UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Agronomy Food Natural Resources Animals and Environment

Second Cycle Degree (MSc) In Sustainable Agriculture

Evaluating an *Ascophyllum nodosum* Extract as an Organic Biostimulant on Tomato

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1. Abstract

Modern agriculture is evolving toward adopting sustainable food production practices around the world because of environmental concerns about rapid climate change and food insecurity. Climate change has had a variety of effects on crop production in previous years. To deal with this problem, traditional agricultural techniques have been accustomed to using excessive agrochemicals (chemical fertilizers, pesticides), which have minor effects but have a bigger impact on pollution, prompting increased environmental concerns. Modern agriculture is looking for environmentally sustainable alternatives to maintain crop output while reducing reliance on chemical fertilizers. In this study present Comprehensive approach using phenomics and RNA-seq analysis for dissecting plant responses to biostimulant treatments. Tomato plants (*Solanum lycopersicum* cv. Micro-Tom) were grown in the growth chamber, greenhouse, and open field (cv. Rio Grande) conditions in 2020 and 2021. Foliar treatments with an *Ascophyllum nodosum* biostimulant extract (ANE) were carried out with two doses (1 l/ha and 2 l/ha) at three different phenological stages (BBCH51, BBCH61, and BBCH65) within the flowering phase. Phenomics profiling highlighted higher net photosynthesis and stomatal conductance in all the growing conditions that resulted in improved fruit yield traits in ANE-treated plants when compared to the untreated ones. RNA-seq analysis revealed the highest number of differentially expressed genes (DEGs) with the lower dose of ANE (1 l/ha). The impact of the first ANE application recorded at the beginning of the flowering stage on the leaf transcript was moderate, whereas the second and third applications resulted in a higher number of DEGs. The functional enrichment analysis of the overall set of DEGs, irrespective of the plant phenological stage and dose of product application, highlighted a significant contribution of pathways related to photosynthesis and response to the stimulus. This molecular result hints at the major role exerted by the product on the plant photosynthetic processes, and this is consistent with the morpho-physiological results. This is the first comprehensive dual-omics approach for profiling plant responses to biostimulant applications across three different growing conditions.

2. Keywords:

Plant Biostimulants development, Plant Biostimulants characterization, RNA sequencing, morpho-physiological traits, genotype-phenotype relationship, controlled environment conditions, open-field trial.

3. Introduction

Global food and agricultural production systems are experiencing unprecedented difficulties owing to increased demand for food for a growing population, rising hunger and malnutrition, negative effects of climate change, overexploitation of natural resources, biodiversity loss, and food loss and waste rising. The environmental concern over rapid climate change and food insecurity shapes modern-day agriculture moving toward adopting sustainable food production practices around the world. To cope with this issue traditional agricultural practices are habituated to the use of excess agrochemicals (chemical fertilizers, pesticides) which are having minimal beneficial effects with a greater impact on environmental pollution with growing concerns about the environmental impact, Modern agriculture is seeking eco-friendly ways to sustain crop productivity and reduce the dependency on chemical fertilizers agriculture (Xu and Geelen, 2018). Conventional agricultural practices relying mainly on synthetic agrochemicals are uneconomical and unsustainable for the environment and human health (Dookie et al., 2021), there is an urgent need to develop more sustainable, environmentally friendly crop production processes. There is a growing desire for sustainably produced food that has fewer synthetic agrochemicals and a higher concentration of biologicals. Over the past decades, Plant Biostimulants (PBs) became novel and sustainable inputs for agriculture (Del Buono, 2021, De Saeger et al., 2020). Increasingly important in agriculture, being considered an environmentally sustainable and economically feasible option to optimize crop productivity (Colla and Rouphael, 2015). The global market of PBs reached $ 2.000 106 in 2019, and it is projected to reach 3.930 Mn USD at an average Compound Annual Growth Rate (CAGR) of 11.54% from 2020 to 2025 (up from 10.95% in 2015-2020) (Dunham and Trimmer, 2020). In this market expansion scenario, the concept of biostimulant activity relates to existing and future legislation and regulatory prescriptions governing the placing of products on the market of of PBs (Lucini and Miras-Moreno, 2020).

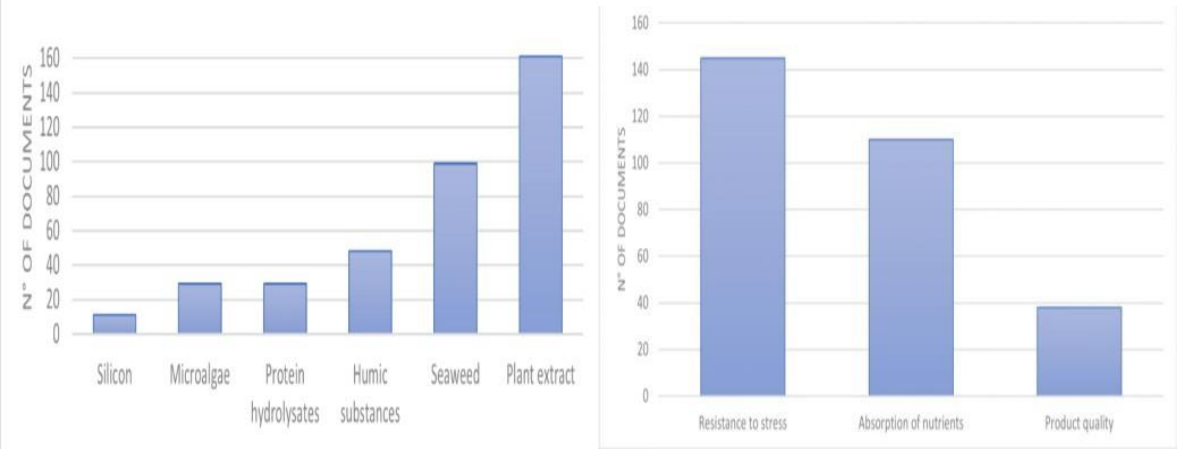

Figure 1: Breakdown of papers by substance, breakdown of papers by biostimulant effects (2021).

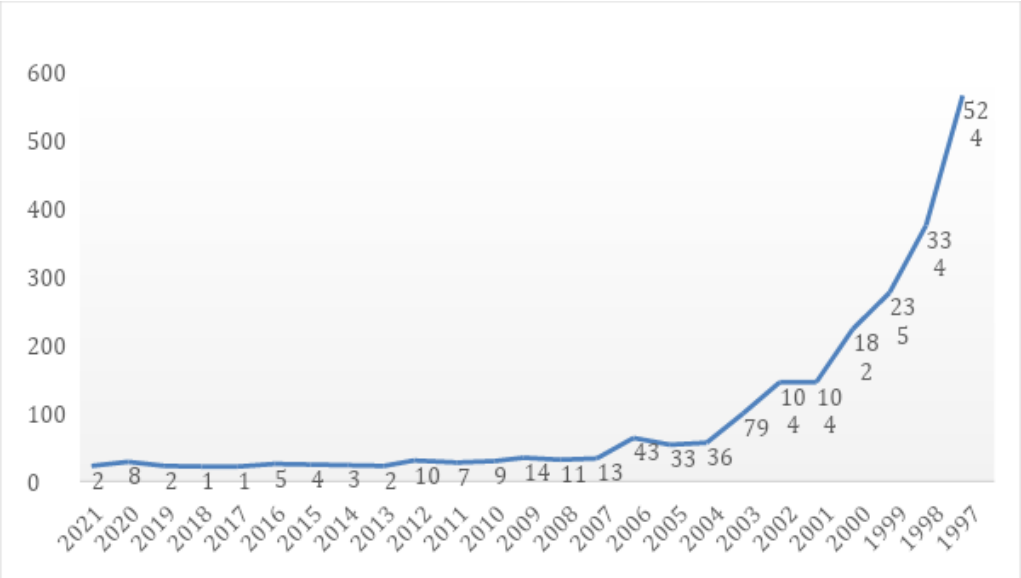

Figure 2: International scientific production 1997-2021(n. of articles per year).

There are currently several definitions of biostimulants. Trailing back to the earliest definition of biostimulants identifies a web journal dedicated to turf maintenance professionals, called Ground Maintenance (Zhang and Schmidt 1997 (http://grounds-mag.com). One of the first formally agreed-upon definitions of Plant Biostimulant was outlined by the EU Fertilizer Regulation 2019/1009, a milestone in recognition of the biostimulation concept, that frames these products in a discrete class of fertilizers based mainly on their function. According to this definition a plant biostimulant is a product that stimulates plant nutrition processes irrespective of the product's nutritional content with the sole goal of increasing one or more of the following plant or plant rhizosphere characteristics: I) nutrient use efficacy, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere.

The discovery of the scientific underpinnings of biostimulant action and science-based product development has therefore become a prerequisite for placing on the market effective and reliable PBS. Nevertheless, most PB products are complex substances or mixtures, and the complex nature of their components raises the challenge of understanding their modes of action. Currently, the implementation of several omics and phenotyping approaches, and physiological analyses, is strongly aiding the PBs research in pursuing this goal by providing key information for the discovery of plant metabolic pathways or developmental processes that are modulated by a given PB (Yakhin et al., 2017, Nardi et al., 2021). Specifically, the integration of omics technologies targeting the molecular and biochemical responses of the plants such as transcriptome profiling and metabolomics with high-throughput

phenotyping, and physiological evaluations of specific photosynthetic parameters enables a comprehensive characterization of the PB activity (Franzoni et al., 2022, Della Lucia et al., 2022).

In the process of describing the effects and investigating the modes of action of a PB, the potentialities of these highly informative tools can be maximized by setting an experimental design that considers different degrees of environmental variability. As the traits associated with the claims defining the bio stimulatory action strongly depend on environmental conditions, the characterization of PB's impact on crops and the definition of an effective technical product positioning require the experiments to be performed under different growing 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).

Indeed, crops grown in the open field confront multiple abiotic stresses and heterogeneous conditions which are hardly reproducible in artificially controlled conditions. Moreover, a plant's phenotype is directly affected by its environment, and observed phenotypic variables are a direct reflection of these interactions. Accordingly, PBs screened in a controlled environment can perform differently in field conditions (Rouphael et al., 2018). Several reasons account for these observed discrepancies. Among them, for instance, is the impact of climatic conditions on leaf uptake that can reduce the biostimulant efficacy in field applications when dealing with foliar treatments (Pecha et al., 2012). Another case regards the dependency upon the native-microbial composition and other soil chemical and physical properties on the capacity of microbial biostimulants to exert specific effects on the plants (Fadiji et al., 2022).

Generally, plant biostimulants are evaluated in controlled environments to speed up the selection process of the most interesting products which will then be tested in the field. However, most research works focus either on laboratory conditions or field trials, therefore not achieving a complete basic functional characterization of the PB.

In this work, complementary experiments conducted in the laboratory, greenhouse and open field were conducted to fully characterise a biostimulant product, through a dual-omics approach involving transcriptomics and phenotyping of key plant physiological and morphological traits. The most widely researched seaweed, used as a source for industrial and commercial plant biostimulants, is the brown, intertidal seaweed *Ascophyllum nodosum*. *Ascophyllum nodosum* pertains to the Fucaceae family. It grows in the North Atlantic and covers the coast that stretches from the United States to Europe. They are particularly looking at seaweeds which have bladders filled with air in the fronds.

Figure 3: Ascophyllum nodosum

Varied commercial extracts from *A. nodosum* have been demonstrated to improve plant growth and mitigate some abiotic and biotic stresses while also improving plant defences by the regulation of molecular, physiological, and biochemical processes. There is evidence that the hormonal effects of extracts from the brown alga *Ascophyllum nodosum* are largely explained by the downregulation and upregulation of hormonal biosynthetic genes in plant tissues and, to a lesser extent, by the hormonal content of the seaweed extract itself (Wally et al., 2013a, b).

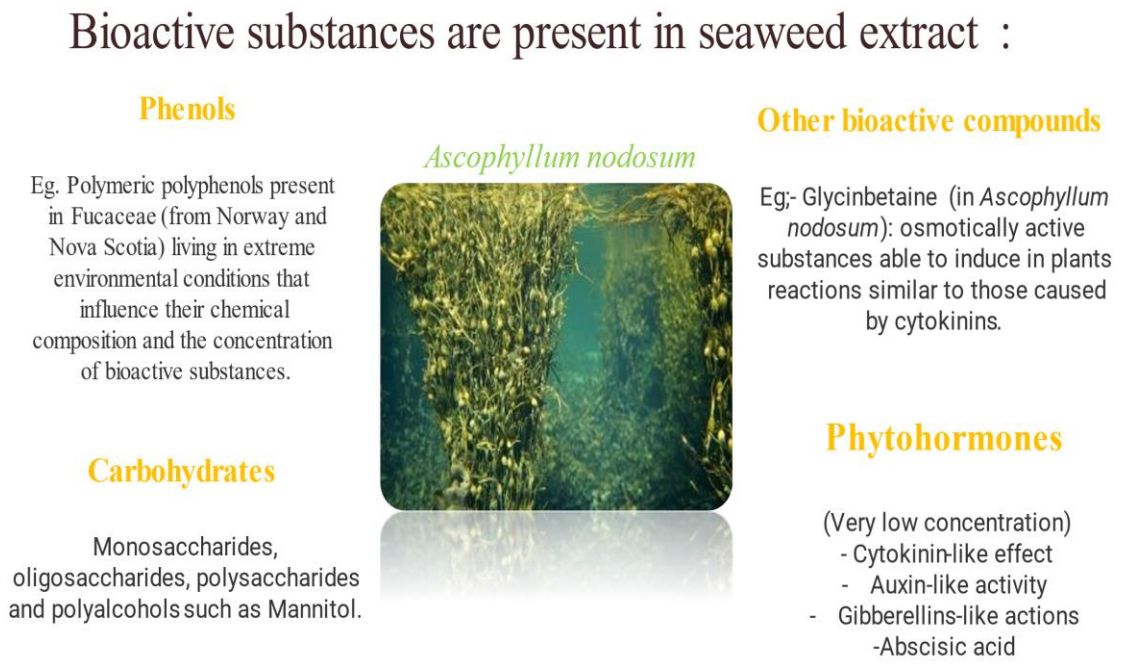

Figure 4 : Bioactive substances present in seaweed extract.

Molecular genetics, i.e., hormone mutants in Arabidopsis and transcript analysis by RT-qPCR, were used to reach this conclusion (Scientia Horticulture, Volume 196,2015). Counting this framework into consideration, using tomato is a model plant for fruit development, a unique trait that classical model plants such as Arabidopsis and rice lack. The tomato genome was sequenced in 2012 and the tomato is becoming very popular as an alternative system for plant research. Among the many tomato varieties, Micro-Tom has been recognized as a model cultivar for tomato research because it shares several key advantages with Arabidopsis, including small size, short life cycle, and ability to grow under fluorescent light with high density. Mutant and transgenic plants are essential materials for functional genomics research, and thus the availability of mutant genetic resources and transgenic methods are important tools to facilitate tomato research. Specifically, in this study, the mode of action of an *Ascophyllum nodosum* extract (ANE) was firstly characterised on tomato plants through transcriptomics and morpho-physiological profiling under controlled conditions in a growth chamber. Further physiological evaluations were subsequently conducted in the greenhouse and open field to achieve a complete description of the product's effects.

This study presents a comprehensive approach using phenomics and RNA-seq analysis for dissecting plant responses to biostimulant treatments.

4. Materials & Methods

4.1. Plant materials and growing condition

To provide a rigorous and detailed method to characterise plant biostimulant activity under different environmental conditions, three separate experiments were conducted in 2020 and 2021, one in a growth chamber (2020), and one in a greenhouse (2021), and one in the open field (2021). The diagram in Figure 5 is displaying the workflow adopted. Tomato plants received the same treatments based on an *Ascophyllum nodosum* extract (ANE) applied to the leaves three times during the reproductive phase, specifically when plants reached BBCH51, BBCH61, and BBCH65. Two doses of biostimulants, 1 l/ha and 2 l/ha, selected among a range of tested concentrations in preliminary experiments (data not shown), were compared.

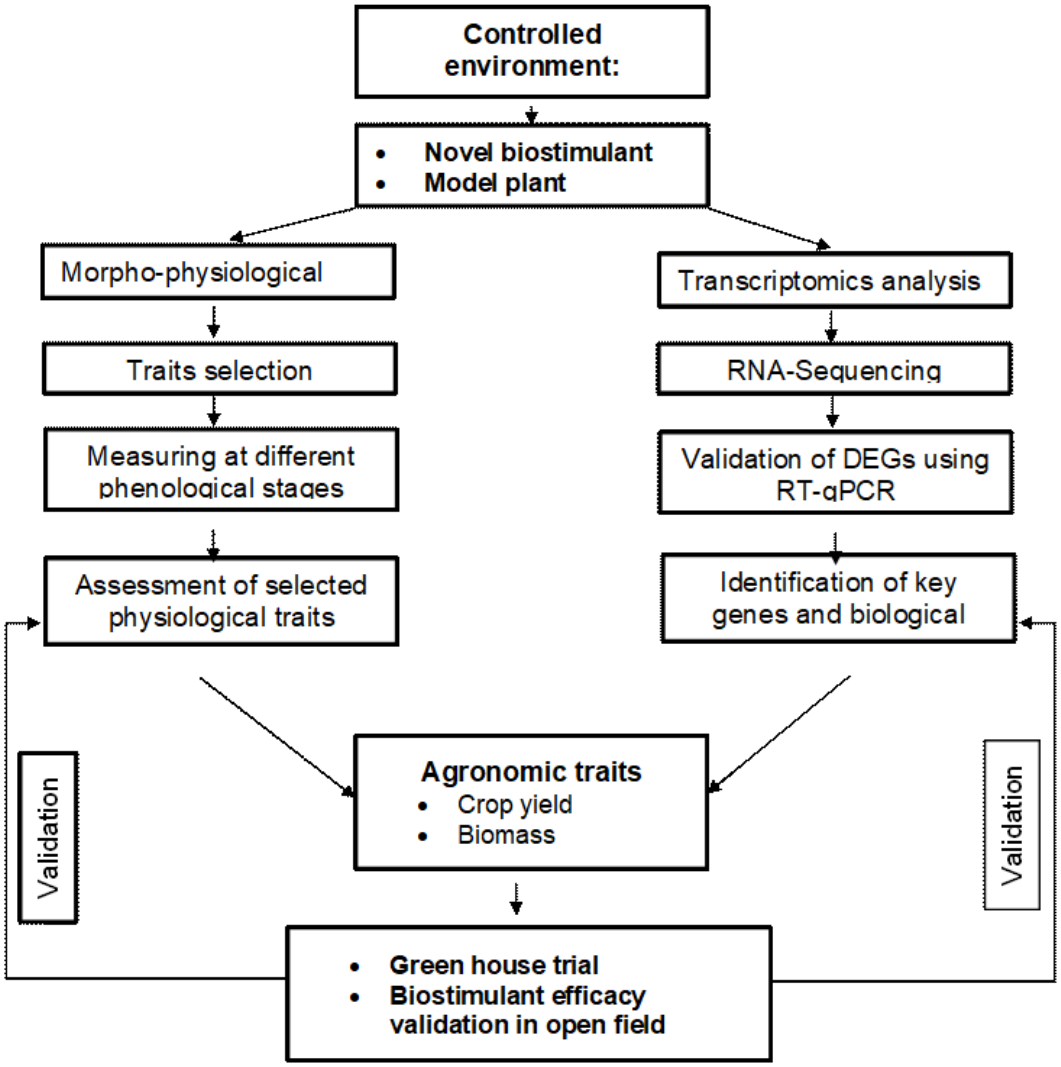

Figure 5: Workflow adopted in the present study to assess the effects of a biostimulant from controlled conditions to the open field.

4.1.1 Growth chamber & greenhouse experiments

In the growth chamber trial, a light-emitting diodes (LED) lighting system was used (PAR photon flux density (PFD) of 250-290 μmol/s (AE100); 210-230 μmol/s (AE80) with a 14 /10 h light/dark photoperiod and photosynthetically active radiation (PAR) efficiency of 2.0 μmol/J and 2.2 μmol/J respectively). Relative humidity and temperature were constantly monitored (60% humidity and 24/20 °C light/dark temperature, respectively). The trial in the greenhouse was carried out in a fully equipped structure with a lighting system with PAR 1500 adjusted to 14/10 h light/dark, controlled temperature 24/20 °C light/dark temperature, 60% relative humidity, natural ventilation roof, lateral openings, and horizontal fan systems for air circulation.

4.1.2 Substrate, plant materials, and treatments

In both experiments, the substrate was composed of peat and perlite (Klasmann-Deilmann) in which Micro-Tom (*Solanum Lycopersicum* L.) seedlings were grown. At 3-4 true leaf stage (30-35 days after sowing date), tomatoes were transplanted in pots 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 solution was also replaced with a more suitable formulation for plant development (NPK 15-15-30 Florti Prod). Plants were maintained under well-water conditions.

Two different doses of *Ascophyllum Nodosum* Extracts (ANE)-based biostimulant (SOB_610.10) (1 l/ha and 2 l/ha) were compared and an untreated control was included. The biostimulant was provided by Sipcam Oxon S.p.A. 10 ml of treatment was applied as a foliar treatment to each plant as a solution diluted in ultra-pure water. The trials, both in the growth chamber and greenhouse, were arranged in a randomized complete block design with seven replicates (pot). Applications of the treatment were carried out three times during flowering time, respectively when plants reached the phenological stages described by the following codes: BBCH51 (first inflorescence visible, first bud erects), BBCH61 (first inflorescence: first flower open), and BBCH65 (5 or more inflorescences with open flowers) (Meier, 2001).

4.2. RNA-Sequencing

RNA-Seq analysis was carried out for two sampling times (24h and 48h after treatment), on treated and untreated plants, at the two doses of product application. Two leaf disks were collected around the mid-vein 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 for direct mRNA isolation from tissue. The amount 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. Their 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). The final double-stranded barcoded cDNA libraries were eluted in 15 µl of nuclease-free water.

4.2.1. 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). 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. 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 log2-fold change $\geq |1.0|$ 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.

4.2.2. 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. We performed the validation on biological replicates of samples collected after 24h from the treatment with the 2 l/ha dose in the three phenological stages from plants grown in the same conditions. Primers were designed using the Primer-BLAST tool on NCBI (Ye et al. 2012). 3 µg of total RNA extracted with the Maxwell® 16 LEV Plant RNA Kit (Promega Corporation, USA) were converted to cDNA using a GoScript Reverse Transcription Mix, Random Primer, according to the manufacturer's protocol (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) 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 (Livak & Schmittgen, 2001).

4.3. Leaf gas exchange measurements

Leaf gas exchange measurements were done before and 48 hours after applications of the biostimulant treatments at three phenological phases (BBCH51, BBCH61, and BBCH65) to detect early physiological responses induced by the treatments' application. Gas-exchange measurements were taken with an infrared gas analyzer (CIRAS 3 PP Systems, Amesbury, MA, USA). Measurements were made under saturating light of 1500 μmol m^{-2} s^{-1} of PPFD (photosynthetic photon flux density) with 400 μmol mol^{-1} of CO_2 surrounding the leaf flux density. 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 and measurements were carried out on the youngest fully expanded leaf throughout every bio-stimulant application.

4.3.1. Biomass and fruit yield

In both growth 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 in each experiment. At harvest, the fresh fruit yield was measured, and the dry weights were recorded after oven-drying the samples at 105 ºC for 24 hours. To determine the percentage of fruit set in the greenhouse, the total number of flowers in the second and third clusters of seven plants (pots) were counted. The fruits were counted at the fruit's development stage on the same clusters where the total flowers were counted. The fruit set percentage was calculated as follows:

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

4.4. Open field condition

Another experiment was carried out to assess the tomato productivity in response to two rates of foliar application of the ANE-based biostimulant (SOB_610.10) in open field conditions. The trial was performed in the Cadriano experimental farm of the University of Bologna, Italy (44° 33' N, 11° 24' E). Tomato certified seeds of *Solanum Lycopersicum* L. cv. Rio Grande was used. Rio Grande is a determinate tomato variety that is widely used in the open field. Seedlings were grown in a greenhouse

under controlled environmental conditions. 4-week-old tomato seedlings were transplanted into the field. Basal fertilization was done with 110 Kg ha^{-1} N (slow-release fertilizer), 100 Kg ha^{-1} P_2O_5, and 200 Kg ha^{-1} K_2O, and during the growing season the plants were enriched with calcium nitrate. Plants were irrigated to create appropriate soil moisture conditions. The first watering was done immediately after transplanting in the field. Each row of plants had its drip line. Drippers were spaced at 40 cm and delivered 5L/m/hr. The amount of water is calculated by both the ETo (reference evapotranspiration (mm/day)) climate conditions and by the crop phenological stage expressed by the Kc factor (crop coefficient). The crop factor, Kc, mainly depends on the type and the crop growth stage of the crop. The crop factor (kc) Values for tomato crop and growth stages were between 0.45 -1.15. The amount of irrigation, crop evapotranspiration or crop water need (ET crop) (mm/day) = ETo × Kc (Brouwer & Heibloem, 1986).

Before the experiment, a composite soil sample was collected to determine the physical and chemical characteristics at 0 - 30 cm depth.

The experimental set-up was a completely randomized design with four replications; each experimental plot consisted of an area of 20 m^2. The space between rows was 115 cm and 40 cm between plants in the row. Two foliar biostimulant levels (1 l/ha and 2 l/ha) were compared and an untreated control was included in the experimental design. The foliar biostimulant treatments were applied again at BBCH51, BBCH61, and BBCH65.

4.5. Leaf gas exchange, biomass, and fruit yield measurements

Leaf gas exchange measurements were carried out as in the case of the controlled environmental conditions (sec. 1.3.) The measurements were carried out on 5 plants per treatment between 9.00 am and 11.00 am. The fruit set percentage and fruit fresh and dry weight were measured in the field trial. 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 number of fruits and the number of flowers. All fruits were harvested from 10 plants of the central rows per treatment with homogeneous development and weighed by an electronic dynamometer. The dry weight of fruits was measured with a precision balance after the samples were oven-dried at 105 °C.

4.6. Statistical Analyses

The repeated measurements ANOVA model used the statistical method with datasets in plant physiological measurements. All the productivity parameters 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. Data analysis of gene expression levels was conducted using RStudio (version R-4.1.0). Principal Component Analysis (PCA) was performed to describe the data after the normalization and Venn diagrams were plotted using the ggVenn package from R to visualize the numbers of DEGs in common between two or more contrasts.

5. Results

5.1. Growth chamber

To characterize the effects of the *Ascophyllum nodosum* Extracts (ANE)-Based Biostimulant under controlled conditions (growth chamber) using transcriptomics and phenomics, we applied the ANE, to leaves of tomato plants at three different phenological phases.

5.1.1. Gene expression

252.549.495 single-end reads were generated by the sequencing runs, with an average of 7.015 million raw reads per sample. The overall alignment rate after mapping to the *S. lycopersicum* genome was on average 78.85%. The transcriptome data obtained are available at the repository link (.

The principal component analysis (PCA) graph of the log2 normalized read counts showed that the samples are well clustered among different phenological stages (panel A). This is less clear for samples collected after the first treatment application at the beginning of the reproductive phase at BBCH51. In this stage, the biostimulant effect was not distinctly detected by the analysis of differentially expressed genes. The behaviour of replicate samples in the PCA plot is pointing out the variability that was registered by our analysis. Only after the second treatment, at BBCH61 and BBCH65, a more treatment-wise consistent clustering is observed.

To detect transcriptional changes induced by the treatment, a differential gene expression analysis was carried out to compare treated with untreated samples for all the three treatments application events, the two different doses of biostimulant (1l/ha and 2l/ha), and the two timings of sample collection (24 and 48 hours after leaf treatment). The number of differentially expressed genes (DEGs) yielded by each comparison is shown in Table 1.

Table 1: Number of DEGs (adj-p < 0.1 and |log2FC| ≥ 1) across different experimental conditions and timings of treatment application. dr stands for downregulated DEGs and ur for upregulated ones.

Number of DEGs	After 1st application (BBCH51)	After 2nd application (BBCH61)	After 3rd application (BBCH65)
1 l/ha 24h vs NT	0	200 (133 dr, 67 ur)	105 (67 dr, 38 ur)
1 l/ha 48h vs NT	19 (11 dr, 8 ur)	209 (144 dr, 65 ur)	1 (1 dr)
2 l/ha 24h vs NT	13 (1 dr, 12 ur)	90 (78 dr, 12 ur)	37 (20 dr, 17 ur)
2 l/ha 48h vs NT	0	17 (9 dr, 8 ur)	28 (10 dr, 18 ur)

After the first application, 62.5% of DE genes are over-expressed, but the total number of DEGs is just 32. Conversely, we observed a greater number of downregulated genes both at BBCH61 (70.5% of

DEGs) and at the full flowering stage (57.3% of DEGs). Given this low number of DEGs and a non-ideal clustering of replicates observed in the PCA plot of samples collected after the first treatment, at BBCH51, we decided to focus on the results obtained after the second and third product leaf application.

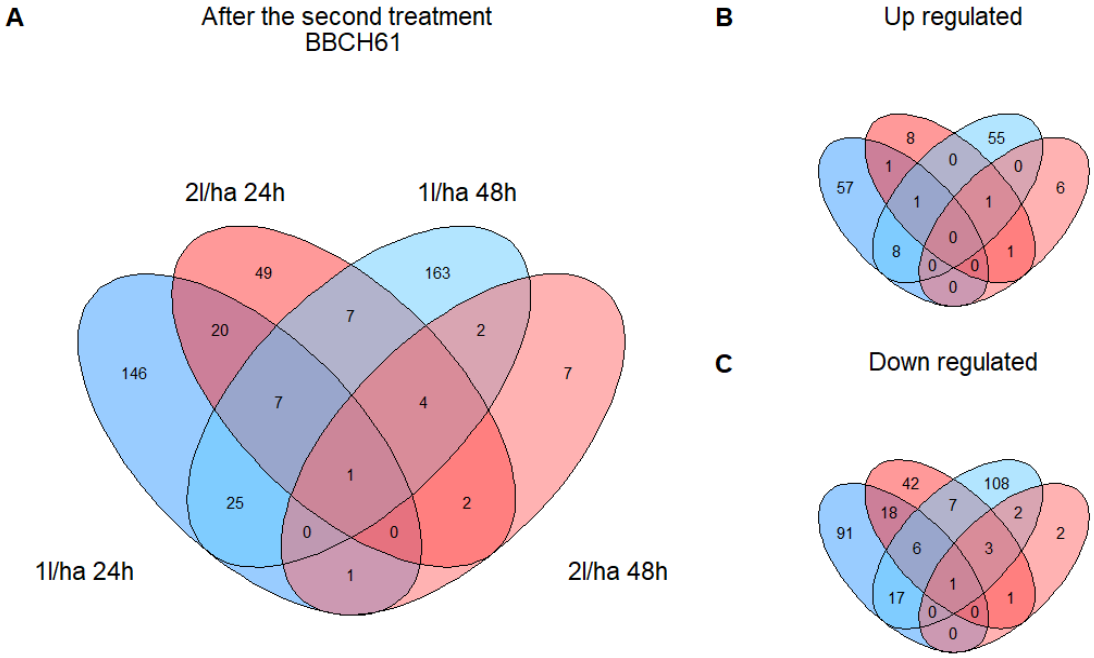

Figure 6: Venn diagrams show the total number of DEGs after the second treatment application (panel A) at BBCH61 Diagrams in panels B and C display respectively the number of upregulated and downregulated genes at BBCH61.

The Venn diagrams in Figures 6 and 7 display the number of genes DE shared among the different time points and volumes of applied product within the same treatment application event. Only one gene, encoding a proline-rich protein 4-like, was always downregulated across all doses and timings at BBCH61, whereas no gene was found to be mutually upregulated at both 24 and 48 hours and with both doses. After the third application, the overall number of dysregulated genes decreases compared to the previous treatment and no gene was found to be up or downregulated by the treatment in more than two conditions, suggesting either a more dose-specific response or an earlier response that was not detected from our sampling time after 24h. Indeed, the number of DEG decreases after 48h compared to the 24h timing, especially for the 2 l/ha dose.

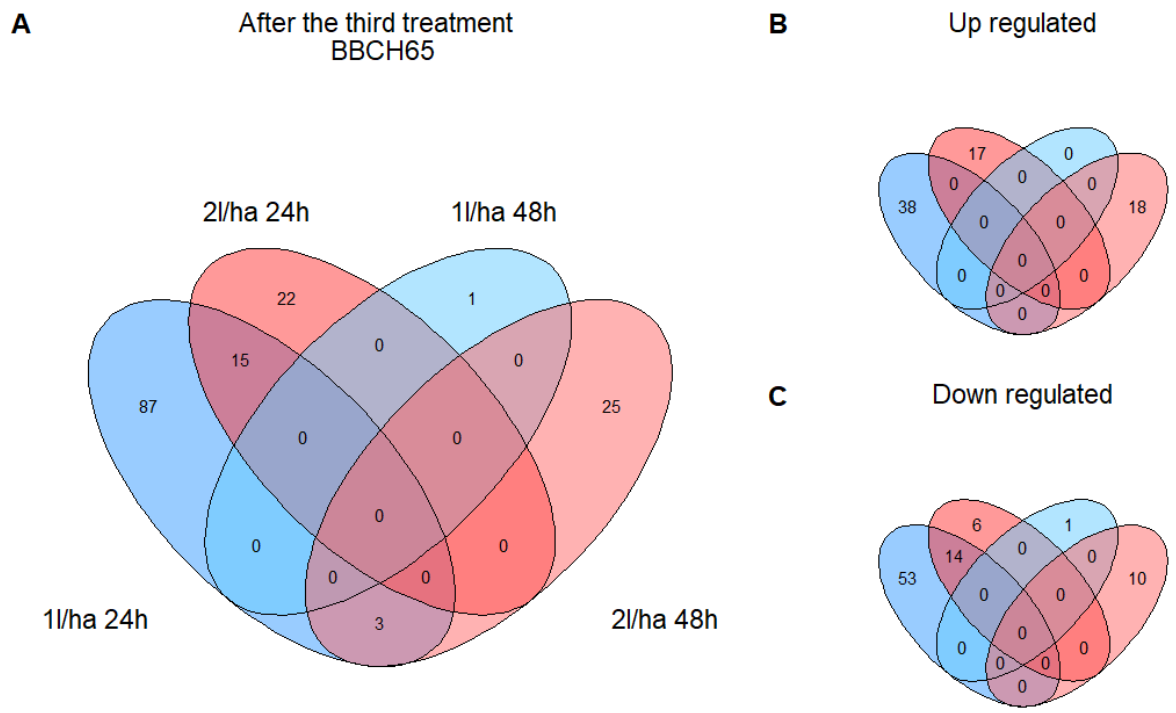

Figure 7: Venn diagrams show the total number of DEGs after the third treatment application at BBCH65 (panel A). Diagrams in panels B and C display respectively the number of upregulated and downregulated genes at BBCH65.

A Gene Ontology (GO) enrichment analysis was carried out separately for DEGs obtained from different comparisons within each phenological stage and for every volume of application and sampling time. The results on the most significantly enriched GO terms related to biological process and molecular function.

Given the high number of different comparisons, we conducted a further GO enrichment analysis on the pool of the total number of DEGs obtained across all to better visualise and characterise the most relevant molecular mechanisms involved in the biostimulant activity pairwise comparisons. The lollipop plots in Figure 8 are showing the significantly enriched biological process (Figure 8A) and molecular function (Figure 8B) GO terms with the highest fold enrichment values. The treatment mainly affected the expression of photosynthesis genes, light and dark reaction, valine biosynthetic process, and response to several stimuli.

The molecular functions most significantly enriched if we consider the overall set of DEGs, are again related to photosynthetic activities, among which are “ribulose-bisphosphate carboxylase activity”, “beta-glucosidase activity”, and “chlorophyll-binding”. Interestingly, the GO terms “chitinase activity” and “water channel activity”, and GO terms related to lipid binding, and oxidoreductase and monooxygenase activity were also among the ones with higher fold enrichment values (Figure 8B).

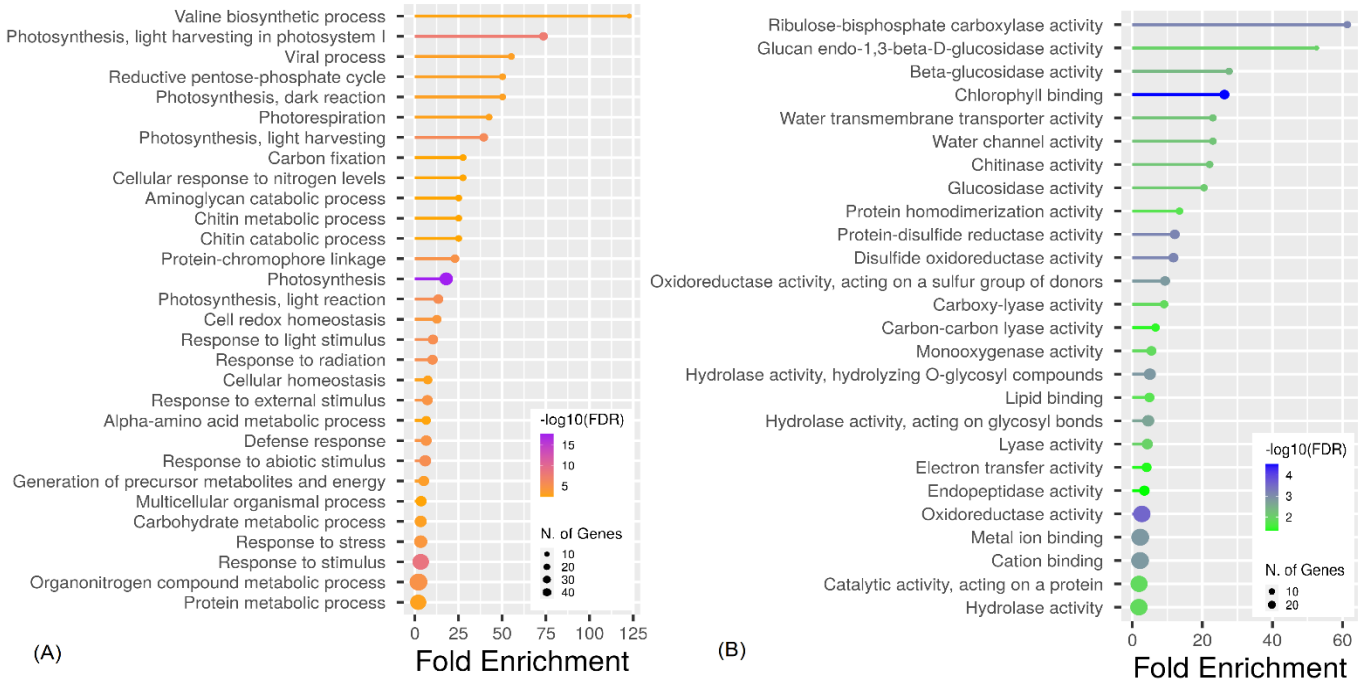

Figure 8: Lollipop plots summarizing the biological process (A) and molecular function (B) GO terms enrichment (FDR ≤ 0.05) of all the DEGs obtained across different comparisons

To identify the main pathways affected by the treatment, we detected 4 main groups of GO terms with the aid of hierarchical clustering (Figure 9) showing the correlation among significant GO terms based on the shared genes within each category. GO terms that are clustering together in the tree plot have more shared genes and bigger 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, defence 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 the stimulus.

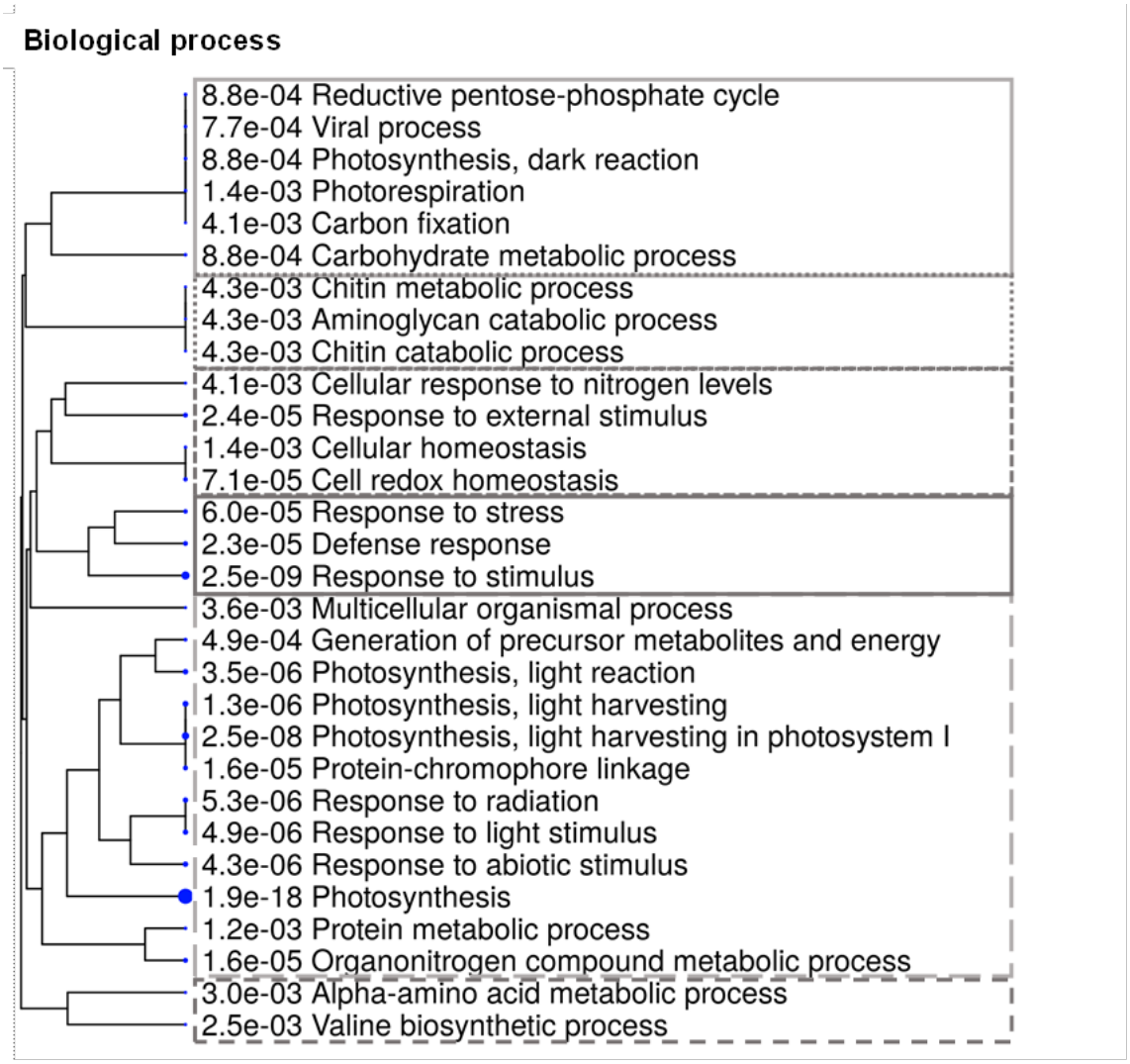

Figure 9: A hierarchical decision tree displaying the degree of association among enriched GO terms in the biological process and its statistical significance. Pathways with more shared genes are closer in the tree plot and bigger dots indicate more significant p-values.

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 2.

Table 2: A selection of representative genes differentially expressed in at least one experimental condition after treatment with ANE at BBCH61 and BBCH65 in the broad enriched biological process categories obtained through hierarchical clustering of GO terms with the highest fold enrichment values

Gene ID	Gene description
Photosynthesis, dark reaction, and carbon fixation	
Solyc02g085950.3	ribulose bisphosphate carboxylase small subunit, chloroplastic 4
Solyc02g063150.3	ribulose bisphosphate carboxylase small chain 1, chloroplastic
Solyc03g034220.3	ribulose bisphosphate carboxylase small subunit, chloroplastic 2
Chitin metabolic process	
Solyc09g098540.3	chitinase-like protein 1
Solyc10g055800.2	endochitinase 4
Solyc10g055810.2	chitinase
Cell redox homeostasis	
Solyc01g087850.2	subtilisin-like protease
Solyc05g015490.3	non-specific lipid transfer protein GPI-anchored 1
Solyc06g008760.1	glutaredoxin-C13
Solyc10g007600.3	glycolate oxidase
Solyc07g042440.3	peroxiredoxin Q, chloroplastic
Response to stimulus and response to stress	
Solyc02g086820.3	Carbonic anhydrase; Reversible hydration of carbon dioxide
Solyc12g099970.2	SNF1 kinase complex anchoring protein
Solyc01g006300.3	peroxidase
Solyc12g011450.2	chlorophyll a-b binding protein 13, chloroplastic
Solyc01g006730.3	calcium-dependent protein kinase 20-like
Solyc07g041720.1	auxin-binding protein ABP19a
Solyc05g055990.3	aquaporin PIP2-4
Solyc10g048030.2	kirola
Photosynthesis	
Solyc01g087040.2	thylakoid lumenal 19 kDa protein, chloroplastic
Solyc01g102770.1	photosystem II protein Z
Solyc02g069460.3	photosystem I reaction centre subunit III, chloroplastic
Solyc05g056050.3	chlorophyll a-b binding protein 6A, chloroplastic
Solyc05g056070.3	chlorophyll a/b binding protein precursor
Solyc10g075160.1	ferredoxin
Solyc07g041720.1	auxin-binding protein ABP19a
Organonitrogen compound metabolic process	
Solyc02g064770.3	probable esterase KAI2
Solyc04g073990.3	annexin p34|calcium-dependent phospholipid-binding protein
Sbt3	subtilisin-like protease
Biosynthesis of secondary metabolites	
Solyc03g044330.1	acetolactate synthase 2, chloroplastic
Solyc04g014510.3	glutamine synthetase cytosolic isozyme 1-1
Solyc04g082030.1	ornithine decarboxylase
Solyc08g007040.3	glycine cleavage system H protein, mitochondrial

Within the biological pathways of "response to stimulus" and "organonitrogen compounds metabolism" we observed the dysregulation of genes encoding proteins with an esterase activity and several protein kinases. DEGs are encoded by the ROS-mediated signalling and oxidative stress response by peroxidase and glutaredoxin family proteins. The defence response was chitinases and pathogenesis-related leaf proteins, whereas key genes in the biosynthesis of secondary metabolites category were acetolactate and glutamine synthase and probable asparaginase.

Genes significantly DE in the photosynthesis process were mainly encoding chlorophyll a-b binding proteins, ribulose bisphosphate carboxylase subunits, and photosystems subunits.

Table 3: RNA-Seq data validation of 5 candidate genes on different biological replicates using RT-qPCR. Fold change in expression is presented using the $2^{-\Delta\Delta Ct}$ *± s.e. for qPCR data and fold change for RNA-Seq data.*

Gene ID	**Gene name**	**Description**	**Treatment application**	**qRT-PCR 2l/ha**	**RNA-Seq 1l/ha**	**2l/ha**
Solyc03g096290	PIP1-7	Aquaporin PIP1-7, 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, catalyzing reversible hydration of carbon dioxide	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).*

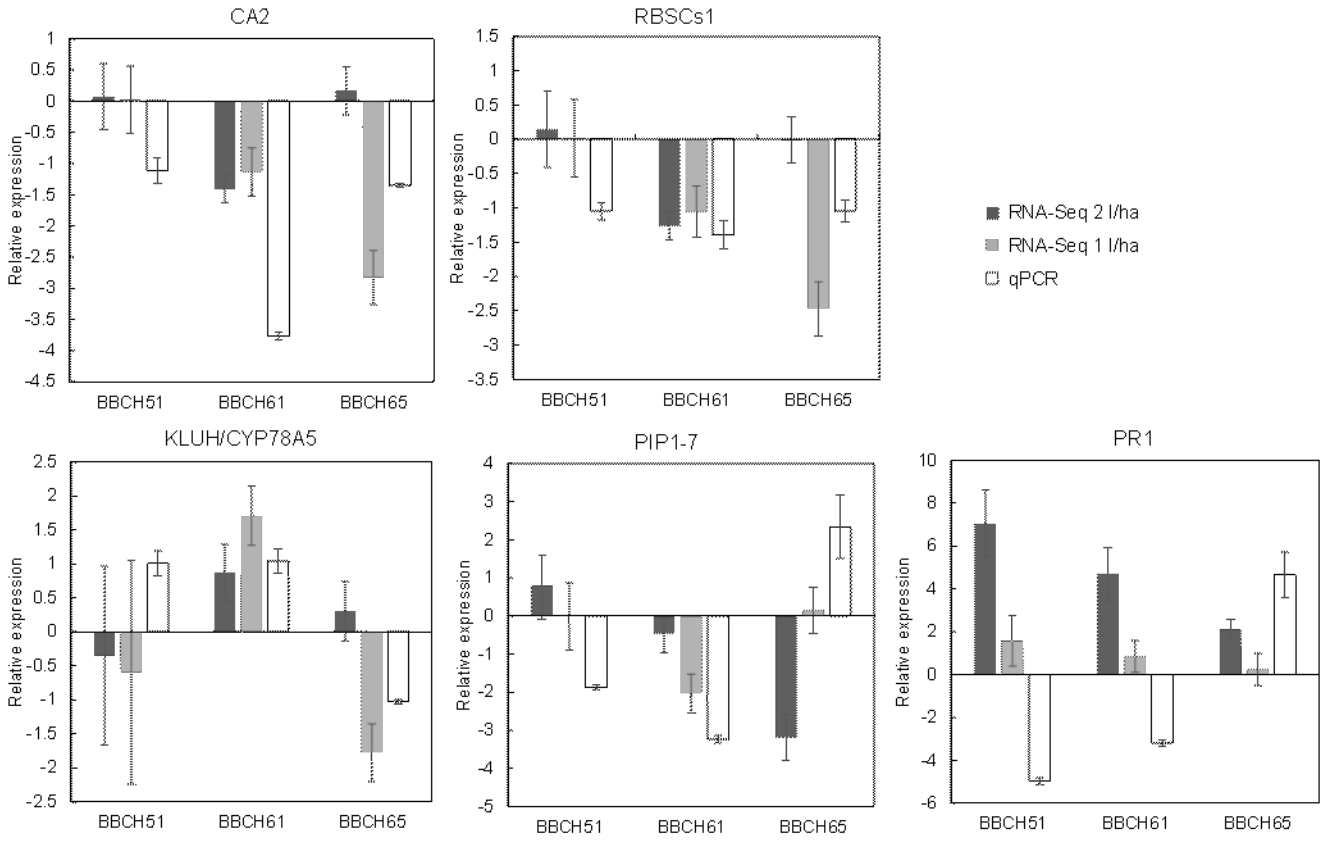

Figure 10: Relative expression values ($2^{-\Delta\Delta Ct}$) from RT-qPCR of plants treated with 2l/ha dosage and log_2 FC from RNA-Seq for both doses of application of five genes in the three different times of treatment application (BBCH51, 61, and 65) after 24h.

To validate RNA-Seq results, five candidate genes involved in photosynthesis and defence response were selected and their expression level was measured through RT-qPCR on different biological Seq analyses for both doses. Despite some discrepancies, we overall observed a 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) compared to the 2 l/ha dose which was the one used in the qPCR validation.

The pattern of expression of RBSCs1, CA2, and the cytochrome P450 (KLUH/CYP78A5) detected by the RNA-Seq data after the second and third application of 1l/ha of ANE biostimulant was generally consistent with the relative expressions obtained through qPCR (Figure 10). However, the folds change in up and downregulation of these genes in the treated samples compared to the control are not fully matching. The downregulation of RBSCs1 and CA2 encoding respectively a ribulose bisphosphate carboxylase small chain and a carbonic anhydrase were observed in leaves of plants treated with 1l/ha at both BBCH61 and BBCH65, In contrast, the only statistically significant downregulation registered

with the higher dose of application (2 l/ha) is for the CA2 gene at BBCH61 (Figure 10). 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. PR1b1 encoding a pathogenesis-related protein 1 was significantly upregulated after every treatment with the highest product dose (2l/ha) in the RNA-Seq results. The same higher level of PR1b1 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 10).

5.1.2. Leaf gas exchange and physiological parameters

Physiological surveys performed during the plant growth showed significant effects of the treatment on stomatal conductance and net photosynthesis. A significant interaction between the different dosages of fertilization and time of application was detected in stomatal conductance and net photosynthesis (Table 4).

Table 4: Analysis of variance of the physiological measured parameters that were affected by foliar application of the different dosages fertilizer (F) (SOB_ 610.10) at different phenological phases in the growth chamber.

Treatment	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Net photosynthesis (µmol CO_2 m^{-2} s^{-1})
Fertilizer (F)		
2 L/ha	302 a	18.7 a
1 L /ha	291 a	18.5 a
Control	214 b	17.5 b
Time (T)		
B51	273 B	22.7 A
A51=B61	418 A	23.9 A
A61=B65	145 C	12.2 C
A65	240 B	14.0 B
Significance		
F	*	*
T	*	**
Fx T	**	*

*SOB_610.10, seaweed formulated biostimulant. BBCH51 (the first inflorescence visible: first bud erects), BBCH61 (first inflorescence: first flower open), BBCH65 (5th inflorescence). ns, non-significant. *, ** significant*

respectively at the 0.05, .01 level of probability. Different letters indicate a significant difference according to LSD Fisher's test (P ≤ 0.05).

The application of ANE significantly enhanced stomatal conductance and net CO_2 assimilation in comparison with the control, but no differences were recorded between different dosages of fertilizer application (Table 4). Positive effects of ANE on stomatal conductance were evident at the stages before BBCH61 and after BBCH65. A significant effect on net photosynthesis was obtained with the application of ANE in comparison to the control only after the last treatment application, but no statistically significant differences were detected between the two doses 1 l/ha and 2 l/ha. Positive effects of SOB_610.10 were evident after BBCH65 (Figure 11).

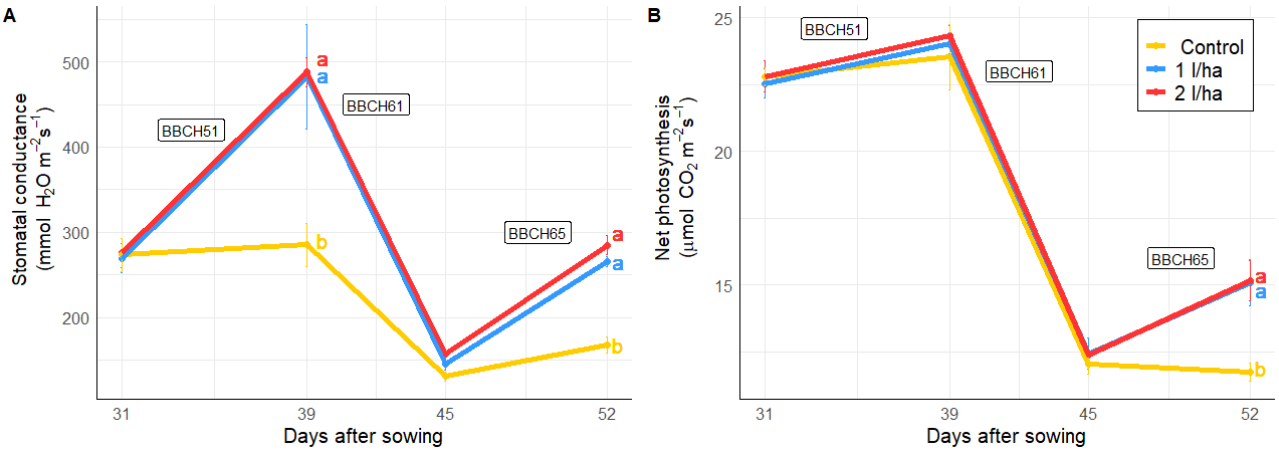

Figure 11:Interaction between different dosages of ANE and time of application on the stomatal conductance (left) and net photosynthesis (right) at plant growth of Micro-Tom in the growth chamber. Different dosages of Bst - ANE and control - no application of biostimulant. Different letters indicate a significant difference according to LSD Fisher's test (P ≤ 0.05).

At the final harvest, ANE application significantly increased the number of fruits per plant compared to the control. The plants treated with both different dosages of ANE showed significantly higher total fruit dry matter than the untreated plants. There was no discernible difference between the different the various biostimulant dosages. (Figure 12).

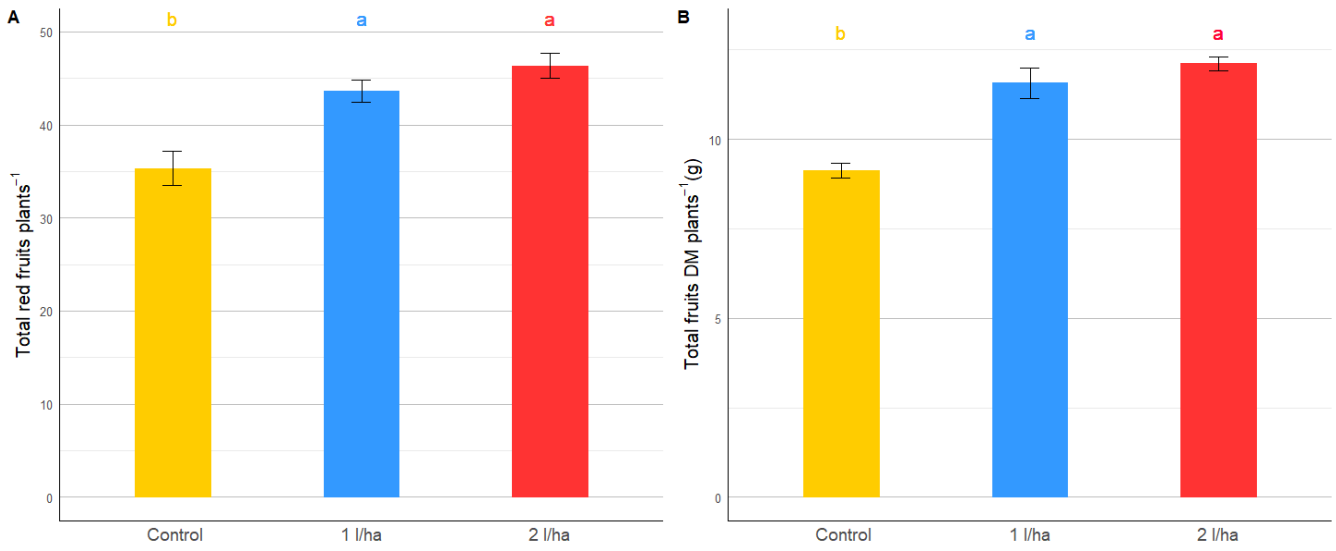

Figure 12: Comparison between the different dosages of biostimulant fertilization and control on the total number of fruits per plant (left) and total fruit biomass per plant (right) in the growth chamber. Different dosages of Bst - ANE and control - no application of biostimulant. Different letters indicate a significant difference according to LSD Fisher's test ($P \leq 0.05$).

5.2. Greenhouse

The physiological analysis carried out in the greenhouse showed significant effects of treatment on net photosynthesis and stomatal conductance and significant interactions between the biostimulant treatment and the time of application on net photosynthesis (Table 5).

Table 5: Analysis of variance of the physiological measured parameters that were affected by foliar application of the different dosages fertilizer (F) (SOB_ 610.10) at different phenological phases in the greenhouse.

Treatment	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Net photosynthesis (µmol CO_2 m^{-2} s^{-1})
Fertilizer (F)		
2 L/ha	198 a	15.1 a
1 L /ha	193 a	14.9 a
Control	160 b	12.9 b
Time (T)		
B51	186 B	12.1 C
A51=B61	232 A	17.0 A
A61=B65	151 C	13.9 BC
A65	165 BC	13.5 BC
Significance		
F	**	**
T	**	**
Fx T	ns	**

*ANE, seaweed formulated biostimulant. BBCH51 (the first inflorescence visible: first bud erects), BBCH61 (first inflorescence: first flower open), BBCH65 (5th inflorescence). ns, non-significant. *, significant at the 0.05 level of probability. Different letters indicate a significant difference according to LSD Fisher's test ($P \leq 0.05$).*

The application of ANE resulted in a significant increase in stomatal conductance and net photosynthesis compared with the control, but no differences were recorded among different dosages of fertilizer application (Figure 13). Net photosynthesis was higher in treated plants compared to the control.

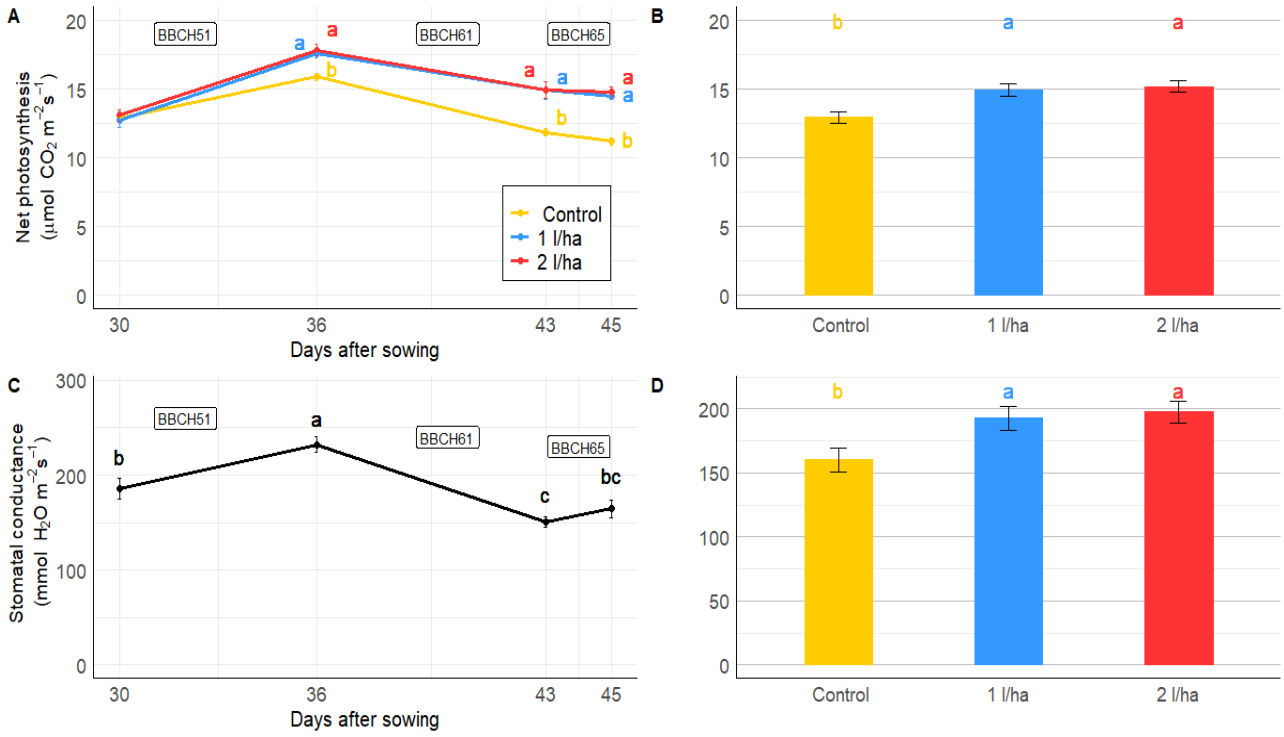

Figure 13: Interaction between different dosages of ANE and time of application on the net photosynthesis at plant growth of Micro-Tom (A) and comparison of different dosages of ANE referring to the average net photosynthesis across all time points (B) and stomatal conductance (C & D) in the greenhouse. Different letters (graph A, B, D) indicate a significant difference according to LSD Fisher's test ($P \leq 0.05$). Different letters (graphs C) indicate a significant difference in different plant growth stages according to LSD Fisher's test ($P \leq 0.05$). BBCH51 (the first inflorescence visible: first bud erects), BBCH61 (first inflorescence: first flower open), BBCH65 (5th inflorescence).

The fruit set percentage was significantly affected by biostimulant application, but no significant difference was found among the different dosages of biostimulant treatments. Plants treated with different dosages of ANE showed significantly higher total fruit yield and biomass than untreated plants. However, there is no discernible difference between the various dosages of biostimulant (Figure 14).

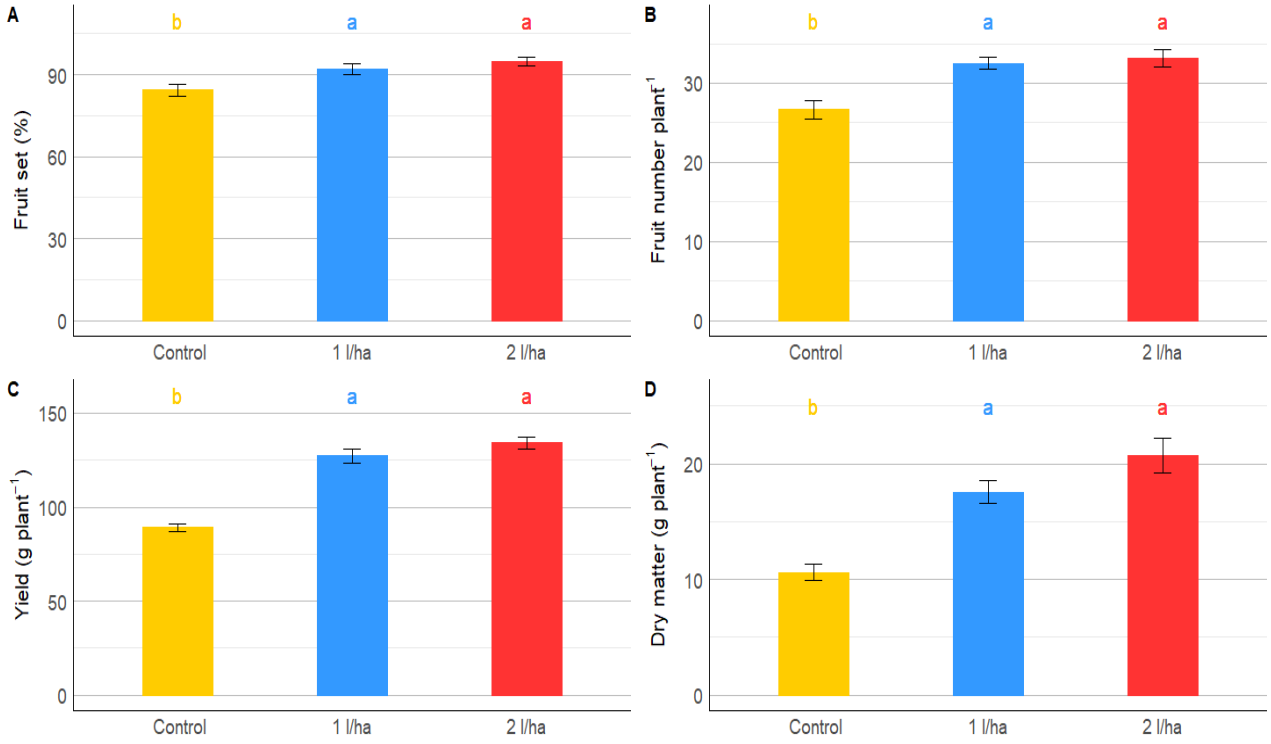

Figure 14: Comparison between the different dosages of biostimulant fertilization and control on the percentage of fruit set per plant (A) and the total number of fruits per plant (B) Total fruit yield per plant (C) total fruit biomass per plant and (D) in the greenhouse. Different dosages of Bst - ANE and control- no application of biostimulant. Different letters indicate a significant difference according to LSD Fisher's test ($P \leq 0.05$).

5.3. Open field

The response induced by ANE was assessed in the open field to validate previous phenotyping experiments carried out under environmentally controlled growth conditions. In the field trial, ANE foliar treatments regardless of the dose applied had a positive effect on tomato plant growth. The positive effects of the treatment on the measured physiological parameters were evident after the biostimulant application (Table 6).

Table 6: Analysis of variance of the physiological measured parameters that were affected by foliar application of the different dosages fertilizer (F) (SOB_ 610.10) before and after the last application (different phenological phases) in the open field.

Treatment	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Net photosynthesis (µmol CO_2 m^{-2} s^{-1})
Fertilizer (F)		
2 L/ha	495 a	25.5 a
1 L/ha	493 a	25.3 a
Control	428 b	20.9 b
Time (T)		
Pre-application	489 A	26.3 A
After last application	454 B	23.5 B
Significance		
F	*	*
T	*	*
Fx T	ns	ns

*SOB_ 610.10, seaweed formulated biostimulant. ns, non-significant. *, significant at the 0.05 level of probability. Different letters indicate a significant difference according to the LSD test.*

The average leaf stomatal conductance and net photosynthesis on plants treated with biostimulant were significantly higher compared to the one detected on non-treated plants. Again, no differences were measured between the two dosages of biostimulant (Fig. 15).

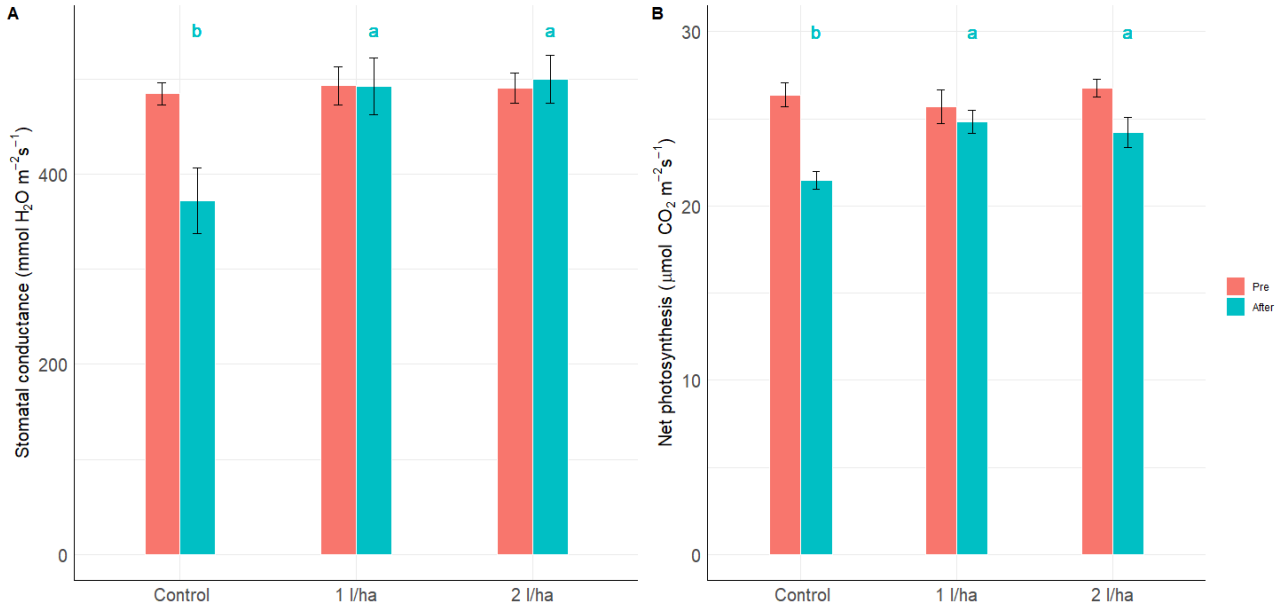

Figure 15: Comparison of different dosages of ANE biostimulant and control in terms of stomatal conductance (A) and net photosynthesis (B) in the open field. Different letters indicate significant differences according to LSD Fisher's test ($P \leq 0.05$). Physiological measurements during plant growth were carried out before the first application and after the last application.

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 application, but these variables did not differ among different dosages of biostimulant application (Table 7).

Table 7: Analysis of variance of the yield and quality measured parameters that were affected by foliar application of the different dosages fertilizer (ANE) in the open field.

Treatments	Fruit yield (Mg/ha)	Fruit DM (Mg/ha)	Fruit set (%)
2 l/ha	13.28 a	5.97 a	96 a
1 l/ha	13.17 a	5.81 a	95 a
Control	9.75 b	4.29 b	82 b

ANE, seaweed formulated biostimulant. Different letters indicate a significant difference according to LSD Fisher's test ($P \leq 0.05$)

The foliar application of biostimulant improved the yield of fresh tomato fruits by 35% (1 l/ha), and 36% (2 l/ha), in comparison with untreated plants, with no significant difference between the biostimulant dosages.

6. Discussion

The application of biostimulants in cultivated plants is hindered by the lack of rigorous evaluations of their effects from laboratory to field conditions. In this work, we are presenting a methodological approach that, from the growth chamber to the open field, aims to describe the effects of a biostimulant product on a given plant through phenomics and transcriptomics. Specifically, we tested two doses of an *Ascophyllum nodosum* extract applied three times through foliar treatment within the flowering stage on tomato plants.

The effects of every treatment application on leaf gas exchange and transcriptome composition were recorded in plants grown in an environmentally controlled ideal situation. The same experimental design was applied to three different growing conditions. The gas exchange measurements and other yield-related morphological parameters were measured in all three trials. The plant responses to the biostimulant treatment in terms of increased stomatal conductance, net photosynthesis, and key yield traits, such as the number of fruits and the fruit biomass, were conserved in the three different growing conditions.

The observation of the effects of the ANE on plants grown in controlled conditions pointed out an enhanced stomatal conductance after the first and the third treatment application, at the early flowering stage and full flowering stage, and an increased rate of net photosynthesis in treated plants throughout all the reproductive phase in the greenhouse trial but only at full flowering stage in the growth chamber trial. Also in the field trial, an increase in the overall rate of leaf gas exchanges was detected. The effects observed in the full flowering phase may partly be due to the residual effects of the biostimulant application in the previous stage, but it is not possible to identify these effects separately. Although the most marked effects on physiological parameters in correspondence with the third application of fertilizers leads us to believe that the cumulative effect of the treatments is a plausible hypothesis.

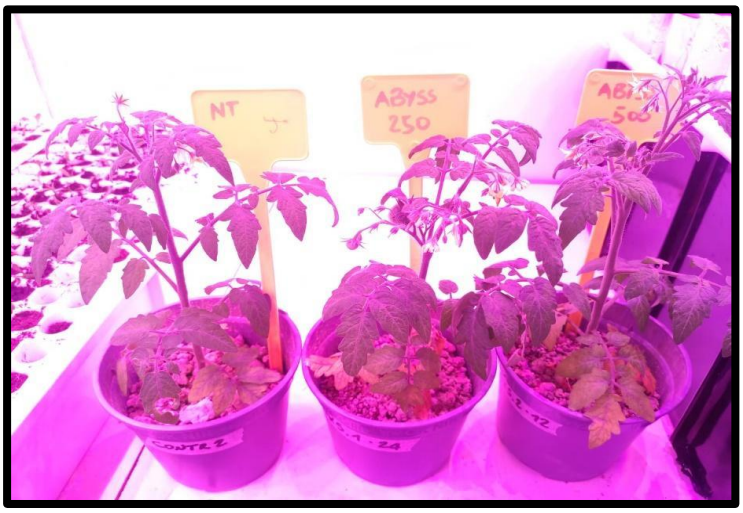

Figure 16: Effect of ANE treatment at full flowering stage in growth chambers.

The analysis of the transcriptome revealed a higher contribution of downregulation compared to upregulation in the overall differential expression of genes in leaves of treated plants compared to control ones. The same trend was recorded by Omidbakhshfard et al. (2020) 48 hours after spraying *Arabidopsis thaliana* plants with an ANE. Also, the GO terms "response to chitin" and "response to oxidative stress" were significantly enriched mainly in downregulated genes both in our experiment and in the previously described one in *Arabidopsis thaliana*.

In terms of regulation of gene expression, the numbers of DEGs after the second and third applications were higher than those recorded after the first ANE application. Even though the number of DEGs 24 and 48 hours after the first treatment was low, the physiological responses were already detected as an increased stomatal conductance in ANE-treated plants. Interestingly, among the DEGs at BBCH51 were some pathogenesis-related leaf proteins and a few endochitinases. As previously reported (Omidbakhshfard et al., 2020, Goni et al., 2016), the recognition of ANE by the plant can induce the differential expression of defence-related genes compared to untreated control plants.

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) seemed to induce a broader response in the plants in terms of the number of DEGs (Table 1). This may be due to the sample collection timing and possibly a lag in the induction of plant molecular responses that may occur when different doses of ANE are sprayed on the leaves. Nevertheless, the final effect in terms of increased leaf gas exchange and fruit yield was achieved with both the volumes tested.

The regulation of the stomatal opening and the modulation of photosynthesis 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 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 (Tombesi et al., 2021; Salvi et al., 2019) or as an opposite reduced stomatal conductance (Santaniello et al., 2017). In the work by Santaniello (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 dysregulation of some salicylic acid (SA)-dependent genes. In the 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). The upregulation of some SA-dependent genes as PR1 (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).

In our study, the higher fruit set and yields can be explained by the higher net photosynthesis detected in treated plants. Crops use photosynthesis to capture solar energy and accumulate nutrients, and plant productivity is directly related to the photosynthetic capacity of leaves. Currently, a limited number of reports have examined the effects of SWE on photosystem II and leaf gas exchange, especially on tomato plants. Nevertheless, the results of Yao et al., (2020) results indicate that SWE produced from *S. horneri* is effective in enhancing tomato yield, chlorophyll content, and photosynthesis capacity. A possible explanation for the increase in net photosynthesis caused by the biostimulant is the increase in leaf chlorophyll, as reported by Schiattone et al., (2018). Accordingly, Kumari and co-workers (2011) observed that the increase in vegetative growth could be due to an increase of photosynthetic pigments (chlorophyll a and carotenoids) in the leaves of SWE treated-tomato plants. On the other hand, Xu & Leskovar, (2015) have found that the inhibition of gas exchange and stomatal conductance induced by drought stress on spinach was reduced by A. *nodosum* extract, but had no effects on leaf chlorophyll content, fluorescence, and gas exchange under full irrigation.

Our 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 reaction and the dark phase of photosynthesis are significantly enriched and mainly downregulated upon treatment application at BBCH61 and upregulated at BBCH65.

Jannin et al. (2013) reported a higher number of downregulated than 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 (Fig. 10). At the same time, the physiological evaluation of the leaf gas exchange on the same plants was revealing a higher rate of stomatal conductance and net photosynthesis. The amount of CO2 that reaches the carboxylation sites can be modulated/altered by the activity of carbonic anhydrase (CA), which catalyzes the reversible hydration of CO2 to HCO3-. Given the overall increase in the net photosynthesis rates measured in treated plants and the parallel downregulation of genes

directly involved in the photosynthetic process, we can hypothesize that the untreated plants were undergoing faster senescence of the photosynthetic machinery and coping with sub-optimal artificial light to demand an increase in transcripts involved in the light reaction of photosynthesis.

The application of ANE significantly affected plants in terms of fruit setting and yield in all three different growing conditions. The total number of red fruits and fruit dry matter per plant were higher in treated plants, irrespective of the dose of biostimulant applied (Figs. 12, 14, and Table 7). In the field trial, treated plants showed a higher number of flowers, 35% increase in fresh fruit yield, and higher dried biomass. Our findings were in line with previous studies showing increased tomato yields following the application of sea-weed extracts (SWE) (Ali et al., 2016; Campobenedetto et al., 2021; Khan et al., 2009; Murtic et al., 2018; Mzibra et al., 2021; Yao et al., 2020; Zodape et al., 2011). In many crops, yield is proportional to the number of blooms at maturity. Because the beginning and development of flowering and the number of flowers produced are connected to the developmental stage of plants, ANE probably promotes flowering by initiating robust plant growth. Moreover, the beneficial effects of seaweed extracts on the reproductive parameters of tomato plants, such as the number of flowers and fruits per cluster, and induction of early flowering have been previously demonstrated (Ali et al., 2016; Shukla et al., 2019).

The modulation of key genes involved in flowering exerted by a seaweed extract on tomato reproductive organs described by Dookie et al. (2021) suggests an effect, either direct or indirect, of such treatments on the regulation of plant architectural development. In our experiment, the transcriptome of plants at the first open flower stage (BBCH61) was characterized by high fold enrichment values for the “flower development” biological process. Indeed, the genes FT (flowering time), CLAVATA, and SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors were upregulated 24 h after the application of 1l/ha dose of ANE. It has been demonstrated that SA has a flower-inducing activity, and SA accumulation can activate the expression of FT, while in SA-deficient plants low levels of FT transcripts are found (Martínez et al., 2004). Again, these observations are suggesting the implication of SA in the responses produced in tomato plants by ANE treatments.

Modern plant biology faces many challenges in establishing genotype-phenotype relationships. A shortage of phenotypic data for many physiological and developmental traits from many individuals is currently the greatest challenge (Furbank and Tester, 2011). In addition to representing the majority of important agronomically important traits, complex traits influence key biological processes that affect overall plant productivity and adaptability (Lynch and Walsh, 1998). A better understanding of these processes would lead to elucidating the mechanisms that contribute to important ecophysiological traits, which could lead to improved crop management decisions that maximize productivity and quality. To unravel and quantify the biostimulant activity of various products, phenotypic variables are crucially important.

Summarizing these results tomato plants grown in three different environmental conditions and treated with the ANE biostimulant showed an improved fruit yield that can be explained by the higher number of flowers and fruit set. Also, higher net photosynthesis and stomatal conductance were detected in all the growing conditions, at least after one treatment application. ANE applications recorded in the first and third flowering stages both resulted in a higher number of DEGs. Regardless of the plant phenological stage or dose of the product applied, a functional enrichment analysis of the overall set of DEGs revealed a significant contribution from pathways associated with photosynthesis and response to the stimulus. Further comparison of morpho-physiological and molecular data collected under laboratory conditions showed consistent results. Our study provides a rigorous and detailed method to highlight plant biostimulants' effects under different growing conditions, using the tomato as a model plant. Based on our findings, the combination of transcriptomics and phenomics approaches here evaluated could become a key system for dissecting the responses of plants to biostimulant treatments.

7. Conclusion

Transcriptomics and physiological analyses have provided a detailed description of the different modes of action. Exerted by the biostimulant effect compared in different growing conditions (controlled environment, Greenhouse& open field) with two different concentrations of biostimulants applications. treated with the ANE biostimulant showed an improved fruit yield by the higher number of flowers and fruit set. Also, higher net photosynthesis and stomatal conductance were detected in all the growing conditions, at least after one treatment application. ANE applications recorded in the first and third flowering stages both resulted in a higher number of DEGs. Regardless of the plant phenological stage or dose of the product applied, a functional enrichment analysis of the overall set of DEGs revealed a significant contribution from pathways associated with photosynthesis and response to the stimulus.

At the molecular level, the modulation of different gene categories both in terms of up-regulation and down-regulation by the biostimulant is compared in different growing conditions. Furthermore, there are some pathogenesis-related leaf proteins and a few endochitinases, pathogenic defence-related genes detected on plants treated with the biostimulant could explain the observed improved physiological parameters in plants subjected to ANE. Our study provides a rigorous and detailed method to highlight plant biostimulants' effects under different growing conditions, using the tomato as a model plant. Based on our findings, the combination of transcriptomics and phenomics approaches here evaluated could become a key system for dissecting the responses of plants to biostimulant treatments. 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.

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9. References

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Appendix- List of Figures