nardi et al., 2021). In addition to this, HS have a role in promoting plant tolerance to abiotic and biotic stresses. Plants treated with HS undergo some modifications in the secondary metabolism mainly by modulating the activity of enzymes implicated in the scavenging of reactive oxygen species generated under multiple stress conditions (Canellas et al., 2015; Schiavon et al. 2010).

Seaweed Extracts

Seaweed extracts (SWEs) are very promising and widely exploited sources of plant biostimulants (Van Oosten et al., 2017). Seaweeds are multicellular algae that grow naturally in marine habitats and can be harvested or, to a lower extent, cultivated. There are three main classes of seaweeds: green (*Chlorophyta*), red (*Rhodophyta*), and brown (*Phaeophyta*) algae, which are the most used in agricultural applications (Khan et al., 2009). Some of the bioactive compounds in seaweed extracts are polysaccharides such as laminarin, alginates, fucan, and carrageenans, amino acids, betaines, macro- and micronutrients, and secondary metabolites (phenolic compounds, vitamins, and their precursors). Moreover, SWEs contain phytohormones which are in part associated with their biostimulant activity on plants (Pereira et al., 2020). The functional characteristics of SWEs depend on the extraction method and the season and area of harvest and can be poorly homogeneous among different products (Carrasco-Gil et al., 2018). In general, the use of several biostimulant products based on SWE is reported to induce plant growth promotion, improvement of flowering and consequently fruit set, and increased yield and

quality of harvested products (Ali et al., 2016; Shukla et al., 2019; Hussain et al., 2021, Battacharyya et al., 2015). Observed plant physiological responses also include stimulated root growth and mineral nutrient uptake and increased chlorophyll and flavonoid content (Calvo et al., 2014; Jannin et al., 2013; Spinelli et al., 2010). Moreover, the plant tolerance to several abiotic and biotic stresses (drought, cold, salinity, nematodes, bacteria, fungi, and insects) is expanded upon SWE treatments (Deolu-Ajayi et al., 2022).

The detailed modes of action by which these algae extracts beneficially influence plant growth and development are not fully understood, however, it is mostly attributed to the hormonal-like activity of some constituents and the presence of elicitors (Sangha et al., 2014). SWEs induce plant growth through the regulation of hormonal metabolism and biosynthesis. Several works report the presence in marine algae extracts of phytohormones, the concentration of which, however, seems to vary across different studies. (Shukla et al., 2019). Wally and co-workers (2013) analyzed different seaweed extracts to determine the concentration of indole acetic acid (IAA), cytokinin, and abscisic acid (ABA). They reported low concentrations of phytohormones in the diluted solution applied to plants, to exclude the direct induced physiological response. A more likely explanation of the observed effect in plants is the elicited rearrangement of hormonal balance by influencing the overall hormone homeostasis through the regulation of expression of hormone metabolism-related genes (De Saeger et al., 2020).

Due to the elaborate picture of biochemical pathways related to plant stress tolerance, the anti-stress effects of SWEs described in several studies are not fully disentangled. Moreover, the observed plant responses may differ based on the different experimental factors, such as plant species, type of seaweed extract, method and dose of application, and applied environmental and stressful conditions. Taken together, the topic reviewed by Deolu-Ajayi et al. (2022), De Saeger et al. (2020), Khan et al. (2009), and Shukla et al. (2019) points to the conclusion that SWE can dampen stress-induced responses, through the regulation of different genes involved in stress responses, increasing the activity of antioxidant enzymes, triggering plant defense pathways against different pathogens.

Protein hydrolysates

Protein-based products (polypeptides, oligopeptides, and amino acids) are considered important sources of plant biostimulants. Indeed, this group of compounds has been shown to enhance crop yield and quality, plant growth, and tolerance to biotic and abiotic stresses (Colla et al., 2015, Calvo et al., 2014). They are obtained by the chemical, enzymatic, or thermal hydrolysis of crop biomass, such as leguminous crops, or industrial by-products of animal or plant origin (Colla et al., 2014). Protein hydrolysates (PH) are entitled to become the ideal sustainable agricultural inputs, because of their potential derivation from wastes and by-products like the animal and plant wastes of agroindustry (e.g. fish wastes, collagen, feathers,

and blood), sewage, wastewater, and compost extracts (Corsi et al., 2022). The efficacy of a PH-based biostimulant is related to the characteristics of the peptide fraction. Higher content in free amino acids accounts for a stronger biological effect on plants, given that amino acids and small peptides can be absorbed by plant roots and leaves (Schiavon et al., 2008).

PHs can influence several pathways of plant metabolism. Irrespective of their direct supply of N and C, they are reported to directly influence plant regulatory processes through the hormone-like activity of signaling peptides (Oh et al., 2018). The stimulation of plant defense responses is also described as resulting in plant protection against salinity and oxidative stress (Ertani et al., 2013), heat (Kauffman et al., 2007), and the presence of heavy metals in soil (Sharma and Dietz 2006). Schiavon and co-workers (2008) showed an effect of alfalfa-derived PHs on N uptake and assimilation through the modulation of genes from the tricarboxylic acid (TCA) cycle and key enzymatic activity related to N assimilation and N metabolism in *Zea mays* L. Moreover, in the same study, they confirmed both auxin- and gibberellin-like activity of the PH-based product. Among the beneficial effects of PH, Colla and co-authors (2017) reviewed the few available contributions in the literature investigating the impact of this treatment on plant and soil microbiome composition. They concluded that the application of PHs to soil or plant can result in a modified microbial community structure and that this feature should be thoroughly studied to develop biostimulants that improve the activity of beneficial microorganisms naturally occurring in the plant ecosystem.

Beneficial microorganisms

Often referred to as biofertilizers and biopesticides, microbial biostimulants include mycorrhizal and non-mycorrhizal fungi, plant growth-promoting rhizobacteria (PGPR), and bacterial endosymbionts (Ali et al., 2022). Beneficial microbial inocula are a broad and diverse category that in general are promoting plant growth and development, and the plant capacity to cope with stressful conditions. The effectiveness of such treatments requires specific understanding and attention when selecting the microbial formulation combined with plant species, soil type, environmental conditions, modes of applications, and other factors (Calvo et al., 2014).

Plant biostimulation upon microbic inoculant application can derive from improved nutrient availability, and nutrient uptake, following soil organic matter mineralization and mobilization of unavailable soil-bound nutrients. Nitrogen fixators can be used as biofertilizer inoculum, either as free-living bacteria or as symbionts for leguminous crops (Bhattacharyya et al., 2012). Moreover, PGPR and beneficial fungi promote nutrient availability through phosphate solubilization, soil acidification, and the release of siderophores (Fadiji et al., 2022). Specifically, mycorrhizal fungi are extensively studied for their beneficial effects on plant nutrition resulting from the symbiotic interaction with the plant. Improved water balance, better soil exploration and availability of macro (especially P) and micronutrients, and greater tolerance to biotic and abiotic

stresses are the main benefits deriving from the inoculation of plants with these fungi (Ma et al., 2022; Stratton et al., 2022). Other beneficial fungi, such as *Trichoderma* spp., are becoming increasingly important for their potential uses in agriculture, not only for their beneficial effects on plant nutrition but also for their function in boosting plant development and defense mechanisms against pathogens. The main limitations to the thorough application of these endophytes as commercial formulates for agriculture are the host specificity, the difficulties of *in vitro* cultivation, and the complexity of microbiome population dynamics (Du Jardin 2015).

The functions of PGP microbes go beyond the increased nutrient availability. Indeed, their utilization is known to directly influence plant metabolism by producing phytohormones, and by triggering induced systemic resistance. Whereas indirect promotion of plant health is obtained through the synthesis of antibiotic metabolites, and indirect competition with potential soil-borne pathogens for the same ecological niche (Ortìz-Castro et al., 2009).

Nonetheless, microbial-based biostimulants represent potentially effective alternatives to some conventional agricultural practices that require further research efforts to achieve a more complete understanding of the mechanisms beyond their action to obtain a wider adoption.

Approaches to the study of plant biostimulants

Predicting the plant responses to plant biostimulant applications taking into consideration the variability of effectiveness that depends upon the different climatic, agronomical, and biological factors is crucial to improve their performing features and the reliability of their claims. The wide variety of constituents and sources of raw materials, and the complexity of the final formulation are making the process of discovering their modes of action and describing the product characteristics very challenging (Calvo et al., 2014). A relevant contribution to the study of these complex biological systems is the combination of different omics sciences and the setting of a robust experimental design. The methodological approaches used to tackle the different scientific questions in the present thesis work consist of the joint use of omics technologies, specifically metagenomics, transcriptomics, and phenomics.

Metagenomics is the study of the structure and function of organisms associated with a specific habitat. It is often used to study bacterial communities through the massive sequencing of genetic material extracted from the analyzed sample (Ramazzotti and Bacci, 2018). The concept of plant-associated microbial communities playing a role in plant health and development is widely accepted. Indeed, the significant contribution of the so-called “phytomicrobiome” to plant fitness and health has been recently assessed and reviewed (Dong et al., 2019; Trivedi et al., 2020). The composition of the rhizosphere (root-soil interface) and phyllosphere (air-plant interface) microbiomes are subjected to the plant status such as

developmental stage and presence of stress factors, and environmental perturbations including the application of plant biostimulants (Thapa et al., 2018). Bioactive compounds provided to the plant leaves or roots not only are sensed and utilized by plants but also by plant-associated microorganisms. Therefore, evaluating the effect of biostimulant treatments on the microbiome composition of epiphytes and endophytes is contributing to the scope of achieving a complete understanding of the biostimulation effect of such products applied to plant-soil ecosystems.

Transcriptomics analysis through RNA-Sequencing allows for comprehensive untargeted gene expression analysis. Modulation of gene expression in plants depends upon several factors and it is regulated during each stage of plant development. Plant responses to the interactions with environmental perturbations, abiotic, and biotic stressors are inducing changes at the gene transcription level (Costa-Silva et al., 2017). Therefore, through RNA-Sequencing and gene expression analysis, it is possible to recognize which are the main metabolic pathways involved in the biostimulation effect, in the given experimental conditions, and perhaps identify molecular markers that can be linked with the plant responses to a specific biostimulant. These molecular markers can be later exploited during biostimulant characterization or commercial product development (González-Morales et al., 2021).

Pairing transcriptomic analysis with plant phenomics allows for picturing both at the molecular and morpho-physiological level the effect of the biostimulant application on plants (Briglia et al., 2019). Moreover, the choice of experimental conditions to apply when functionally characterizing a biostimulant is crucial. Empirical knowledge acquired in a diverse range of experimental conditions in which many environmental and agronomic factors are included is adding robustness to the acquired knowledge that is ultimately transferred to the farmers (Li et al., 2022). This points to the importance of combining both lab and field trials when assessing the responses of a biostimulant product on a given crop (Baghdadi et al., 2022; Jindo et al., 2020, Rouphael et al., 2018).

RESEARCH OBJECTIVES

The overall objective of this research is to contribute to the development of the understanding of biostimulation processes in plants. In particular, the aim is to evaluate the effects of different biostimulant substances in two important crops: sugar beet (*Beta vulgaris* L.) and tomato (*Solanum lycorpesicum* L.).

The three specific objectives of the thesis are:

I. Contributing to the exploration of the effects of leonardite applications to sugar beet. Specifically, the question addressed was whether leonardite treatments could affect the leaf microbiome composition of field-cultivated plants, together with stimulating sugar beet yield traits and modulating the expression of target genes.

II. Assessing the effectiveness of a novel calcium-based biostimulant in reducing the detrimental effects of drought stress in tomato and investigating the mechanisms underpinning such activity.

III. Developing and testing a methodological approach for characterizing the effects of a biostimulant product on a model crop. Given the importance of this emerging category of products, and the growing body of scientific literature contributing to the progress of describing their functions, effects, and mechanisms of action, it can be useful to propose and define a methodological approach that can achieve a reliable description of the effects on crops of a specific product.

STRUCTURE OF THE THESIS

The first part of the thesis provides the background and justification of the present research work. The introduction covers in general the current agricultural challenges in the framework of climate change and sustainable development and provides an overview of available definitions and categories of plant biostimulants. Moreover, it presents the research aims and the structure of the thesis.

The body of the thesis is structured as a collection of three scientific papers (contributions). They are presented in chronological order and published in Frontiers in Plant Science (Impact factor: 6.627; Category: Plant Science, 20/238) (**Table 1**).

Table 1: Contributions included in the thesis

	Reference
Contribution 1	**Della Lucia M.C**., Bertoldo G., Broccanello C., Maretto L., Ravi S., Marinello F., Sartori L., Marsilio G., Baglieri A., Romano A., Colombo M., Magro F., Campagna G., Concheri G., Squartini A., Stevanato P. (2021). Novel effects of leonardite-based applications on sugar beet. Frontiers in Plant Science, 12:290, https://doi.org/10.3389/fpls.2021.646025
Contribution 2	**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, 12:781993, https://doi.org/10.3389/fpls.2021.781993
Contribution 3	Baghdadi A., **Della Lucia M.C**., Borella M., Bertoldo G., Ravi S., Zegada-Lizarazu W., Chiodi C., Pagani E., Hermans C., Stevanato P., Nardi S., Monti A., Mangione F. A dual-omics approach for profiling plant responses to biostimulant applications under controlled and field conditions. Frontiers in Plant Science, https://doi.org/10.3389/fpls.2022.983772

Finally, some overall conclusions and research recommendations based on the three contributions are drawn in the last part of the thesis.

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CONTRIBUTION I

NOVEL EFFECTS OF LEONARDITE-BASED APPLICATIONS ON SUGAR BEET

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ABSTRACT

The present study aimed to explore the effects of foliar application of a leonarditebased product on sugar beet (Beta vulgaris L.) plants grown in the field. The approach concerned the evaluation of the community compositional structure of plant endophytic bacteria through a metabarcoding approach, the expression level of a gene panel related to hormonal metabolism and signaling, and the main sugar beet productivity traits. Results indicated that plants treated with leonardite (dosage of 2,000 ml ha^{-1}, dilution 1:125, 4 mg C l^{-1}) compared with untreated ones had a significant increase ($p < 0.05$) in (i) the abundance of Oxalicibacterium spp., recognized to be an endophyte bacterial genus with plant growth-promoting activity; (ii) the expression level of LAX2 gene, coding for auxin transport proteins; and (iii) sugar yield. This study represents a step forward to advance our understanding of the changes induced by leonardite-based biostimulant in sugar beet.

Keywords: sugar beet, leonardite, 16S rRNA metabarcoding, gene expression, sugar yield.

INTRODUCTION

Biostimulant products, applied to soil or plants, are recognized for improving plant health, quality, and yield (Nardi et al., 2018). They have been shown to influence plant metabolism through the enhancement of photosynthesis, water use efficiency, nutrient uptake, and assimilation (Calvo et al., 2014; Yakhin et al., 2017). Although the study of biostimulation mechanisms is still an ongoing task, available research highlighted a hormone-like activity and an enhancement of root and organ growth and development (Canellas et al., 2011). Moreover, biostimulants have an important role in promoting tolerance to abiotic stresses and plant recovery (Halpern et al., 2015; Van Oosten et al., 2017). Humic substances (HSs), such as leonardite, have prominent importance among biostimulant products. They are a dark brown natural organic compounds, ubiquitous in water, soil, and sediments (Piccolo, 2002). Particularly, leonardite, originating from the atmospheric oxidation of lignite, is very rich in humic acids (David et al., 2014). Leonardite application has been shown to improve nutrient uptake, such as Fe, N, and K, and increase plant yield and quality (Ece et al., 2007; Fascella et al., 2015; Cieschi et al., 2017). Therefore, leonardite is generally used in agriculture as a soil conditioner, increasing the permeability of the stem cell membrane, nutrition rate, fruit quality, and crop yield (Ratanaprommanee et al., 2017). An improved production has been reported for leonardite-treated cherry, potato, corn, and ornamentals (Eyheraguibel et al., 2008; Sanli et al., 2013; Fascella et al., 2018; Demirer, 2019). Sugar beet (Beta vulgaris L.) plays a key role in the

agricultural and economic scenario of 52 countries. In 2017, the world area harvested with sugar beet reached almost 5 Mh for a total production of 314 Mt (Food and Agriculture Organization (FAO), 2019), and the increasing trend is to move toward a sustainable cultivation. In this context, biostimulant products are classified as ecofriendly, minimizing the agricultural impact on the environment. Furthermore, these products not only protect microbes already present in the soil but also foster the growth of new rhizosphere bacteria communities and the related soil enzymatic activity (Du Jardin, 2015). Thus, the use of biostimulants is based on the knowledge of plant root and shoot bacterial communities.

The compositional structure of plant endophytic microbes is influenced by many factors. External environmental conditions, climate, biotic stresses, human practices, and the soil environment are the most important key factors altering the composition of plant endophytic communities (Reinhold-Hurek et al., 2015). The role of endophytic bacteria is crucial. Several studies revealed protective function from plant abiotic stresses, accelerating plant immune response following pathogen infection (Miliute et al., 2015). Furthermore, they can promote plant growth, development, and nutrient uptake (Liu et al., 2017). However, significant knowledge gaps remain, involving the cross-talk between plant and microbes and how the microbiome modulates gene expression in the plant (Liu et al., 2020).

Analysis of plant microbial communities requires suitable techniques and reproducible protocols. A rapidly emerging technique to explore complex bacterial populations is presented by the 16S rRNA gene metabarcoding. This approach, common between different sequencing platforms, involves the PCR amplification of the most taxonomically informative region of 16S rRNA gene followed by high-throughput sequencing. The 16S gene includes nine hypervariable regions (V1—V9) that are taxon-specific, flanked by conserved sequences. The selection of the most informative region is still a matter of scientific debate. V3 and V4 are the most commonly used regions for taxon identification (Yarza et al., 2014).

The present work aimed to explore the effects of leonardite treatment on sugar beet. For this purpose, we firstly compared the microbiome profiles of plants cultivated in hydroponics and field conditions. Then, we exploited the effect of foliar application on plants grown in the open field. Therefore, we investigated (i) the consequences of leonardite application on the composition of plant endophytic communities, (ii) the expression level of key genes related to hormonal and signaling metabolism, (iii) and its impact on yield traits using sugar beet (B. vulgaris L.) as a model crop.

MATERIALS AND METHODS

Plant Material

The sugar beet variety used for the experimental trials, both in the field and in hydroponics, was Smart-Briga (KWS, Einbeck, Germany), diploid and resistant to the herbicide Conviso, Cercospora leaf spot, Rhizomania, and nematodes.

Field Experiment

The field trials were carried out in four locations for 6 months, between March and August 2020. The geographical coordinates of the four locations involved are Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E); Loreo, Rovigo, Italy (45°04'33.6"N, 12°10'36.2"E); Cavarzere, Venezia, Italy (45°06'37.7"N, 12°03'05.1"E); and San Martino di Venezze, Rovigo, Italy (45°06'12.9"N, 11°53'52.5"E). An experimental design constituted of four randomized blocks was applied. Each of the randomized blocks was divided into four subplots whose size was 2.7 × 10 m. A control plot was placed outside the randomized block, and plants were kept without treatments. Plants were subjected to foliar spray treatments with leonardite solution using a dosage of 2,000 ml ha^{-1} (dilution 1:125, 4 mg C l^{-1}). The novel leonardite formulation and non-commercial product used in this work was provided by Sipcam SpA (Italy). The leonardite formulation was analyzed by combustion (Elementar vario MACRO CNS, Elementar Analysensesystemse GmbH, Germany) for C, N, and S contents, ionomic analysis (inductively coupled plasma optical emission spectrometry, SPECTRO ARCOS II MV, SPECTRO, Germany) for elemental analysis, and NMR analysis (solid-state 13C MAS NMR spectra, fully proton-decoupled using a Bruker Avance II 400 MHz instrument, Bruker Corp., United States) for spectra and the distribution of the diverse forms of carbon. The results of this analysis were previously described by Barone et al. (2019). The first application was set for the stage BBCH 38 (leaves cover 80% of the ground), the second treatment was performed 40 days after the first, and the last treatment was applied 20 days after the second one. The untreated control plants were sprayed only with water. A 50-l backpack sprayer was used to uniformly distribute the leonardite solution. Four biological replicates consisting of three-leaf discs taken by plants randomly picked, inside the same subplot, were collected 48 h after treatment. Samples of approximately 50 mg of leaf tissue were placed in dry ice and taken to the laboratory for DNA extraction.

Hydroponic Experiment

Sugar beet seeds were sterilized by dipping in 76% ethanol for 5 min. The washing procedure with distilled water was repeated three times. To promote germination, seeds were kept inside

a growing chamber in the dark on distilled water-moistened filter paper for 48 h at 25°C. Six days after germination, plants were transferred inside 500 ml glass pots with complete Hoagland solution (Arnon and Hoagland, 1940). After 6 days, plants were divided into two different pots containing, respectively, 1 ml l^{-1} of leonardite (treated plants) and complete Hoagland solution (control plants). Leaf sampling was done 2 days after leonardite treatment. The experiment was repeated three times for validation aims.

DNA Extraction

DNA was extracted from 50 mg of fresh leaf material. Samples were homogenized inside the collection microtubes with 300 ml of Buffer RLT and 3 mm stainless steel beads. The homogenization step involved the use of Tissue Lyser (Qiagen, Hilden) for 5 min at 30 Hz. Homogenized samples were then transferred in a 96-well S-block plate containing also 200 ml of isopropanol and 20 ml of MagAttract magnetic beads (Qiagen). This plate was used for automatic DNA extraction using Biosprint 96 (Qiagen) together with five other plates respectively composed of 500 ml of Buffer RPW, 500 ml of 0.02% Tween, and two plates filled with 500 ml of 96% ethanol. DNA was eluted in 100 ml of nuclease-free water. Nucleic acid quantification was performed using Qubit (Thermo Fisher Scientific, Carlsbad, CA) with Qubit DNA High Sensitivity Assay Kit (Thermo Fisher Scientific).

RNA Extraction

mRNA was isolated using Dynabeads mRNA Direct Micro Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, starting from 50 mg of leaf material. mRNA was immediately analyzed with qPCRBIO SyGreen 1-step kit (Resnova-PCR Biosystem).

Metabarcoding of Bacterial 16S rRNA Gene by High-Throughput Sequencing

Library preparation was carried out using the 16S Ion Metagenomics Kit (Thermo Fisher Scientific). Briefly, the protocol consists of a first PCR amplification using two different primer sets (V2, V4, V8 and V3, V6, V7, V9) for the amplification of seven different hypervariable regions. The PCR program consisted of an initial denaturation of 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 20 s, and a hold stage at 72°C for 7 min. Amplicons were quantified and pooled together to obtain a final concentration of 30 ng ml^{-1}. Subsequently, the protocol involved the use of the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific) and Ion Express Barcode Kit (Thermo Fisher Scientific) for bar code ligation. The library was amplified with six cycles of PCR at 58°C for 15 s and 70°C for 1 min, then 4°C for up to 1 h. The library was diluted to a concentration of 25 pM and used to prepare

the template positive Ion sphere particles with Ion One Touch 2 instrument (Thermo Fisher Scientific). The enrichment process was done with the Ion ES instrument (Thermo Fisher Scientific) and the sequencing with Ion GeneStudio S5 using the Ion 520 chip kit (Thermo Fisher Scientific). The data were analyzed using the Ion Torrent Suite software, and the taxonomical assignment was performed by comparing operational taxonomic units (OTUs) against the Greengenes database (version 13.5) and the curated MircoSeq reference library v2013.1 on the Ion Reporter cloud (Thermo Fisher Scientific).

Real-Time PCR for Bacterial Detection

The obtained bacterial sequences were used to design Real-Time PCR primers with the software Primer Express V3.0 (Thermo Fisher Scientific). The primer sequences used in this work are reported in **Table 1**. Real-Time PCR was conducted using QuantStudio 5 (Life Technologies, United States) with the following mix: 5 ml of SYBR Green Real-Time PCR Master Mix, 0.1 ml of forward primer, 0.1 ml of reverse primer, 1.4 ml of nuclease-free water, and 1 ml of each sample. The PCR program was set as follows: 10 min of preincubation at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C.

TABLE 1. List of forward and reverse primer sets used for quantification of bacterial genera by Real-Time PCR on leonardite-treated and untreated samples.

Name	Forward primer 5' – 3'	Reverse primer 5' – 3'
Pseudomonas	GCGCGTAGGTGGCTTGATAA	GGATGCAGTTCCCAGGTTGA
Burkholderia	CCTCTGCCATACTCTAGCCC	ATGTGAAATCCCCGGGCTTA
Oxalicibacterium	GCGCAACCCTTGTCATTAGT	TGTCACCGGCAGTCTCATTA
Massilia	CAATGCCGCGTGAGTGAA	GAACCGTTTCTTCCCTGACAAA
Propionibacterium	GGGTTAAGTCCCGCAACGA	ACCATAACGTGCTGGCAACA
Methylobacterium	CTTCCGGTACCGTCATTATCG	GTGATGAAGGCCTTAGGGTTGT
Hymenobacter	AGGTGGCCCCGCAAGT	TCCATGGCAGTTCTGTAGTTGAG
Xanthomonas	AAGGTGGGGATGACGTCAAG	TGTGTAGCCCTGGTCGTAAG

Real-Time Quantitative RT-PCR for Expressed Plant Genes

Eight sugar beet genes were used to test leonardite effects on plants. Primer design with Primer Express V3.0 (Thermo Fisher Scientific) was done starting from mRNA sequences downloaded from RefBeet_1.2 (http://bvseq.molgen.mpg.de). **Table 2** shows the complete list of genes, their functional category, and gene product. Quantitative RT Real-Time PCR amplification and detection were conducted on a Quant Studio 12K Flex Real-Time PCR (Thermo Fisher

Scientific) using qPCRBIO SybrGreen 1-step kit (Resnova-PCR Biosystem). The 10 ml of reaction mixture contained 5 ml of SYBR Green, 0.5 ml retrotranscriptase, 0.4 ml of forward and reverse primers, 0.7 ml of nuclease-free water, and 1 ml of RNA. The threshold cycle (Ct) values obtained were normalized against the average transcript abundance of three housekeeping genes (*Tubulin*, Bv2_037220_rayf; *GAPDH*, Bv5_107870_ygnn; *Histone* H3, Bv6_127000_pera) using the formula: $2^{-\Delta Ct}$ in which ΔCt is obtained from the difference between the Ct of the target gene and the Ct of the control gene (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

TABLE 2. Details of genes used for quantitative RT Real-Time PCR showing their functional category and gene product.

Gene	Category	Gene product
AREB1	Hormone metabolism	Abscisic acid-insensitive 5-like protein
HAB1	Hormone metabolism	Serine/threonine phosphatases Mg dependent
AHG3	Hormone metabolism	Phosphatases 2C
AUX1	Hormone metabolism	Auxin transporter-like protein 1
ATTIR1	Hormone metabolism	Protein transport inhibitor response 1, auxin binding
LAX2	Hormone metabolism	Auxin transporter-like protein 2
PIN3	Hormone metabolism	Auxin efflux membrane carrier protein, component 3
CSD2	Hormone metabolism	Superoxide dismutase [Cu-Zn]

Yield Traits

The effect of leonardite on sugar beet yield traits such as root yield, sugar yield, and processing quality-related traits were evaluated betweenMarch and August 2020 in Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E). The experimental design was divided into four randomized blocks, each one divided into four subplots whose size was 2.7 × 10 m. Outside the randomized block, a control plot was placed, and plants were kept without any treatments. The foliar spray treatments with leonardite solution were done using a dosage of 2,000 ml ha^{-1} (dilution 1:125, 4 mg C l^{-1}). Topped sugar beets from each subplot were collected after BBCH 49 (beet root has reached harvestable size) and analyzed to detect the mean of root yield, sugar yield, and processing qualityrelated traits as influenced by leonardite application. Roots from each collected plant were washed, and using a special sawing machine (AMA-KWS, AMA Werk GmbH, Alfeld, Germany), 1 kg of micronized tissues (brei) was obtained. About 70 g of representative homogenized brei samples were immediately frozen at –40°C. Sugar content and the main non-sugars were analyzed after cold digestion of the brei in lead acetate 0.75% (w/w) solution (Schneider, 1979) using an automated brei mixer (Venema Automation b.v.,

Groningen, Netherlands). To quantify the sugar content, a Thorn-Bendix 243 polarimeter (Bendix Corp., Nottingham, United Kingdom) was used, whereas K and Na concentrations were measured by a flame photometer (Model IL 754, Instrumentation Laboratory S.p.A., Milan, Italy). The a-amino N was quantified by colorimetric analysis (PM2K; Carl Zeiss GmbH, Oberkochen, Germany) following the procedure proposed by Kubadinow and Wieninger (1972) and Stevanato et al. (2010). The purity was calculated as the percentage of sugar from the roots extractable by the factory according to Wieninger and Kubadinow (1971) and Stevanato et al. (2010).

Data Analysis

Data analysis of community compositional structure of plant endophytic bacteria was conducted using Ion Torrent Suite software 5.16. This included the use of BaseCaller module to filter out low-quality sequences marked during the signal processing step followed by base calling, barcode assignment, and adaptor trimming at 3' end. The preprocessed fastq files were analyzed using Quantitative Insights into Microbial Ecology (QIIME) 1.9.1 pipeline. OTU clustering was done using a unique read abundance threshold of 10 and 97% sequence similarity against the curated Greengenes database v.13.8 and Curated MicroSEQ 16S Reference Library v2013.1. Microbial diversity was assessed using alpha and beta diversity using QIIME. The relative abundance of OTUs was calculated for both the family and genus level. Permutational multivariate analysis of variance (PERMANOVA), to test significance between groups, was performed using QIIME.

Data analysis of expression level of the gene panel and the main sugar beet productivity traits was conducted using Statistica v13.4 (Dell, Round Rock, TX, United States). Significant differences among the mean values were evaluated with Student *t*-test followed by *post hoc* analysis (Duncan's test). Significance was estimated at the $p < 0.05$ level. Data are expressed as mean ± standard error of the mean.

RESULTS

Bacterial 16S rRNA metabarcoding was performed on 14 untreated samples. We chose to sequence two groups of untreated plants, seven coming from the field (located in Pozzonovo, Padua, Italy) and seven grown in hydroponic solution, to study and compare the microbiome composition of sugar beet grown in two different environments without any treatment. Sequences have been deposited in the European Nucleotide Archive (ENA) browser under accession numbers PRJEB42500 and ERP126366.

A total number of 2,145,785 paired-end sequences were obtained, with an average length of 258 bp, and among them, 635,152 (29.6%) were rejected after the filtering process with the Torrent Suite software. Sequences were clustered into 139 OTUs at 97% identity cutoff. The remaining OTUs, divided into 34 different families and 37 genera, were subjected to the characterization of the endophytic bacterial communities. Alpha diversity, corresponding to the number of species or OTUs within samples (Willis, 2019), showed the highest number of sequences in samples grown in the field compared to hydroponics using the Chao indexes (**Figure 1**). A principal component analysis based on Euclidean distance was used to show how bacterial communities were distributed between field and hydroponics (**Figure 2**). Plants grown in hydroponic conditions (yellow dots) clustered separately from plants grown in the field (red dots) (PERMANOVA, $p < 0.05$).

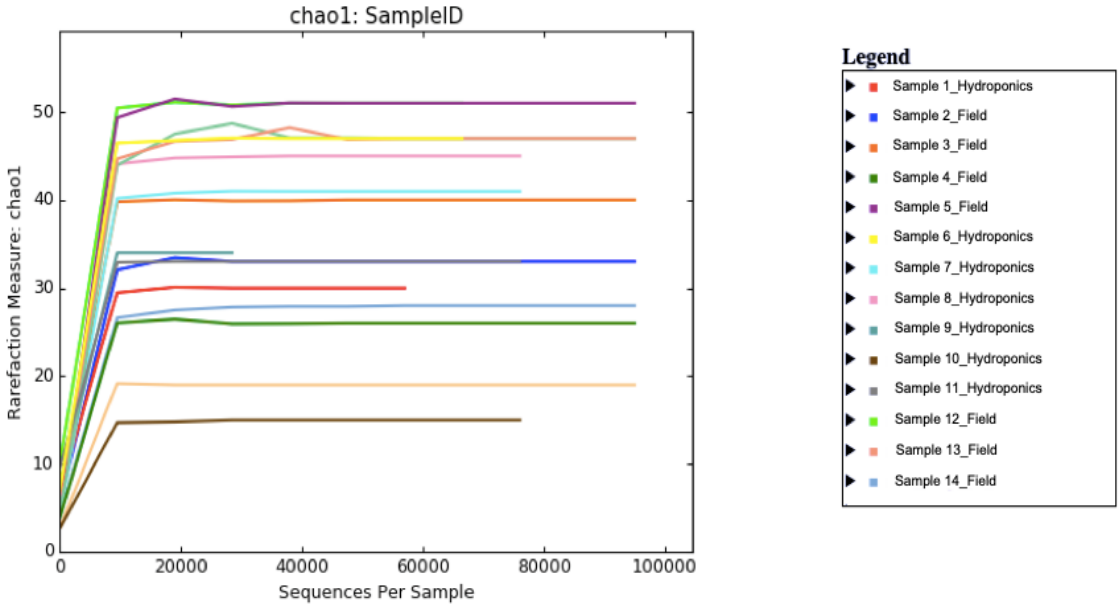

FIGURE 1. Alpha diversity in seven field and hydroponics-grown plants calculated with the Chao diversity index.

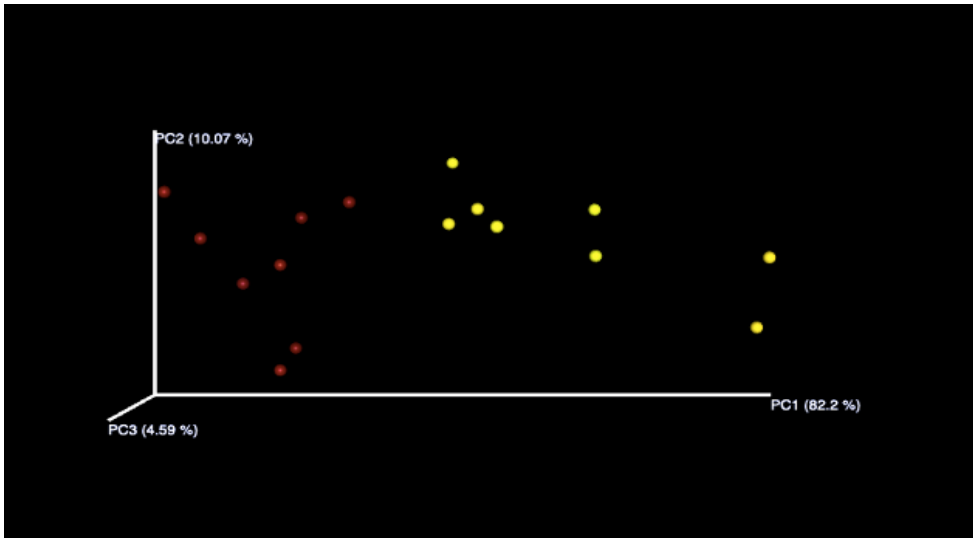

FIGURE 2. Evaluation of beta diversity in field (red) and hydroponic (yellow) plants. The principal component analysis was performed using Quantitative Insights into Microbial Ecology (QIIME).

The complete microbial profiles generated are shown in **Figure 3**. Bar-plot analysis showed that the majority of OTUs in the two groups were assigned to the genera *Pseudomonas*, followed by *Sphingomonas*, *Hymenobacter*, and *Methylobacterium*, as reported also by the percentage listed in Table 3. The minority of the OTUs found belonged to *Propionibacterium*, *Burkholderia*, *Massilia*, *Oxalicibacter*, and *Xanthomonas* (**Table 3**). Moreover, the bar plot represented a remarkable variability in the field-grown plants at the genus level. This variability is directly related to a higher number of genera identified, 20 in the field-grown plants compared to the 14 genera identified in hydroponics-grown ones. Particularly, these additional genera included *Duganella*, *Stenotrophomonas*, *Ralstonia*, *Delftia*, *Microbacterium*, *Acidovorax*, *Aurantimonas*, *Spirosoma*, and *Rhizobium*. In **Figure 3**, "Others" represents bacterial genera that formed less than 1% of the total abundance.

TABLE 3. Mean relative abundance (%) in each group at the genus level.

Genera	Field (%)	Hydroponics (%)
Pseudomonas	47.0	46.2
Sphingomonas	23.6	24.4
Hymenobacter	4.0	5.3
Methylobacterium	2.9	2.4
Massilia	2.2	4.1
Propionibacterium	1.8	1.4
Oxalicibacterium	1.4	1.9
Burkholderia	1.1	3.3
Xanthomonas	1.0	1.0

Bacteria with relative abundance higher than 1.0% are reported.

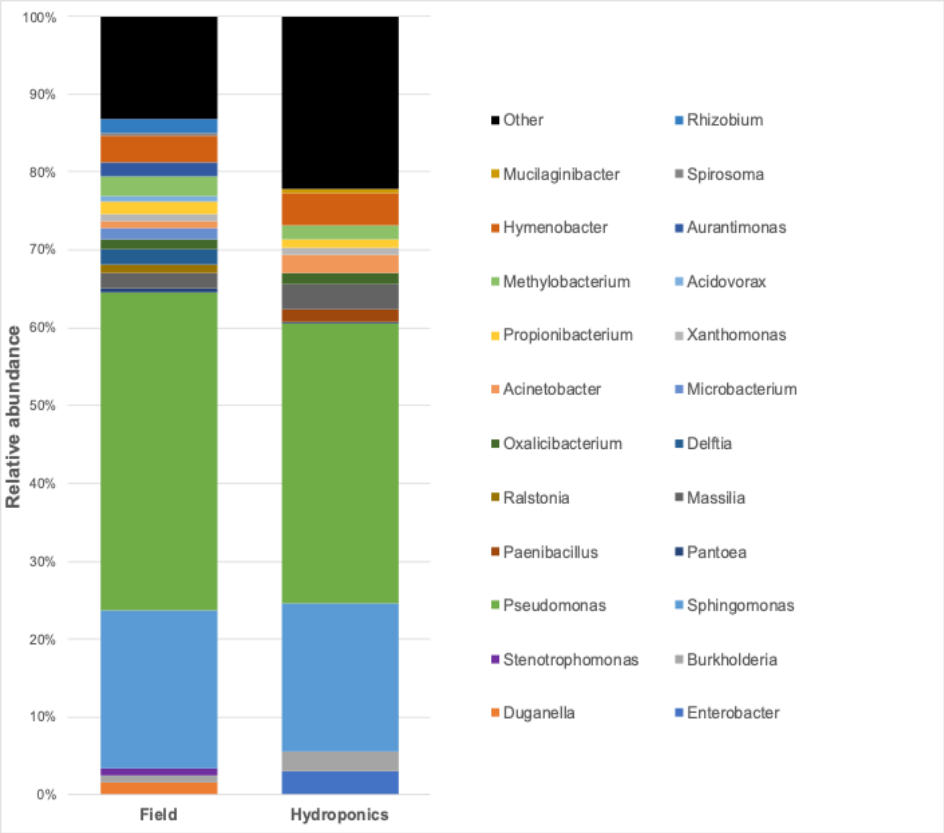

FIGURE 3. Relative sequence abundance of bacterial genera associated with field and hydroponics-grown plants. The most represented operational taxonomic units (OTUs), with relative abundance higher than 1%, are reported. OTUs with less than 1% are assigned as "Others."

Specific Real-Time PCR primer pairs were designed to detect eight genera, constituting the core microbiome of sugar beet, on leaf samples collected under field conditions (in four different locations) on 48 h leonardite-treated plants and untreated ones. All genera tested by Real-Time PCR were detected in both treated and untreated plants, without showing any significant variation, with exception of *Oxalicibacterium* spp. The average threshold cycle obtained for untreated samples was 24.20 with a standard error of 0.33, while samples treated with a dosage of 2,000 ml ha^{-1} (dilution 1:125) showed an average of 23.32 and a standard error of 0.29. Ct resulted from the mean of three biological replicates. Using a *p*-value threshold at 0.05, the treated samples have a significantly lower Ct value (indicating higher amounts of the template related to the presence of *Oxalicibacterium* spp.) compared to the untreated ones.

Quantitative RT Real-Time PCR was carried out to identify changes in gene expression profile between untreated and treated plants of the four locations. The selected genes had been detected in a previously published paper by Barone et al. (2019), where they were found responsive to leonardite treatment in hydroponic conditions. Among the complete dataset of 53 genes, we choose the ones involved in hormone metabolism. **Table 4** shows the percentage of

variation in the gene expression level of treated samples with respect to the untreated ones. Samples were collected after 24 h from leonardite treatment using a dosage of 2,000 ml ha^{-1} (dilution 1:125). One of the analyzed genes, *LAX2*, showed a significantly different level of expression ($p < 0.05$) in treated vs. untreated samples. This gene encodes for an auxin transport protein. Particularly, 24 h after the leonardite application, an expression level of 38% over the control of the *LAX2* was observed.

TABLE 4. Percentage variation in the gene expression level of treated samples with respect to the untreated ones.

Genes	Percentage of variation	*p*-value
AREB1	31%	n.s.
HAB1	8%	n.s.
AHG3	16%	n.s.
AUX1	-4%	n.s.
ATTIR1	13%	n.s.
LAX2	38%	0.025
PIN3	-7%	n.s.
CSD2	37%	n.s.

Student t-test was applied to verify the statistical significance between groups ($p < 0.05$; n.s., not significant). Samples were collected after 24 h from leonardite treatment using a dosage of 2,000 ml ha^{-1} (dilution 1:125), in four different locations.

Table 5 shows yield values and quality parameters as obtained from laboratory analyses on leonardite-treated and untreated sugar beet coming from Pozzonovo, Padua, Italy. The sugar yield of plants treated with leonardite (12.30 ± 1.13 t ha^{-1}) was significantly higher ($p < 0.05$) compared to that of the untreated ones (11.40 ± 1.56 t ha^{-1}). No significant differences can be observed in quality parameters of juice such as Na, K, α-amino N content, and sugar purity.

TABLE 5. Mean of root yield, sugar yield, and processing quality-related traits in leonardite-treated and untreated sugar beet grown in Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E).

Samples	**Root weight (t ha^{-1})**	**Sugar yield (t ha^{-1})**	**Potassium (meq % °S)**	**Sodium (meq % °S)**	**Alpha-N (meq % °S)**	**Sugar purity (%)**
Untreated	75.7	11.4	24.38	8.07	6.69	93.7
Treated	80.7	12.3*	23.54	7.73	7.04	93.8

DISCUSSION

Maintaining a healthy environment, while increasing plant yield and quality, is one of the key aspects of sustainable agriculture. The application of chemical pesticides and fertilizers can undermine soil quality and invertebrate population (Liu et al., 2015). Therefore, the scientific community is studying the role and specific effects of organic plant biostimulants as a gradual and promising replacement of chemical products.

Among biostimulants, leonardite, due to the high percentage of humic acids, is considered a bioactive compound suitable to preserve soil integrity (Turgay et al., 2010). Organic molecules (phenolic and alcohol compounds) contained in leonardite can be used by microbes as a source of nitrogen and carbon (Conselvan et al., 2017; Zhang et al., 2020). Consequently, the microbiome change following leonardite applications may be useful in elucidating the mechanism of action of this product (Yu et al., 2015). Therefore, the monitoring of bacterial species and their relative abundance is fundamental to understand the changes induced by biostimulant application.

In this study, the 16S rRNA metabarcoding analysis was performed on the pretreated microbiota of seven sugar beets grown in the field and seven grown in hydroponics. This comparison revealed nine shared bacterial genera between the two groups of plants. *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Propionibacterium*, *Burkholderia*, *Massilia*, *Oxalicibactirium*, *Hymenobacter*, and *Xanthomonas* constituted the core microbiome of seedlings grown in the two different environments. These, being found also in hydroponically grown seedlings, qualify as plant-borne and seed sterilization-resistant endophytes. As a result, these bacteria outline the seed microbiome of the sugar beet genotype used to compare the changes brought by leonardite treatments. These common bacteria are recognized to be seed endophytes with plant growth-promoting activity (Truyens et al., 2015), such as *Pseudomonas* and *Sphingomonas*, found also to be the most abundant genera. Other genera, including *Propionibacterium* and *Burkholderia* are involved in seed germination and root and shoot growth (Johnston-Monje and Raizada, 2011; Rodríguez et al., 2020). Among total bacteria found through sequencing, many of them were unique of field-grown sugar beet, originating from soil and environment. These are *Duganella*, *Stenotrophomonas*, *Ralstonia*, *Delftia*, *Microbacterium*, *Acidovorax*, *Aurantimo nas*, *Spirosoma*, and *Rhizobium.* They can be mostly divided into disease suppressive, such as *Duganella*, *Microbacterium*, *Rhizobium*, *Delftia*, and *Stenotophomonas* that also have beneficial activity on plant growth and, on the other hand, *Acidovorax* and *Ralstonia* are recognized to be plant pathogens (Bergna et al., 2018; Woźniak et al., 2019).

The shared bacteria between the two groups were analyzed using quantitative Real-Time PCR on leonardite-treated and untreated sugar beet. Specific primers were designed to quantify their

abundance. The results obtained showed that *Oxalicibacterium* spp. revealed a significant increase in abundance in plants treated with leonardite. *Oxalicibacterium* spp. belongs to the *Oxalobacteraceae* family, and among this family, we detected also the genus *Massilia*. *Massilia* is the richest genus of the *Oxalobacteraceae* family, isolated from roots and leaves, with plant growth-promoting activity and disease-suppressive abilities, while *Oxalicibacterium* is considered the most specialized oxalate degrader (Bonanomi et al., 2018; Raths et al., 2020). Oxalate is a secondary metabolite, widely reported in plants and soils, and a major component of root exudate with a key role in the recruitment of soil microbial species (Martin et al., 2012; Baldani et al., 2014). Typically, the root exudates contain acetate, succinate, lactate, fumarate, malate, citrate, isocitrate, aconitate, and oxalate. The release of these organic compounds increases microbial activity and nutrient exchange (Jones, 1998). Oxalotrophic bacteria metabolize oxalic acid, and the product of their metabolism leads to a strong local increase of soil pH (Martin et al., 2012). In *Arabidopsis thaliana* and *Phaseolus vulgaris* L., the degradation of oxalic acid has a protective function against pathogens, making the environment less favorable to fungi growth (Müller et al., 2016). Oxalate degrader microorganisms can increase the number of available phosphates influencing the phosphorus cycle and intensify the absorption of metals such as Fe and Al from soil (Morris and Allen, 1994). Other bacteria have been reported as oxalate degraders including *Propionibacterium*, *Burkholderia* spp., *Pseudomonas* spp., *Ralstonia*, and *Methylobacterium* spp. that we found as constituents of the core seed microbiome. Microbiome changes following leonardite treatment have already been studied in other plants, such as grapevine and potato (Cappelletti et al., 2016; Akimbekov et al., 2020). Also, Moreno et al. (2017) observed an increase of Gram-negative bacteria, such as *Proteobacteria*, as a consequence of the application of leonardite in barley.

The molecular analysis conducted in this work was done to evaluate hormonal gene responses, induced by leaf application of leonardite. The analyzed gene, belonging to hormonal metabolism, was selected among a larger set of 53 genes related to leonardite treatment on sugar beet and more generally based on the already known activity of humic acids on plant growth and development (Canellas et al., 2015; Nardi et al., 2016; Barone et al., 2019; Hajizadeh et al., 2019). However, the aforementioned genes were tested only on plants grown in hydroponic conditions, showing significant variation compared to untreated samples after 24 h of treatment. Thus, a first evaluation of the data obtained revealed the complexity of leonardite effects on sugar beet grown in a dynamic and variable context such as the open field. Among eight evaluated genes, the *LAX2* gene, encoding for auxin transport protein, showed a significant change between treated and untreated plants, while the others showed high variability among replicates. The overexpression of the *LAX2* transporter at 24 h from the foliar

application could be explained as a particular consequence of the ascertained auxin-like activity of humic substances contained in the product (Pizzeghello et al., 2001; Canellas et al., 2002). However, 72 h from leonardite treatments, the increasing trend in *LAX2* expression of treated samples is no longer observable (data are not shown). High variability, due to the open-field growth conditions, was observed for the other hormone-related genes and, although they showed a high percentage of variation, the statistical test resulted in no significant difference. However, these auxin-like substances are mainly transported through the phloem but are also exported and imported from cell to cell thanks to specific membrane transporters (Petrášek and Friml, 2009). The movement of auxins and the regulation of homeostasis of these substances within the plants are key processes in the modulation of growth and development such as tropism, embryogenesis, and organogenesis of roots, shoots, and vascular tissues.

Regarding the relationship between sugar beet yield traits and leonardite treatment, we did not find significant differences in the impurity content between control and treated plants unlike Rahimi et al. (2020) who observed a decrease in Na, K, and α-amino N following treatment with humic acid. However, we reported higher values of sugar yield on treated plants. This improvement in production is confirmed also in other treated crops with higher tuber yield in potato, higher root growth and yield in tomato, and a higher dry matter in canola (Akinremi et al., 2000; Pertuit et al., 2001; Sanli et al., 2013).

The present study provides important evidence for understanding the effects induced by leonardite-based biostimulant in sugar beet. Initially, the microbial populations of plants grown under hydroponic and field conditions were compared. After leonardite treatment, the most responsive genus was *Oxalicibacterium*, comprising endophytes with plant growth-promoting activity. Also, an upregulation of the *LAX2* gene, coding for auxin transport proteins, has been observed. This finding is in agreement with our previous work (Barone et al., 2019), which was entirely conducted on hydroponics-grown seedlings and the same gene was overexpressed after leonardite treatment. A significant increase in sugar yield was also observed in plants treated with leonardite compared with untreated ones. Thus, the present study represents a step forward to understand the changes induced by leonardite-based biostimulant in sugar beet.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: European Nucleotide Archive (ENA) Browser, https://www.ebi.ac.uk/ena/browser/home, PRJEB42500 and ERP126366.

AUTHOR CONTRIBUTIONS

AB, FM, AS, and PS: conceptualization. GCo, LS, and FM: supervision. MCDL, GB, LM, GM, SR, MC, CB, and GCa: methodology. CB and PS: writing original draft. AR: writing, review, and editing. All authors contributed to the article and approved the submitted version.

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CONTRIBUTION II

TRANSCRIPTIONAL AND PHYSIOLOGICAL ANALYSES TO ASSESS THE EFFECTS OF A NOVEL BIOSTIMULANT IN TOMATO

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ABSTRACT

This work aimed to study the effects in tomato (*Solanum lycopersicum* L.) of foliar applications of a novel calcium-based biostimulant (SOB01) using an omics approach involving transcriptomics and physiological profiling. A calcium-chloride fertilizer (SOB02) was used as a product reference standard. Plants were grown under well-watered (WW) and water stress (WS) conditions in a growth chamber. We firstly compared the transcriptome profile of treated and untreated tomato plants using the software RStudio. Totally, 968 and 1,657 differentially expressed genes (DEGs) (*adj-p-value* < 0.1 and $|\log_2(\text{fold change})| \geq 1$) were identified after SOB01 and SOB02 leaf treatments, respectively. Expression patterns of 9 DEGs involved in nutrient metabolism and osmotic stress tolerance were validated by real-time quantitative reverse transcription PCR (RTqPCR) analysis. Principal component analysis (PCA) on RTqPCR results highlighted that the gene expression profiles after SOB01 treatment in different water regimes were clustering together, suggesting that the expression pattern of the analyzed genes in well water and water stress plants was similar in the presence of SOB01 treatment. Physiological analyses demonstrated that the biostimulant application increased the photosynthetic rate and the chlorophyll content under water deficiency compared to the standard fertilizer and led to a higher yield in terms of fruit dry matter and a reduction in the number of cracked fruits. In conclusion, transcriptome and physiological profiling provided comprehensive information on the biostimulant effects highlighting that SOB01 applications improved the ability of the tomato plants to mitigate the negative effects of water stress.

Keywords: plant biostimulant, functional characterization, drought stress, tomato, transcriptomics, differentially expressed genes, physiological traits

INTRODUCTION

Biostimulants are increasingly important in agriculture, being considered environmentally sustainable and economically favorable answers to optimize crop productivity (Rouphael and Colla, 2020). There are currently several definitions of biostimulants. Conceptually they can be defined as non-nutrient substances or microorganisms applied to plants to promote plant growth, nutrient use efficiency, and stress tolerance (Calvo et al., 2014; Du Jardin, 2015).

Their action on plants is exerted through several mechanisms among which are the capacity to produce a hormone-like activity, the enhancement of photosynthesis, and the promotion of the activity of plant-soil microorganisms (Nardi et al., 2016; Van Oosten et al., 2017; Hellequin et al., 2020; Della Lucia et al., 2021).

Biostimulants are derived from a broad variety of compound classes that include mainly humic and fulvic substances, seaweed extracts, beneficial microorganisms, protein hydrolyzates and other nitrogen-containing compounds, carbohydrates, and inorganic compounds (Rouphael and Colla, 2020).

They are increasingly studied and used to mitigate the negative effects of environmental stresses such as lack of water and nutrients on cultivated plants (Van Oosten et al., 2017). Drought stress is one of the major problems of crops and especially limited water availability is a frequent suboptimal condition encountered by horticultural crops as tomato (Bulgari et al., 2019). One of the main biochemical impairing conditions occurring in plants under drought stress is the oxidative damage brought on by the overproduction of reactive oxygen species (ROS) (Fahad et al., 2017). The physiological responses induced by water stress include decreased cell turgor (Le Gall et al., 2015), leaf rolling (Kadioglu et al., 2012), inhibited CO_2 exchange, decreased photosynthetic efficiency and chlorophyll contents (Mao et al., 2015), and, finally, a drop in overall crop performance.

The diverse nature of many biostimulants and the wide variety of their constituents are adding complexity to the processes of modes of action discovery, product description, production, legislation, and use (Yakhin et al., 2017). Owing to the advancements in omics sciences, relevant steps forward have been made in the last years in studying the modes of action of plant biostimulants (Ertani et al., 2009; Przybysz et al., 2014; Colla et al., 2017a; Lucini et al., 2020). The joint use of omics technologies, such as transcriptomics, metabolomics, and phenomics, can comprehensively clarify the biological basis underlying the biostimulation activity. Moreover, product screening strategies using omics technologies are considered efficient and cost-effective for developing and testing biostimulant substances (Paul et al., 2019).

The mRNA sequencing technology has become a crucial tool for differential gene expression analysis. It is advantageously used to monitor plant status and it has been adopted to study the biostimulants' function in several works in a wide variety of crop species and biostimulant compounds in different environmental conditions (Briglia et al., 2019; González-Morales et al., 2021). Plant gene expression is dependent upon a multitude of factors. It is regulated during plant development, and it changes in response to environmental factors, and abiotic or biotic stresses (Costa-Silva et al., 2017).

To thoroughly study the biostimulant effects on plants, the combination of transcriptome profiling with plant phenomics, which measures specific physiological parameters, has been exploited and suggested (Briglia et al., 2019). The use of chlorophyll fluorescence images combined with phenotyping structures enables rapid screening of the overall photosynthetic performance and characterization of a plant's potential to harvest light energy, which is related to biomass

formation and plant structure (Tschiersch et al., 2017). Photosynthesis prediction is the first step to preannounce crop growth, yield, and quality in response to environmental changes (Zhang et al., 2018) and predict the onset of abiotic stresses (Murchie and Lawson, 2013).

This work aimed to study the effects in tomato of foliar applications of a novel calcium-based biostimulant (SOB01) in well-watered and water scarcity conditions by means of an omics approach involving transcriptomics and physiological profiling. A calcium-chloride fertilizer (SOB02) was used as a product reference standard.

Firstly, we analyzed the transcriptome profiling mRNA sequencing of treated and untreated tomato plants after the first treatment application, at one developmental stage (5th inflorescence, BBCH65). We then selected nine mRNA transcripts and we evaluated their expression patterns by real-time qPCR on plants grown under well-watered and water stress conditions at three different plant phenological stages (BBCH65, BBCH75, and BBCH85) to evaluate the effects of the treatments in two different water regimes. At the same time, we conducted physiological evaluations to functionally validate the selected transcripts potentially relevant for plant growth and yield and to describe the plant physiological responses.

MATERIALS AND METHODS

Plant Material, Growing Conditions, and Experimental Setup

Tomato (*Solanum lycopersicum* L.) seeds, var. Micro-Tom, were provided by Sipcam Oxon S.p.A. Seedlings were grown individually in 1.2 L pots filled with a mixture of 90% peat (white sod peat (10–25 mm), white peat (0–25 mm), and peat fiber) and 10% perlite with a concentration of N (140 mg L^{-1}), P (160 mg L^{-1} P_2O_5), K (1,680 mg L^{-1} K_2O), Mg (100 mg L^{-1}), and all necessary trace elements. Pots were placed within a growth chamber under controlled environmental conditions of 20–24°C temperature, 60% relative humidity, and LED lighting for 12 h/day. A soluble commercial fertilizer with 20% N, 20% P, 20% K content by weight was added twice a week to each pot. A randomized complete block design with two blocks was set up with two water regimes and two foliar treatments in a factorial combination. A total of 30 plants including 6 biological replicates (plants) for each experimental condition were used. The two applied treatments were a novel calcium-based mixture with a concentration of 5 ml L^{-1} (SOB01) and a calcium-chloride solution with a concentration of 10.05 gr L^{-1} (SOB02). These products were provided by Sipcam Oxon S.p.A. and the composition of SOB01 is described in **Supplementary Table 1**. Each solution was diluted in ultra-pure water and was applied as a foliar spray, at a volume of 10 ml per plant at three different phenological stages BBCH65 (5th inflorescence), BBCH75 (5th fruit cluster), BBCH85 (50% of fruits show typical fully ripe color).

Rates of application of both SOB01 and SOB02 were defined to achieve the same amount of Ca per hectare following the label recommendations in three key developmental stages of tomato crop. The two water regimes applied were well-watered at pot water capacity (WW) and water stress, 65% of pot water capacity (WS). Water stress was induced before the flowering stage, after 2 weeks from transplant for those plants subjected to drought stress and kept throughout the experiment. The water content of the pots was continuously measured through an automatic moisture content monitoring system of independent loading cells and data were recorded as hourly average in a data logger (Gmr Strumenti Sas, Italy).

Transcriptome Profiling

Sample Harvest

For transcriptome sequencing well-watered plants were sampled just before (untreated, $t = 0$) and after 48 h from treatment application (treated) at BBCH65. The choice of this specific sampling time was based on previous similar experiments that allowed the detection of molecular responses in the early hours following a foliar treatment. Each plant sample was made by two leaf disks collected per single plant. Three biological replications were analyzed for each entry. For gene expression analysis through quantitative RT-PCR, we collected four sample replicates (2 leaf disks per single plant) just before and 48 h after each treatment application at the three developmental stages previously described (BBCH65, BBCH75, and BBCH85), from well-watered and water-stressed plants. Samples were immediately stored at −80°C until RNA extraction.

Direct mRNA Isolation

mRNA sequencing was carried out at the phenological stage BBCH65 (5th inflorescence) before and after 48 h of treatment application. mRNA was directly isolated using the Dynabeads mRNA Direct Micro Kit (Thermo Fisher Scientific, Carlsbad, CA, United States) following the protocol for mRNA isolation from tissues. Briefly, we ground 30 mg of frozen leaf samples for 3 min with the Tissue Lyser (Qiagen, Germany) together with 100 µl of lysis-binding buffer. The lysates were then combined with 20 µl of pre-washed Dynabeads Oligo (dT) and mixed by pipetting up and down three times. The sample tubes were placed in a mixer for 5 min to allow the mRNA to anneal to the Dynabeads Oligo (dT) and successively placed in DynaMag-2 Magnet (Thermo Fisher Scientific) for 1 min to discard the supernatant. Samples were then removed from the magnet and the Dynabeads-mRNA complex was resuspended in 100 µl of Washing Buffer A. Again, the supernatant was removed by placing the sample tubes in the DynaMag-2 Magnet and this step was repeated. 100 µl of Washing Buffer B was added to the remaining Dynabeads-mRNA complex and washed two times by discarding the supernatant using the DynaMag-2 Magnet. Finally, the Dynabeads-mRNA complex was eluted in 20 µl of ice-cold 10 mM Tris–HCl

and incubated at 65–80°C for 2 min. Once the tubes were placed in the magnetic rack, we transferred the supernatant containing the purified mRNA into a new tube and its quality and quantity were checked by Agilent TapeStation 1500 (Agilent Technologies Inc., Santa Clara, CA, United States). The average mRNA yields obtained were 2,150 ± 479 pg μl^{-1}. Once extracted, the quantification method showed contamination from 18S or 28S sequences that were removed to avoid unwanted amplicons by performing an additional washing step at the end of the mRNA extraction protocol.

Sequencing Library Preparation

Sequencing libraries were prepared using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific). mRNA was fragmented with Rnase III and the reaction was assembled as follows: 10 µl of poly(A) RNA, 1 µl of 10× Rnase III Reaction Buffer, and 1 µl of Rnase III. The incubation was done in a thermal cycler at 37°C for 3 min. Immediately after incubation, we added 20 µl of nuclease-free water to stop the reaction. After fragmentation, we proceeded to purify the 32 µl fragmented RNA by adding 5 µl of beads, 90 µl of Binding Solution Concentrate and 150 µl of 100% ethanol. After 5 min of incubation, samples were placed in the magnetic rack for 6 min to separate the beads from the solution, then we discarded the supernatant. Beads were washed with 150 µl of Wash Solution Concentrate for 30 s and the supernatant was discarded. We eluted the RNA from the beads by adding 12 µl of pre-warmed (37°C) nuclease-free water to each sample. At the end of the purification steps, we quantified the fragmented RNA with Agilent TapeStation 1500 (Agilent Technologies). The second step involved the hybridization and ligation of RNA. The hybridization master mix was prepared as follows: 3 µl of fragmented RNA, 2 µl of Ion Adapter mix v2, and 3 µl of Hybridization solution. The thermal cycler was set at 65°C for 10 min and 30°C for 5 min. The ligation master mix was composted by 8 µl of hybridization reactions, 10 µl of 2× ligation buffer, and 2 µl of ligation enzyme mix and incubated 30°C for 1 h. Then, we performed the reverse transcription with 2 µl of nuclease-free water, 4 µl of 10× RT buffer, 2 µl of 2.5 mM dNTP Mix, 8 µl of Ion RT Primer v2, together with 20 µl of the ligation reaction. The reaction was incubated at 70°C for 10 min, then added 4 µl of 10× SuperScript III Enzyme Mix and incubated again at 42°C for 30 min. cDNA was purified with the same procedure described above. The third step involved the amplification of cDNA with the following mix: 6 µl of cDNA, 45 µl of Platinum PCR SuperMix High Fidelity, and 1 µl of Ion Xpress RNA 3′ Barcode Primer. The reaction was set at 94°C for 2 min, 2 cycles at 94°C for 30 s, 50°C for 30 s, 68°C for 30 s, then 16 cycles at 94°C for 30 s, 62°C for 30 s, 68°C for 30 s, and the final hold at 68°C for 5 min. The amplified cDNA was purified as described above, eluted with 15 µl nuclease-free water, quantified through D1000 screen Tape (Agilent TapeStation 1500), normalized, and pooled.

mRNA Sequencing

The sequencing run was performed using an Ion Torrent S5 (Thermo Fisher Scientific) with Ion 540 kit OT2. 8 µl of the 100 pM cDNA library were diluted in 100 µl of nuclease-free water and used to prepare the template positive Ion sphere particles with the Ion One Touch 2 instrument (Thermo Fisher Scientific). The template positive Ion sphere particles were used for the enrichment process with the Ion ES instruments (Thermo Fisher Scientific). Samples were then sequenced with Ion 540 chip kit (Thermo Fisher Scientific).

Sequencing Data Analysis

Raw RNA-Seq reads were filtered to remove low-quality reads (phred-like *Q*-value ≤ 20). Filtered reads were mapped to *S. lycopersicum* genome (SLv3.0) (publicly available from NCBI, GenBank accession GCA_000188115.3) using Bowtie2 (v2.4.2) (Langmead and Salzberg, 2012). Mapped files were processed using samtools (v1.11) (Li et al., 2009) and raw read counts were counted for all predicted genes using bedtools multiBamCov (Quinlan and Hall, 2010). To remove less informative data, we filtered genes with an overall expression level smaller than 20. DESeq2 R package (v.1.30.0) (Love et al., 2014) was used to perform the inferential analysis and obtain differentially expressed genes (DEGs) across the biological conditions. An adjusted *p*-value < 0.1 and a range of log2-fold change ≥ 1.0 to ≤ –1.0 were set as thresholds of significance to select DEGs. Genes with a log2-fold change > 1 were regarded as up-regulated DEG, while genes with a log2-fold change < −1 were regarded as down-regulated DEG. DEGs were then subjected to enrichment analysis of Gene Ontology (GO) terms at an FDR threshold of 0.05 using ShinyGO v0.66 (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) to functionally categorize the genes by Biological Process, Cellular Component, and Molecular Functions.

Real-Time Quantitative Reverse Transcription PCR

Nine gene sequences were selected for further characterization of their expression on the three timings of treatment application and in WW and WS conditions. The genes annotations and primers sequences used in this study are reported in **Table 1**. Primer design with Primer Express V3.0 (Thermo Fisher Scientific), was done starting from mRNA sequences downloaded from Tomato Genome SLv3.0. The gene sequences belonged to the following significantly enriched functional categories: protein serine/threonine kinase activity, ion transmembrane transporter activity, response to stress, response to stimulus, and RNA binding. All these categories have emerged as relevant in the in-depth study of biostimulant applications (Ertani et al., 2017; Barone et al., 2019).

TABLE 1. Selected genes used for validation of RNA-Seq data using real-time quantitative RT-PCR.

Gene ID	Gene name	Gene description	Gene ontology term	Forward Primer 5',3' and Reverse Primer 3',5'
Solyc02g090510	*CRK*	CDPK-related protein kinase	GO:0004683 – calmodulin-dependent protein kinase activity	AATGCCAGCACTAATTCTACTC
				CCCTCTTCCAACTTCCTCTC
Solyc01g080300	*ABCI12*	ATP binding cassette	GO:0009236 – cobalamin biosynthetic process	TCTTTTCTCCTCTTCTTCCTCC
				ACGACTTCAATGCTCATCAC
Solyc06g071500	*SlBOR02*	Boron transporter	GO:0080139 – borate efflux transmembrane transporter activity	AGAGGAGAAAGAAGCCCCAG
				AGACACACAAACAAGGAAACAC
Solyc01g103890	*MRS2-4*	Magnesium transporter	GO:0015095 – magnesium ion transmembrane transporter activity	TCCCTTTTCGTTTTTTCCCC
				TTCCCCATCTTACCCAGTTC
Solyc01g067740	*SODCC1*	Superoxide dismutase [Cu-Zn]	GO:0004784 – superoxide dismutase activity	CTATTACCGACAAGCAGATTCC
				AATACCACAAGCAATCCTTCC
Solyc03g006680	*5203_PHOS32*	Universal stress protein	GO:0006950 – response to stress	ACTCAATAAGTCCCAACTCCC
				TTCTACCACCAACCATCCC
Solyc07g053200	*3369_PHOS32*	Universal stress protein	GO:0006950 – response to stress	CGTCCAAAACTACCTCCGTC
				TCAATCTCAACCTCTCCACTTC
Solyc10g079820	*ERD15*	Dehydration-induced protein	GO:0005515 – protein binding	ACCCAAATACTTTGAGAAGCC
				TGACACCTACCTTGCTCTATAC
Solyc12g056790	*NAC1*	NAC1 stress-related	GO:0045449 – regulation of transcription	AACCTCTCTCTACATCCATACC
				GAAACTAACCTCCAACCAACC

Real-time quantitative reverse transcription PCR (RT-qPCR) amplification and detection were conducted on a Quant Studio 12K Flex Real-Time PCR (Thermo Fisher Scientific) using qPCRBIO SyGreen 1-step kit (Resnova-PCR Biosystem, Rome, Italy). The 10 µl of reaction mixture contained 5 µl of SYBR Green, 0.5 µl retrotranscriptase, 0.4 µl of forward and reverse primers, 0.7 µl of nuclease-free water, and 1 µl of RNA. The threshold cycle (Ct) values obtained were normalized against the average transcript abundance of three housekeeping genes (GAPDH *Solyc05g014470*, Actin *Solyc11g005330*, and UBI *Solyc01g056940*), using the formula: $2^{-(\Delta Ct)}$ in which ΔCt is obtained from the difference between the Ct of the target gene and the Ct of the control gene (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Physiological Analysis

Every physiological measurement was carried out during the experiment before, and 48 h after the applications of the two products at the three aforementioned phenological phases to detect early physiological responses induced by the treatments' application.

Dry Matter and Fruit Cracking Measurements

Tomato fruits were harvested when fruits reached the maturity stage. At harvest fruit's fresh and dry weights were recorded for each plant. The dry matter (DM) of tomato fruit was measured by oven-drying a sub-sample at 60°C until a constant weight was obtained. At the harvest time, the number of cracked tomato fruits was counted, and cracking rates were calculated.

Gas Exchange Analysis

Gas-exchange measurements were taken with an infrared gas analyzer (CIRAS-3, PP Systems, Amesbury, MA, United States). The rates of net photosynthesis (Pn) and stomatal conductance (gs) were measured on the youngest fully expanded leaf in all the plants. The leaf was marked and the measurement after the treatment application occurred on the same leaf within the same treatment event. A different leaf was selected in every biostimulant application time due to plant growth. Measurements were made under saturating light at a PPFD (photosynthetic photon flux density) of 1,500 μmol m^{-2} s^{-1} with 390 μmol mol^{-1} of CO_2 flux density surrounding the leaf. The leaf cuvette had a 2.5 cm^2 window, and the light was provided by red, green, and blue light-emitting diodes. Leaf temperature for all measurements was kept at ambient temperature.

Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence measurements were estimated with a Hansatech Handy Plant Efficiency Analyzer (Handy PEA, Hansatech Instruments, King's Lynn, Norfolk, United Kingdom) on two intact leaves per pot before every treatment application and after approximately 48 h. The Handy-PEA sensor was placed on the leaf clip with the shutter open. The leaves on which the measurements were carried out were similar to the ones used for photosynthesis measurements. The leaf changed among application times due to plant growth. The saturated light level of the instrument was set at 3,000 μmol m^{-2} s^{-1} to generate a true fluorescence intensity of maximum value. Key fluorescence parameters were analyzed: the minimum fluorescence (F_0, dark-adapted leaf pre-photosynthetic fluorescent state), the maximum fluorescence (Fm, measured under a pulse of super-saturating light after the leaves were dark-adapted), and the fluorescence variable (Fv) which is the variable component of fluorescence obtained by the difference between Fm and F_0. The ratio Fv/Fm is proportional to the quantum yield of photochemistry in photosystem II (PSII) and shows a high degree of correlation with the quantum yield of net photosynthesis.

Soil Plant Analytical Division Measurements

The leaf chlorophyll content index was determined using a Soil Plant Analytical Division (SPAD) chlorophyll meter (SPAD 502, Konica Minolta Sensing, Inc., Ramsey, NJ, United States). It calculates the SPAD value based on the intensity of light transmitted around 650 nm (red band), where absorption by chlorophyll is high, and a reference wavelength around 940 nm (Markwell et al., 1995). Measurements took place on two fully expanded leaves per plant selected one at half-height of the plant and one among the uppermost leaves.

Statistical Analyses

Analysis of variance (ANOVA) of the data collected on physiological parameters of plants was performed using Statistix 10 (Analytical Software, Tallahassee, FL, United States).

Data analysis of genes expression levels was conducted using Statistica v13.4 (Dell, Round Rock, TX, United States). Statistically significant differences between the mean values of SOB01 and SOB02 treated plant samples were determined using the *t*-Student test ($p < 0.05$).

RESULTS

Transcriptomics Analysis

Direct mRNA Isolation and Library Preparation

The mean mRNA content of the extracted samples was 2,150 ± 479 pg μl^{-1}. The fragmentation step produced sequences of an average length of about 160 bp as represented in **Supplementary Figure 1C**. The library preparation protocol allows obtaining a quantity of amplified cDNA of approximately 15,000 pg μl^{-1}. **Supplementary Figure 1D** reports the final quantification of the cDNA library obtained from a tomato leaf sample with a read length ranging from 100 to 700 bp and a mean of 200 bp.

Sequencing and Data Analysis

Sequencing data were downloaded from the Torrent server which provides a preliminary run and samples quality check. The information available is the chip loading density, the percentage of loading, enrichment, clonal sequence, and final library (**Supplementary Figure 2**). In total, 139,979,951 single-end sequences were obtained across all samples. Sequences were filtered to remove polyclonal, low quality, and adapter dimer, for a total of 32% of removed sequences. The remained 96,700,462 sequences, with an average length of 205 bp, were used to measure the relative abundances of the transcripts.

Totally, 968 significantly DEGs (adj-*p* < 0.1 and |FC| ≥ 2) were identified from the comparison between samples of SOB01 treated plants after 48 h from the product application and untreated plants before application (T0) (**Figure 1A**). Among the DEGs we identified 173 up-regulated genes and 795 down-regulated genes. The comparison between SOB02 and untreated plants showed 1,657 DEGs divided into 1,348 down-regulated and 309 up-regulated genes (**Figure 1B**). The comparison between the two treatments showed that 16 genes were significantly up-regulated by both treatments, 157 were uniquely up-regulated by SOB01, and 293 uniquely by SOB02 (**Figure 2B**, red diagram). Down-regulation was observed in 186 genes common to both treatments and 1,162 and 609 uniquely down-regulated respectively in SOB01 and SOB02 treated plants (**Figure 2B**, blue diagram).

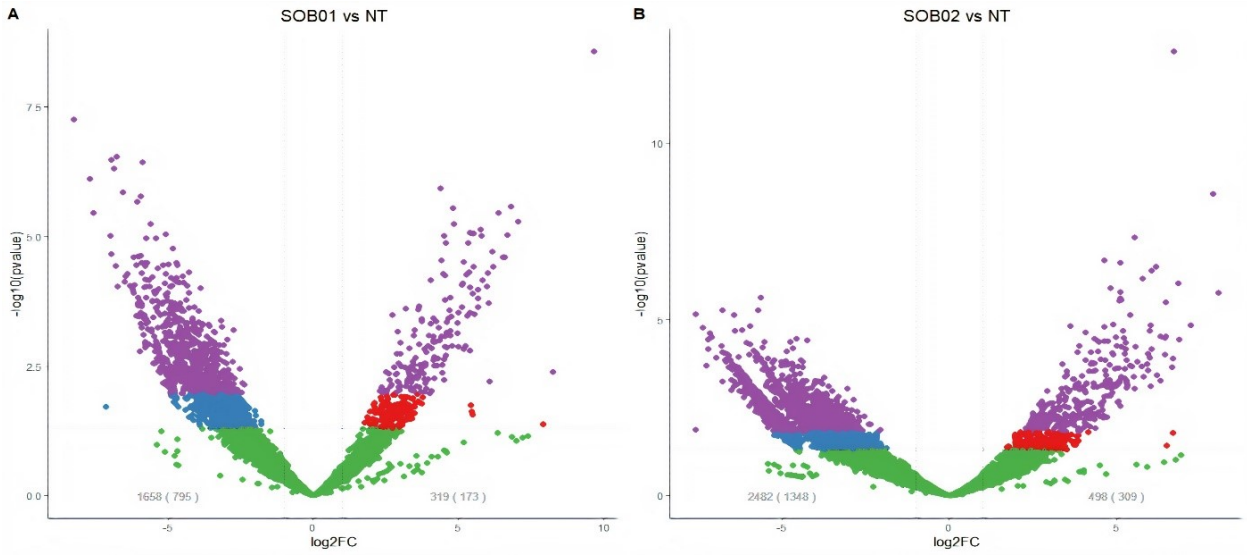

FIGURE 1. Volcano plots of significantly differentially expressed genes (DEGs) in tomato plants 48 h after treatment. Purple dots represent significantly up- and down-regulated DEGs (adj-*p* < 0.1 and | log2FC| > 1), red dots represent up-regulated DEGs (*p*-value < 0.05); blue dots represent down-regulated DEGs (*p*-value < 0.05); green dots are genes considered not differentially expressed according to the applied thresholds. Gray numbers display the number of DEGs according to the *p*-value threshold and in brackets according to the adj-p threshold. **(A)** DEGs in tomato plants 48 h after biostimulant treatment (SOB01) and non-treated (NT). **(B)** DEGs in tomato plants 48 h after calcium chloride treatment (SOB02) and non-treated (NT).

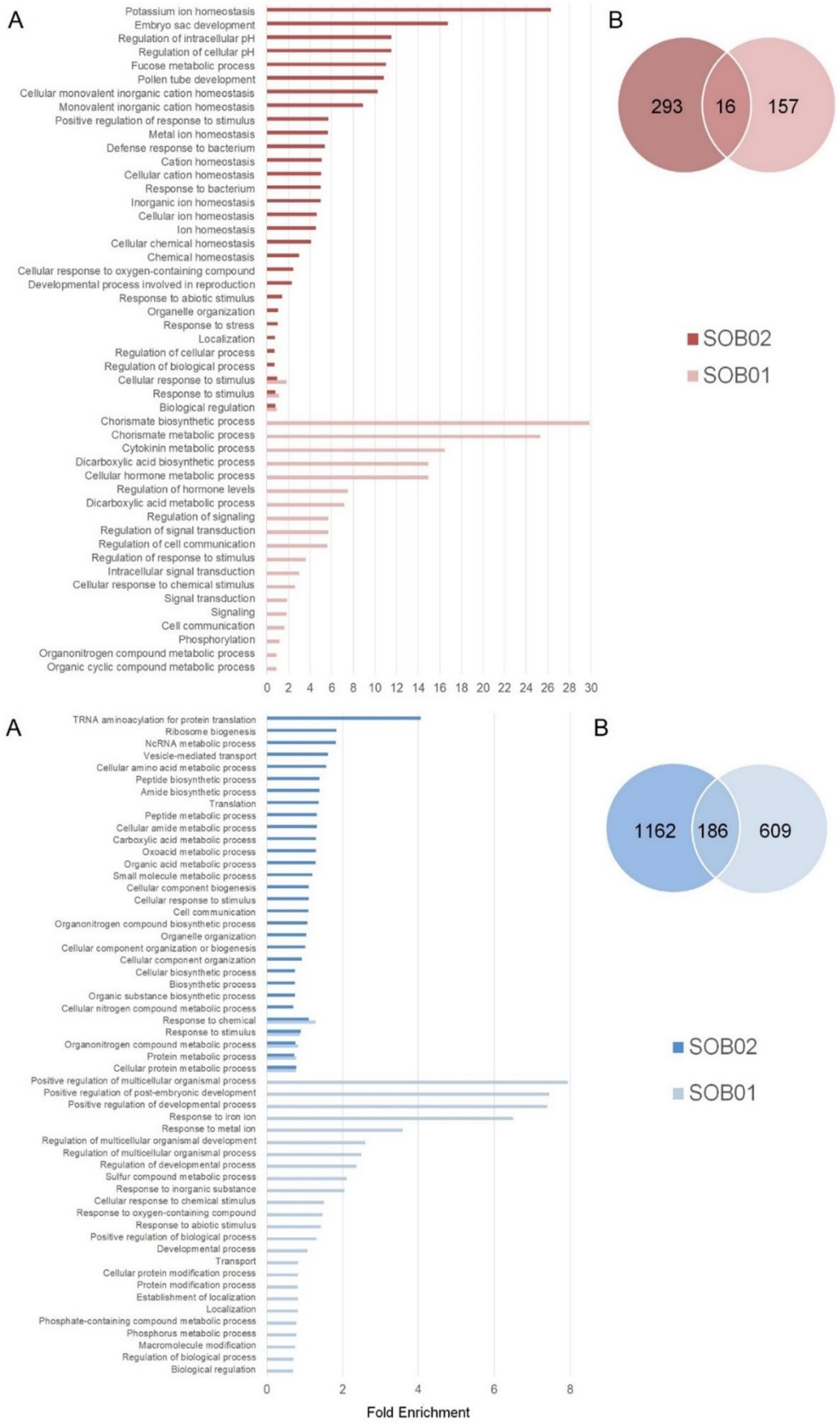

FIGURE 2. Categorization of DEGs (adj-p < 0.1 and $|\log_2 FC| \geq 1$) in tomato plants in response to SOB01 and SOB02 treatments. (A) Bar plots showing the significant enriched GO terms

(FDR < 0.05), describing biological processes, for up-regulated DEGs (red) and down-regulated DEGs (blue). GO enrichment analysis is obtained with ShinyGO (http://bioinformatics.sdstate.edu/go/) and *Solanum lycopersicum* as a reference genome. (B) Venn diagrams displaying the comparison of number of DEGs between SOB01 and SOB02. The number of uniquely DEGs are shown in the non-overlapping section and mutual DEGs among the two treatments in the overlapping section. Results for down-regulated genes (blue) and up-regulated genes (red) are shown in dark colors and light colors, respectively for SOB02 and SOB01 treatment in both Venn diagrams and bar plots.

Differentially regulated genes after SOB01 and SOB02 treatments were classified based on their functional category and a GO enrichment analysis was performed. The complete list of GO terms related to DEGs is given in **Supplementary Tables 2**, **3** (available online at the link https://www.frontiersin.org/articles/10.3389/fpls.2021.781993/full#supplementary-material). **Figure 2A** is showing the significantly enriched GO terms related to biological process in response to SOB01 and SOB02 (FDR < 0.05). The "positive regulation of developmental and multicellular organismal processes" related GO terms were the main categories significantly enriched in the down-regulated genes after SOB01 treatment (**Supplementary Table 2**). Various "metabolic and biosynthetic process" GO terms, concerning peptides, organonitrogen compounds, organic substances, amino acids, were observed in the down-regulated genes in response to the calcium chloride standard fertilizer SOB02 alone.

The highest folds enrichment in up-regulated genes associated just with SOB01 were related with chorismate metabolism and regulation of hormone levels and signaling. Up-regulated genes in SOB02 treated plants were mostly related to ion homeostasis.

Among the significantly enriched gene categories commonly down-regulated in SOB01 and SOB02 treatments, were organonitrogen compound and protein metabolic processes and again "response to stimulus." Whereas the few SOB01 and SOB02 commonly up-regulated genes encoded proteins involved in the cellular response to stimulus and biological regulation (**Figure 2A**).

Quantitative Reverse Transcription PCR Assay Results

Nine genes selected within significantly enriched functional categories and emerged as relevant in the in-depth study of biostimulant applications were selected for further characterization of their expression on the three timings of treatment application and in WW and WS conditions. The selected genes are: *CRK*, *ABCI12*, *ERD15*, *NAC1*, *5203_PHOS32*, 3369_PHOS32, *BOR02, SODCC1*, and *MRS2-4* (**Table 1**).

TABLE 2. Relative expression levels of nine selected genes in tomato leaves after 48 h from the treatment application.

	Well-watered			Water stress		
	SOB01	**SOB02**	***p*-value**	**SOB01**	**SOB02**	***p*-value**
Gene CRK						
BBCH65	0.260 ± 0.150	0.260 ± 0.062	0.8380	0.300 ± 0.090	0.320 ± 0.110	0.6845
BBCH75	0.160 ± 0.035	0.130 ± 0.051	0.0871	0.120 ± 0.026	0.90 ± 0.032	**0.0214** *
BBCH85	0.113 ± 0.040	0.111 ± 0.041	0.9536	0.095 ± 0.048	0.112 ± 0.051	0.3960
Gene ABCI12						
BBCH65	0.196 ± 0.011	0.182 ± 0.022	0.7216	0.248 ± 0.023	0.261 ± 0.088	0.4872
BBCH75	0.291 ± 0.024	0.275 ± 0.028	0.4015	0.300 ± 0.021	0.163 ± 0.015	**0.0002** *
BBCH85	0.100 ± 0.097	0.072 ± 0.008	**0.0032** *	0.095 ± 0.011	0.115 ± 0.009	0.1855
Gene ERD15						
BBCH65	0.045 ± 0.005	0.043 ± 0.005	0.7470	0.031 ± 0.006	0.038 ± 0.010	0.4377
BBCH75	0.068 ± 0.004	0.066 ± 0.006	0.9579	0.047 ± 0.006	0.056 ± 0.008	0.1156
BBCH85	0.100 ± 0.011	0.056 ± 0.004	**0.0007** *	0.076 ± 0.012	0.082 ± 0.009	0.6534
Gene NAC1						
BBCH65	0.100 ± 0.008	0.069 ± 0.006	**0.0044** *	0.080 ± 0.009	0.100 ± 0.010	0.1150
BBCH75	0.080 ± 0.005	0.078 ± 0.006	0.4367	0.078 ± 0.010	0.090 ± 0.010	0.2248
BBCH85	0.035 ± 0.002	0.022 ± 0.001	**0.0001** *	0.026 ± 0.004	0.044 ± 0.003	**0.0126** *
Gene 5203_PHOS32						
BBCH65	0.034 ± 0.004	0.033 ± 0.003	0.8324	0.020 ± 0.002	0.025 ± 0.006	0.4394
BBCH75	0.060 ± 0.005	0.050 ± 0.004	0.1926	0.055 ± 0.004	0.053 ± 0.007	0.6282
BBCH85	0.130 ± 0.020	0.080 ± 0.010	**0.0078** *	0.180 ± 0.020	0.130 ± 0.010	0.0942
Gene 3369_PHOS32						
BBCH65	0.05 ± 0.002	0.06 ± 0.008	0.0938	0.005 ± 0.001	0.120 ± 0.013	**0.0010** *
BBCH75	0.110 ± 0.003	0.120 ± 0.004	0.1590	0.082 ± 0.010	0.090 ± 0.010	0.6643
BBCH85	0.100 ± 0.020	0.060 ± 0.002	**0.0009** *	0.090 ± 0.010	0.098 ± 0.009	0.5895
Gene BOR.02						
BBCH65	0.075 ± 0.005	0.037 ± 0.002	**0.00004**	0.060 ± 0.008	0.075 ± 0.010	0.3346
BBCH75	0.360 ± 0.100	0.390 ± 0. 120	0.4083	0.390 ± 0.190	0.330 ± 0.290	0.0913
BBCH85	0.080 ± 0.007	0.045 ± 0.003	**0.0006** *	0.050 ± 0.004	0.070 ± 0.008	**0.0145** *
Gene SODCC.1						
BBCH65	0.0010 ± 0.0002	0.0014 ± 0.0009	0.0885	0.0015 ± 0.0004	0.0015±0.0008	0.9700
BBCH75	0.0016 ± 0.0020	0.0011 ± 0.0010	0.1348	0.0007 ± 0.0002	0.0024±0.0021	**0.0265** *
BBCH85	0.0017 ± 0.008	0.0015 ± 0.0005	0.6066	0.0024 ± 0.0006	0.0024±0.0010	0.9514
Gene 7864_MRS2-4						
BBCH65	0.040 ± 0.005	0.055 ± 0.004	**0.0276** *	0.035 ± 0.002	0.090 ± 0.011	**0.0149** *
BBCH75	0.250 ± 0.030	0.230 ± 0.070	0.4788	0.240 ± 0.020	0.267 ± 0.031	0.1730
BBCH85	0.210 ± 0.018	0.200 ± 0.005	0.2298	0.250 ± 0.070	0.280 ± 0.020	0.2690

Data are means ± S.D. and *p*-values of Student *t*-test comparisons ($p < 0.05$) between SOB01 and SOB02 in the two water regimes (WW and WS). Significantly different means ($p < 0.05$) are marked with a * and respective *p*-values are written in bold.

Real-time RT-qPCR was conducted on 48 samples divided into well-watered and water stress samples, two treatments, and three phenological stages. The experiments were repeated three

times, and representative data are reported. In **Table 2** relative expressions of the nine selected genes revealed significantly different expression levels between SOB01 and SOB02 for all the genes in at least one experimental condition (phenological stage or water regime).

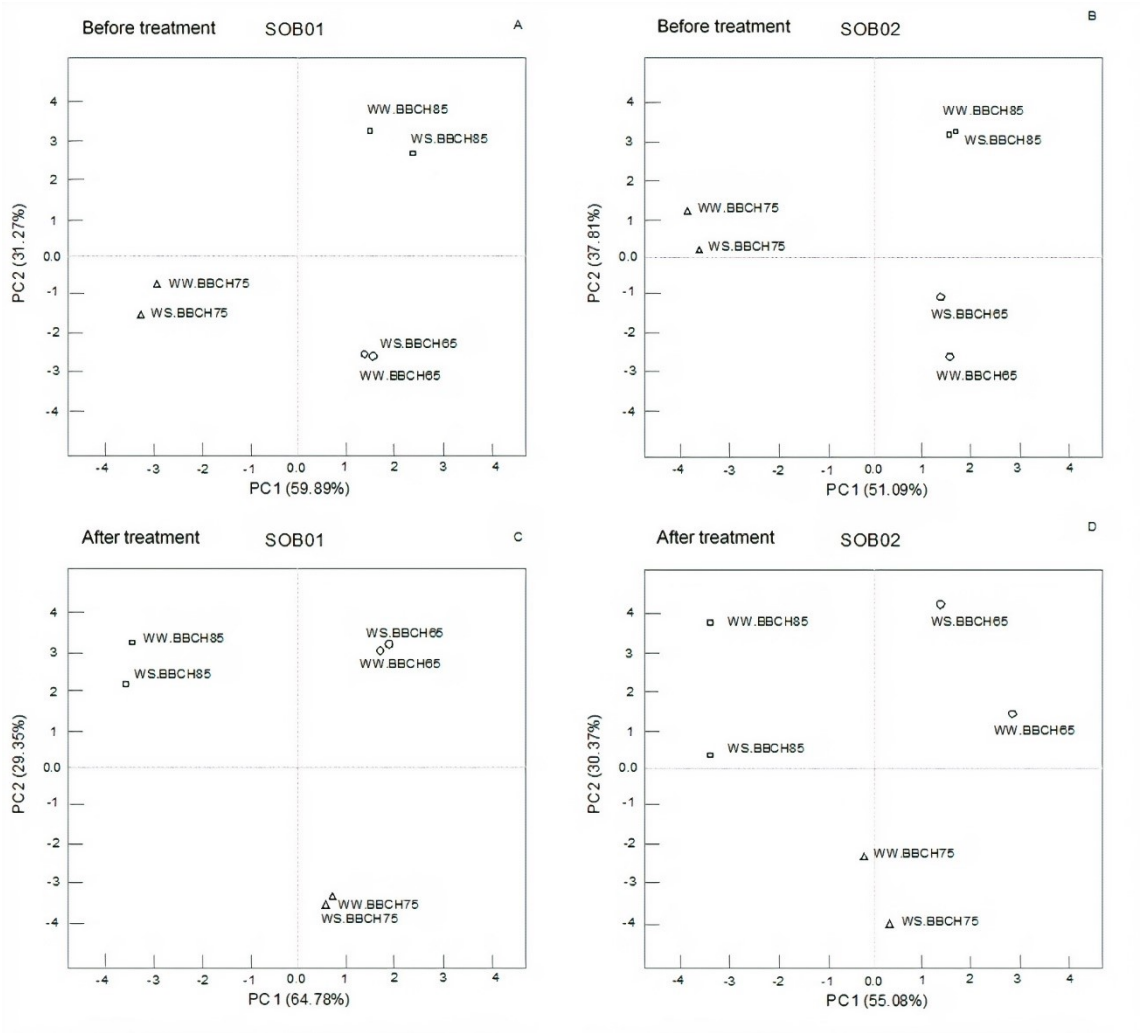

FIGURE 3. Principal component analysis of gene expression data of well-watered (WW) and water stress (WS) samples before treatment **(A,B)** and after treatment **(C,D)** with SOB01 **(A,C)**, and SOB02 **(B,D)** in three different phenological stages (BBCH65, BBCH75, and BBCH85).

Figure 3 shows the results of the principal component analysis (PCA) accomplished on gene expression data from RT-qPCR, pooling the results of 24 WS and 24 WW samples, each group composed of 12 plants treated with SOB01 and 12 treated with SOB02, and divided by three phenological phases. **Figures 3A** and **3B** display the PCs obtained from gene expression data divided by phenological stages before SOB01 and SOB02 treatments, respectively. **Figures 3C** and **3D** show the effect after SOB01 and SOB02 application, respectively, in the three phenological stages on WW and WS samples. PCA is often used in exploratory data analysis and pattern recognition as a tool to highlight differences among several types of samples.

The PCAs in **Figure 3** distinguish different groups based on phenological stage for each different sampling time. WW and WS in the different plant stages are clustering together after SOB01 treatment (**Figure 3C**), whereas gene expression data of WW and WS plants after treatment with the standard fertilizer SOB02 are not clustering together (**Figure 3D**).

Physiological Responses to Biostimulant Application and Water Stress

To assess the physiological responses of tomato plants to water deficit and biostimulant application, physiological traits including tomato fruit dry matter, number of cracked fruits, leaf gas exchange parameters, chlorophyll fluorescence, and SPAD were evaluated.

The physiological measurements taken in BBCH65 showed no significant effects of the treatments. A significant interaction was observed between the fertilizers and water regimes only at the fruit development phase (BBCH75). Although, there is no significant interaction between the two factors at BBCH65 and BBCH85 (**Table 3**). Except for the net photosynthesis rate, the water regime never influenced the plant response to fertilizers treatments.

TABLE 3. Analysis of variance of the physiological measured parameters that were affected by foliar application of fertilizers (F) and the Water regimes (W) at three phenological phases.

	BBCH											
	65				**75**				**85**			
	gs	**A**	**Fv/Fm**	**SPAD**	**gs**	**A**	**Fv/Fm**	**SPAD**	**gs**	**A**	**Fv/Fm**	**SPAD**
Fertilizer (F)	ns	ns	ns	ns	*	*	ns	*	*	ns	ns	ns
Water regime (W)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FxW	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns

SOB01, novel calcium-based biostimulant. SOB02, calcium-chloride fertilizer. A, net photosynthesis. Gs, stomatal conductance. Fv/Fm, chlorophyll fluorescence. BBCH65 (5th inflorescence), BBCH75 (5th fruit cluster), BBCH85 (50% of fruits show typical fully ripe color). ns, non-significant. *, Significant at the 0.05 level of probability.

Results indicated that the water regime did not affect the response of the fertilizers treatment on fruit dry matter content and the number of cracked fruits. In general, tomato plants treated with SOB01 showed significantly higher fruit dry matter production (5.35 g plant^{-1}) than those treated with SOB02 fertilizer (3.67 g plant^{-1}) (**Figure 4A**). Furthermore, the biostimulant treatment decreased the rate of cracked fruits (0.16 fruit plant^{-1}) compared to SOB02 fertilizer (0.19 fruit plant^{-1}) (**Figure 4B**).

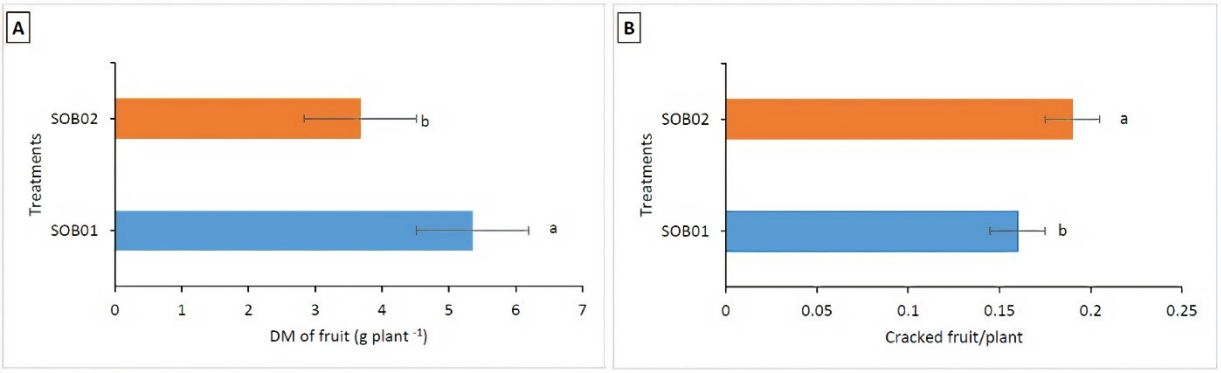

FIGURE 4. Average tomato fruit dry matter **(A)** and number of cracked fruits **(B)** per plant in response to different treatments. SOB01, novel calcium-based biostimulant. SOB02, calcium-chloride fertilizer. DM, dry matter. Different letters indicate a significant difference according to LSD test ($p < 0.05$).

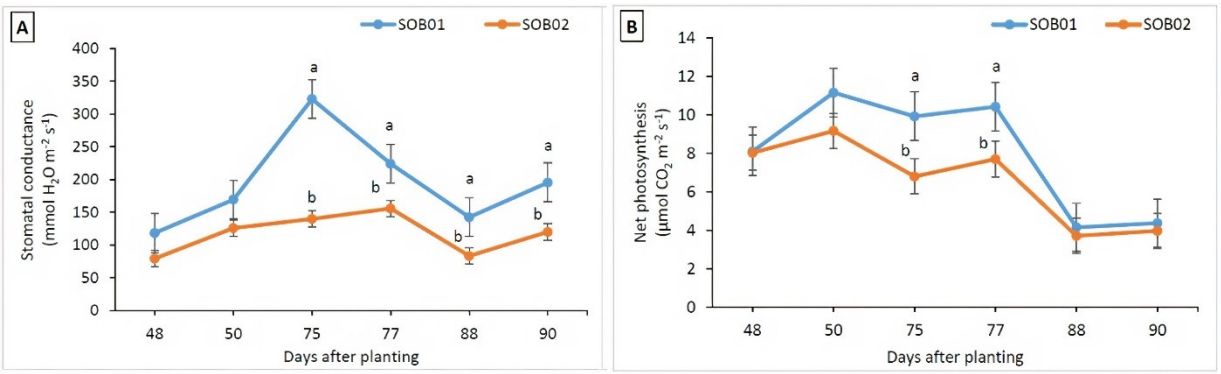

FIGURE 5. Effect of foliar fertilizer SOB01 and SOB02 on stomatal conductance **(A)** and net photosynthesis **(B)** at different phenological stages of tomatoes. Physiological measurements during plant growth were carried out before the application and 48 h after treatments application. SOB01, novel calcium-based biostimulant. SOB02, calcium-chloride fertilizer. Phenological stages BBCH65 (5th inflorescence = flowering), BBCH75 (5th fruit cluster = fruit development), BBCH85 (50% of fruits show typical fully ripe color = maturity). Different letters indicate a significant difference according to LSD test ($p < 0.05$).

The positive effects of SOB01 in terms of stomatal conductance and net photosynthesis were evident 48 h after the application at BBCH75 and BBCH85. The leaf stomatal conductance detected on plants treated with SOB01 was significantly higher than the one detected on plants treated with SOB02 before and after the second (BBCH75) and the third (BBCH85) application (**Figure 5A**). Stomatal conductance in plants treated with SOB01 reached its maximum value earlier in time compared to what was observed in plants treated with calcium chloride SOB02. Moreover, the net photosynthesis rate on plants treated with SOB01 was significantly higher than SOB02 treatment only at BBCH75, before and after application (**Figure 5B**).

A significant interaction between the different fertilizers and water regimes was detected on net photosynthesis at the fruit development stage (BBCH75) only (**Figure 6**). The application of the novel calcium-based biostimulant on plants partially compensated for the effect of water deficiency on net photosynthesis. The net photosynthesis of stressed plants treated with SOB01 showed almost a 2-fold increase compared to the plants treated with calcium-chloride fertilizer (SOB02) in the same water deficit conditions. Similar patterns were observed for well-watered plants (**Figures 6A,B**).

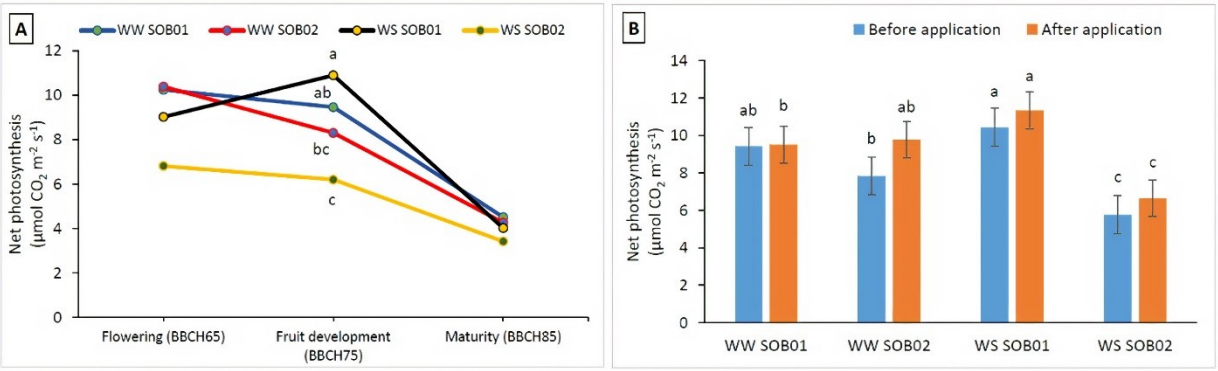

FIGURE 6. Interactions between different water and fertilizer treatments on net photosynthesis. **(A)** At different phenological stages and **(B)** at the fruit development stage (BBCH75) before and after treatment application. Measures were taken before the treatment application and 48 h after application. SOB01, novel calcium-based biostimulant. SOB02, calcium-chloride fertilizer. WW, well water. WS, water stress. Different letters indicate a significant difference according to LSD test ($p < 0.05$).

Physiological surveys performed during the plant growth showed significant effects of the treatment on the SPAD values. No significant changes in plant SPAD value were observed after treatments application at flowering and maturity stages. Conversely, the novel biostimulant (SOB01) induced a significant increase in this parameter, after the application at BBCH75 (fruit development stage) (**Figure 7A**). In contrast, no significant response of plants' chlorophyll fluorescence (Fv/Fm) to SOB01 and SOB02 application was observed at any developmental stage (**Figure 7B**).

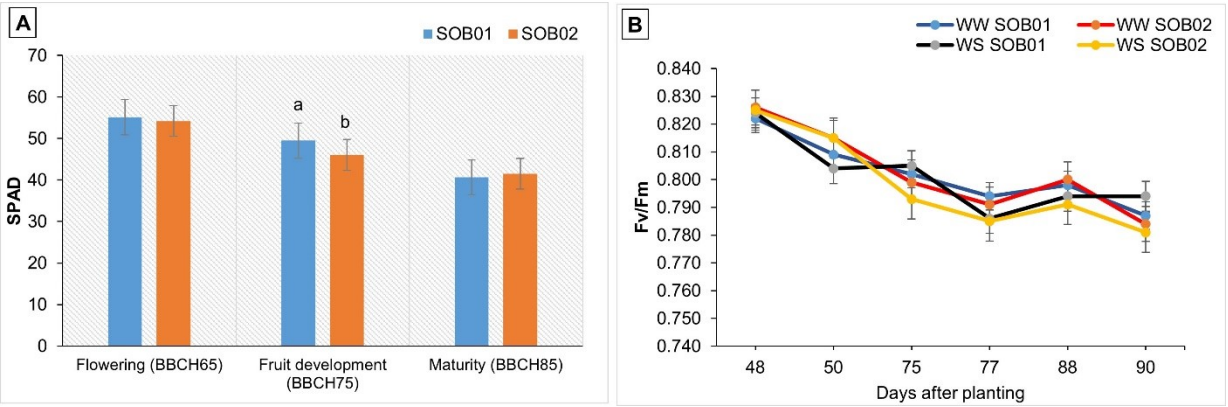

FIGURE 7. Soil Plant Analytical Division (SPAD) value **(A)** and Fv/Fm (maximum potential quantum efficiency of PSII) **(B)** in leaves of tomato at different phenological stages. Measures were taken before the application and 48 h after treatment application. SOB01, novel calcium-based biostimulant. SOB02, calcium-chloride fertilizer. WW, well water. WS, water stress. Different letters indicate a significant difference according to LSD test ($p < 0.05$).

DISCUSSION

Plant biostimulants constitute an emerging class of agricultural inputs that help to improve crop yield and quality while protecting from biotic and abiotic stresses (Rajput et al., 2019). The effects are dependent on application and dosage and act differently on different cultivars and environmental conditions (Di Mola et al., 2019). The ability to predict plant response to biostimulants is a high priority in the development of sustainable agriculture.

In this study, the applications of a novel calcium-based biostimulant (SOB01) and a calcium-chloride fertilizer (SOB02) were compared to elucidate their different effects on tomato plants under well-watered and water stress conditions. Since biostimulants have broad-spectrum activity, involving many plants' metabolic pathways, we initially focused on the analysis of the plant's transcriptome. This analysis allows the identification of changes in gene expression providing evidence regarding the pathways and the biological processes involved in the treatment-induced responses.

To describe all the effects produced by the biostimulant, we identified DEGs among plants before and after treatment with SOB01 and SOB02. The analysis output only 16 commonly up-regulated genes, indicating a dissimilarity in the transcriptome modulation after the fertilizer and the biostimulant application. For both the products, the number of down-regulated genes was greater than the number of up-regulated ones.

We then identified a panel of nine genes belonging to nutrient transport and metabolism and involved in the response to osmotic and water stress. The extensive characterization of the expression levels of the selected genes in the three different phenological stages corresponding to the treatment application times, on well-watered and water-stressed samples, was carried

out. The PCA plot is highlighting how the expression data of these nine genes in WW and WS plants after treatment with the standard fertilizer SOB02 are not clustering together in the same phenological stage. This pattern of expression could suggest that the applied water stress or the treatment SOB02 caused a variation in the expression of the nine genes, leading to the separation of WS samples from the WW ones.

We observed that two genes involved in chorismate metabolism were up-regulated and significantly enriched in SOB01 treated plants. Chorismic acid represents a key step in the shikimate pathway of aromatic acid biosynthesis, being a precursor to the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Maeda and Dudareva, 2012). Being crucial for the synthesis of aromatic amino acids and other secondary metabolites, the gene encoding the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), is fundamental for plant growth and development. Chorismate is also a common precursor of the three phytohormones auxin, salicylic acid, and melatonin (Pérez-Llorca et al., 2019). This observation could be explained as an activation of the plant signaling pathway in response to the application of SOB01 and its sugar fraction in particular. It is known that oligo- or polysaccharides are signaling molecules acting as elicitors of several pathways involved in plant secondary metabolisms (Zhao et al., 2005). This possibility is further reinforced by the observed significant enrichment associated with SOB01 up-regulation of the biological process described as “regulation of signal transduction” in which a gene coding for an ethylene receptor is over-expressed.

In the leaves treated with SOB01 we also observed an up-regulation of two genes involved in the cytokinin metabolic process which had a significant fold enrichment. Specifically, the gene LOG3 encoding a cytokinin riboside 5′-monophosphate phosphoribohydrolase is converting inactive cytokinin nucleotides to their biologically active forms. Cytokinins are a class of purine-based molecules with hormonal activity in plants which is promoting not only cell division and differentiation, but also growth, delay of senescence, and protection from oxidative stress (Mok and Mok, 2001). Cytokinins at leaf-level affect in several ways the photosynthetic process by promoting cell differentiation, increasing stomatal conductance, and improving the number and differentiation of chloroplasts (Hönig et al., 2018). The upregulation of key genes involved in the cytokinins activation following SOB01 treatment is positively correlated with the higher stomatal conductance measured in plants treated with SOB01 compared with the ones treated with SOB02. Moreover, the protection of the photosynthetic machinery potentially exerted by an increased cytokinins activation in the leaves, together with the antioxidant activity of these phytohormones, could have played a role in the overall mitigation of drought stress-induced detrimental effects.

Among the DEGs, obtained by the RNA-Seq analysis, down-regulated by SOB01 application we found SODCC.1 (*Solyc01g067740*) that encodes a Cu-Zn superoxide dismutase. It belongs

to the significantly enriched GO terms of response to abiotic stimulus, chemical, metal ions, and biological regulation. It was not differentially expressed in SOB02 treated plants. In the RT-qPCR analysis validation, the expression level of the same gene was significantly lower (3.4-folds lower) in SOB01 treated plants, sampled at the 5th fruit cluster (BBCH75) in water stress, compared to SOB02. Water stress is known to cause a wide range of plant responses. One of the most important is the increase in oxidative stress (Rao and Chaitanya, 2016). Superoxide dismutases (SODs) are a group of antioxidant metalloenzymes that protect cells from oxidative stress by catalyzing the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide. To regulate the ROS levels and restore normal physiological status, variations in the expression of SOD-encoding genes in response to environmental stresses are expected (Wang et al., 2016). Specifically, in young tomato plants, the expression of *Solyc01g067740* gene was previously evaluated by Feng et al. (2016) in response to high salinity and polyethylene glycol-induced drought stress. The former stress induced significant up-regulation of SODCC.1, whereas in drought conditions the level of expression did not change with respect to the control. In general, the activity of ROS scavenger enzymes in plants increases as a response to drought stress (Das and Roychoudhury, 2014). Moreover, responses to biostimulants involving the dysregulation of the superoxide dismutase gene family were observed in several works. Biostimulant application has been observed to either promote this antioxidant enzymatic activity as a response to the water-limited conditions (Liu et al., 2013) or, even before the stress application, exerting a priming effect that restrains the negative effects of the incoming stress (Goñi et al., 2016; Santaniello et al., 2017). In other cases, it can conversely slow down the activation of such metabolic pathways, compared to what happens in untreated plants, suggesting a process of adaptation to the drought stress due to the biostimulant treatment (Murtic et al., 2019; Campobenedetto et al., 2021). Our results on the lower expression levels of SODCC.1 in water-stressed plants treated with the novel biostimulant, compared to plants treated with the standard CaCl2 fertilizer, are following these last observations. Anyway, several studies on calcium chloride applications have shown the capacity of this mineral fertilizer to induce increased activities and gene expressions of superoxide dismutase and catalase which are protecting from oxidative stress produced by cold, pathogens, and drought (Xu et al., 2013; Shi et al., 2014; Chakraborty et al., 2017; Hou et al., 2021). Nevertheless, we did not observe a significant up-regulation of superoxide dismutase following SOB02 treatments in WW conditions and the physiological results in our experiment seem to encourage the interpretation of the lower expression of SODCC.1 gene in SOB01 plants as a reduction of the water stress susceptibility of the plants.

The application of the novel calcium-based biostimulant (SOB01) enhanced dry matter yield and fruit quality. The dry matter content of tomato plants treated with SOB01 was 50%

significantly higher than the one of plants treated with calcium chloride (**Figure 4A**). Furthermore, the biostimulant treatment decreased the rate of cracked tomato fruits per plant compared to SOB02 treated plants (**Figure 4B**). Tomato fruit cracking is a serious problem that results in significant financial losses. The fruit development rate during the ripening stage, maybe sustained by internal turgor pressure, is a key factor in fruit cracking (Domínguez et al., 2012). Fruit cracking can occur during fruit growth and/or ripening time. The cracking of the fruit is the result of a physiological imbalance determined by the action of multiple factors and physical nature linked to the plant. In addition to genetic susceptibility, water stress is one of the main determinants of cracking. Therefore, it is likely that fruit cracking was reduced in SOB01 treated plants through the positive effects on drought tolerance that allowed sustaining the plant during fruit development (Chrysargyris et al., 2020; Petropoulos et al., 2020).

Enhanced photosynthetic efficiency and a greater level of plant water content under drought stress conditions indicate an improved metabolic activity of plants (**Figures 6A,B**). In our experiment, it is likely that the biostimulant improved plant water status under drought and promoted cell enlargement, preventing ROS damage to pollen viability, with a beneficial effect on fruit development. Drought stress in plants leads to inhibition of photosynthesis and respiration, accumulation of ROS, and reprogramming of gene expression (Selote et al., 2004; Hayano-Kanashiro et al., 2009; Meng et al., 2020). The improved water status and the protection of cellular membranes under drought could be the reason for the higher yield reported in plants treated with SOB01, which was mediated by the higher drought tolerance of these plants during the sensitive stages of fruit development (BBCH75) and enlargement (**Figure 6**; Francesca et al., 2021). Additionally, our results on leaf gas exchange (**Figure 5**) were consistent with the findings of other researchers (Colla et al., 2017b; Parađiković et al., 2019; Soppelsa et al., 2019; Francesca et al., 2021), who observed that biostimulants application can enhance the leaf gas exchange characteristics to maintain plant water status under the water deficiency, improve nutrient uptake in plants, promote plant vigor and uniformity, be effective in regulating flowering, and stimulate fruit set and ripening.

Of the many biological processes activated when plants encounter environmental stresses, the photosynthesis-related processes and gas exchange responses are the most sensitive to water deficit (Hayano-Kanashiro et al., 2009; Huo et al., 2016; Min et al., 2016). Since photosynthesis is one of the main physiological processes affected by drought, photosynthetic parameters have been universally used to evaluate plant drought tolerance (Chaves et al., 2009; Osakabe et al., 2014; Zhang et al., 2018; Zhao et al., 2020).

Another common physiological response in plants suffering from drought stress is stomata closure. The closing of stomata is a well-known mechanism that plants use to avoid water loss in response to drought stress (Yan et al., 2016), but this adaptation also results in decreased

CO_2 flux (Ying et al., 2012). As water stress continues, the stomata remain closed for longer during the day. This leads to a reduction in carbon assimilation rate and water loss, which results in the maintenance of carbon assimilation at the expense of low water availability (Zhao et al., 2020).

The stomatal limitation is generally considered a major factor in the weakening of photosynthesis under water stress (Jones, 1998; Tardieu and Simonneau, 1998). In the case of water deficit, the reduction of leaf relative water content and water potential causes the stomata to close, leading to a decrease in the effectiveness of CO_2 and net photosynthesis (Bota et al., 2004; Zhao et al., 2020). Scientific evidence shows that photosynthesis, photochemical efficiency, and gas exchange processes are significantly less affected by stresses when biostimulants are applied (Van Oosten et al., 2017).

In this research, stomatal conductance was affected by the treatment application at fruit development and ripening stages, but stomatal closure was not significantly affected by water stress, resulting in no obvious reduction in photosynthesis. On the other hand, net photosynthesis was significantly affected by water stress and increased with the application of SOB01 at the fruit development stage (**Figures 6A,B**). This significant biostimulant-induced enhancement in photosynthesis of plants grown under WS conditions may be attributed to the changes in the photosynthetic machinery, chlorophyll content, leaf area, temperature, and leaf relative water content. These data are consistent with previous reports by Xu and Leskovar (2015) and Kałużewicz et al. (2017), who concluded that biostimulant application improved leaf water relations and helped to maintain cell turgor pressure and reduced stomatal closure, increased photosynthetic rate, and consequently enhanced growth. Furthermore, this seems to be consistent with the PCAs of genes expression results which show that, after the application at BBCH75, plants treated with SOB01 have a similar expression profile both in conditions of correct irrigation and under a reduced water regime (**Figure 3C**).

The positive effect of plant biostimulants is also based on increasing the content of chlorophyll in leaves and thus increasing the efficiency of photosynthesis. Chlorophyll is the main pigment carrying out photosynthesis in plants, involving the process of light energy absorption, transfer, distribution, and transformation (Biswal et al., 2011). The decrease in PSII photochemical efficiency in environmental stress conditions may be related to a reduction of chlorophyll content (Song et al., 2014). Indeed, this index is considered a major indicator of green pigment biosynthesis efficiency and thus improved crop yields (Di Mola et al., 2019). In the current study, leaf SPAD measurements recorded in biostimulant-treated plants were significantly higher than in calcium chloride-treated plants after the flowering stage, which might contribute to the improved photosynthetic rate of tomato leaves under water deficit. The higher SPAD values may have been caused by different mechanisms: (i) increased N uptake efficiency, (ii) reduced

chlorophyll degradation and leaf senescence, and (iii) modified hormonal metabolism (Jannin et al., 2013; Battacharyya et al., 2015; Ertani et al., 2017). Different phytohormones are involved in leaf senescence and stomatal conductance (Luo et al., 2019). We have previously mentioned how the role of cytokinins, acting as antagonists of abscisic acid, can delay leaf senescence and promote gas exchanges at leaf level (Hönig et al., 2018). Hormone metabolic process and regulation of hormone levels are among the enriched GO terms category of up-regulated genes after SOB01 application (**Figure 2A**). Therefore, the increased stomatal conductance and SPAD value measured in plants treated with the biostimulant can be caused by an alteration of the hormone profile (Russell et al., 2006).

CONCLUSION

The main goal of this preliminary research was to provide a rigorous multidisciplinary approach to the characterization of the activity of a plant biostimulant, using tomato (*S. lycopersicum* L.) as a model crop.

Transcriptomics and physiological analyses have provided a detailed description of the different modes of action exerted by the biostimulant product compared to a classic fertilizer both in water stress and well-watered conditions.

At the molecular level, the modulation of different genes categories both in terms of up-regulation and down-regulation by the biostimulant compared to the standard calcium-chloride fertilizer suggests a peculiar activity exerted by the novel product. Furthermore, the mitigation of some stress-related genes detected on plants treated with the biostimulant could explain the observed improved physiological parameters in plants subjected to water stress.

Consistent with this, physiological measurements demonstrated that biostimulant application increased the photosynthetic rate and the chlorophyll content under water deficiency, helping the plant to cope with drought and resulting in the higher production of fruit dry matter and reduction of cracked fruits. Moreover, the biostimulatory action of the new calcium-based biostimulant resulted in improved stomatal response at tomato fruit development and ripening stage.

To validate this multidisciplinary approach for the characterization of the plant biostimulant activity at different levels of environmental and genetic variability, further studies are required.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ENA–ERX6700091-ERX6700098.

AUTHOR CONTRIBUTIONS

FM, PS, and AM: conceptualization and supervision. FM, PS, AM, GC, MD, AB, MB, WZ-L, SR, SD, and CB: methodology. AB, CB, MD, and PS: writing the original draft. FM and SN: writing, review, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.781993/full#supplementary-material

Supplementary Tables

Supplementary table 1. Chemical composition and properties of the calcium-based biostimulant SOB01 provided by Sipcam-Oxon 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
Saccharides mixture* (% w/w)	20
pH	4.25
Electrical conductance (mS/cm)	28.8
Density (kg/l)	1.407

*composed by monosaccharides (30.53%), disaccharides (26.02%), polysaccharides (20.48%).

Supplementary table 2. Gene Ontology enrichment analysis of DEGs after SOB01 treatment. Biological process, molecular function and cellular component GO terms, obtained with ShinyGO online tool, for down-regulated DEGs (blue) and up-regulated DEGs (red) (adj-p < 0.1 and $\log_2$FC < -1) in SOB01 treated plants compared to the untreated ones.

Available online

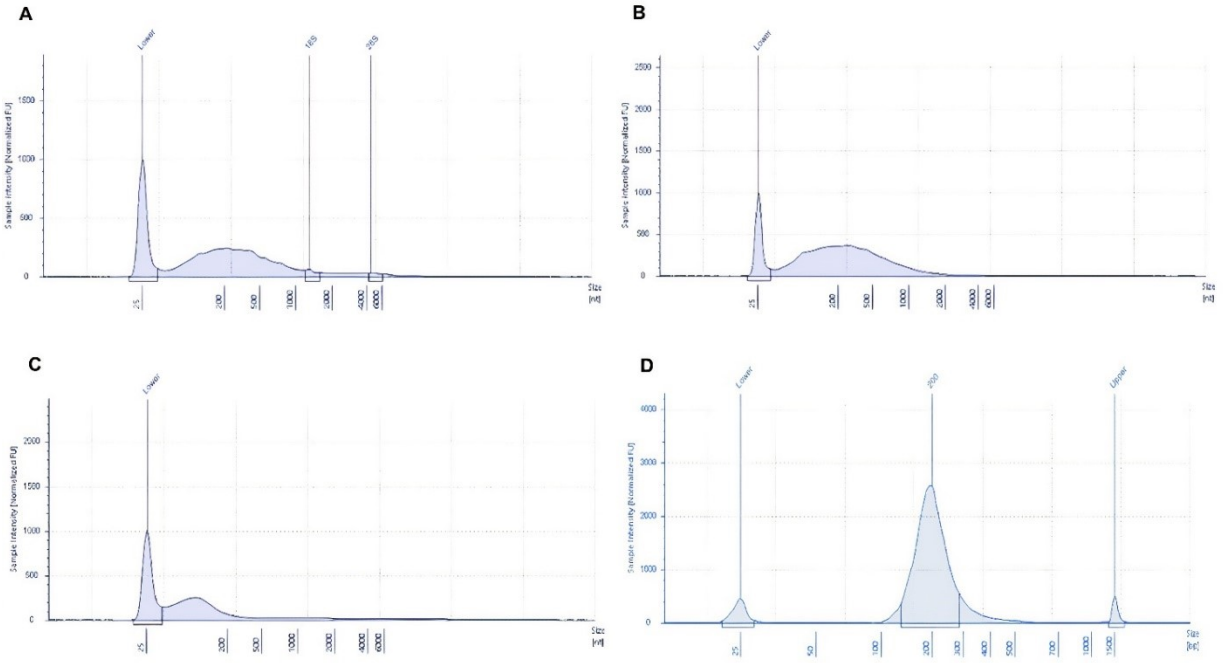
https://www.frontiersin.org/articles/10.3389/fpls.2021.781993/full#supplementary-material

Supplementary table 3. Gene Ontology enrichment analysis of DEGs after SOB02 treatment. Biological process, molecular function and cellular component GO terms, obtained with ShinyGO online tool, for down-regulated DEGs (blue) and up-regulated DEGs (red) (adj-p < 0.1 and $\log_2$FC < -1) in SOB02 treated plants compared to the untreated ones.

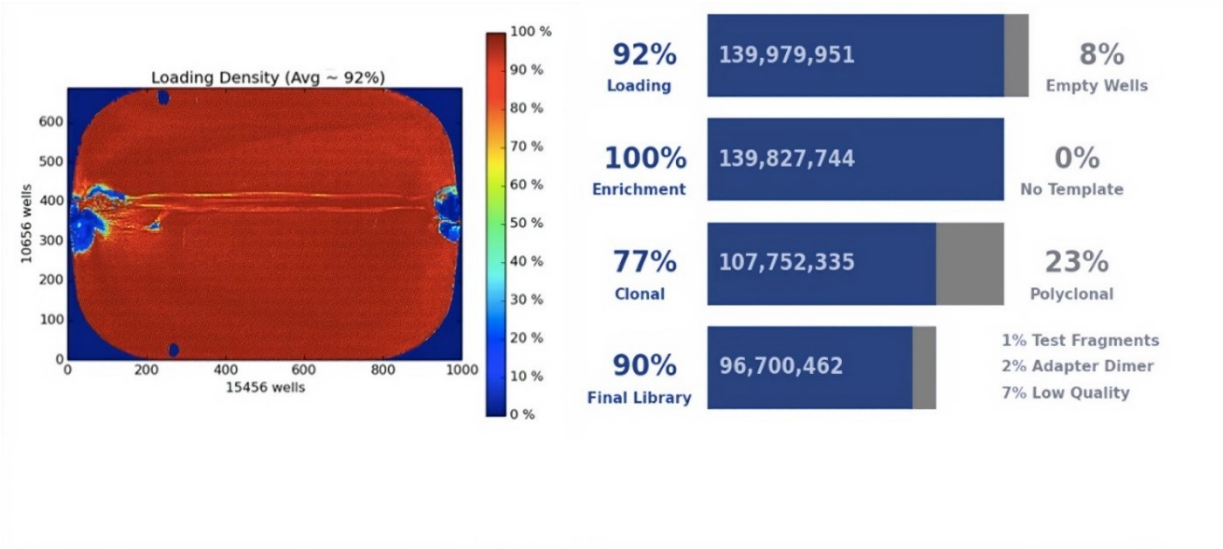
Available online

https://www.frontiersin.org/articles/10.3389/fpls.2021.781993/full#supplementary-material

Supplementary figures



Supplementary Figure 1. Figure S1A (**A**) reports the electropherogram obtained with Agilent TapeStation 1500 of one mRNA sample (1,930 pg/µl) showing a contamination from 18S and 28S sequences while Figure S1B (**B**) shows the electropherogram of the same tomato mRNA sample after the additional washing step. Figure S1C (**C**) and S1D (**D**) display the electropherogram of fragmented mRNA and of the final obtained cDNA library, respectively.



Supplementary Figure 2. Chip loading and sequencing details from Ion S5 server.

CONTRIBUTION III

A DUAL-OMICS APPROACH FOR PROFILING PLANT RESPONSES TO BIOSTIMULANT APPLICATIONS UNDER CONTROLLED AND FIELD CONDITIONS

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ABSTRACT

A comprehensive approach using phenomics and global transcriptomics for dissecting plant response to biostimulants is illustrated with tomato (*Solanum lycopersicum* cv. Micro-Tom and Rio Grande) plants cultivated in the laboratory, greenhouse, and open field conditions. Biostimulant treatment based on an *Ascophyllum nodosum* extract (ANE) was applied as a foliar spray with two doses (1 or 2 l ha^{-1}) at three different phenological stages (BBCH51, BBCH61, and BBCH65) during the flowering phase. Both ANE doses resulted in greater net photosynthesis rate, stomatal conductance, and fruit yield across all culture conditions. A global transcriptomic analysis of leaves from plants grown in the climate chamber, revealed a greater number of differentially expressed genes (DEGs) with the low ANE dose compared to the greater one. The second and third applications induced broader transcriptome changes compared to the first one, indicating a cumulative treatment effect. The functional enrichment analysis of DEGs highlighted pathways related to stimulus-response and photosynthesis, consistent with the morpho-physiological observations. This study is the first comprehensive dual-omics approach for profiling plant responses to biostimulants across three different culture conditions.

Keywords: *Ascophyllum nodosum*, biostimulant, crop yield, plant physiology, tomato, transcriptome

INTRODUCTION

Modern agriculture is seeking eco-friendly ways to sustain crop productivity and reduce the dependency towards chemical fertilizers (Xu and Geelen, 2018). Conventional agricultural practices mainly rely on synthetic agrochemicals. They are uneconomical and harmful to the environment and human health (Dookie et al., 2021). Over the past decades, plant biostimulants have become sustainable inputs for agriculture (De Saeger et al., 2020; Del Buono, 2021). The global market of plant biostimulants reached up to USD 2 billion in 2019, and it is projected to reach USD 3.93 billion, with an average Compound Annual Growth Rate (CAGR) of 11.54% between 2020 and 2025 (previously 10.95% between 2015 and 2020) (Dunham and Trimmer, 2020). In this expansion scenario, the concept of biostimulant activity relates to current and future regulations and regulatory prescriptions regarding the placement of plant biostimulants in the market (Lucini and Miras-Moreno, 2020).

One of the first formally agreed-upon definitions of plant biostimulant was outlined by the EU Fertilizer Regulation 2019/1009. This was a milestone in recognition of the biostimulation

concept, that frames these products in a discrete class of fertilizers based mainly on their function. Accordingly, a plant biostimulant is a product stimulating plant nutrition processes independently of the product nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits or iv) availability of confined nutrients in the soil or rhizosphere.

Another aspect to consider when evaluating the effects of biostimulants is the method of application. Biostimulants can be applied as a seed treatment, soil preparations -or drenches-, or sprayed on leaves and other aerial organs (Drobek et al., 2019). Different factors should be considered, like the type of substance applied, the expected effects on the plant, the crop species and phenological stage, the growing conditions, and the agricultural practices. Plant nutrient absorption happens both through leaves and roots: seaweed-based extracts can be utilized as root treatments for the soil and/or foliar sprays. Both application methods can be equally effective to improve plant stress tolerance, growth, and yield (Ali et al., 2016). Soil applications can modify the biological and physical soil properties by stimulating soil microflora, improving water retention and nutrient availability (Battacharyya et al., 2015). Nonetheless, the foliar application is more convenient for characterizing biostimulant effects on plant biochemistry and physiology because it directly targets the aerial organs. On the contrary, soil application introduces more complexity due to the buffer effect exerted by the biological, chemical, and physical soil properties.

The physiological characterization of biostimulant function and the science-driven product development have become a prerequisite for introducing effective and reliable plant biostimulants on the market. Nevertheless, most of these products are complex substances or mixtures. Such complexity raises the challenge of understanding the modes of action. Currently, the implementation of phenotyping with omics approaches moves research on plant biostimulants forward to identify key information on plant metabolic pathways and developmental processes (Yakhin et al., 2017; Nardi et al., 2021). Precisely, the integration of omics technologies (i.e. metabolomics, phenomics, transcriptomics) enables a comprehensive molecular and physiological characterization of plant biostimulant effects (Della Lucia et al., 2022; Franzoni et al., 2022). Such technologies are very informative tools, whose potentialities can be maximized by setting an experimental design that considers different degrees of environmental variability to better describe plant biostimulants modes of action. However, the traits associated with the biostimulant action strongly depend on the environmental conditions. Therefore, the characterization of the impact of the product on crops and its technical definition requires the experiments to be carried out in different field conditions and with dedicated

multidisciplinary study plans, aimed at dissecting the complexity of the plant response in the open field (Ashour et al., 2021; Della Lucia et al., 2021).

Undeniably, crops grown in the open field are exposed to multiple abiotic stresses and heterogeneous conditions which are hardly reproducible in laboratory conditions. Moreover, the plant phenotype is directly affected by the environment, and observed phenotypic variables reflect these interactions. Accordingly, plant biostimulants screened in a controlled environment can perform differently than in field conditions (Rouphael et al., 2018). Several reasons account for these observed discrepancies. For instance, weather conditions can reduce the biostimulant efficacy after foliar treatment (Pecha et al., 2012). Furthermore, soil chemical and physical properties as well as the native-microbial composition exert specific effects on plants (Fadiji et al., 2022). In practice, plant biostimulants are evaluated first in controlled environment to speed up the selection process of the most interesting products and eventually in the field. However, studies are usually focusing on one or another environment, without gaining a complete functional characterization of plant biostimulants.

Among biostimulants, seaweed-based extracts are widely adopted in cultivated plants. Especially the brown inter-tidal seaweed *Ascophyllum nodosum* is widely used for the formulation of commercial products and it has shown to beneficially influence the plant ability to face biotic and abiotic stresses and to improve plant growth (Shukla et al., 2019). The bioactivity of seaweed extracts is not homogeneous among different products, as it strongly depends upon the extraction method and the harvest season and geographic location. (Carrasco-Gil et al., 2018). The main constituents of seaweed extracts are polysaccharides, fatty acids, amino acids, mineral compounds, phytohormones, and secondary metabolites (phenolic compounds, vitamins, and their precursors) (Pereira et al., 2020). The application of *Ascophyllum nodosum* extract (ANE)-based biostimulants is reported to increase chlorophyll content and yield in tomato and pepper, to improve the yield and quality of the harvested product in grapevine (improved berry size, weight, and firmness) and olive (increased oil content and fatty acids composition), to enhance photosynthetic rates and antioxidant enzymes activities of soybean, and to promote net photosynthetic rate, water and nutrient use efficiency, and sucrose accumulation in sugarcane (Battacharyya et al., 2015; Chen et al., 2021; Chandra and General, 2022).

This study focuses on transcriptomic and physiological responses in tomato plants, after a foliar application of *Ascophyllum nodosum* extract (ANE). Through a dual-omics approach, molecular targets of ANE were identified by RNA-Seq analysis, and the expression level of the most representative genes was confirmed by qPCR. Complementary morpho-physiological experiments were conducted in a climate chamber, greenhouse, and open field conditions to achieve a comprehensive characterization of the ANE biostimulant.

MATERIALS & METHODS

Experimental design and growing conditions

During the years 2020 and 2021, experiments were conducted in three different environments: (i) climate chamber (first year), (ii) greenhouse (second year), and (iii) open field (second year). The adopted workflow is presented in **Figure 1**. The plant material was *Solanum lycopersicum*. The Micro-Tom cv. was grown both in climate chamber and greenhouse, while Rio Grande cv. in the open field.

A biostimulant product based on one extract of the brown alga *Ascophyllum nodosum* (ANE) provided by Sipcam Oxon S.p.A. (Pero, Italy) was applied as a foliar spray. The chemical composition is reported in **Table 1**. We tested different doses of seaweed extract obtained by serial dilutions (from 1:10000 to 1:100) to identify a range of optimal product efficacy in terms of the promotion of plant biomass and fruit yield. These preliminary experiments (data not shown) permitted the selection of two ANE doses: 1 or 2 l ha^{-1}. Foliar applications were repeated three times during the reproductive phase at the specific stages: BBCH51 (first inflorescence visible, first bud erected), BBCH61 (first inflorescence: first flower open), and BBCH65 (five or more inflorescences with open flowers) (Meier, 2001).

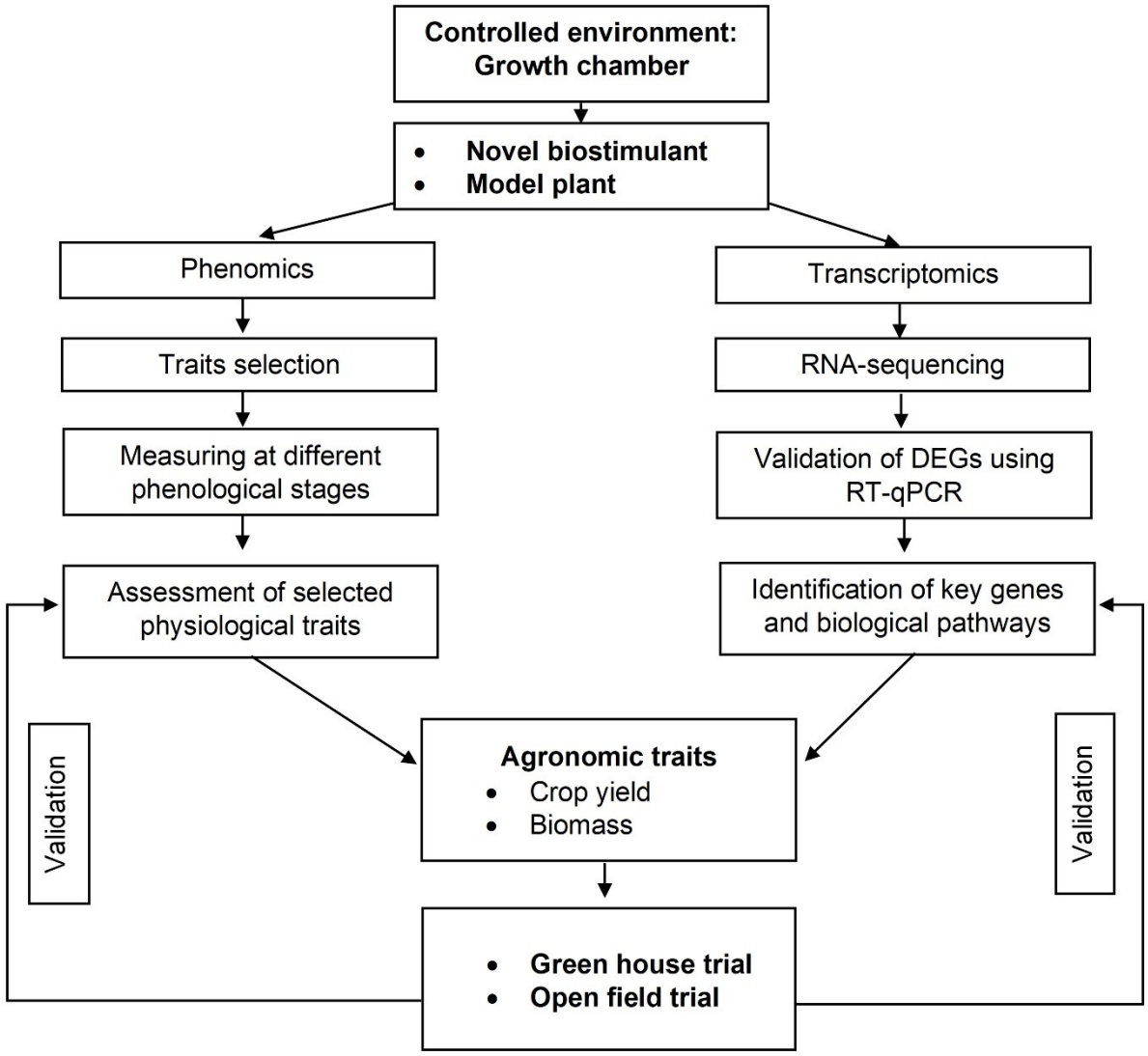

FIGURE 1 Workflow adopted to assess the effects of a biostimulant in controlled environments and open field. The main steps are briefly described. The first step includes phenomic and transcriptomic analyses conducted in the laboratory and the evaluation of agronomic traits. The last step is the validation of the observed biostimulant effects in the greenhouse and field.

Table 1. *Ascophyllum nodosum* extract (ANE)-based biostimulant composition, provided by Sipcam Oxon S.p.A.

Characteristics	Values	Unit
Dry matter content	10.9	%
Density	1.046	Kg l^{-1}
pH (t.q.)	4.6	
Sieve residue at 150 µm-45 µm	0.01 - 0.1	%
Conductivity	4.2	mS cm^{-1}
Organic Carbon of biological origin	3.8	%
(% TQ) Mannitol	1.3	%
	13.6	g l^{-1}
Trace Elements		
Zn	10	ppm
Co	<1	ppm
B	14	ppm
Al	20	ppm
Cu	6.5	ppm
Fe	35.5	ppm
Mo	<0.2	ppm
Mn	4.7	ppm
Macro-, meso-nutrients		
N tot	0.11	%
P_2O_5	0.05	%
K_2O	0.62	%
Na	0.4	%
Mg (ppm)	895	ppm

Assay in climate chamber

In the climate chamber, two types of light-emitting diodes (LED) were used: an AE100 and an AE80 at a photon flux density (PFD) of 250-290 and 210-230 µmol photons m^{-2} s^{-1}, respectively. The photoperiod was 14h light and 10h darkness. Relative humidity was set to 60% and temperature to 24°C (light)/20°C (darkness). Tomato plants cv. Micro-Tom were cultivated on Klasmann-Deilmann (Germany) substrate: 35% white sod peat 10-25 mm, 45% white peat 0-25 mm, 5% peat fiber, and 15% perlite. The substrate physical and chemical properties are given in **Supplementary Table 1**. Plants with three to four true leaves (30-35 d after sowing)

were transplanted in pots with a capacity of 1.2 l. From the third week after sowing, plants were fertilized twice a week with Flortis (Energy blue) NPK (20:20:20). Upon reaching the biostimulant treatment application time, the standard maintenance fertilization was replaced with a formulation entitled to be more suitable for plant development (NPK 15-15-30 Flortis Prod). Each pot was irrigated with 150 ml of water, three times per week. For preparing spray solution, 1.375 g or 2.750 g of ANE were diluted in 1 l of ultra-pure water, respectively corresponding to 1 l ha^{-1} or 2 l ha^{-1} doses. A volume of 10 ml was sprayed on leaves. Control plants were sprayed with an equal volume of ultra-pure water. The trial was arranged as a completely randomized design with seven replicates (pots) each containing one plant.

Leaf gas exchange measurements

Leaf gas exchange measurements were done on the youngest fully expanded leaves below the nearest inflorescence, before the first ANE application and 48h after every other application at three phenological phases (BBCH51, BBCH61, BBCH65). Gas-exchange measurements were taken with an infrared gas analyzer (CIRAS 3 PP Systems, Amesbury, MA, USA), under ambient temperature, saturating light of 1,500 µmol photons m^{-2} s^{-1} and 400 µmol CO_2 surrounding the leaf flux density. The size of the leaf cuvette window was 2.5 cm^2, and the light was provided by red, green, and blue light-emitting diodes.

Yield parameters

In both climate chamber and greenhouse experiments, plants were harvested at the fruit maturity stage. The number of fruits, the fruit weight per plant, and their total biomass were recorded. At harvest, the fresh fruit yield was measured, and the dry weights were recorded after oven-drying the samples at 105°C for 24h.

RNA sequencing

Samples treated with two ANE doses were harvested 24h and 48h after treatment for RNA-Seq analysis together with controls.

Two leaf disks were collected around the mid-vein of the distal leaflets of the most recently fully expanded leaf below the nearest inflorescence, from four different plants for each experimental condition. Messenger RNA was directly isolated from frozen and powdered leaf disk pools using the Dynabeads mRNA Direct Micro Kit (Thermo Fisher Scientific, Carlsbad, CA) following the manufacturer's instruction. The concentration and quality of mRNA were assessed by an Agilent 4150 TapeStation system (Agilent Technologies, USA). Sequencing libraries were prepared from a range of 10-50 ng of poly(A) RNA using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) following the manufacturer's protocol. The final double-stranded barcoded cDNA libraries were eluted in 15 µl of nuclease-free water. The concentration and size distribution 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).

Sequencing data and differential gene expression analysis

Raw reads were filtered to remove the low-quality ones and use reads with a phred-like Q value > 20 for downstream analysis. Bowtie2 (v2.4.2) (Langmead and Salzberg, 2012) was used for mapping the filtered reads to *Solanum lycopersicum* genome (SLv3.0) (NCBI, GenBank accession GCA_000188115.3). The raw transcriptome data obtained are available at the ENA Browser under the name "PRJEB53962 (ERP138777)". Raw read counts were calculated for all predicted genes using bedtools multiBamCov (Quinlan and Hall, 2010) after processing mapped reads with samtools (v1.11) (Li et al., 2009). To remove less informative data, we filtered out genes with an overall expression level smaller than 20. The DESeq2 R package (v.1.32.0) (Love et al., 2014) was used to perform the inferential analysis and obtain differentially expressed genes (DEGs) across the biological conditions. An adjusted *p*-value < 0.1 and a $\log_2$ fold change ≥ |1.0| were set as thresholds of significance to select DEGs. Gene Ontology (GO) enrichment analysis was performed with the web-based toolkit ShinyGO v0.66 (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.

Validation of DEGs using RT-qPCR

Genes differentially expressed across different time points were selected to evaluate their expression levels through RT-qPCR for validation of RNA-Seq results. The validation was performed on biological replicates collected 24h after treatment with 2 l ha^{-1} ANE in the three phenological stages. Primers were designed using the Primer-BLAST tool on NCBI (Ye et al., 2012). The list of primers is shown in **Supplementary Table 2**. A quantity of 3 µg total RNA extracted with a Maxwell® 16 LEV Plant RNA Kit (Promega Corporation, USA) was converted into cDNA using a GoScript Reverse Transcription Mix, Random Primer (Promega Corporation, USA). The RT-qPCR assay was performed using a reaction mix composed of 5 µl of GoTaq qPCR Master Mix (Promega Corporation, USA), 1 µl of cDNA (4 ng μl^{-1}), and 0.25 µl of each gene-specific primer in a final volume of 10 µl. Three biological and two technical replicates were performed for each gene. The average Ct values of two internal reference control genes *EFI1* (Solyc06g005060.2; Forward: 5'-CTGTGAGGGACATGAGGCAG-3', reverse: 5'-CTGCACAGTTCACTTCCCCT-3') and *UBI* (Solyc07g064130.1; Forward: 5'-GGACGGACGTACTCTAGCTG-3', reverse: 5'-TCGTCTTACCCGTGAGAGTC-3') were measured for relative expression analysis using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Greenhouse experiment

The greenhouse experiment was carried out in a fully equipped structure with a lighting system (PFD: 300 ± 20 µmol photons m^{-2} s^{-1}) adjusted to 14/10h light/dark, 24/20°C light/dark temperature, 60% relative humidity, natural ventilation roof, lateral openings, and horizontal fan systems for air circulation. All the methodological parameters on plant material, growing conditions, treatments, and experimental design were the same as previously described in the climate chamber experiment. After seed germination, Micro-Tom plants with three to four true leaves were transplanted to individual 1.2 l-capacity pots that were arranged in a completely randomized design with seven replications per treatment. Treatments consisted of untreated control and two ANE doses (1 and 2 l ha^{-1}) applied as a foliar spray in three phenological stages (at BBCH51, BBCH61, and BBCH65). Leaf gas exchange and yield traits were measured as above described in paragraphs “Leaf gas exchange measurements” and “Yield parameters”. The percentage of fruit set was computed on six plants (pots) by counting the total number of flowers in the second and third clusters and later, on the same clusters, at full maturity, the number of fruits. The fruit set percentage was calculated as follows:

$$\textit{Fruit set}\ (\%) = \frac{\textit{Number of fruits}}{\textit{Number of flowers}} \times 100$$

Field experiment

A field trial was conducted at the experimental farm of the University of Bologna located in Cadriano (Italy) (44° 33’ N, 11° 24’ E) during the growing season of 2021. The cv. Rio Grande was used. Four-week-old seedlings cultivated in a greenhouse on the soil substrate previously described in 2.2 were transplanted to the field. Pre-transplant mineral fertilization consisted of 110 kg ha^{-1} N (slow-release fertilizer), 100 kg ha^{-1} P_2O_5, and 200 kg ha^{-1} K_2O. During the fruit setting plants were enriched with calcium nitrate (foliar, 2 kg 1000 l^{-1}). Water was applied by drip irrigation at a rate of 5 l m^{-1} h^{-1} with drippers spaced 40 cm. The first watering was done immediately after transplanting. The amount of water supplied was calculated by both the ETo (reference evapotranspiration (mm day^{-1}) climate conditions and by the crop phenological stage expressed by the Kc factor (crop coefficient), using the following formula: crop evapotranspiration or crop water need (ET crop) (mm day^{-1}) = ETo × Kc (Brouwer and Heibloem, 1986). Values of the crop factor (Kc) for tomato crop and growth stages were between 0.45-1.15. The monthly and long-term mean (10 years), maximum, and minimum temperature and precipitation during the experimental period are presented in **Supplementary Figure 1**. A composite soil sample was collected before the experiment to determine the physical and

chemical characteristics at 0-30 cm depth. The physical and chemical properties of the soil are presented in **Supplementary Table 3**. The experimental set-up was a completely randomized block design with three blocks and four replications per treatment (1 l ha^{-1}, 2 l ha^{-1} of ANE, and control). Each plot had a surface of 20 m^2 (4x5 m) and consisted of four rows. The space between rows was 115 cm and between plants in one row 40 cm. A buffer zone of 3 m spacing was provided between plots. Two ANE doses (1 l ha^{-1} and 2 l ha^{-1}) were applied using a hand sprayer three times, specifically at BBCH51, BBCH61, and BBCH65, and were compared with untreated control.

Leaf gas exchange, biomass, and fruit yield measurements

Leaf gas exchange measurements were done before the first ANE application (BBCH51) and 48 h after the last one (BBCH65). The measurements were done on the youngest fully expanded leaves below the nearest inflorescence on five plants per treatment in the morning (9.00-11.00 am). The fruit set percentage and fruit fresh and dry weight were measured. To assess the tomato fruit set in the field, the total number of flowers in the second and third clusters were counted in five randomly selected plants within the plot. The fruits were counted at the fruit's development stage on the same clusters where the total flowers were counted. The fruit set (measured as a percentage) was calculated as a ratio between the fruits and flowers numbers. Fruits harvested at full ripening from 10 plants from the central rows were weighed with an electronic dynamometer. The dry weight of fruits was measured after the samples were oven-dried at 105°C.

Statistical analysis

The statistical method applied to physiological traits data was the repeated measurements ANOVA model. Productivity traits were subjected to a one-way analysis of variance ($p< 0.05$), and the differences between samples were determined by the least significant difference (LSD) test. Statistical analyses were carried out using RStudio (version R-4.1.0). Venn diagrams were plotted using ggVenn package from R.

RESULTS

Physiological and molecular characterization of the ANE-based biostimulant effects were first assessed in laboratory conditions with plants cultivated in a climate chamber and treated at three growth stages. Eventually, plant physiological and yield-related traits were evaluated in greenhouse and open field conditions.

Effects of ANE treatment on tomato plants grown in culture chamber

Leaf gas exchange and yield

Stomatal conductance and net photosynthesis were measured across three different time points after applying the ANE. The average rates were increased significantly ($p \leq 0.05$) by the treatment but were not significantly different between the two doses (**Table 2**). The average stomatal conductance was 41% and 36% greater than the control in plants treated with the 2 l ha^{-1} and 1 l ha^{-1} dose, respectively. A significant interaction ($p \leq 0.05$) between different doses of ANE and time of application was detected in stomatal conductance and net photosynthesis (**Table 2**).

TABLE 2. Mean values and analysis of variance of photosynthetic parameters after foliar application of ANE (biostimulant, B) at different phenological phases (time, T) in a climate chamber.

Treatment	Stomatal conductance (mmol m^{-2} s^{-1})	Net photosynthesis (µmol m^{-2} s^{-1})
Biostimulant (B)		
2 l ha^{-1}	302 ± 25.5 a	18.7 ± 1.1 a
1 l ha^{-1}	291 ± 29.5 a	18.5 ± 1.1 a
Control	214 ± 16 b	17.5 ± 1.2 b
Time (T)		
Before first treatment	273 ± 9.6 B	22.7± 0.3 A
BBCH51	418 ± 31.4 A	23.9 ± 0.4 A
BBCH61	145 ± 4.1 C	12.2 ± 0.2 C
BBCH65	240 ± 13.4 B	14.0 ± 0.5 B
ANOVA significance		
B	*	*
T	*	**
B x T	**	*

Data are means ± standard error. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$). *, ** significant respectively at 0.05 or 0.01 levels according to ANOVA. BBCH51 (the first inflorescence visible: first bud erects), BBCH61 (first inflorescence: first flower open), BBCH65 (fifth inflorescence).

Stomatal conductance was greater in treated plants after the first ANE applications at BBCH51 and BBCH65. A significant effect on net photosynthesis was obtained only after the last ANE application. The difference between the two doses is not significant for both leaf gas exchange parameters (**Figure 2**). At the final harvest, ANE application significantly ($p < 0.05$) increased the fruit number per plant compared to the control. The plants treated with the two ANE doses

showed significantly greater total fruit dry matter than untreated ones. No significant difference was found between the different doses of biostimulant (**Figure 3**).

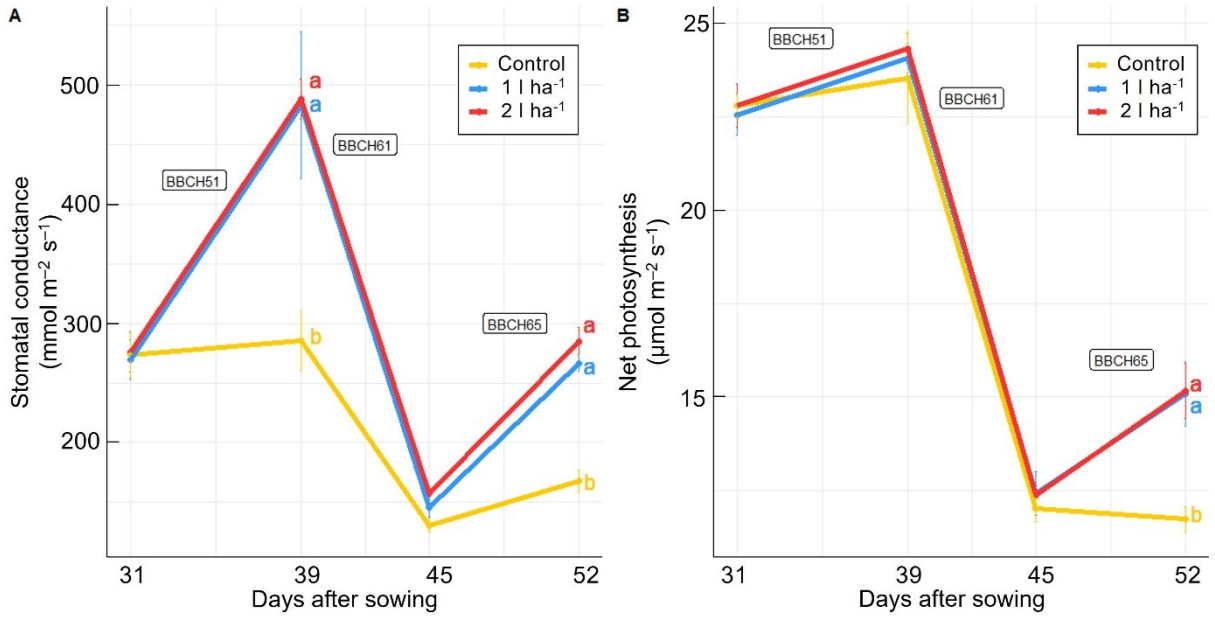

FIGURE 2. Effect of ANE treatment on photosynthetic parameters in tomato plants cultivated in a climate chamber. Stomatal conductance **(A)** and net photosynthesis **(B)** were measured before the first treatment application and 48h after every ANE leaf application at BBCH51, BBCH61, and BBCH65 in Micro-Tom plants untreated (control) or treated with ANE (1 or 2 l ha^{-1}). Each value is the mean of n = 6 observations ± s.e. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$).

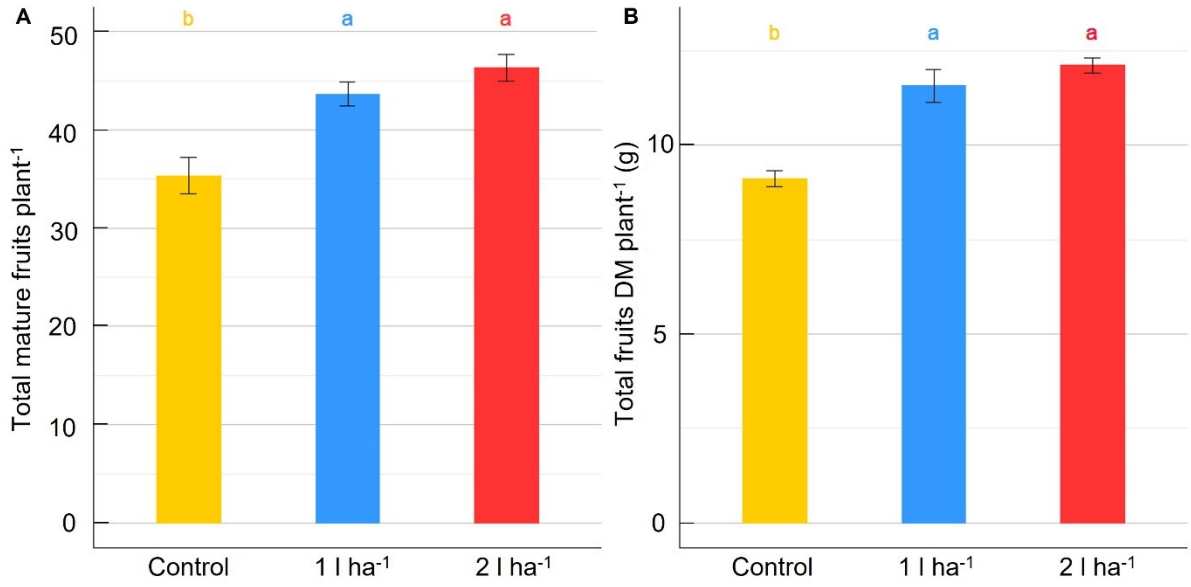

FIGURE 3. Effect of ANE treatment on fruit production in tomato plants cultivated in a climate chamber. The total number of mature fruits per plant **(A)** and the total fruit dry biomass per plant **(B)** were measured in Micro-Tom plants untreated (control) or treated with ANE (1 or 2 l ha^{-1}). Each value is the mean of n = 6 observations ± s.e. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$).

Leaf transcriptome responses

To detect transcriptional changes induced by ANE treatment, mRNA sequencing was conducted on leaves collected 24h and 48h after each application. A total of 252,549,495 single-end reads were generated by the sequencing runs, with an average of 7.015 10^6 raw reads per sample. The overall alignment rate after mapping to the *S. lycopersicum* reference genome was on average 78.85%.

A principal component (PCA) plot with the log2 normalized read counts (**Supplementary Figure 2**) shows samples are mainly clustered according to the collection phenological stages (**Figure S2A**). However, samples collected at the beginning of the reproductive phase (BBCH51) are not tightly clustered in the plot. Moreover, PCA analysis is showing that replicate samples have high variability in this phase (**Figure S2B**). Only after the second treatment, at BBCH61, and the third one at BBCH65, a more treatment-wise consistent clustering is observed.

The analysis of DEGs was set to compare samples across three treatment applications, two ANE doses, and two sampling time points (24h and 48h after treatment). The number of DEGs yielded by each comparison is shown in **Table 3**. Most of the genes were upregulated (62.5% of DEGs) after the first ANE application. Conversely, a greater number of down-regulated genes were identified after the second (70.5% of DEGs) and the third (57% of DEGs) applications. A few genes were differentially expressed after the first treatment (**Table 3**), consistently with a non-ideal clustering of replicates observed in the PCA at the same stage. We assumed a weak biostimulant effect at BBCH51 and decided to focus on the results obtained from the second and third ANE applications, which yielded a higher number of DEGs and a more consistent PCA (**Table 3** and **Figure S2**). The number of DEGs shared between the two time points (24h and 48h) and two ANE doses within the same treatment application event, for both BBCH61 and BBCH65 were analyzed (**Figures 4, 5**). Only one gene, encoding a proline-rich protein 4-like, was consistently downregulated across all time points and ANE doses at BBCH61, whereas no gene was found to be mutually upregulated at both 24h and 48h and with both doses.

TABLE 3. Number of differentially expressed genes with adj-p < 0.1 and $|\log_2 FC| \geq 1$, across treatment applications (1st, 2nd, and 3rd applications), ANE doses (1 l ha^{-1} or 2 l ha^{-1}), and sampling time (24h or 48h).

	1st application		**2nd application**		**3rd application**	
	Up	**Down**	**Up**	**Down**	**Up**	**Down**
1 l ha^{-1} 24h *vs* NT 24h	-	-	67	133	38	67
1 l ha^{-1} 48h *vs* NT 48h	8	11	65	144	1	1
2 l ha^{-1} 24h *vs* NT 24h	12	1	12	78	17	20
2 l ha^{-1} 48h *vs* NT 48h	-	-	8	9	18	10

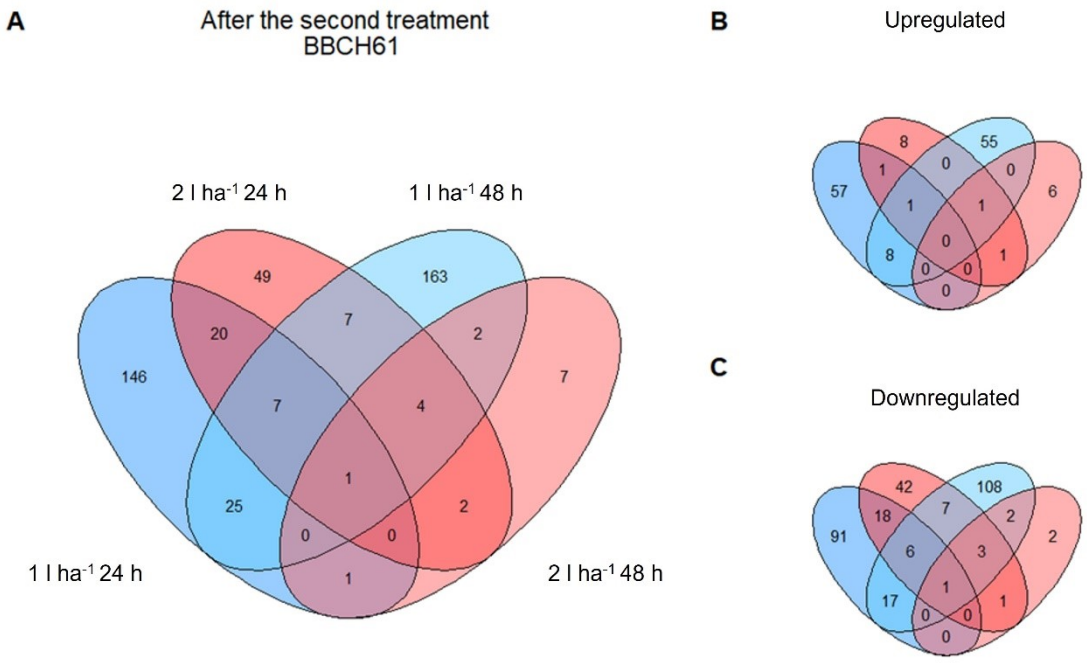

FIGURE 4. Venn diagram showing shared and unique DEGs of different comparisons after the second ANE application. The diagrams show the total number **(A)** and the breakdown between up- **(B)** and down- **(C)** regulated DEGs after the second ANE application at BBCH61.

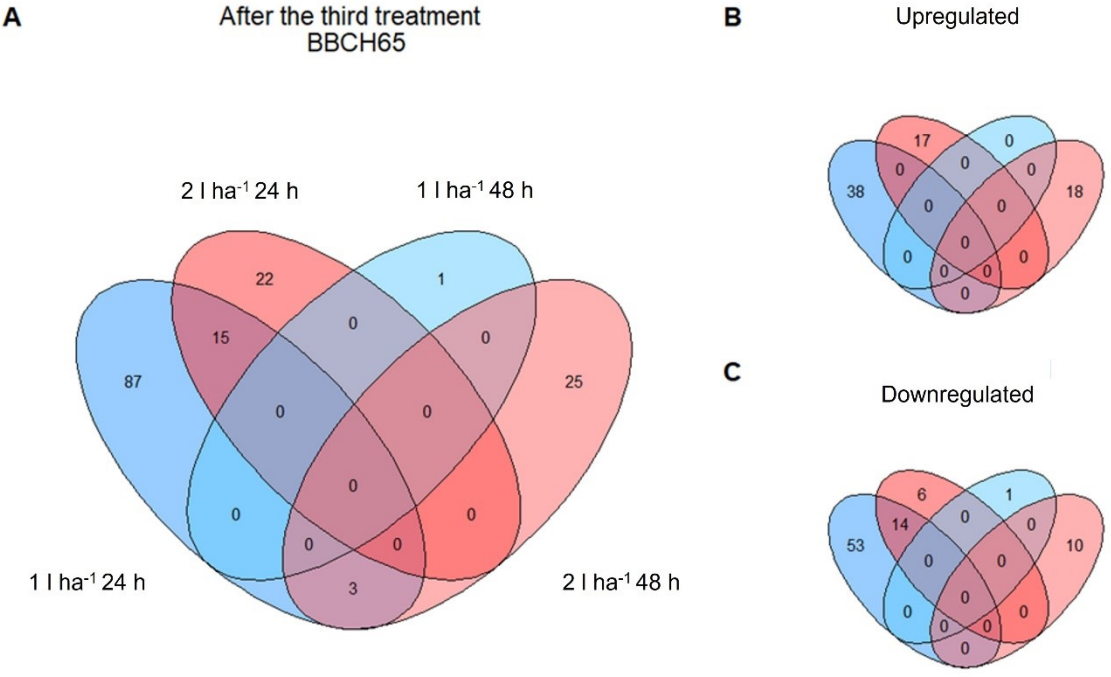

FIGURE 5. Venn diagram showing shared and unique DEGs of different comparisons after the third ANE application. The diagrams show the total number **(A)** and the breakdown between up- **(B)** and down- **(C)** regulated DEGs after the third ANE application at BBCH65.

A Gene Ontology (GO) enrichment analysis was conducted separately for DEGs obtained from different comparisons within each phenological stage and for every ANE dose and sampling time. The output for the most significantly enriched GO terms related to biological process and molecular function is presented in **Supplementary Table 4** (available online at the link https://www.frontiersin.org/articles/10.3389/fpls.2022.983772/full#supplementary-material). To better visualize and characterize the most relevant molecular mechanisms involved in the biostimulant activity, given the large number of different comparisons, we further conducted one GO enrichment analysis on the pool of the total number of DEGs obtained across all pairwise comparisons. The treatment mainly affected the expression of genes related to photosynthesis (both light and dark reactions), valine biosynthetic process, and response to several stimuli (**Figure 6**). The molecular functions GO terms with the greatest enrichment values were related to photosynthetic activity, among which are "ribulose-bisphosphate carboxylase activity", "beta-glucosidase activity", and "chlorophyll binding". Interestingly, the GO terms "chitinase activity" and "water channel activity", and those related to lipid binding and oxidoreductase and monooxygenase activity were also among the ones with greater fold enrichment values (**Figure 6B**).

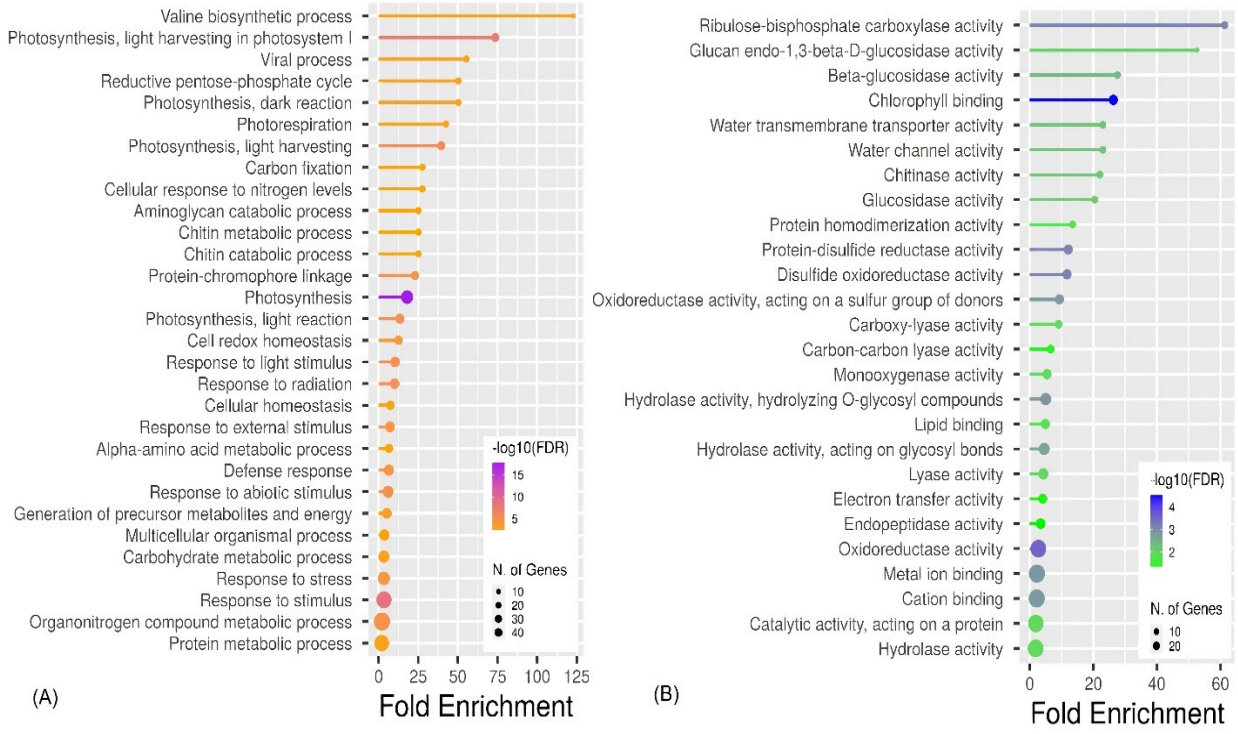

FIGURE 6. Gene ontology enrichment analysis for all the DEGs obtained across different comparisons. Lollipop plots show GO fold enrichment, significance (FDR ≤ 0.05), and number of genes in each pathway. GO categories analyses are biological process **(A)** and molecular function **(B)**. Analysis was perfomed with the online tool ShinyGO, v.0.66 (http://bioinformatics.sdstate.edu/go/).

GO terms were hierarchically clustered based on shared genes. Such clustering produced six main groups (**Figure 7**) that show the main pathways affected by the ANE treatment. GO terms that are clustering together in the tree plots have more shared genes and larger dots indicate a lower p-value. This helps reduce the redundancy of GO terms and focus on the main broad categories enriched. They can be summarized in dark and light reactions in photosynthesis, chitin metabolic process, response to external stimulus, defense response, and biosynthesis of secondary metabolites. The broader categories and the ones with the highest significance in the decision tree are the categories of genes involved in photosynthesis and response to stimulus. To have an overview of the genes differentially expressed in each enriched broad category, a list of annotations and gene descriptions is provided in **Table 4**.

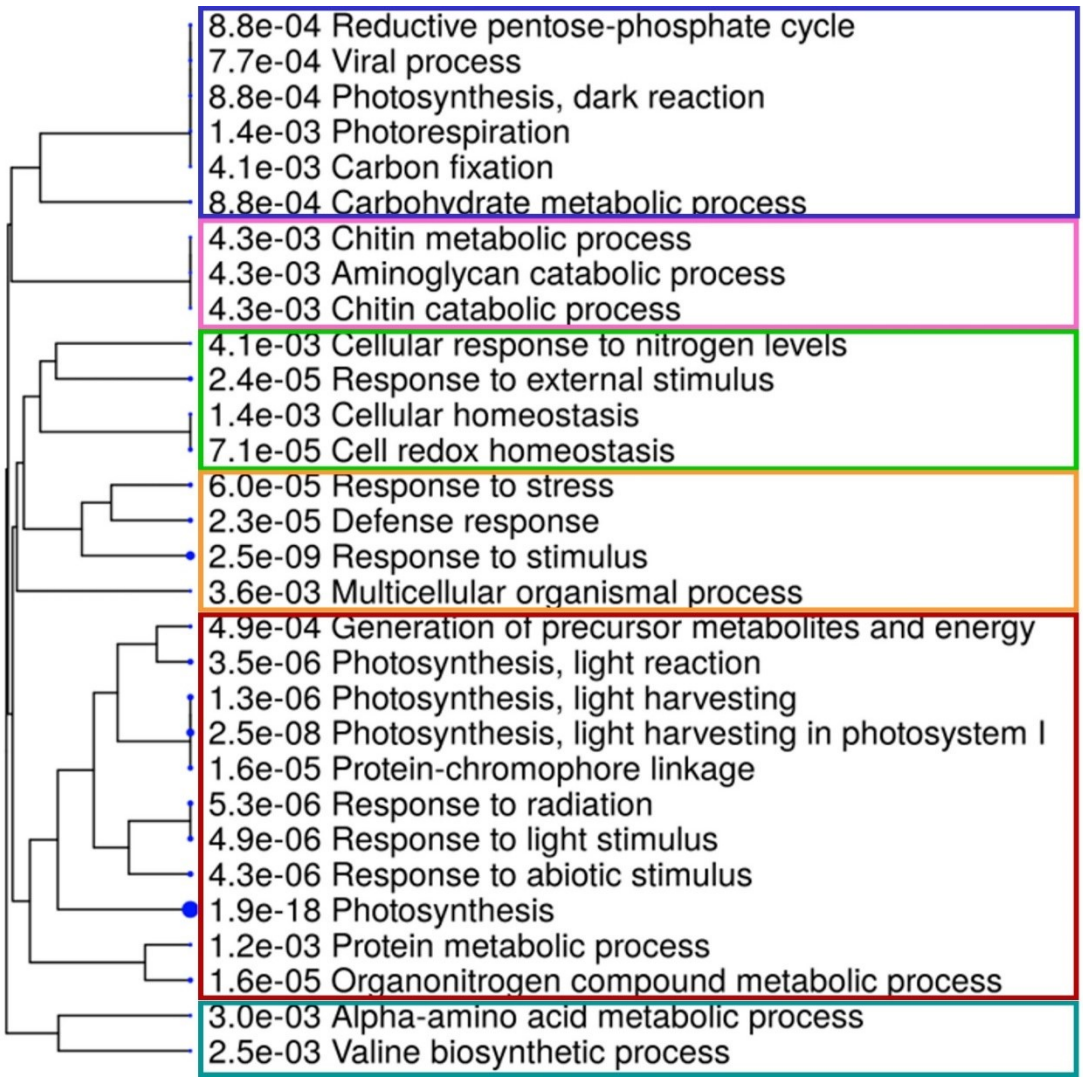

FIGURE 7. Hierarchical decision tree displaying the degree of association among enriched GO terms in the biological process and its statistical significance. Pathways with more shared genes are closer in the tree plot and visually grouped by different colors. Bigger dots indicate more significant p-values (ShinyGO, v.0.66, http://bioinformatics.sdstate.edu/go/).

TABLE 4. A selection of representative genes differentially expressed in ANE-treated plants in at least one comparison.

Gene ID	Gene description
Photosynthesis, dark reaction, and carbon fixation	
Solyc02g085950	Ribulose bisphosphate carboxylase small subunit, chloroplastic 4
Solyc02g063150	Ribulose bisphosphate carboxylase small chain 1, chloroplastic
Solyc03g034220	Ribulose bisphosphate carboxylase small subunit, chloroplastic 2
Chitin metabolic process	
Solyc09g098540	Chitinase-like protein 1
Solyc10g055800	Endochitinase 4
Solyc10g055810	Chitinase
Cell redox homeostasis	
Solyc01g087850	Subtilisin-like protease
Solyc05g015490	Non-specific lipid transfer protein GPI-anchored 1
Solyc06g008760	Glutaredoxin-C13
Solyc10g007600	Glycolate oxidase
Solyc07g042440	Peroxiredoxin Q, chloroplastic
Response to stimulus and stress	
Solyc02g086820	Carbonic anhydrase
Solyc12g099970	SNF1 kinase complex anchoring protein
Solyc01g006300	Peroxidase
Solyc12g011450	Chlorophyll *a*/*b* binding protein 13, chloroplastic
Solyc01g006730	Calcium-dependent protein kinase 20-like
Solyc07g041720	Auxin-binding protein
Solyc05g055990	Aquaporin
Solyc10g048030	Kirola
Photosynthesis	
Solyc01g087040	Thylakoid lumenal 19 kDa protein, chloroplastic
Solyc01g102770	Photosystem II protein Z
Solyc02g069460	Photosystem I reaction center subunit III, chloroplastic
Solyc05g056050	Chlorophyll *a*/*b* binding protein 6A, chloroplastic
Solyc05g056070	Chlorophyll *a*/*b* binding protein precursor
Solyc10g075160	Ferredoxin
Solyc07g041720	Auxin-binding protein
Solyc02g064770	Probable esterase
Solyc04g073990	Annexin p34
Solyc01g087850	Subtilisin-like protease
Biosynthesis of secondary metabolites	
Solyc03g044330	Acetolactate synthase 2, chloroplastic
Solyc04g014510	Glutamine synthetase cytosolic isozyme 1-1
Solyc04g082030	Ornithine decarboxylase
Solyc08g007040	Glycine cleavage system H protein, mitochondrial

The categories are obtained through the clustering of GO biological processes most significantly enriched.

To validate the RNA-Seq data set, the expression levels of five candidate genes involved in photosynthesis and defense response selected among DEGs in at least two conditions, were measured by RT-qPCR on samples treated with ANE (2 l ha^{-1}) after 24 h. Relative expression values of selected genes obtained with qPCR using the $2^{-\Delta\Delta Ct}$ method on plants treated with the 2 l ha^{-1} dose were compared with fold changes (FC) obtained from RNA-Seq analysis of plants treated with both ANE doses (**Table 5**).

TABLE 5. RNA-Seq data validation of five candidate genes using RT-qPCR. Fold change in expression is presented using the $2^{-\Delta\Delta Ct}$ ± s.e. for RT-qPCR data and fold change for RNA-Seq data.

Gene ID	Gene name	Description	Treatment application	qRT-PCR	RNA-Seq	
				2 l ha^{-1}	1 l ha^{-1}	2 l ha^{-1}
Solyc03g096290	*PIP1-7*	Aquaporin, plasmamembrane intrinsic protein 1.7	BBCH51	-1.88 ± 0.06	-1.01	1.74
			BBCH61	-3.24 ± 0.10	-4.11 *	-1.38
			BBCH65	2.34 ± 0.83	1.11	-9.13 *
Solyc03g114940	*KLUH/ CYP78A5*	Cytochrome P450 78A5-like	BBCH51	1.01 ± 0.19	-1.51	-1.27
			BBCH61	1.04 ± 0.18	3.27 *	1.84
			BBCH65	-1.03 ± 0.03	1.22 *	1.23
Solyc02g063150	*RBSCs1*	Ribulose bisphosphate carboxylase small chain 1, chloroplastic	BBCH51	-1.05 ± 0.13	1.01	1.11
			BBCH61	-1.39 ± 0.20	-2.08 *	-2.39 *
			BBCH65	-1.05 ± 0.16	-5.55 *	-1.01
Solyc02g086820	*CA2*	Carbonic anhydrase	BBCH51	-1.11 ± 0.20	1.02	1.05
			BBCH61	-3.77 ± 0.06	-2.20 *	-2.66 *
			BBCH65	-1.35 ± 0.03	-7.09 *	1.13
Solyc09g007010	*PR1b1*	Pathogenesis-related leaf protein	BBCH51	-4.98 ± 0.16	2.95	132.52 *
			BBCH61	-3.19 ± 0.15	1.79	25.77 *
			BBCH65	4.65 ± 1.07	1.18	4.29 *

* Indicates genes significantly differentially expressed according to the adjusted *p*-value cutoff ($p < 0.1$).

We observed some discrepancies between qRT-PCR and RNA-Seq data, particularly for *PIP1-7*, *KLUH/CYP78A5*, and *PR1b1*. We anyway observed an overall positive correlation between the relative expression values measured with qPCR and the FC obtained through sequencing. However, the use of biological replicates and the different normalization methods adopted may account for the differences observed in gene expression responses to the treatment. Moreover, the correlation was stronger for the RNA-Seq data obtained from samples treated with the lower dose of application (1 l ha^{-1}) compared to the 2 l ha^{-1} dose which was the one used in the qPCR validation. The expression pattern of *RBSCs1*, *CA2*, and *KLUH/CYP78A5* detected by the RNA-Seq data after the second and third ANE applications (1 l ha^{-1}) was generally consistent with the qPCR results (**Supplementary Figure 3**). However, the fold changes in up- and down-regulation of these genes in the treated samples compared to the control are not fully matching.

Lower transcript levels of *RBSCs1* and *CA2*, encoding respectively a ribulose bisphosphate carboxylase small chain and a carbonic anhydrase, were observed in leaves of plants treated with 1 l ha^{-1} at both BBCH61 and BBCH65, whereas the only statistically significant down-regulation registered with higher ANE dose (2 l ha^{-1}) is for *CA2* at BBCH61 (**Figure S3**). The *KLUH* gene, a member of the cytochrome P450 family, that controls fruit size and mass, modulates plant architecture, and ripening time (Chakrabarti et al., 2013), was upregulated in treated plants after the second application but was found downregulated in the same conditions at BBCH65. The *PR1b1* gene encoding a pathogenesis-related protein 1 was significantly upregulated after every treatment with the highest product dose (2 l ha^{-1}) in the RNA-Seq results. The same higher level of the *PR1b1* gene transcript was observed in treated plants compared to untreated at BBCH65 in different biological replicates used for qPCR analysis, but not in the other two previous product applications in which we observed the downregulation of the same gene (**Figure S3**).

Effects of ANE treatment on tomato plants grown in greenhouse

The physiological analysis carried out in the greenhouse showed significant effects of treatment on the average net photosynthesis and stomatal conductance, and a significant interaction between the biostimulant treatment and the time of application on net photosynthesis (**Table 6**).

TABLE 6. Mean values and analysis of variance of photosynthetic parameters after foliar application of ANE (biostimulant, B) at different phenological stages (time, T) in the greenhouse.

Treatment	Stomatal conductance (mmol m^{-2} s^{-1})	Net photosynthesis (µmol m^{-2} s^{-1})
Biostimulant (B)		
2 l ha^{-1}	198 ± 8.7 a	15.1 ± 0.4 a
1 l ha^{-1}	193 ± 9.3 a	14.9 ± 0.4 a
Control	160 ± 9.6 b	12.9 ± 0.4 b
Time (T)		
Before first treatment	186 ± 10.9 B	12.9 ± 0.3 C
BBCH51	232 ± 8.1 A	17.1 ± 0.3 A
BBCH61	151 ± 5.5 C	13.9 ± 0.5 BC
BBCH65	165 ± 9.3 BC	13.5 ± 0.4 BC
ANOVA Significance		
B	**	**
T	**	**
B x T	ns	**

Data are means ± standard error. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$). *, ** significant respectively at 0.05 or 0.01 levels according to ANOVA. BBCH51 (the first inflorescence visible: first bud erects), BBCH61 (first inflorescence: first flower open), BBCH65 (fifth inflorescence).

After the first application of ANE, the stomatal conductance and the net photosynthesis were both higher than the control at every time point, and the comparison between the two ANE doses revealed no statistically significant differences (**Figure 8**). Treated plants had an improved fruit set percentage, total fruit yield, and fruit dry biomass compared to untreated ones, and again no significant differences were found among the different ANE doses. (**Figure 9**).

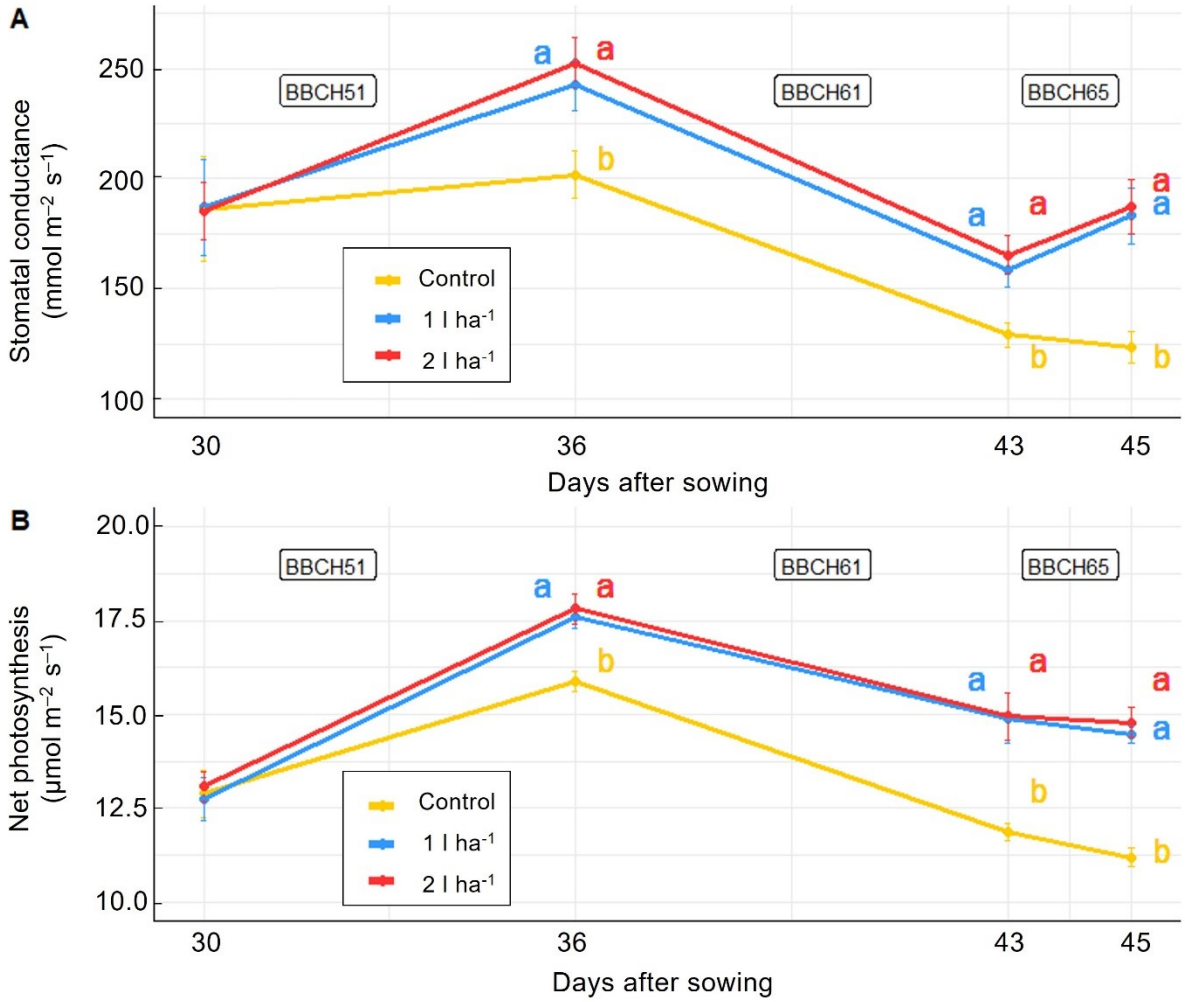

FIGURE 8. Effect of ANE treatment on photosynthetic parameters in tomato plants cultivated in greenhouse. Stomatal conductance **(A)** and net photosynthesis **(B)** were measured before the first treatment application and 48h after every ANE leaf application at BBCH51, BBCH61, and BBCH65 in Micro-Tom plants untreated (control) or treated with ANE (1 or 2 l ha^{-1}). Each value is the mean of n = 6 observations ± s.e. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$).

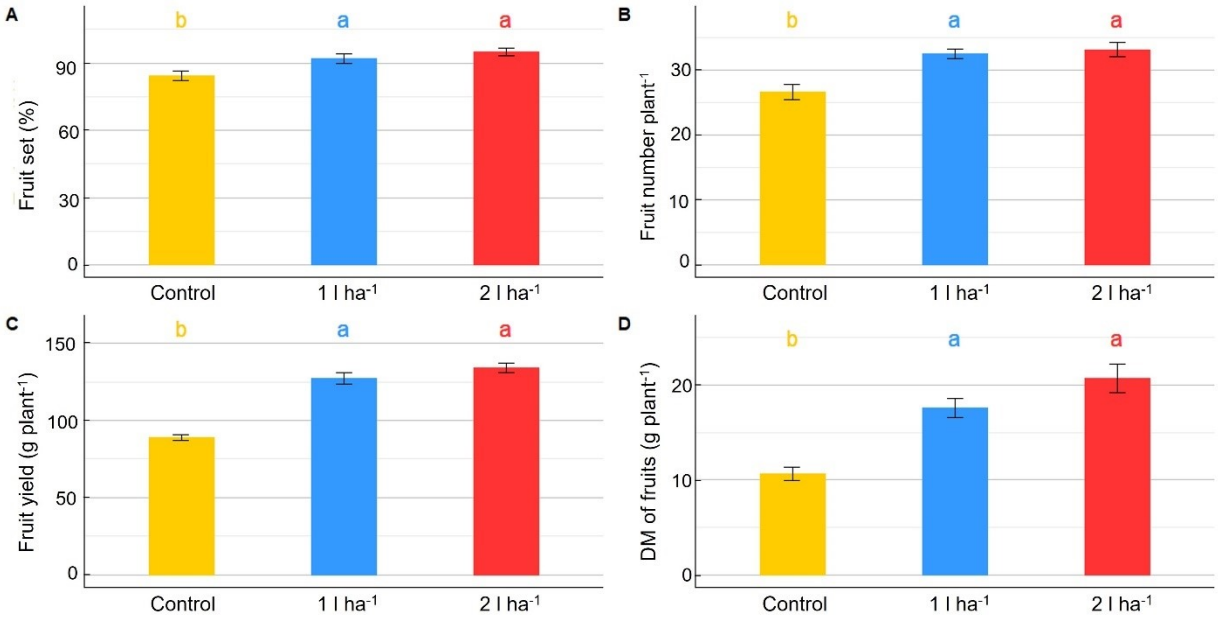

FIGURE 9. Effect of ANE treatment on fruit production in tomato plants cultivated in the greenhouse. The percentage fruit set **(A)**, the total number of mature fruits **(B)**, the total fruit yield **(C)**, and the total fruit dry biomass **(D)** were measured per plant in Micro-Tom cv untreated (control) or treated with ANE (1 or 2 l ha^{-1}). Each value is the mean of n = 6 observations ± s.e. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$).

Effects of ANE treatment on tomato plants grown in open field

Finally, the effects of ANE treatment were assessed in the open field. This step was to validate findings from the two previous experimental settings in controlled growth conditions. Regardless of the dose, the leaf gas-exchange parameters stomatal conductance and net photosynthesis were significantly ($p \leq 0.05$) more important in treated plants at BBCH65 (**Table 7**). The leaf stomatal conductance and net photosynthesis of ANE-treated plants measured at the full flowering stage after the last ANE application were significantly ($p \leq 0.05$) greater compared to non-treated plants. Before the beginning of the treatment, these leaf gas exchange parameters were similar among all groups of plants. Again, no difference was found between the two ANE doses.

Crop fruit yield and total biomass are important parameters in the open field. The total fruit yield and biomass of the total fruits were significantly affected by the biostimulant applications, but these variables did not differ between ANE doses (**Table 8**). The foliar application of biostimulant improved the yield of fresh tomato fruits by 35% (1 l ha^{-1}), and 36% (2 l ha^{-1}), in comparison with untreated plants, with no statistically significant difference between these two values.

TABLE 7. Mean values and analysis of variance of photosynthetic parameters after foliar application of ANE (biostimulant, B) at different phenological phases (time, T) in the field.

Treatment	Stomatal conductance (mmol m^{-2} s^{-1})	Net photosynthesis (µmol m^{-2} s^{-1})
Biostimulant (B)		
2 l ha^{-1}	495 ± 13.9 a	25.5 ± 0.7 a
1 l ha^{-1}	493 ± 16.5 a	25.3 ± 0.6 a
Control	428 ± 27.2 b	23.9 ± 1.0 b
Time (T)		
Pre-application	489 ± 8.6 A	26.3 ± 0.4 A
After last application	455 ± 23.6 B	23.5 ± 0.6 B
ANOVA Significance		
B	*	*
T	*	*
B x T	ns	ns

Data are means ± standard error. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$). *, ** significant respectively at 0.05 or 0.01 levels according to ANOVA.

TABLE 8. Analysis of variance of the yield and quality measured parameters that were affected by foliar application of the different ANE doses in the open field.

Treatments	Fruit yield (kg ha^{-1})	Fruit DM (kg ha^{-1})	Fruit set (%)
2 l ha^{-1}	132,750 ± 2,612 a	5,965 ± 170 a	95 ± 1.41 a
1 l ha^{-1}	131,650 ± 7,800 a	5,807 ± 474 a	96 ± 1.49 a
Control	97,500 ± 6,611 b	4,294 ± 345 b	82 ± 2.93 b

Data are means ± standard error. Different letters indicate a significant difference according to LSD Fisher's test ($p \leq 0.05$)

DISCUSSION

The perspective of using plant biostimulants is hindered by the lack of knowledge translation from laboratory to field. A methodological framework is here presented, which includes different experimental settings in controlled and field conditions, to describe the effects of one biostimulant product through phenomics and transcriptomics. As a case study, an extract from the brown alga *Ascophyllum nodosum* was sprayed on tomato plants and applied at three time points during the flowering period.

The same experimental design was applied across three culture conditions. Firstly, a comprehensive picture of the plant responses induced by ANE, including physiological evaluations and global transcriptome analysis, was obtained in a climate chamber. Then, leaf gas exchange measurements and other yield-related morphological parameters were measured

on plants grown in the greenhouse and field. Seaweed extracts can be applied as foliar spray or soil solutions. In this work, foliar applications were chosen with the aim of directly targeting the plant aerial organs at specific phenological stages.

The plant responses to the biostimulant treatment were conserved in the three different growing conditions, in terms of increased stomatal conductance, net photosynthesis, and key yield traits, such as the number of fruits and fruit biomass. Enhanced leaf stomatal conductance and rate of net photosynthesis were always detected after the third treatment application at full flowering. Also, in terms of regulation of gene expression, the response detected after the first ANE application was moderate compared to the one recorded after the second and third applications. These marked effects detected after the third ANE application suggest a cumulative effect of the treatments.

Overall, our observations were in line with previous studies showing increased tomato yields following the application of seaweed extracts (SWE) (Khan et al., 2009; Zodape et al., 2011; Ali et al., 2016; Murtic et al., 2018; Yao et al., 2020; Campobenedetto et al., 2021; Mzibra et al., 2021). This activity is partially explained by the reported presence in ANE of several hormones (e.g., auxins, cytokinins, gibberellins, abscisic acid (ABA), brassinosteroids, ethylene, and strigolactones) (De Saeger et al., 2020). Moreover, some ANE substances are known to stimulate the biosynthesis of endogenous phytohormones including auxins, cytokinins, and gibberellic acid, which leads to improved plant growth (Ali et al., 2019).

In many crops, including tomato, yield is associated with the number of flowers at maturity. Moreover, the cellular division phase leading to the fruit formation starts during flowering. Precisely, seaweed extracts were previously reported to promote flowering, increasing the number of flowers and fruits per cluster in tomato plants and yield parameters of other crops (Ali et al., 2016; Shukla et al., 2019; Hussain et al., 2021). Indeed, the product was applied during the flowering stage to evaluate possible effects on the fruit setting and the fruit yields eventually. The application of ANE improved fruit setting and yield across all experimental settings. Consistently, seaweed extracts modulated the expression of key genes involved in flowering (Dookie et al., 2021). Our results from the transcriptomic study point to “flower development” biological process at BBCH61 as a key functional category (**Table S4**). Indeed, *FLOWERING TIME* (*FT*), *CLAVATA* (*CLV*), and *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) were up-regulated 24h after the lower ANE dose application. Salicylic acid (SA) has a widely reported flower-inducing activity and its accumulation can activate *FT* expression: in fact, SA-deficient plants show low levels of *FT* transcripts (Martínez et al., 2004). This suggests SA could be involved in flowering response to ANE.

In addition, greater fruit setting and yield in treated plants could be explained by greater photosynthesis and enhanced allocation of assimilates to the fruit. A possible explanation could be the increase in leaf chlorophyll content and photosynthetic capacity (Blunden and Gordon, 1986; Schiattone et al., 2018; Yao et al., 2020). Accordingly, Kumari et al. (2011) observed that the increase in vegetative growth could be due to an increase of photosynthetic pigments (chlorophyll and carotenoids) in the leaves of tomato plants treated with seaweed extracts. On the other hand, Xu and Leskovar (2015) described how the inhibition of gas exchange and stomatal conductance induced by drought stress on spinach, was reduced by *A. nodosum* extract but had no effect on leaf chlorophyll content, chlorophyll fluorescence, and gas exchange under full irrigation. The stomatal opening regulation and the photosynthesis modulation are primarily involved in the widely documented mitigation of drought stress detrimental effects exerted by seaweed extracts on plants (Santaniello et al., 2017; Shukla et al., 2018). When plants are grown in optimal conditions or in the field, without the environmental pressure of water stress, the effect of ANE treatment on the stomatal conductance was previously described either as an increased stomata opening (Salvi et al., 2019; Tombesi et al., 2021) or as an opposite reduced stomatal conductance (Santaniello et al., 2017). In the work by Santaniello et al. (2017), the decrease in the transpiration rate of ANE-treated *Arabidopsis thaliana* plants went with the reduced expression of the MYB60 transcription factor responsible for stomatal movements regulation, and a higher expression of two ABA-responsive genes, suggesting a priming effect on the plants that produced higher sensitivity of stomata to changes in ABA concentration.

The ANE used in the present work seemed not to target ABA-responsive genes. On the contrary, the stomatal conductance was promoted, and we observed the modulation of some SA-dependent genes. In the context of plant responses to biotic and environmental stresses, ABA is known to act antagonistically to SA, and to jasmonic acid and ethylene (Cao et al., 2011). Moreover, as previously reported, the recognition of ANE by the plant can induce the differential expression of defense-related genes compared to untreated control plants (Goñi et al., 2016; Omidbakhshfard et al., 2020) and among the genes dysregulated after the first ANE application in our study were some pathogenesis-related leaf proteins and a few endochitinases. The upregulation of some SA-dependent genes as *PR1b1* (Solyc09g007010), *FT* (flowering time, Solyc03g077920), and one *WRKY* transcription factor (Solyc03g095770) upon the ANE treatment encourages the hypothesis of the activation of the SA signaling pathway. Given the observed antagonistic interaction between SA and ABA, we hypothesize a diminished sensitivity to ABA that leads to reduced stomatal closure (Mosher et al., 2010).

The RNA-Seq results, when considering the pool of DEGs obtained from all the different comparisons, and the GO enrichment analysis output, are suggesting a substantial contribution

of genes involved in several photosynthetic pathways. Both the biological processes of light-dependent reaction and the dark phase of photosynthesis are significantly enriched and mainly downregulated upon treatment application at BBCH61 and upregulated at BBCH65 (**Table S4**). Overall, the transcriptome analysis revealed a major number of downregulated genes than upregulated ones. The same trend was recorded by Omidbakhshfard et al. (2020) 48 hours after spraying *Arabidopsis thaliana* plants with an ANE. Jannin et al. (2013) reported a greater number of downregulated compared to upregulated genes related to the photosynthetic pathways in shoots of *Brassica napus* after applying ANEs to the roots. In their work, the downregulation affected nuclear genes encoding chloroplast precursor proteins involved in biosynthesis and degradation of chlorophyll or a plastid division regulator. To the same group of chloroplast precursors, belonged upregulated genes (such as ferredoxins and carbonic anhydrase 1) encoding mainly proteins implicated in the electron transport chain.

Our results suggested an opposite regulation of two similar genes: a carbonic anhydrase gene (*CA2*) and a subunit of the Rubisco enzyme (*RBSCs1*). After one day from the leaf application of the ANE used in the present work, we recorded a downregulation of both genes in the early flowering stage and at full flowering (**Table 5**). At the same time, the physiological evaluation of the leaf gas exchange on the same plants was revealing a greater rate of stomatal conductance and net photosynthesis. The amount of CO_2 that reaches the carboxylation sites can be modulated by the activity of beta carbonic anhydrases (CA*)*, which catalyzes the reversible hydration of CO_2 to HCO_3^-. The improved stomatal conductance of ANE-treated plants could account for optimal availability of CO_2 reaching leaves carboxylation sites, thus resulting in a decreased *CA2* transcription. An overall increase in net photosynthesis rates was observed in treated plants as well as a downregulation of genes directly involved in the photosynthetic process. Thus, we can hypothesize that the untreated plants were undergoing photosynthetic apparatus early senescence. Possibly, coping with sub-optimal artificial light caused an increase in transcripts involved in the light reaction of photosynthesis.

Despite the physiological parameters measured and the yield traits never being influenced by the dose of the product, the lower dilution dose (1 l ha^{-1}) seemed to induce a broader response in the plants in terms of the number of DEGs. Moreover, after the third application, the overall DEGs number decreased compared to the previous treatment. No gene was found to be up or downregulated by the treatment in more than two conditions (doses and sample timing). These patterns of gene expression modulation suggest either a dose-specific response to ANE, or an earlier common response that was not detected by sampling at 24h. Indeed, the DEG number decreased after 48h compared to 24h, especially for the greater ANE dose. Nevertheless, the final effect in terms of increased leaf gas exchange and fruit yield was achieved with both doses. Future applications of a similar methodology for biostimulant characterization could include

more sample collection timings to achieve a more complete time-wise description of the molecular mechanisms involved in the plant response to the treatment.

CONCLUSION

Across three growing conditions, tomato plants treated with ANE showed a greater number of flowers and fruit sets, resulting in a greater fruit yield. Also, net photosynthesis and stomatal conductance were improved after one ANE application. There was a transcriptome reprogramming caused by ANE treatment and particularly, after the second and the third leaf ANE application.

This study provides a detailed and robust methodology to evaluate plant biostimulant effects under different growing conditions. It also suggests that ANE application to tomato plants during flowering time can foster yield increases in greenhouse and field conditions. Furthermore, the combination of transcriptomic and phenomic approaches could become a key system for dissecting the plant response to any biostimulant. A comparison of morpho-physiological and molecular data collected under laboratory conditions showed coherent results. Such scientifically consistent methodological approaches to achieve the functional characterization of a biostimulant may support the whole stakeholders' chain involved in developing, describing, registering, and commercializing plant biostimulants. Ultimately, farmers applying biostimulant products would greatly benefit from such a complementary study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB53962 (ERP138777).

AUTHOR CONTRIBUTIONS

FM, AM, PS: conceptualization. FM, AM, and PS: supervision. FM, PS, AM, AB, ML, MB, GB, WZ-L, SR, CC, EP, and CH: methodology. AB, ML, CC, and PS: writing the original draft. FM, CC, SN, CH: writing, reviewing, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:

https://www.frontiersin.org/articles/10.3389/fpls.2022.983772/full#supplementary-material

Supplementary tables

Supplementary Table 1. Physical and chemical properties of the substrate of cultivation.

Soil characteristics	Values
Composition	
White sod peat 10-25 mm (% vol.)	35%
White peat 0-25 mm (% vol.)	45%
Peat fiber (% vol.)	5%
Perlite (% vol.)	15%
Structure	
Medium (0-30 mm)	
Chemical data	
H value (H_2O, v/v 1:2:5)	6
Fertilizer level (g l^{-1})	1
Nitrogen (mg N l^{-1})	140
Phosphorous (mg P_2O_5 l^{-1})	160
Potassium (mg K_2O l^{-1})	180
Magnesium (mg mg l^{-1})	100
+ all necessary trace elements	
Iron added as EDTA chelates	
Physical data	
Dry matter	<10%
Water capacity	65-70%
Air capacity	20-25%

Supplementary Table 2. Selected genes used for validation of RNA-Seq data using real-time quantitative RT-PCR.

Gene ID	Gene name	Gene description	Forward Primer 5',3' and Reverse Primer 3',5'
Solyc03g114940.3.1	*P450*	Cytochrome P450 78A5-like	ACGCTGAAGTTGGAACCGAT
			GCCTTGCCCACGAGAGTAAT
Solyc02g086820.2.1	*CA2*	Carbonic anhydrase	AGGGTGGATTTGAGCTGTGG
			GAAGGAAATTGTGAGGGCCA
Solyc03g096290.3.1	*PIP1-7*	Aquaporin	TACAAAGAGCCACCACCAGC
			TTAGAAACGCCCATGACGGT
Solyc02g063150.2.1	*RBCS-1*	Ribulose bisphosphate carboxylase small chain 1	GCCGCTTCTTTTCCCGTTAC
			CATGCATCTAACGCGTCCAC
Solyc09g007010.1.1	*PR1b1*	Pathogenesis-related leaf protein	TGACATATGAATCAAGTCAAACTCC
			AATCAACTTAAGCCCATTATGAACA

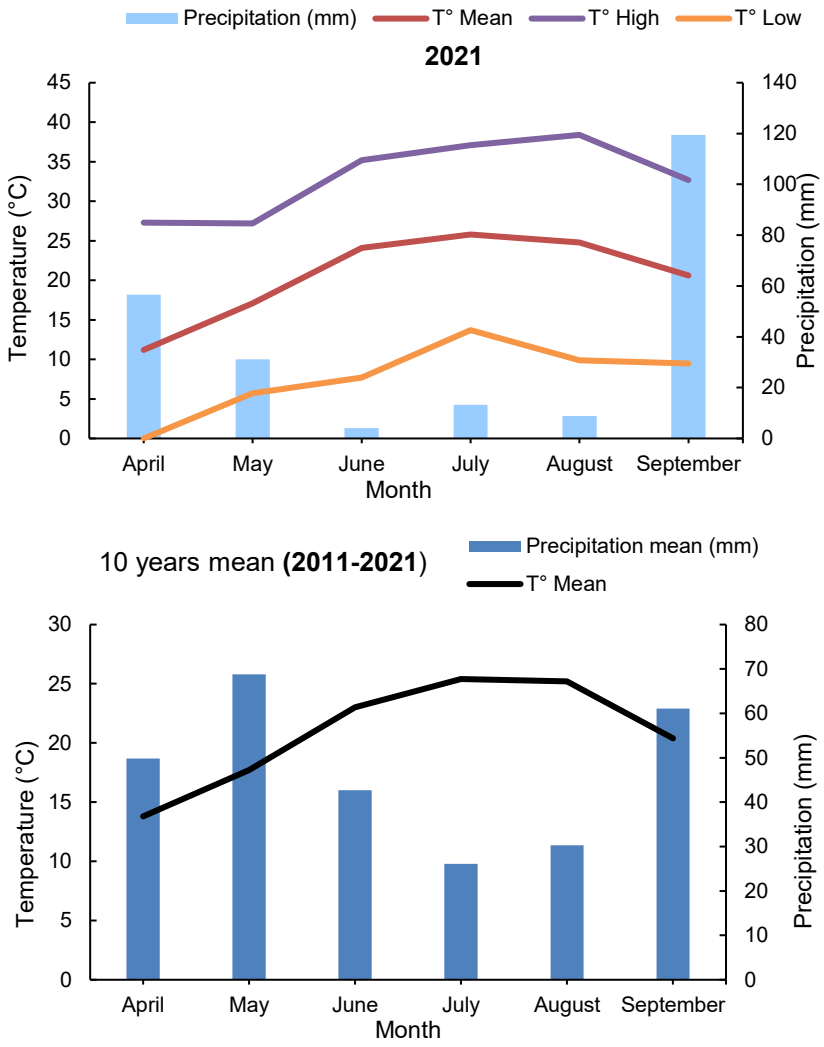
Supplementary Table 3. Physical and chemical properties of the soil (0 - 30 cm depth).

Soil characteristics	UM	Value
Sand	% dm	24
Silt	% dm	48
Clay	% dm	28
pH		7.23
Total $CaCO_3$	%	1.02
Active $CaCO_3$	%	0.94
Organic C	g kg^{-1} dm	8.85
Organic matter	% dm	1.53
Total N	g kg^{-1}	1.16
P_2O_5	mg kg^{-1}	72
K_2O	mg kg^{-1}	170
C/N		7.63

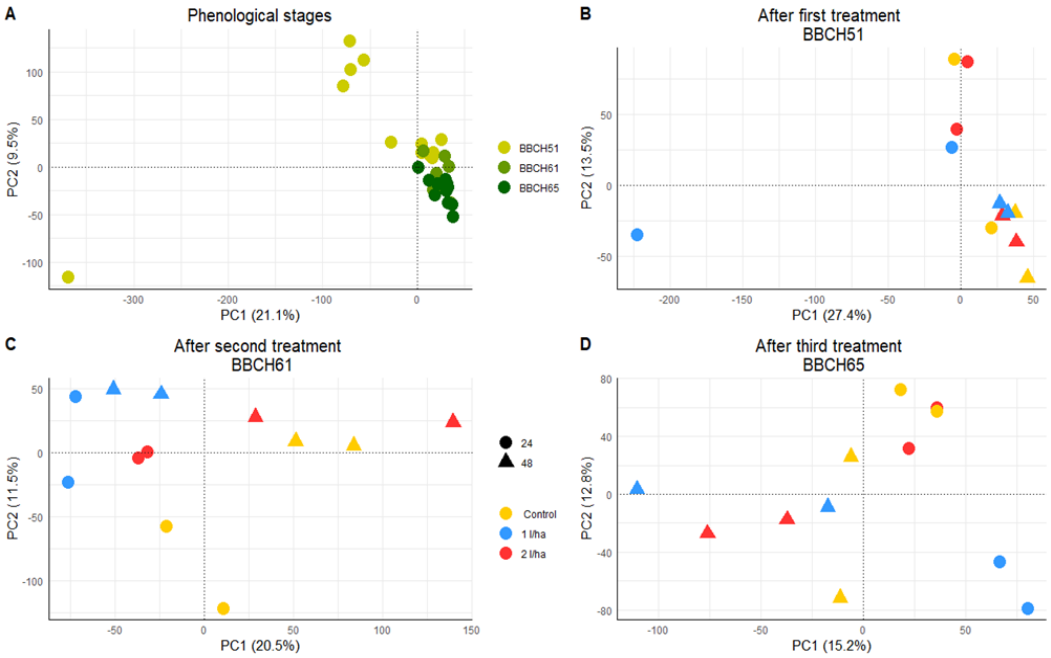
Supplementary table 4. Gene Ontology enrichment analysis of DEGs after ANE treatment at BBCH51 (**Table S4.A**), at BBCH61 (**Table S4.B**), and at BBCH65 (**Table S4.C**), in different comparisons according to dose (1l ha-1 and 2l ha -1) and sampling time (24 h and 48h). Biological process, molecular function and cellular component for down-regulated (blue) and up-regulated (red) genes (adj-p < 0.1 and log2FC ≤ -1) in treated plants compared to the untreated ones.

Available at the link
https://www.frontiersin.org/articles/10.3389/fpls.2022.983772/full#supplementary-material

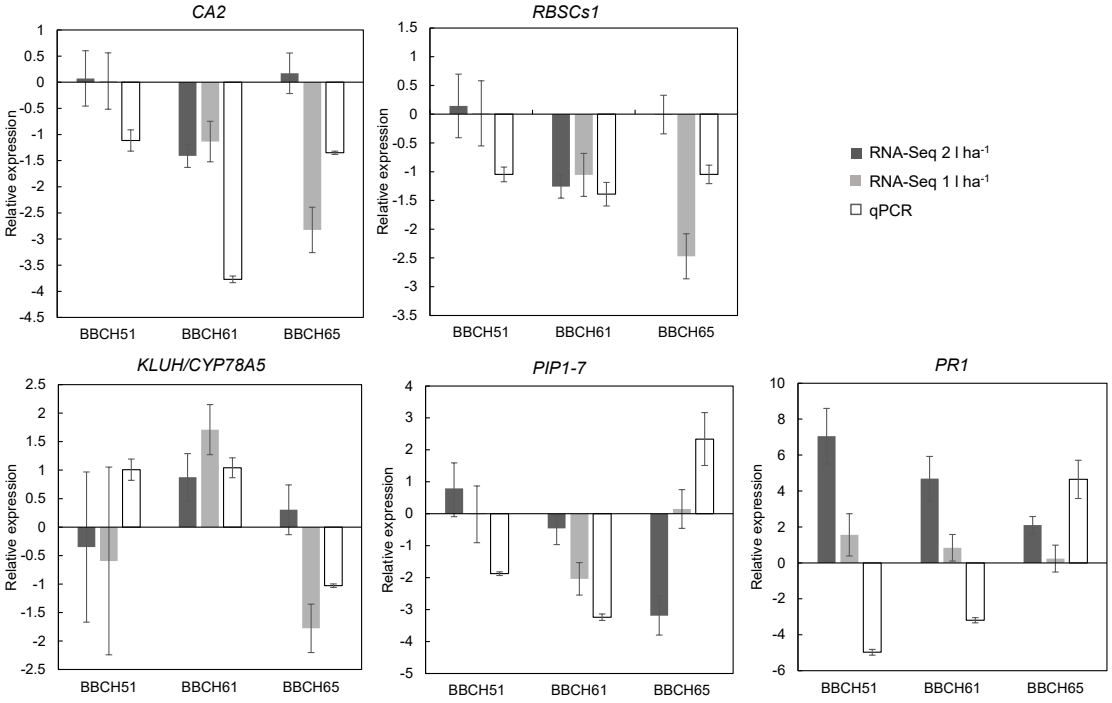
Supplementary figures



Supplementary Figure 1. Average monthly temperature, maximum and minimum temperatures, and rainfall precipitation during the experimental period in 2021 and in the previous ten years (2011-2021) at Cadriano, Bologna (Italy).



Supplementary Figure 2. Principal component analysis plots of log_2 normalized read counts in the three different treatment applications, at BBCH51, BBCH61, and BBCH65. Different colors refer to the treatment variable: blue and red are treated samples respectively with 1 and 2 l ha^{-1}. The untreated ones are yellow. Triangles and circles are used to distinguish sampling time, respectively samples collected 48 h and 24 h after the treatment.



Supplementary Figure 3. Relative expression values ($2^{-\Delta\Delta Ct}$) from RT-qPCR of plants treated with 2l ha^{-1} dosage and log_2 FC from RNA-Seq for both doses of application of five genes in the three different times of treatment application (BBCH51, 61, and 65) after 24h.

GENERAL CONCLUSIONS

Modern agriculture is seeking a balance between high yields and quality of production and low environmental impact. Plant biostimulants are an emerging class of agricultural inputs. They can contribute to the sustainability of agricultural practices improving crop nutrient use efficiency, growth, and tolerance to stress, while reducing the heavy reliance on chemical fertilizers and pesticides (Rajput et al., 2019). However, these products may result highly variable in responses, due to the lack of understanding of modes of action and the relatively scarce availability of rigorous independent validations (Yakhin et al., 2017). To overcome legitimate possible skepticism and to optimize their agricultural application, it is fundamental to constantly improve our understanding of the modes of action of each specific formulation.

The present thesis contributed to the body of literature pursuing this specific goal. Our focus was more oriented to the thorough description of the plant responses following biostimulant-based treatments. This work may set the basis for new research questions to be answered.

In the first contribution, we assessed the yield-promoting effect of a leonardite-based biostimulant on sugar beet cultivated in the North-East of Italy. Also, the increased abundance of a bacterial genus, namely Oxalicibacterium spp., reported in leaf tissues after leonardite treatment should be further validated to clarify the causes and the implications of our observation.

Moreover, the thesis proposes a methodological approach that combines transcriptomics and phenomics for the functional description of a plant biostimulant. In the third contribution, we present an experimental strategy through the presentation of a case study in which a seaweed extract is applied to tomato plants during the flowering stage. Tomato was used as a model crop in our studies. However, the presented methodology could become a useful system for dissecting the response of other crops to any biostimulant. Indeed, including trials across three growing conditions, from lab to field, and comparing morpho-physiological and molecular data collected after the treatment, allows for a robust characterization of the biostimulant effects on the studied crop.

Overall, this thesis is providing a contribution to the current knowledge on the plant responses to three selected plant biostimulants: one derived from leonardite and rich in humic acids, a calcium-and-polysaccharides-based formulation, and an *Ascophyllum nodosum* extract. Data presented are both collected from plants grown in laboratory conditions and from field-cultivated crops. Characterizing biostimulant effects on plants and disentangling their modes of action are critical ongoing challenges that will benefit from the joint effort of scientific community and agriculture stakeholders.

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