UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare
Ph.D. in Agro-Food System

Cycle XXXIV
S.S.D. AGR/02

Biostimulants at a crossroads: tools and techniques for the evaluation of emerging products

Candidate:

Giulia Antonucci

Matriculation n:

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Biostimulants at a crossroads: tools and techniques for the evaluation of emerging products

Coordinator:
Ch.mo Prof. Paolo Ajmone Marsan

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Tutor: **Stefano Amaducci**

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“If agriculture is founded upon life, upon the use of living energy to serve human life, and if its primary purpose must therefore be to preserve the integrity of the life cycle, then agricultural technology must be bound under the rule of life.
It must conform to natural processes and limits rather than to mechanical or economic models. The culture that sustains agriculture and that it sustains must form its consciousness and its aspiration upon the correct metaphor of the Wheel of Life. The appropriate agricultural technology would therefore be diverse; it would aspire to diversity; it would enable the diversification of economies, methods, and species to conform to the diverse kinds of land. (…)

We create and sustain environments where we can come back to ourselves, where we can return home, stand on solid ground, and be a true witness.”

bell hooks -

Earthbound: On Solid Ground

General Abstract

Drought management largely depends on timely, accurate and integrated information about its characteristics. Concurrently, biostimulants could represent a sustainable measure to foster the resilience of cropping systems under water-limited conditions. Drought-prone regions, where urbanisation and climate change are decreasing the stability of water availability, could benefit from the promise of PBs to increase drought tolerance and WUE. Nevertheless, scientific recognition of the potential of biostimulants has not grown as fast as the interest from industry. Therefore, there is an urgent need to investigate biostimulant action. This work seeks to explore different combinations of analytical techniques based on biostimulant effects on plants: dynamic in time, they elicit shared plant responses such as stress priming, better shoot and root growth, improved germination, bloom and fruit set, enhanced nutrient uptake and improved stress tolerance. This was achieved by a combination of greenhouse and open field trials, featuring the following high-throughput techniques chosen based on biostimulant expected and documented effects: continuous gas exchange acquisition, metabolomics and UAV imaging. Moreover, this thesis objective was to identify a viable statistical tool to properly analyse the generated high-throughput data: generalised additive modelling (GAM) was selected to achieve this. This resulted in insights on the fitness of continuous gas exchange acquisition and snapshot metabolomic profiling for detecting biostimulants effects in a crop at advanced phenological stages (flowering, in this case); innovative statistical analyses strategies (GAM) to evaluate dynamic biostimulant effects and the potential of PROSAIL retrieved biophysical parameters to model these effects. While PBs were demonstrated to be valuable tools in counteracting the effects of water stress through continuous gas exchange measurement and metabolomics in greenhouse conditions, it was not possible to transfer such findings to the open field via UAV imaging. This might be due to intrinsic characteristics of the biostimulant tested: for example, their activity threshold being below detectability in the field and their dose or stage of application not being optimal. On these grounds, future research endeavours shall concentrate on furthering the knowledge on biostimulant activity thresholds and biostimulant mechanisms of action to better identify the instruments to investigate biostimulant effects and the related acquisition timeframes. Overall, this study serves as a stepping stone to explore biostimulant evaluation techniques.

Content

Chapter I

Introduction

1.1. Drought stress in plants

Adaptation to climate change is becoming central to the conversation about water management for agriculture (Iglesias and Garrote, 2015). In this context, drought management represents an essential tool to achieve sustainable agriculture while minimising drought-related losses of crop plant productivity (Osmolovskaya et al., 2018). Plants experience two possible water stress timeframes in nature: slowly developing water shortage (within days to weeks or months) or short-term water deficits (hours to days): the two dynamics generate different results in terms of physiological response or adaptation (McDonald and Davies 1996). In the former case, plants apply one of two techniques: shortening their life cycle or acclimating to optimise their resources in the long term. In the latter case, the reaction of plants includes the minimisation of water losses, metabolic activation against dehydration and oxidative stress (Chaves et al., 2003).

The earliest response of plants to drought is represented by stomatal closure and reduced stomatal conductance, resulting in photosynthesis inhibition (Michaletti et al., 2018). This, in turn, leads to CO_2 uptake and concentration reduction (Medrano et al., 2002), causing hampered leaf growth (Chaves et al., 2003). Plant response to environmental stresses includes the activation of a highly-regulated cascade of signal transduction events, both gene and hormone (e.g. abscisic acid, salicylic acid) activated. This triggers the accumulation of reactive oxygen species (ROS) and antioxidant mechanisms, the production of defence-related metabolites and the related physiological and morphological changes, eventually leading to abiotic stress resistance (Chaves et al., 2003). Plant cellular metabolism is reconfigured based on this chain of molecular and cellular events, analogous to the response mechanisms elicited by biotic stress (Nephali et al., 2020).

In addition to stomatal closure and reduced stomatal conductance, typical responses to drought stress are root swelling to promote nutrient uptake, decreased chlorophyll content, leaf abscission and decreased leaf area (Chaves et al., 2003; Hussain et al., 2019). The decrease in chlorophyll content, in particular, is an indicator of severe stress: it is a consequence of extreme drought stress leading to the ultimate break down of chloroplast, which in turn allows for the proportion of nitrogen resources tied up in leaves (mostly in chloroplasts) to be redistributed elsewhere (Lawlor, 1993). In this sense, water stress is considered to accelerate leaf nitrogen and chlorophyll loss, therefore enhancing senescence, as observed in wheat (Yang et al., 2001). At a chloroplast level, ROS accumulation occurs when the plant cannot efficiently dissipate the excess energy due to water stress impairment of photosynthesis, photorespiration and thermal dissipation. These can cause oxidative damage to the photosynthetic apparatus (Apel & Hirt, 2004; Foyer, 2018). In order to contrast this phenomenon, the xanthophyll cycle plays a protective role in non-photochemical energy quenching (Zhao et al., 2017). Additionally, it has been demonstrated to play an active antioxidant action by enhancing the tolerance of thylakoid membranes to lipid peroxidation (Johnson et al., 2007).

In light of this, the crucial aspects of achieving plant tolerance to water stress are identical to those stated by Sobhanian and colleagues (2010) for salt stress:

(1) damage prohibition, namely the minimisation of damage

(2) reestablishment of homeostatic condition under stress,

(3) continuation of growth, maybe at a reduced rate.

1.2. Biostimulants

Among the suggestions to improve resiliency and adaptive capacity, the most cited approach is the introduction of drought-resistant crops. While this can be achieved through classical breeding and biotechnology, such efforts have so far produced little results (Nuccio et al., 2018). Moreover, in the second case, even when plants are successfully genetically modified (GM), resistant plants typically represent a restricted number of crops. In this sense, a technology applicable to multiple crops in multiple locations would represent a desirable alternative. Del Buono (2020) pointed out that biostimulants could represent a sustainable measure to foster the resilience of cropping systems under climate change-related environmental stress.

Indeed, biostimulants are increasingly used in agricultural production systems to optimise productivity (Yakhin et al., 2017). Attention for biostimulants has been on the rise both in academia and industry in the last two and a half decades (Crouch and van Staden, 1993; Herve, 1994; Zhang and Schmidt, 1999; Maini, 2006; Khan et al., 2009; Apone et al., 2010; Craigie, 2011; Sharma et al., 2014; Brown and Saa, 2015). Concerning academic interest, several reviews have been published in the last ten years to provide scientific evidence of the potential use of biostimulants (Calvo et al., 2014; du Jardin, 2015; Colla et al., 2017; van Oosten et al., 2017; Bulgari et al., 2019; Juárez-Maldonado et al., 2019; Pylak et al., 2019; Nephali et al., 2020; Cristofano et al., 2021).

At the European level, biostimulants have recently undergone the regulatory process for the first time, under the regulation of fertiliser products. The regulation includes a standard definition of what plant biostimulants (PB) are while at the same time stating the requirements for biostimulant commercialisation, thereby laying the foundation for the introduction of mandatory claim testing.

The new regulation (EU 2019/1009) has defined PBs (including microbial ones) as follows:

"an EU fertilising product the function of which is to stimulate 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:

nutrient use efficiency,

tolerance to abiotic stress,

quality traits, or

availability of confined nutrients in the soil or rhizosphere."

On the other hand, the U.S. have a legal definition of biostimulants but still do not have any specific regulations in place: the 2018 U.S. Farm Bill described biostimulants as "a substance or microorganism that, when applied to seeds, plants, or the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, or crop quality and yield" (US Congress 2018). The Environmental Protection Agency (EPA) of the U.S. has published a draft with the results of a public comment period opened with the current description, along with an updated comprehensive outline for what distinguishes biostimulants from plant growth regulators (US EPA 2020).

The recent EU definition emphasises PBs as distinguished and specified in their agricultural functions: they can be obtained from a wide range of raw materials having different bioactive substances and resulting in different functions (Rouphael and Colla, 2020). However, one of the main functions of these substances is to enhance plant capacity to resist abiotic stresses (Bulgari et al., 2019).

As mentioned earlier, PB use showed common responses independent of the plant species considered: better shoot and root growth, improved germination, bloom and fruit set, enhanced nutrient uptake and improved stress tolerance (Calvo et al., 2014; Wozniak et al., 2020). Moreover, PBs can act as plant priming agents, namely by activating plant defences and resistance against different environmental stresses, drought included (Fleming et al., 2019). Agronomically speaking, the most sought-after effect of PBs is the increase of crop production, measured by increased yield and improved harvest quality. A recent review by Wozniak and colleagues (2020) analysed more than 380 papers on treatments with PBs: about 59% of them were statistically significant, with an average yield increase of 29%, and about 41% were not statistically significant, with an average yield increase of 8%.

Drought prone regions in particular, where urbanisation and climate change are decreasing the stability of water availability, could benefit from the promise of PBs to increase drought tolerance and WUE (Van Oosten, 2017). Interestingly, Sible and colleagues (2021) further argue that thanks to the heightened awareness of both governments and the general public on agronomic practices and the influence of such practices on water quality and nutrient management, PBs can represent a viable solution even in the absence of measurable yield increases.

In this sense, osmotically active biostimulant molecules are particularly interesting. While it has been argued that osmolyte accumulation does not entail positive effects on yield under drought conditions (Serraj and Sinclair, 2002), many reports demonstrate the general positive effect of osmolyte accumulation (notably Blum, 2016). Osmotic adjustment has been observed to enhance soil moisture extraction and, therefore, transpiration (Blum, 2016). At the same time, higher-yielding plants are characterised by high stomatal conductance over time and higher transpiration (Blum, 2009).

1.2.1 Common use

Commercially available biostimulants are usually targeted to specialty crops (Neill and Morgan, 2021), mainly because of their higher return on investment than row crops and their heightened susceptibility to environmental stress (Kistner et al., 2018). However, biostimulant application has the potential to be extended to all crops susceptible to climate change-related losses: in Europe, for example, arable land totalled 98.8 million hectares while permanent crops accounted for 12 .2 million hectares (ha) in 2020. Therefore, arable land occupies 8.1 times more land than permanent crops. In this context, the total area under tomato crop production was 233'200 ha over 2.08 million ha total fresh vegetable area (Eurostat 2020). As pointed out by Sible and colleagues (2021), the main opportunity for application of PBs in crop row production is the joint application with scheduled established agronomic management practices, such as the pesticide application or planting stages. In this sense, biostimulant compatibility with these inputs must be checked. The most common application method, according to the review by Wozniak and colleagues (2020), is foliar spraying (60%), followed by application to the soil, roots or hydroponic media (30%) and seed treatment 10%. While the application method primarily depends on the nature of the biostimulant composition, a critical issue to the implementation of PBs is represented by identifying the optimal time of treatment, which also depends on the purpose of the application (Sible et al., 2021). Multiple parameters require consideration, among which the phenological stage of interest, climate conditions, and crop physiology are the most important. However, there is a considerable knowledge gap on the modes of action of biostimulants resulting from limited fundamental research. The efficient employment of PBs is conditional on acquiring such knowledge (Sible et al., 2021).

Up to now, PBs have been grouped either based on their beneficial effects on plants or based on their constituent class. Indeed, biostimulants are a vast class of products, including bioactive substances (humic acids, fulvic acids, protein hydrolysates and algal extracts) as well as microorganisms (plant growth-promoting bacteria and mycorrhizal fungi).

For this reason, the mode and mechanisms of PB action are still under investigation. Due to the vast range of molecules and possible combinations, and since the legislation requiring PB claims to be validated is recent and has yet to be enforced, there is no consensus over the best ways to test biostimulant products. For example, at the industrial level, the early stages of molecule selection are usually conducted through a "lab to field" pipeline by SMEs, while big industries tend to apply the opposite "field to lab" approach (Rouphael et al., 2018) due to lesser time and investment constraints. While the techniques substantially differ in their nature, the core approach suggested from available literature to test biostimulant action often include phenotyping, whether at field or lab level. Jindo and colleagues (2020) reported, for example, that most of the scientific publications available on humic substances feature hydroponic assays and growth chamber conditions (Nardi et al., 2000, 2018; Russell et al., 2006), while field and greenhouse experiments are less explored. This is mainly due to the variety of underlying factors in crop fields, including weather variability and climate fluctuations, soil type, and field management. Moreover, while considering lab techniques, transcriptomics and proteomics were more abundantly reported than metabolomics studies. Nevertheless, there is still significant variability in the efficacy of PBs and limited understanding of the underlying mechanisms responsible for it in field-tested scenarios where differences are expected to be observed (Sible et al., 2021).

The choice of technique to test PBs must be informed by the mode of action of PBs. At the same time, this influences the best application time, and the optimal dose. The combination of the two determines the range for which the crop can positively respond to biostimulant application (Toscano et al., 2018): too high or low concentrations can nullify the biostimulant effect (Vernieri et al., 2005).

While, at present, the scientific community is focusing on the mechanism of action of PBs, namely their effects on plant productivity, intended as the induction of photosynthesis, plant growth, uptake of nutrients and improved use of water (Shekhar et al.,2012; Yakhin et al., 2017), attention should be put in distinguishing between the mode of action and mechanisms of action (Del Buono et al., 2020). Further fundamental research on biostimulants using new technologies is needed to elucidate the mechanisms and mode of actions of PBs. Comprehensive reviews on the role of biostimulant substances are available for both biostimulant classes (Jindo et al., 2020; el Boukhari et al., 2020; Mannino et al., 2021) and biostimulant mode of action: among others, abiotic stress effectors (Van Oosten 2017) and potential effects on soil health indicators (Sible et al., 2021).

1.3. High-throughput technologies and their significance

PBs can act as plant priming agents and influence phenotypic traits by enhancing crop stress tolerance and nutrient uptake and assimilation (Rouphael et al., 2018). Literature sources are available where PB application under adverse environmental conditions resulted in modified leaf pigmentation, photosynthetic efficiency, leaf number and area, shoot and root biomass, as well as fruit number and mean weight (Ertani et al., 2013, 2014; Colla et al., 2015; Lucini et al., 2015, 2018; Rouphael et al., 2017).

Non-destructive phenotyping techniques characterised by high accuracy (high-throughput techniques) have gained popularity in the scientific community and have been successfully employed in plant breeding (Araus and Cairns, 2014; Halperin et al., 2017; Tardieu et al., 2017; Campbell et al., 2018; Mir et al., 2019), precision agriculture (Chawade et al., 2019), and biostimulant activity testing (Petrozza et al., 2014; Rouphael et al., 2018; Paul et al., 2019a,b). High-throughput phenotyping technologies have attracted attention for their potential in: (1) screening and monitoring multiple morpho-physiological traits; (2) time-series measurements, crucial in the acquisition and interpretation of high spatial and temporal resolution data; and (3) labour automation, time, and cost efficiency (Rouphael et al., 2018).

In recent years, the use of emerging digital technologies such as sensors, automatic image acquisition, and connected algorithms and models has increased, resulting in increasing volumes and complexity of data. Large-scale acquisition of data has allowed data interpretation to shift from a model-based to a data-based paradigm by improving the possibility to generalise the data acquired and consequently allowing for an increase in model accuracy. On the other hand, the main challenge lies in data management: the massive amount of data generated needs to be handled both at the acquisition and analysis stage through suitable, often custom, tools (Coppens et al., 2017).

Moreover, field phenotyping and remote sensing are undergoing a process of convergence. Formally, field phenotyping refers to a quantitative description of a plant's phenotype devoid of spatial effects, and remote sensing refers to the site-specific observation of vegetation by a remote device and the retrieval of its qualitative or quantitative properties. Therefore, traditionally, both disciplines concentrate on the interactions of plant growth with the surrounding environment with two different scopes: the first to generalise them, the second to explore site-specific interactions and describe spatially explicit traits (Machwitz et al., 2021). The adoption of hybrid instruments, such as radiative transfer models, often rooted in artificial intelligence, to estimate plant traits from remotely sensed data can be considered the link between generalisation and data spatialisation.

1.3.1 Precision agriculture: proximal and remote and sensing

Since its establishment in the 1980s, through the integration of the global positioning system (GPS), geographic information system (GIS) and remote sensing technologies, precision agriculture has revolutionised agricultural operations (Zhang et al. 2002), evolving into a tool for tactical monitoring of crops, ranging from regional to field-scale site-specific treatment (Huang et al., 2018).

In recent decades, precision agriculture has been successfully applied to crop growth and stress monitoring (Barbedo, 2019; Xie and Yang, 2020; Galieni et al., 2021). The use of unmanned aerial vehicles (UAVs) in particular has been on the rise: representing an economical and efficient method to meet the increasing requirements of spatial, temporal, and spectral resolutions (Yue et al., 2017; Zheng et al., 2018; Heinemann et al., 2020; Qiao et al., 2020), they can provide flexible high information resolution.

As of today, drought stress is mainly detected through thermal UAV: the most common drought stress signals in plants (i.e. temperature) are susceptible to detection by thermal camera technology, namely by exploiting the inverse linear relation between transpiration and surface temperature of leaves (Maes and Steppe, 2012; 2019).

Remote sensing represents a highly flexible and widely used instrument to assess various plant traits. Nevertheless, the assessment of complex traits such as identifying and quantifying abiotic and biotic stress is still regarded as challenging: there is a rising necessity to establish reliable retrieval techniques enabling the spatiotemporally explicit quantification biophysical variables. Among others, precision agriculture (Zhang and Kovacs, 2012; Tao et al., 2020), crop phenotyping, monitoring of crop traits (Domingues Franceschini et al., 2017; Jay et al., 2017) and the improvement of yield prediction (Cilia et al., 2014; Goffart et al., 2008) all rely on the possibility to quantitatively estimate bio-physical/-chemical crop parameters accurately (Roosjen et al., 2018). Remote sensing in particular, and spectroscopy data in general, are suited for the cost-effective estimation of biophysical plant traits through the dense information content contained in a few spectral bands, either narrow or broad (Verrelst et al., 2018).

While remote sensing approaches in the past have mainly involved hyperspectral cameras (Kanning et al., 2018; Duan et al., 2014; Kalisperakis et al., 2015; Li et al., 2015; Roosjen et al., 2018; Yue et al., 2018; Tao et al., 2020), crucial biophysical parameters such as leaf area index (LAI) have also successfully been retrieved through the use of multispectral cameras. Among multiple parameters that can be retrieved, LAI is of particular interest: a key canopy structural variable, it is used to model crop growth (Zhao et al., 2013¸ Potgieter et al., 2017), variability (Mueller et al., 2012), monitor crop growth (Duveiller et al., 2011), predict the crop yield (Geipel et al., 2014), estimate the amount of aboveground biomass (Yue et al., 2017), and evaluate the effects of field management (Baez-Gonzales et al., 2005). At the same time, chlorophyll content, defined either at the leaf level (leaf chlorophyll content, LCC) or at the canopy level (canopy chlorophyll content, CCC), is used as a bioindicator of vegetation state, crop productivity, health status and photosynthetic capacity (Gitelson et al., 2006; Clevers et al., 2011; Hoeppner et al., 2020; Mutanga et al., 2004; Wu et al. 2008; Houborg et al., 2007). These variables are also good proxies of the general health state of the crop, and the use of remote sensing allows to estimate variations in these physical parameters at a relatively low cost compared to field measurements (Mutanga et al., 2004; Pu et al., 2014; Zhang et al., 2008; Wong and He, 2013).

Remote sensing, thanks to its characteristics of flexibility, cost-effectiveness and customizability, is an untapped resource in characterising biostimulant activity and mode and mechanisms of action in field conditions.

1.3.2 Gas exchange analysis

Plants face the crucial dilemma 'lose water to fix carbon' (Chaves et al., 2003). When exposed to water stress, they can close stomata as a first measure to prevent excessive water loss. This entails the limitation of CO2 uptake into the chloroplasts, causing a decrease in photosynthesis and assimilation, reducing overall growth while the risk of photo-oxidative stress increases (Urban et al., 2017). Several research works have reported decreased gas exchange and water status under drought stress (Ali & Ashraf, 2001; Yan et al., 2016; Wang et al., 2018). Nevertheless, the extent of this decrease varied widely among the studies, making it difficult to compare the results. Yan and colleagues (2016) carried out a meta-analysis to characterise the effect of drought on gas exchange: they observed that drought-related decrease in the gas exchange and water status under mild drought is minor while under severe stress it is substantial. Therefore, regulating gas exchange parameters is essential to increase crop resistance to various biotic and abiotic stress conditions (Chaves et al., 2008). This results in gas exchange being an effective tool both to model plant response to drought and quantify PB efficiency in counteracting drought-related effects.

Related to this, Halperin et al. (2017) have proposed a platform that uses a custom algorithm to select genotypes based on their abiotic stress resistance characteristics. The system coupled with single-leaf gas exchange acquisition provides high-resolution whole plant transpiration, biomass gain, stomatal conductance, and root flux. Overall, research featuring gas exchange quantification to test biostimulant efficacy has recently been on the rise (Rouphael et al., 2017; Schiattone et al., 2018; Jalakas et al., 2019; Mateus-Cagua et al., 2019; Hamani et al., 2020; Mudo et al., 2020; Nazar et al., 2020), mostly at leaf level.

1.3.3 Metabolomics

Metabolomics is commonly defined as a holistic analysis of the metabolites (≤1500 Da in size) within a biological system under specific conditions (Sumner et al., 2003; Nephali et al., 2020).

The metabolome renders the effects of perturbations related to both genetic and environmental factors. For this reason, its application to PB effect analysis would enhance knowledge on both modes and mechanisms of action of PBs at a cellular and molecular level (Nephali et al., 2020). As pointed out by Teklić et al. (2020), in addition to investigating the effect of biostimulants on the plant stress response, there is a growing necessity to elucidate stress and interactions of biostimulants in terms of metabolic changes. To further explore the variation in physiological traits, the integration of phenotyping data and omics data represents the next frontier and a promising tool to bridge the phenotype-genotype gap (Coppens et al., 2017) and to understand dynamic plant stress responses in a changing environment (Gosa et al., 2019). Specifically, high-throughput phenotyping data combined with metabolomics have already been applied successfully to biostimulant testing (Lucini et al., 2015; Paul et al., 2019a,b; Rouphael et al., 2020). In metabolomics, two commonly used analytical strategies can be distinguished: untargeted and targeted. Untargeted metabolomics, aiming to detect as many metabolites as possible in a biological sample, is particularly suited to identifying metabolite abundance variations connected to either environmental stimuli or genetic variations (Cheng et al., 2018). Metabolomics has a recognised potential to provide significant insights into the mechanisms of the stress response (Shulaev et al., 2008; Arbona et al., 2013) by identifying different compounds, such as the molecules involved in stress acclimation (e.g., secondary metabolites) and by-products of stress metabolism, and has been successfully applied to the investigation of biostimulant action in general (Lucini et al., 2015, 2018; Paul et al., 2019a,b) and especially under abiotic stresses (Nephali et al., 2020).

1.3.4 Big data: handling volumes and analysis

The rapid development of digital and high throughput technologies for agriculture, increasing both data frequency and resolution, has generated a dramatic increase in data volumes, coupled with much higher complexity (Huang et al., 2018). According to Stubbs (2016), though, the term big data in agriculture is employed to describe the new combination of technology and advanced analytics employed to process information in a more useful and timely way rather than to define the size of data. Handling and combining this information in a useful way requires new instruments. Responding to the necessity to analyse the volumes and complexity of data produced through high-throughput phenotyping, non-linear regression models are emerging. These models need to capture dynamic data, often time series, as well as the interaction and effects between multiple factors (Ohana-Levi et al., 2020). Among these, generalised additive mixed models (GAMMs) have been successfully featured in several applied science fields (Murase et al., 2009; Zuur et al., 2009; Pedersen et al., 2019; Boswijk et al., 2020; Ohana-Levi et al., 2020) including ecology at large and plant ecophysiology. In particular, Ohana-Levi et al. (2020) highlighted the potential of GAMs to model non-linear relationships between evapotranspiration (ETc) drivers and evaluate their impacts on grapevine transpiration. GAMMs are regression models, which allow for the modelling of non-linear regressions (Wieling, 2018). Unlike linear models, which feature a sum of linear terms, GAMMs are characterised by a sum of smooth functions. Among the multiple advantages of this approach is the possibility to handle the complexity of the data without oversimplifying them, the determination of the relationship between the dependent variable and the predictors as a function of the algorithm (which can be linear but is not necessarily linear), the inclusion of multiple numeric predictors and the possibility to include autoregressive AR(1) error model for Gaussian models in order to handle the autocorrelation component of the error (van Rij et al., 2019).

1.4. Objectives of the thesis

The main objective of this thesis is to respond to the need to develop comprehensive, reliable and accurate technical instruments in order to test biostimulants, particularly under drought conditions. In order to do this, different combinations of techniques, both in greenhouse and in open field conditions, need to be investigated. Therefore, this results in the following research questions:

1. **Can high-throughput analytical procedures efficiently detect the effects of biostimulant application at later plant developmental stages? Which are more suited?**
2.

   Biostimulant effects are mainly investigated through screenings conducted at the early stages of plant development, especially those contrasting abiotic stresses. There is a need for testing and developing analytical pipelines to establish methods for later development stages.

3. **Which statistical instrument is the most fitting for the modelisation of dynamic biostimulant effects?**

Biostimulant effects are dynamic in time, especially when considering progressive abiotic stresses such as drought. Tailored statistical instruments are needed to modelise their effect taking into account the dynamic nature of their action.

4. **Are biostimulant effects detectable via UAV imaging?**

UAV imaging is being more and more widely used for stress detection and growth monitoring. Therefore, the question to be answered was whether it is suitable to detect biostimulant effects under abiotic stress conditions.

1.4.1 Outline and experimental approach

The chapters of this thesis are peer-reviewed publications or in phase of submission.

Chapter 2 examines the effects of a glycinebetaine (GB)-based biostimulant on gas exchanges and metabolite profile of greenhouse-grown tomato plants under drought stress. In this chapter, we carried out high-throughput gas exchange measurements which we then analysed via generalized additive modeling (GAMM). The effects of changes in the metabolite profile were described via unsupervised hierarchical cluster analysis, OPLS-DA and volcano analysis.

Chapter 3 explores the possibility of utilizing radiative transfer model (PROSAIL) inversion in order to model growth and health status parameters such as LAI, LCC and CCC for biostimulant treated processing tomato under three different levels of water stress in a field experiment. Field data were used in order to generate a dataset based on which PROSAIL was built. Afterwards PROSAIL was inverted based on hyperspectral data collected via UAV. Finally, resulting LAI, LCC and CCC were analysed via GAM.

Chapter 4 draws conclusions in relation to the research questions. Furthermore, this chapter discusses the implications of the results and provides an outlook for future research avenues.

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Chapter II

Integration of Gas Exchange With Metabolomics: High-Throughput Phenotyping Methods for Screening Biostimulant-Elicited Beneficial Responses to Short-Term Water Deficit

Antonucci Giulia[1]. Croci Michele[1], Miras-Moreno Begona, Fracasso Alessandra[1], Amaducci Stefano[1]

[1] Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore (UCSC), Piacenza, Italy

[2] Department for Sustainable Food Process, Research Centre for Nutrigenomics and Proteomics, Università Cattolica del Sacro Cuore, Piacenza, Italy

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Abstract

Biostimulants are emerging as a feasible tool for counteracting reduction in climate change-related yield and quality under water scarcity. As they are gaining attention, the necessity for accurately assessing phenotypic variables in their evaluation is emerging as a critical issue. In light of this, high-throughput phenotyping techniques have been more widely adopted. The main bottleneck of these techniques is represented by data management, which needs to be tailored to the complex, often multifactorial, data. This calls for the adoption of non-linear regression models capable of capturing dynamic data and also the interaction and effects between multiple factors. In this framework, a commercial glycinebetaine- (GB-) based biostimulant (Vegetal B60, ED&F Man) was tested and distributed at a rate of 6 kg/ha. Exogenous application of GB, a widely accumulated and documented stress adaptor molecule in plants, has been demonstrated to enhance the plant abiotic stress tolerance, including drought. Trials were conducted on tomato plants during the flowering stage in a greenhouse. The experiment was designed as a factorial combination of irrigation (water-stressed and well-watered) and biostimulant treatment (treated and control) and adopted a mixed phenotyping-omics approach. The efficacy of a continuous whole-canopy multichamber system coupled with generalized additive mixed modeling (GAMM) was evaluated to discriminate between water-stressed plants under the biostimulant treatment. Photosynthetic performance was evaluated by using GAMM, and was then correlated to metabolic profile. The results confirmed a higher photosynthetic efficiency of the treated plants, which is correlated to biostimulant-mediated drought tolerance. Furthermore, metabolomic analyses demonstrated the priming effect of the biostimulant for stress tolerance and detoxification and stabilization of photosynthetic machinery. In support of this, the overaccumulation of carotenoids was particularly relevant, given their photoprotective role in preventing the overexcitation of photosystem II. Metabolic profile and photosynthetic performance findings suggest an increased effective use of water (EUW) through the overaccumulation of lipids and leaf thickening. The positive effect of GB on water stress resistance could be attributed to both the delayed onset of stress and the elicitation of stress priming through the induction of H2O2-mediated antioxidant mechanisms. Overall, the mixed approach supported by a GAMM analysis could prove a valuable contribution to high-throughput biostimulant testing.

2.1 Introduction

Adaptation to climate change is becoming central to the conversation on water management for agriculture (Iglesias and Garrote, 2015). Among the suggestions to improve resilience and adaptive capacity, the most cited approach is the introduction of drought resistant crops. While this can be achieved through classical breeding and biotechnology, such efforts have so far produced little results (Nuccio et al., 2018). Moreover, in the second case, even when plants are successfully genetically modified (GM), resistant plants typically represent a restricted number of crops. In this sense, a technology applicable to multiple crops in multiple locations would represent a desirable alternative: as pointed out by Del Buono (2020), biostimulants could represent a sustainable measure to foster the resilience of cropping systems under water-limited conditions. The earliest response of plants to drought is represented by stomatal closure, which then results in the inhibition of photosynthesis (Michaletti et al., 2018) and therefore leads to CO_2 uptake and concentration reduction (Medrano et al., 2002). Multiple osmolytes can be found among biostimulant constituents targeting water stress resistance in plants, such as glycinebetaine (GB). GB not only acts as an osmoregulator, but also stabilizes the structures and activities of enzymes and protein complexes via detoxification of reactive oxygen species (Papageorgiou et al., 1991; Papageorgiou and Murata, 1995), while maintaining the integrity of membranes against the damaging effects of excessive salt (Mbarki et al, 2018; Tang et al., 2014, Tian et al., 2017, Yang and Lu, 2005a) , cold (Quan et al., 2004), heat (Allakhverdiev et al., 2007; Yang et al., 2006) and freezing (Razavi et al., 2018; Wang et al., 2019), as well as drought (Ma et al., 2006). The role of GB in plant resistance to abiotic stressed has been widely investigated and documented (Ashraaf and Foolad, 2007; Chen and Murata, 2011; Gorham, 1995; Huang et al., 2020; Sakamoto and Murata, 2002), ranging from exogenous application to genetic engineering (Fariduddin et al., 2013), to it biosynthesis and the underlying molecular mechanisms behind in planta accumulation under stress (Annunziata et al., 2019). While it has been argued that osmolyte accumulation does not entail positive effects on yield under drought conditions (Serraj and Sinclair, 2002), many reports demonstrate the positive effect of osmolyte accumulation generally (notably Blum, 2016) and GB specifically on plant growth and final yield. Among drought resistance mechanisms activated by GB, osmotic adjustment has been observed to enhance soil moisture extraction and, therefore, transpiration (Blum, 2016). At the same time, higher yielding plants are characterized by high stomatal conductance over time and higher transpiration (Blum, 2009). Indeed, Mäkelä and colleagues (1999) found that GB could enhance photosynthetic efficiency by reducing photorespiration and enhancing stomatal conductance in tomato plants grown under drought and salinity. This resulted in increased net photosynthesis of stressed plants. This is of particular relevance since tomato plants (*Solanum lycopersicum* L.) do not naturally accumulate GB (Wyn Jones and Storey, 1981). GB foliar application was also found to increase the yield of tomato plants under saline or heat stress (Mäkelä et al., 1998). Likewise, Agboma and colleagues (1997a, 1997b) found that exogenous application of

GB was indeed involved in the maintenance of a higher yield in maize and sorghum grains and tobacco leaves and resulted in improved water use efficiency. GB was found to have a positive effect on yield also in soybean (Agboma et al., 1997), common bean (Xing and Rajashekar, 1999), wheat (Agboma, 1997), sunflower (Hussain et al., 2008) and cotton (Ahmad et al., 2014). Moreover, Park and colleagues found that genetically engineered GB (2004) and exogenously applied GB (2006) increased tomato plants tolerance to chilling stress. Interestingly, they suggest that in addition to its macromolecule and membrane protecting action, GB-enhanced chilling tolerance might imply stress priming through the induction of H_2O_2-mediated antioxidant mechanisms.

According to Fleming and colleagues (2019), scientific recognition of the potential of biostimulants has not grown as fast as the interest from industry. This has been due to limited fundamental research of their modes of action and the speed at which new multi-compound products have entered the market. In the investigation of biostimulant activity, the necessity of accurate assessment of phenotypic variables is emerging as a critical issue (Rouphael et al., 2018) while at the same time the combined phenotypic-omic approach is gaining momentum. In recent years, emerging digital technologies such as sensors, automatic image acquisition and the connected algorithms and models have seen an increasing adoption, resulting in increasing volumes and complexity of data. The large-scale acquisition of data has allowed data interpretation to shift from a model-based to a data-based paradigm, by improving the possibility to generalize the data acquired and consequently allowing for an increase in model accuracy. On the flip side of the coin, the main challenge lies in data management: the huge amount of data generated needs to be handled both at acquisition and at analysis stage through proper, often custom, tools (Coppens et al., 2017). In this context, non-destructive phenotyping techniques characterized by high accuracy (high-throughput techniques) have gained popularity in the scientific community and have been successfully employed in plant breeding (Araus and Cairns, 2014; Halperin et al., 2016; Tardieu et al., 2017, Campbell et al., 2018; Mir et al, 2019), precision agriculture (Chawade et al., 2019), and biostimulant activity testing (Petrozza et al., 2014; Rouphael et al., 2018; Paul et al., 2019a, 2019b). High-throughput phenotyping technologies have attracted attention for their potential in:1) screening and monitoring multiple morpho-physiological traits; 2) time-series measurements, crucial in the acquisition and interpretation of high spatial and temporal resolution data; 3) labor automation, time and costs efficiency (Rouphael et al., 2018). Halperin and colleagues (2016) have proposed a platform, which uses a custom algorithm, to select genotypes based on their abiotic stress resistance characteristics. The system provides high resolution whole-plant transpiration, biomass gain, stomatal conductance, and root flux, coupled with single-leaf gas exchange acquisition. As pointed out by Teklic et al. (2020), in addition to the investigation of the effect of biostimulants on plant stress response, there is a growing necessity to elucidate stress and biostimulants interactions in terms of metabolic changes. To further explore the variation in physiological traits, the integration of phenotyping data and -omics data represents the next frontier and a promising tool to bridge the phenotype-genotype gap (Coppens et al., 2017) and to understand dynamic plant stress responses in a changing environment (Gosa et al., 2019). Specifically, high-throughput phenotyping data in combination with metabolomics have already been successfully applied to biostimulant testing (Lucini et al., 2015; Paul et al., 2019a, 2019b; Rouphael et al., 2020). In metabolomics, two commonly used analytical strategies can be distinguished: untargeted and targeted strategies. Untargeted metabolomics, by aiming to detect as many metabolites as possible in a biological sample, is particularly suited to identifying metabolite abundance variations connected to either environmental stimuli or genetic variations (Cheng et al, 2018). Metabolomics has a recognized potential to provide significant insight in the mechanisms of stress response (Shulaev et al., 2008, Arbona et al., 2013) by identifying different compounds, such as molecules involved in stress acclimatation (e.g. secondary metabolites) and by-products of stress metabolism and has been successfully applied to the investigation of biostimulant action in general (Lucini et al., 2015; Lucini et al., 2018; Paul et al. 2019a, 2019b) and especially under abiotic stresses (Nephali et al., 2020). Lastly, responding to the necessity to analyze the volumes and complexity of data produced through high-throughput phenotyping, non-linear regression models are emerging. These models need to be capable of capturing dynamic data, often time series, as well as the interaction and effects between multiple factors (Ohana-Levi et al., 2020). Among these, generalized additive mixed models (GAMMs) have been successfully featured in several applied sciences fields (Murase et al., 2009; Zuur et al., 2009; Pedersen et al., 2019; Boswijk et al., 2020; Ohana-Levi et al., 2020) including ecology at large and plant ecophysiology. In particular, Ohana and colleagues (2020) highlighted the potential of GAMs to model non-linear relationships between evapotranspiration (ETc) drivers and evalutating their impacts on grapevine transpiration.

In light of this, GB is a particularly fit biostimulant molecule to test the suitability and accuracy of GAMMs in modeling dynamic plant response to drought: widely investigated, its protective action on the photosystem is documented for several abiotic stresses (Huang et al., 2020) . At the same time, while available research concentrates mostly on the effect of GB application on yield traits and cell-level effects (Annunziata et al., 2019), correlation between GB treatment and modifications in photosynthetic rates has received little attention: published research mainly concerned enhanced photosynthetic performance under salt stress (Yang and Lu, 2015; Hamani et al., 2020) and drought (Nawaz and Wang, 2020). Likewise, the effects of GB on water use efficiency (WUE) have been scarcely investigated (Ahmed et al., 2019) and although both its abundance (natural or GM) in plant tissues and its exogenous application have been widely linked to stress response, the effects of exogenous application of GB on the metabolomic profile of leaves are yet to be investigated.

Based on current literature, the hypothesis tested here was whether a biostimulant (GB-based) treatment can be efficiently modeled through GAMM and whether the treatment would result in tangibly different metabolite expression profiles, thereby completing the information derived from GAMM. In order to achieve this, the duration and dynamics of the effect on photosynthesis, transpiration and WUE were investigated, jointly with the snapshot analysis of metabolic profile.

2.2 Material and Methods

2.2.1 Plant material and growing conditions

The experiments were conducted in a greenhouse and the metabolomics analysis in the laboratory of the Department of Sustainable Crop Production of the Università Cattolica del Sacro Cuore, Piacenza. Tomato seeds (H1281 variety, Heinz) were directly sown in a greenhouse at 20°C at 2 cm in a seeding tray (35 ml cell plug) containing a commercial complete mixture of sand – blonde peat – humus. Seeds were kept in dark conditions for 5 days until germination, thereafter they were provided with a PPFD of 800 µmol m^{-2} s^{-1} through LED lighting. 7 days after emergence (DAE), 50 uniformly developed seedlings, at second true leaf unfolded (BBCH 12), were transplanted in 2.3 L pots filled with a commercial complete mixture of sand – blonde peat – humus (1000 g ±1 g). Plants were fertilized at transplant with 40 ml of a complete commercial solution (COMPO, Concime Liquido Universale) at 1.05% w/v (7% N; 5% P; 6% K; 0,01% B; 0,004% Cu; 0.04% Fe; 0,02% Mn; 0,001% Mo; 0,002% Zn). Fertilization was provided every two weeks.
The pots were placed under LED lamps to provide a PPFD of 800 µmol m^{-2} s^{-1} to the top canopy with a photoperiod of 16 h light and 8 hours dark. Pots were watered to field capacity every second day. Temperature was not controlled and ranged between 35°C during the day and 8°C during the night (19.5 °C average). Of the 50 plants, 32 homogeneously developed plants were selected, of which 12 plants were destined to gas exchange analysis and 20 to metabolomics analysis, while the rest was discarded.

The experiment was designed as a factorial combination of irrigation (water stressed and well-watered) and biostimulant treatment (treated and control). The water stressed plants were allowed to dry down for three consecutive days by withdrawal of irrigation. Thereafter, all plants were irrigated. Gas exchange analysis was carried out on three replicates for each treatment, for a total of 12 plants, while metabolomics analyses were carried out on five replicates per each treatment, totaling 20 plants. Plants dedicated to gas exchange analyses were kept in the gas exchange acquisition system for nine days until the end of the experiment (3 initial days to adapt to the ventilation and lighting, followed by dry down) while plants dedicated to metabolomics analysis were kept in the greenhouse under a tunnel replicating the conditions of the gas exchange acquisition system (same air inlet and LED lighting).

2.2.2 Biostimulant characteristics and treatment

The day before the beginning of the gas-exchange analysis, the pots were irrigated to saturation and allowed to drain overnight. The treatment with the biostimulant was carried out on 36 DAE (Day After Emergence) on half of the plant while the other half was sprayed with distilled water. Biostimulant was sprayed at a rate of 6 kg/ha, with a dilution of 300 L/ha to a total of 10 g/plant. Dose and dilution were chosen based on commercial use of the product (Vegetal B60®, ED&F MAN Liquid Products Italia). Vegetal B60® is an organic product extracted exclusively from sugar beet without any added chemical additives. It contains 30% of GB and 5 % L – amino acids, 5% total organic nitrogen and 12% organic carbon.

All pots were sealed in plastic bags fitted around the base of each plant stem to prevent soil evaporation. Well-watered plants were kept at 80% field capacity (FC) throughout the experiment, while for water stressed plants irrigation was interrupted 3 days after the treatment (on 39 DAE). 200 g of water were reintegrated to WS plants on 41 DAE, 2 days after irrigation was stopped, to contrast the high rate of soil drying and allow for a longer dry-down period and a more gradual onset of drought stress (a transpiration exceeding 200 g d^{-1} would have brought the plant at wilting point on DAE 41). Leaf area (LA) of each plant was calculated every 3 days from the start of stress imposition (39 DAE). Leaves were placed on squared paper (square of side 0.5 cm, used as reference of known size) reinforced with a rigid base and photographed with a phone camera, taking care that no leaves overlapped. Images were then processed using ImageJ (Schneider et al., 2012) to extract leaf area and compute total leaf area of each plant.

2.2.3 Gas exchange analysis

Gas exchange was evaluated through a semi-automated multi-chamber whole canopy system (slightly modified from what previously described in Fracasso et al., 2017). In particular, the system computes net photosynthetic rate (*Pn*), transpiration rate (E) and water use efficiency (WUE). Every pot was measured every 12 minutes for a total of 120 measurements per day. *Pn* (µmol s^{-1} m^{-2}) and E (mmol s^{-1} m^{-2}) were calculated based on flow rates and CO_2 and water vapor differentials using the formula provided in Long and Hällgren (1985). Data were acquired 24 h per day at intervals of 1 minute. The semi-automated multi-chamber system is composed of twelve adjustable open

chambers connected to a CIRAS-DC double channel absolute CO_2/H_2O infrared gas analyser (PP-System, Amesbury, MA). Air drawn at 3 m above ground from outside the greenhouse is collected in a buffer tank (0.44 m3 capacity) to ensure the stability of inlet CO_2 concentration and then, forced by one centrifugal blower (Vorticent C25/2M Vortice, Milan, Italy), it is distributed to the chambers through 50 mm diameter rigid plastic pipes. The air flow rate of each chamber is controlled by a baffle and is constantly measured at least 50 cm downstream of the baffle itself with digital flowmeters (50 Pa D6F-PH0026AD1, OMRON, Japan) according to a flow-restriction method (Osborne, 1977). Each chamber is connected to a set of 12 solenoid valves in series (model 177 B04/Z610, Sirai, Bussero, Italy) through a sampling tube (Ø 10mm). Air sampling is switched from one chamber to another at programmed time intervals (Raspberry Pi B+, Raspberry Pi Foundation, United Kingdom).
In order to ensure air flushing inside the sampling tubes and the complete air exchange inside CIRAS-DC, rotary vane pumps (model G 6/01-K-LCL; Gardner, Denver Thomas, Pucheim, Germany) with 33.3 cm^3 s^{-1} of flow rate were added before CIRAS-DC. Both the inlet air temperature and the air temperature of each chamber (outlet) were measured by digital temperature sensors (Dallas DS18B20, Dallas Semiconductor Corp., Dallas, USA).

On 36 DAE 12 plants were transferred under the gas exchange acquisition system and were kept in the system for 9 days until the end of the experiment (as mentioned above), while 20 plants were kept under a tunnel replicating the conditions of the gas exchange acquisition system and were sampled for metabolomics analyses. Both the tunnel and the gas acquisition system were provided with the same air inlet, namely forced air collected from outside (as described above), and the same LED lighting, namely 800 µmol m^{-2} s^{-1} PPFD.

2.2.4 Sample Harvest and Metabolomic Analysis

Gas exchange was measured throughout the duration of the stress: as soon as the *Pn* of stressed plants started decreasing, plants were sampled for metabolomics. Sampling of the 20 plants kept under the tunnel replicating the conditions of the gas acquisition system was carried out on 42 DAE. The second and third fully expanded leaves from the top of each plant were excised and dipped into liquid nitrogen. Samples were kept at -20°C and subsequently analysed.
Plant samples were homogenized in pestle and mortar using liquid nitrogen and extracted as previously reported (Paul et al., 2019b). Briefly, an aliquot (1.0 g) was extracted in 10 ml of 0.1% HCOOH in 80% aqueous methanol using an Ultra-Turrax (Ika T-25, Staufen, Germany). The extracts were centrifuged (12,000 × g) and filtered into amber vials through a 0.22-µm cellulose membrane for analysis. Thereafter, metabolomic analysis was carried out through ultra-high

performance liquid chromatograph (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS). The metabolomic facility included a 1290 ultra-high-performance liquid chromatograph, a G6550 iFunnel Q-TOF mass spectrometer, and a JetStream Dual Electrospray ionization source (all from Agilent Technologies, Santa Clara, CA, United States). The untargeted analysis was carried out as previously described (Rouphael et al., 2016). Briefly, reverse-phase chromatography was carried out on an Agilent PFP column (2.0 x 100 mm, 3 μm) and using a 33-min linear elution gradient (6% to 94% acetonitrile water, with a flow of 200 μL min−1 at 35°C). The mass spectrometric acquisition was done in SCAN (100–1,000 m/z) and positive polarity (Pretali et al., 2016). Quality controls (QCs) were analyzed in data-dependent MS/MS mode using 12 precursors per cycle (1 Hz, 50–1200 m/z, positive polarity, active exclusion after 2 spectra), with collision energies of 10, 20 and 40 eV for collision-induced decomposition.

Raw spectral data were processed as described by Miras-Moreno et al. (2020) using Agilent Profinder B.07 software, (Santa Clara, CA, USA). The PlantCyc 12.6 database (Plant Metabolic Network; Release: April 2018) were used to putatively annotate compounds according to Level 2 with reference to the COSMOS Metabolomics Standards Initiative (Salek et al., 2015). Quality controls (QCs) were analyzed by using the MS-DIAL 3.98 (RIKEN Center for Sustainable Resource Science: Metabolome Informatics Research, Yokohama, Japan) to compare the MS/MS spectra to the publicly available MS/MS experimental spectra built in the software (e.g., MoNA) (Tsugawa et al., 2015).

2.2.5 Data analysis

2.2.5.1 Gas exchanges curve fitting using GAM(M)

The experiment was evaluated from the day prior to stress imposition (38 DAE) for three consecutive days of water stress (DAE 39-41) until the start of stress recovery (42 DAE). Statistical analyses on gas exchange data were carried out via Generalized Additive Mixed Models (GAMMs). GAMMs are regression models which allow for modelling non-linear regressions (Wieling, 2018). Unlike linear models (1), which feature a sum of linear terms, GAMMs (2) are characterized by a sum of smooth functions. GAMMs are used to estimate smooth functional relationships between predictor variables and the response. GAMM data fitting is characterized by a penalized fit: the fit of the data is balanced with the complexity of the model by penalizing wiggliness, namely the deviation from total smoothness, and thus avoiding overfitting (Pedersen et al., 2019).

1) $Y_{ij} \quad \beta_0 + \sum \beta_j x_i + \epsilon_{ij}$

2) $Y_{ij} \quad \beta_0 + \sum f_j(x_i) + \epsilon_{ij}$

Among the multiple advantages of this approach are the possibility to handle the complexity of the data without oversimplifying them, the determination of the relationship between the dependent variable and the predictors as a function of the algorithm- which can be but is not necessarily linear-, the inclusion of multiple numeric predictors and the possibility to include autoregressive AR(1) error model for Gaussian models in order to handle the autocorrelation component of the error (van Rij et al., 2019). The inclusion of autocorrelation is particularly relevant in time series datasets, where each datapoint is clearly correlated to (and therefore dependent on) the previous and the next datapoints. Therefore, this analysis is particularly useful to datasets characterized by dynamic and time-series data (Boswijk et al., 2020).

After Wieling (2018), the analysis started from a simple generalized additive model, the sophistication of which was progressively increased and extended step-by-step. While one would normally directly choose the model reflecting the hypothesis, this approach was chosen to shed light on the process using generalized additive modeling.

Net photosynthesis (*Pn*), transpiration rate (E) and WUE were used as the response variable for the following series of GAM(M)s:

a. Sole irrigation as a fixed factor, including a smooth for day-of-treatment and hour in the day, based on treatment.

b. Irrigation treatment and biostimulant treatment as fixed factors, no interaction between the factors. Including a smooth for day-of-treatment and hour in the day, based on both treatments.

c. Irrigation treatment and biostimulant treatment as fixed factors, interaction between the factors. Including a smooth for day-of-treatment and hour in the day, based on both treatments.

d. Introduction of tensor product-based smooths between hour and day.

e. Inclusion of a smooth for day-of-treatment and hour in the day, based on both treatments, and allowing for random effect per individual.

f. Addition of non-linear random effects per individual for day-of-treatment.

g. Introduction of individual-based autocorrelation.

Models d:g are nested within model c, allowing for comparison of methods using log-likelihood/F tests. This allowed us to evaluate AIC changes among models and, thus, to find the model with the most explanatory power given the degrees of freedom, while at the same time assessing whether better or worse models explained significantly different amounts of the deviance in the data.Statistical tests were performed using the software R version 4.0.2 (R Development Core Team).

GAMM models were fitted in R using a cubic regression spline smoother, with the package itsadug (van Rij J et al.,2020). Mixed model selection, fitting and validation followed Zuur et al. (2009). Biostimulant and stress treatment factors were used in mixed linear models for hypothesis testing.

2.2.5.2 Chemometric Interpretation of Metabolites

Chemometric interpretation of metabolites was performed using Mass Profiler Professional B.12.06 from Agilent (Santa Clara, CA, USA) as previously described (Corrado et al., 2020). The unsupervised hierarchical cluster analysis (HCA - distance measure: Euclidean; clustering algorithm: Ward's) was produced based on the normalized molecular features. The supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out with SIMCA 16 (Umetrics, Sweden) at default parameters. CV-ANOVA ($p < 0.01$) and permutation testing ($n = 100$) were also applied to validate and to exclude overfitting. Goodness-of-fit R2Y and goodness-of-prediction Q2Y were also calculated from the OPLS-DA model. Outliers were investigated according to Hotelling's T2 (95% and 99% confidence limit for suspect and strong outliers, respectively). The most discriminant compounds in separation were selected by performing the variables importance in projection (VIP) analysis.

Thereafter, differential compounds were investigated through Volcano plot analysis, combining fold-change (FC > 2) and ANOVA (p < 0.0,1, Bonferroni multiple testing correction) and were then uploaded into the Omic Viewer Pathway Tool of PlantCyc (Stanford, CA, USA) to identify pathways affected by the treatments as in Caspi and colleagues (2013).

2.3 Results

2.3.1 Gas exchange analysis

On 36 DAE 12 plants were transferred under the gas exchange acquisition system and were kept in the system for 9 days until the end of the experiment. Plants were treated with a factorial combination of irrigation (well-watered and water stressed) and biostimulant treatment (treated and control). Water stressed plants were allowed to dry down for three consecutive days by withdrawal of irrigation. Gas exchange data were processed via Generalized Additive Mixed Models (GAMMs). GAMMs are non-linear regression models which are characterized by a sum of smooth functions. Data were analysed by building models of growing complexity (a-g, Table 2.1), as previously explained. The regression models allowed to explore the dynamics of the influence of the biostimulant and water treatments on photosynthetic performance.

Among the candidate GAMMs, the optimal model was model g, including Photosynthesis ~ irrigation treatment * biostimulant treatment and nonlinear interactions between both treatments and the duration of the experiment (expressed in days) and the time of the day (expressed in hours), smooths for duration of the experiment and the time of the day on both treatments and non-linear variability of the individuals over the duration of the experiment. Moreover, model g included autocorrelation for time over individual. The model explained 87.8% of the total deviance with an Adj.R^2 of 0.872. While the next best model, which did not include autocorrelation (model f), explained 89% of the deviance (Adj.R^2 0.883), when the models were confronted via Akaike information criterion (AIC) (Akaike, 1974), model g scored consistently lower in terms of AIC score although bearing more degrees of freedom (Table 1).

Table 2.1. Characteristics of the nine compared models of influence on photosynthesis.

Model	Resid. Df	Resid. Dev	AIC	dAIC	Dev. Expl (%)
a	3391.454	11624.1257	13974.67	5006.285	61.5
b	3378.586	10206.6696	13552.31	4583.922	66.2
c	3377.564	9648.388	13361.94	4393.553	68
d	3265.717	7618.79372	12724.36	3755.977	74.7
e	3231.964	4454.29886	10950.84	1982.455	85.2
f	3182.686	3307.85823	10024.21	1055.83	89
g	3214.175	3679.9617	8968.384	0	87.8

Model names as in the text: a has the sole irrigation as a fixed factor, including a smooth for day-of-treatment and hour in the day, based on treatment, b has both irrigation and biostimulant treatment as fixed factors, c adds the interaction between the factors and includes a smooth for day-of-treatment and hour in the day, based on both treatments. d introduces tensor product-based smooths between hour and day. e includes a smooth for day-of-treatment and hour in the day, based on both treatments, and allowing for random effect per individual. f adds non-linear random effects per individual for day-of-treatment. g introduces individual-based autocorrelation. Resid.Df = residual degrees of freedom. Resid.Dev = residual deviance. dAIC = difference in AIC based on model g. Dev. Expl. (%) = deviance explained.

The inclusion of the sole irrigation treatment factor (model a) explained 61.5% of the deviance in the data. The addition of the biostimulant treatment factor (model b) increased the deviance explained by 4.7%. The addition of further information increased the deviance explained as follows: the inclusion of the interaction between the fixed factors (model c) resulted in an additional 1.8%. The further introduction of tensor product-based smooths between hour and day (model d) added 6.7% deviance explained, while allowing for random effect per individual (model e) further added 10.5% to it. Lastly, the addition of non-linear random effects per individual for day-of-treatment (model f) resulted in 1.8% more deviance explained.

Under water stressed conditions, model g highlighted statistically higher Pn (Fig. 1.1) in plants treated with biostimulant compared to control plants starting shortly before 39 DAE and lasting all throughout the experiment until 42 DAE. Conversely under well-watered conditions there was no significant difference between treatments. When considering biostimulant treated plants, the difference between water stressed and well-watered plants was significantly lower for water stressed plants starting half 40 DAE to half 41 DAE. Lastly, well-watered and water stressed untreated plants displayed a significant negative difference from mid-39 DAE to 42 DAE.

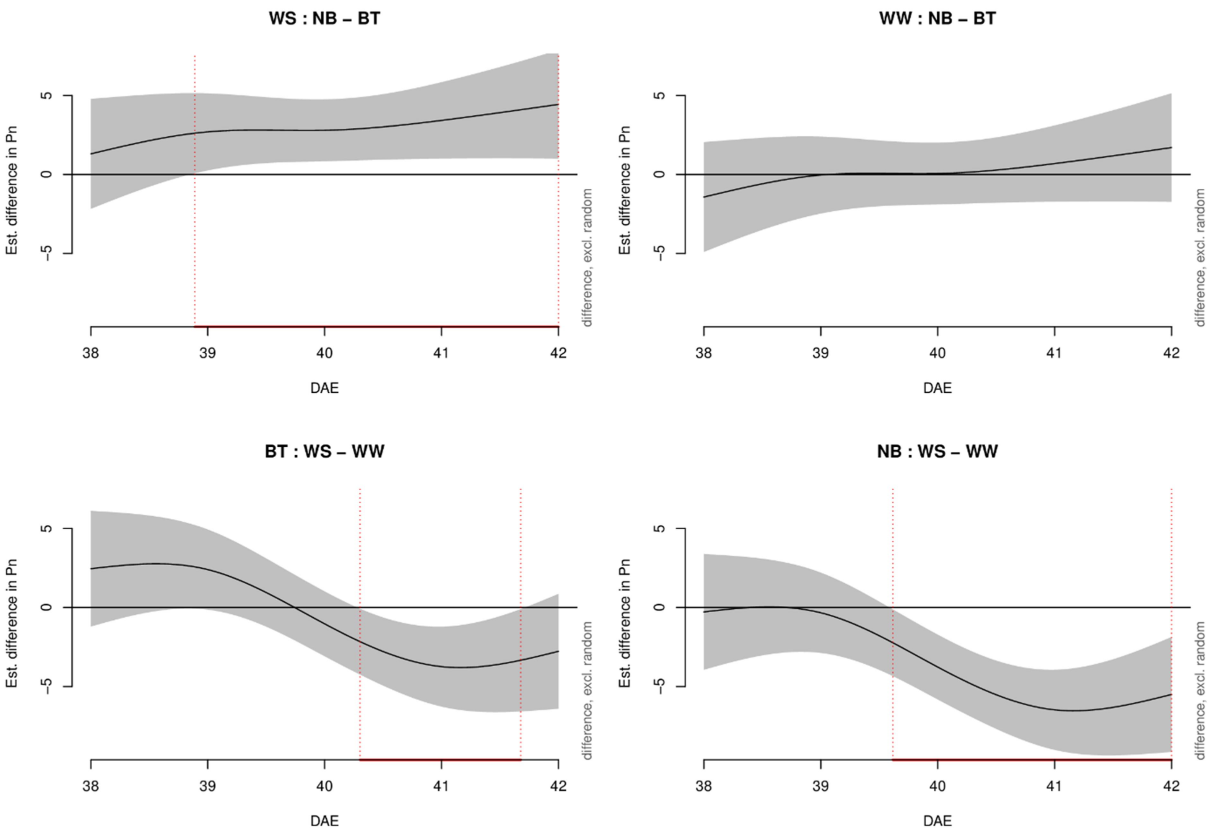


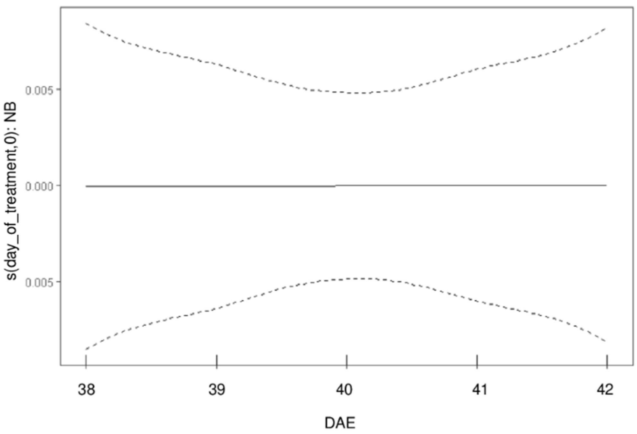
Figure 2.1. Graphs showing differences in *Pn* according to model g among all combination of treatments (irrigation and biostimulant). Random effects are excluded. The baseline above is represented by untreated plants, below by watered (control) plants. The pointwise 95%-confidence interval is shown by a shaded band. When the shaded confidence band does not overlap with the x-axis (i.e. the value is significantly different from zero), this is indicated by a red line on the x-axis (and vertical dotted lines). Upper left (A): water stressed, comparation between no biostimulant and biostimulant treatment. Upper right (B): well-watered, comparation between no biostimulant and biostimulant treatment. Lower left (C): biostimulant treated, comparation between water stressed and well-watered. Lower right (D): control treated, comparation between water stressed and well-watered.

The influence of day of treatment (38-42 DAE), starting from the day prior to stress imposition, and hour were evaluated separately for each treatment (biostimulant and water treatment). Through the use of GAMMs, the significance of the effect of day and hour on biostimulant and water treatment was further investigated singularly for each dimension of both factors (biostimulant treated/control treated and water stressed/well-watered) (Table 2.2). The results show that all the partial correlations were statistically significant for at least one of the two levels of the factors at the *P*-value < 0.001 level, except for the effect of hour and day of week on biostimulant treatment, which was significant at *P*-value < 0.05. While the significance of the smooth terms does not provide information on whether the patterns are statistically significant, this suggests that each individual variable has a statistically significant influence on modeling the wiggliness of *Pn*, which in turns confirms the distance of Pn from a linear function.

Table 2.2. Influence of partial effects on *Pn* using GAMM multivariate analysis.

Partial effect	edf	F	p-value	
te(day_of_treatment,hour):WellWatered	1.819e+01	0.481	0.000509	***
te(day_of_treatment,hour):WaterStressed	3.084e+01	5.554	<2e-16	***
te(day_of_treatment,hour):NoBiostimulant	6.220e+00	0.219	0.000288	***
te(day_of_treatment,hour):BiostimulantTreated	2.847e+01	1.636	<2e-16	***
s(day_of_treatment): WellWatered	1.000e+00	2.872	0.090207	.
s(day_of_treatment): WaterStressed	3.803e+00	17.018	8.28e-12	***
s(day_of_treatment): NoBiostimulant	9.612e-05	0.666	0.993127	
s(day_of_treatment): BiostimulantTreated	3.415e+00	3.049	0.010673	*
s(hour): WellWatered	1.632e+01	11.215	<2e-16	***
s(hour): WaterStressed	6.051e+00	3.626	0.000573	***
s(hour): NoBiostimulant	1.000e+00	1.258	0.262130	
s(hour): BiostimulantTreated	5.880e+00	2.523	0.012176	*
s(day_of_treatment,ID_plant)	3.805e+01	26.618	<2e-16	***

Of particular interest is the partial effect (referred solely to biostimulant treatment) of biostimulant treatment on *Pn* observed for each day of treatment (Fig. 2.2): while no effect can be detected for the control treatment (which is represented by a linear function), the biostimulant treatment has a detectable effect on the wiggliness of the *Pn* curve.



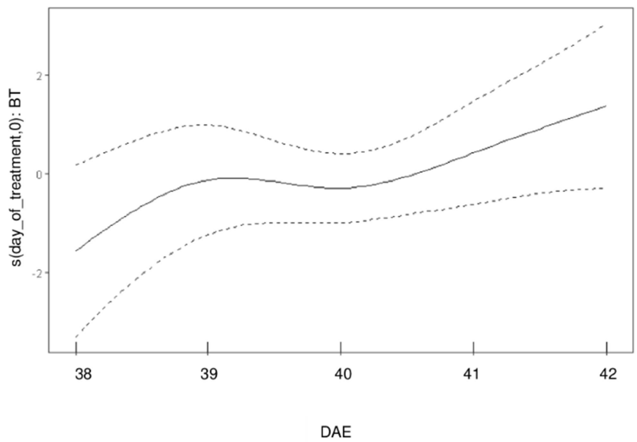


Figure 2.2. Uncoupling of the partial effects of biostimulant treatment/control treatment on the wiggliness of P*n* curve. Above (A): partial effects of control treatment (no biostimulant) over the day of treatment (in DAE) on P*n*. Right (B): partial effects of biostimulant treatment over the day of treatment (in DAE) on Pn.

Transpiration (E, mmol m^{-2} s^{-1}) was evaluated through increasingly complex models, as *Pn*. Model g was once more the best fitting model, with 88.2% of deviance explained and 0.875 Adj.R^2. As for *Pn*, water stressed biostimulant treated plants performed significantly better than untreated plants (Fig. 2.3). In particular, the performance of biostimulant treated plants was significantly higher from mid-38 DAE to mid-40 DAE. Conversely, non-stressed plants (biostimulant treated/untreated) did not show any significant difference in transpiration. Water stressed biostimulant treated plants had a higher E than well-watered plants between mid-41 DAE until 42 DAE. Untreated plants differed from late 39 DAE throughout the end of the experiment.

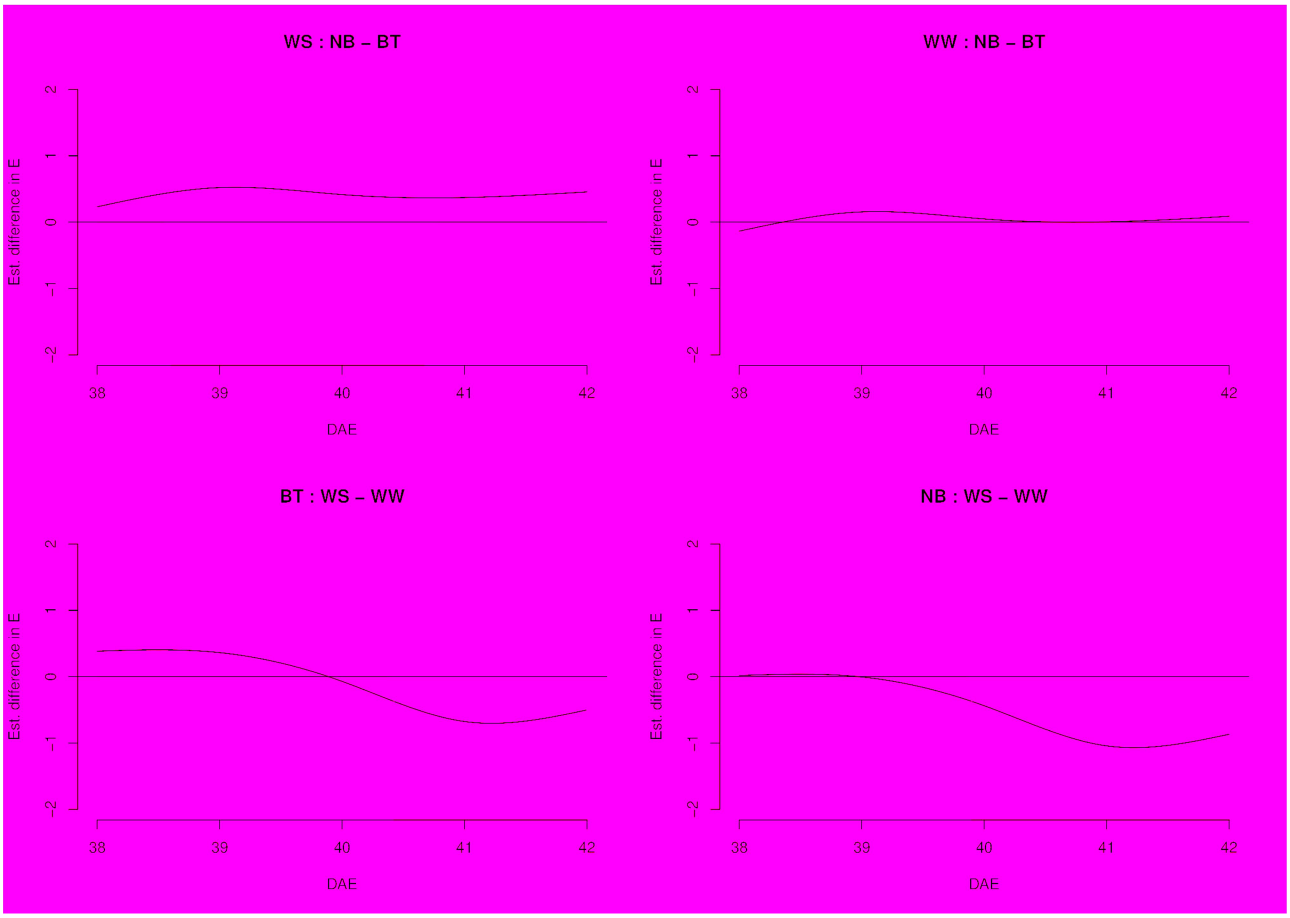


Figure 2.3. Graphs showing differences in Transpiration (mmol m^{-2} s^{-1}) according to model g among all combination of treatments (irrigation and biostimulant). Random effects are excluded. The baseline above is represented by untreated plants, below by watered (control) plants. The pointwise 95%-confidence interval is shown by a shaded band. When the shaded confidence band does not overlap with the x-axis (i.e. the value is significantly different from zero), this is indicated by a red line on the x-axis (and vertical dotted lines). Upper left (A): water stressed, comparation between no biostimulant and biostimulant treatment. Upper right (B): well-watered, comparation between no biostimulant and biostimulant treatment. Lower left (C): biostimulant treated, comparation between water stressed and well-watered. Lower right (D): control treated, comparation between water stressed and well-watered.

As for *Pn* and E, WUE was evaluated through increasingly complex models (Fig. 2.4). Model g was confirmed as the best model, with 85.3% deviance explained and 0.843 Adj.R^2 : As for *Pn* and E, no significant difference was highlighted among well-watered plants (both biostimulant treated and not). The difference in WUE among treated and untreated plants spanned from shortly before the day of stress imposition (39 DAE) to the third day (41 DAE). The duration of the difference among biostimulant treated plants (well-watered/water stressed) extended from early 40 DAE to 42 DAE while the difference among untreated well-watered and water stressed plants went from 40 DAE to 42 DAE.

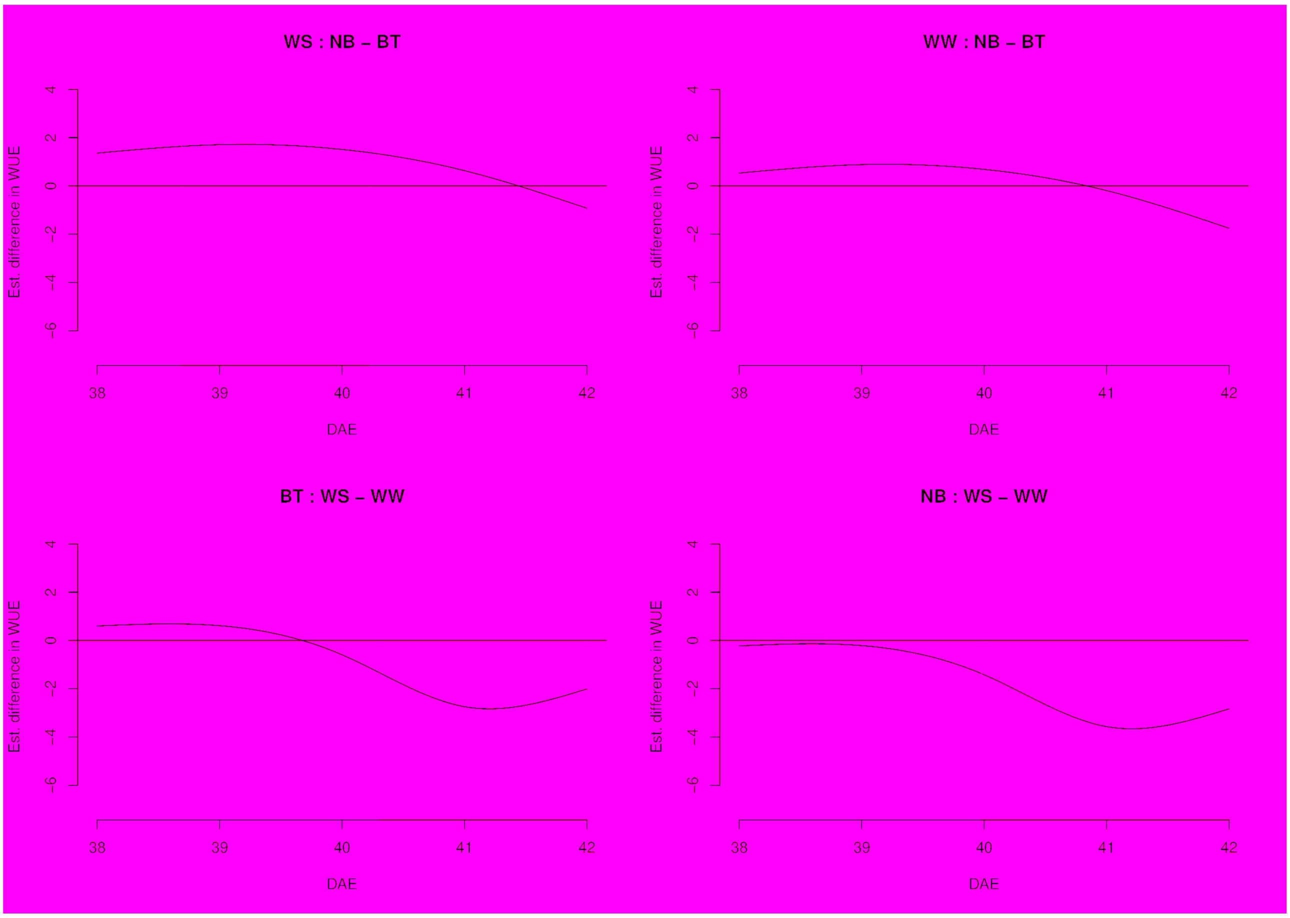


Figure 2.4. Graphs showing differences in WUE among all combination of treatments (irrigation and biostimulant). Random effects are excluded. The baseline above is represented by untreated plants, below by watered (control) plants. The pointwise 95%-confidence interval is shown by a shaded band. When the shaded confidence band does not overlap with the x-axis (i.e. the value is significantly different from zero), this is indicated by a red line on the x-axis (and vertical dotted lines). Upper left (A): water stressed, comparation between no biostimulant and biostimulant treatment. Upper right (B): well-watered, comparation between no biostimulant and biostimulant treatment. Lower left (C): biostimulant treated, comparation between water stressed and well-watered. Lower right (D): control treated, comparation between water stressed and well-watered.

2.3.2 Untargeted Metabolomics

In this study, the untargeted metabolomics approach was able to reveal more than 3400 molecular features. The annotated compounds and composite mass spectra (mass and abundance combinations), together with compounds confirmed by MS/MS, are listed in Supplementary Table 1. The dataset was first interpreted through an unsupervised hierarchical clustering. This unsupervised clustering approach enabled the description of similarities/dissimilarities among treatments, as shown in Figure 2.5A.

Two main clusters were generated – one including the biostimulant treated water stressed treatment and the other including both irrigated and stressed untreated treatments and the treated irrigated one. Two distinct subclusters, one including the untreated water stressed and the other both the treated and untreated irrigated treatments, could be identified. The water stressed, biostimulant treated (WS, BT) profile differed starkly from the others: the naive (unsupervised) hierarchical clustering of metabolomic signatures revealed distinctive profiles in tomato leaves under limited water availability, result of the application of the biostimulant.

Orthogonal partial least squares-discriminant analysis (OPLSDA) analysis allowed separating predictive and orthogonal components (i.e., the components ascribable to technical and biological variation) of variance. The subsequent supervised statistics were used to discriminate the tomato samples according to the treatment. The OPLS-DA (Fig.1.5B) effectively separated the stressed from the non-stressed plants pointing out irrigation as the main separation factor. Among the stressed plants, treated plants presented the most distinct profile, as suggested by the HCA, and were clearly separated from the non-treated one. Similar metabolic profiles were found for non-stressed plants regardless of the treatment. The model parameters of the OPLS-DA regression were $R2Y = 0.89$ and $Q2Y = 0.73$, respectively. The model was validated (CV-ANOVA $p = 1.50 \times 10^{-5}$) and overfitting was excluded through permutation testing ($N = 100$). Given the validated model outcomes, the VIP (Variable Importance in Projection) variable selection method was used to identify compounds explaining the differences observed. The discriminating compounds with a VIP score >1.3 were considered as discriminants. 147 compounds resulted from this selection and are summarized in Supplementary Table 2. Thereafter, a Volcano analysis ($p<0.01$; $FC>2$) was performed and the significant compounds were then uploaded into the Omics Dashboard tool from PlantCyc to facilitate the discussion of results (Supplementary Table 3).

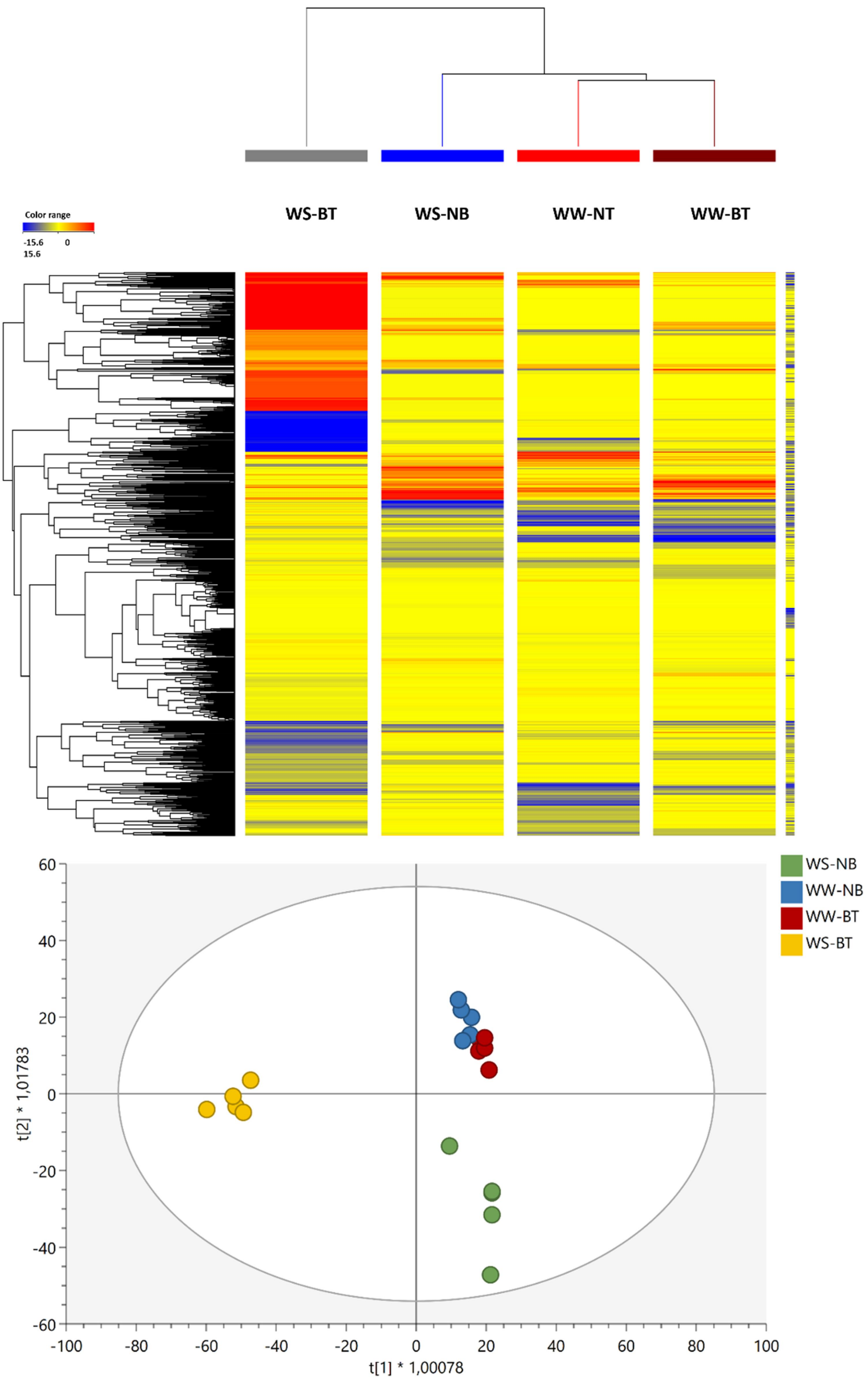


Figure 2.5. Above (A): Unsupervised hierarchical cluster analysis (Euclidean similarity; linkage rule: Ward's). The analysis has been carried out from metabolite profiles in tomato leaves from the factorial combination of biostimulant treatment and water treatment as gained from UHPLC liquid chromatograph coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) untargeted metabolomics. Compound intensity was used to produce fold-change-based heat maps, based on which clustering was done. Below (B): Score plot of Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised analysis carried out from metabolite profiles in tomato leaves treated or untreated plants, as gained from UHPLC/QTOF-MS untargeted metabolomics.

Notably, relatively few biochemical classes included most of the discriminant metabolites (Fig. 2.6).

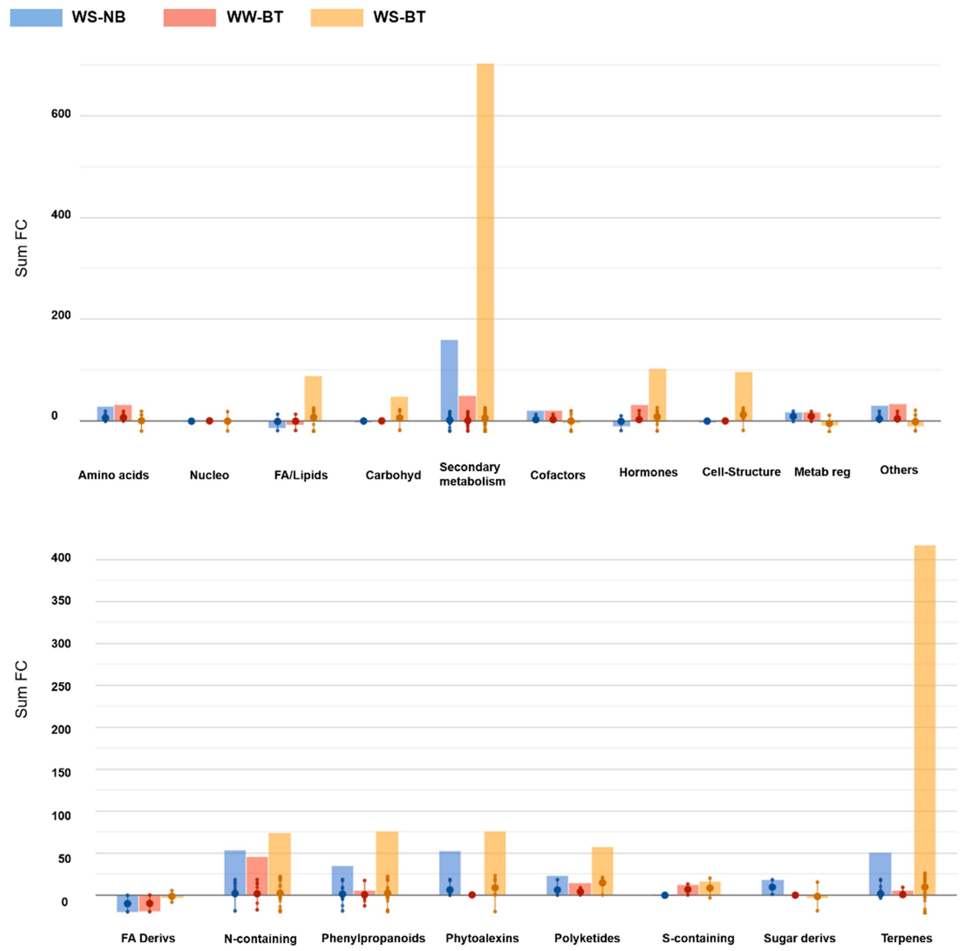


Figure 2.6. Bar graphs resulting from the import of volcano analysis data (p<0.01; FC>2) on the Omics Dashboard tool from PlantCyc. The treatments FC difference is calculated based on well-watered, control treatment (WW – NB). The first bar (in blue) depicts water stressed- biostimulant treatment results (WS – BT), the second (in red) depicts well-watered biostimulant treatment results (WW – BT), the last (in yellow) depicts water stressed biostimulant treatment results (WS – BT).

The water stressed biostimulant treated plants metabolic profile sharply differed from the others. The most affected classes of compounds were secondary metabolites, particularly in their biosynthesis, followed by cellular structure synthase and fatty acids and lipids synthesis. Regarding secondary metabolite biosynthesis, terpenoid biosynthesis, nitrogen-containing secondary compound biosynthesis and phenylpropanoid derivative biosynthesis were the most affected. Compounds related to terpenoid biosynthesis were strongly over-accumulated, mostly represented by triterpenoids and tetraterpenoids, with a high abundance of carotenoids: this is particularly relevant considering the role of carotenoids in photosynthetic organisms. At the same time, compounds responsible for terpenoid degradation were significantly underaccumulated. Interestingly, phytoalexins were also found to be over-accumulated. Cell structure synthase metabolites, among which stearate and oleate, were strongly over-accumulated. Concerning vitamin biosynthesis, molecules responsible for thiamine biosynthesis were sharply over-accumulated. Among fatty acids and lipids, cutin synthase metabolites were strongly over-accumulated, together with epoxylated and hydroxylated fatty acids, stearate, and unsaturated fatty acids. Sphingolipids were also found to be over-accumulated. Concerning degradation, amino acids degrading molecules showed a generalized under-accumulation, for instance regarding glutamate, lysine and tryptophan. Lastly, interestingly gibberellin degrading pathways were under-accumulated in biostimulant treated plants, but differences could be detected among water treatments: the control group showed a sharp under-accumulation of molecules involved in epoxidation, while the stressed group showed a sharp decrease in succinate content, involved in the hydroxylation of gibberellins.

2.4 Discussion

One of the primary adverse effects of water deficit stress is the inhibition of photosynthesis triggered by stomatal closure, which represents the earliest response to drought (Michaletti et al., 2018). As a result, CO2 uptake and concentration in leaves is reduced (Medrano et al., 2002). In view of this, the results from the GAMM analysis confirmed that biostimulant treated plants performed better in terms of *Pn*, E and WUE compared to untreated plants under water stress. The use of generalized additive modeling enabled the analysis of the full set of dynamic data, without the need to reduce time resolution (i.e. average over time or select specific time points). Moreover, with this analysis the effect of treatments (i.e. drought and biostimulant application) on the patterns (i.e. wiggliness) of gas exchange data was accurately modelled and discriminated, also considering data autocorrelation. Using GAMM allowed the extraction of information on the effect of the biostimulant devoid of temporal correlation and random errors due to individual replicates, nonlinear interactions between both treatments and the duration of the experiment (expressed in days) and the time of the day (expressed in hours). At the same time, GAMM analysis allowed to consider different smooths for day (duration of the experiment) and hour (time of the day) for both factors (water and biostimulant). Lastly, the use of GAMM allowed for the inclusion of non-linear variability of the individuals over the duration of the experiment. While the significance of the smooth terms does not provide information on the statistical significance between patterns,each individual variable has a statistically significant influence on modeling the wiggliness of *Pn*, which in turns confirms the distance of Pn from a linear function. This resulted in a model explaining

87.8% of the deviance. Further, the use of GAMMs enabled the comparison between the curves of stressed and well-watered plants, both with and without biostimulant, thereby providing both a visual screening tool and a statistical tool to further confirm or disprove the effect of biostimulant treatment. The positive effect of the biostimulant treatment observed through GAMM analysis is in line with literature findings on the potential of glycinebetaine to increase photosynthetic performance under water stress (Yang and Lu, 2015; Hamani et al., 2020; Nawaz and Wang, 2020), especially in tomato (Mäkelä et al., 1999). Differences in the length of significance windows among *Pn*, E and WUE were detected. While the photosynthetic rate was constantly higher for treated plants compared to the untreated ones, from stress imposition (39 DAE) to the end of stress (42 DAE), the positive effect in the biostimulant treated water stressed plants for transpiration rate, and consequently WUE, was shorter compared to *Pn*. Specifically, under water stressed conditions, the positive effect of the biostimulant treatment on E was reduced in duration, indicating that higher transpiration could only be supported until late, on 40 DAE (second day of water stress) and efficacy on WUE was further reduced to early on 40 DAE. This indicates that the increased photosynthetic rate after the first day of water stress imposition is followed by increased transpiration, resulting in the reduction of the WUE advantage. WUE is a largely diffused performance indicator for crop yield and water consumption. Water stressed plants typically exhibit a higher WUE due to a more conservative use of water, resulting in improved resource utilization efficiency under conditions of water scarcity (Zao et al., 2020.) Nevertheless, high stomatal conductance over time is essential to high plant production, translating into maximized soil water-use for transpiration, or effective use of water (EUW) for transpiration. It is therefore evident that, under drought conditions, higher stomatal conductance over time will result in lower WUE (Blum, 2009). Indeed, drought resistant plants display minimization of leaf permeability, for example by means of higher epicuticular wax deposition, in order to maximize soil water capture while diverting water to stomatal transpiration (Blum, 2009). Besides, Blum indicates effective use of water (EUW) as the key objective in maximizing biomass production under a limited water regime.

The increased photosynthetic rate was in line with the protective role of glycinebetaine in terms of cellular osmotic adjustment, detoxification of reactive oxygen species, and protection and stabilization of membrane integrity (Ashraaf and Foolad, 2007) and is further supported by the sharp separation of the metabolic profile of the water stressed biostimulant treated thesis. At the same time, the smaller reduction in net photosynthesis in leaves subjected to water stress can be attributed to an increased stomatal conductance, as well as the maintenance of chloroplast ultrastructure (Ma et al., 2006) and Rubisco activity (Yang et al., 2005b). Indeed, the over-accumulation of carotenoids is of particular interest, given their photoprotective role in preventing the overexcitation of photosystem II (Uarrota et al., 2018), while at the same time acting as toxic oxygen species scavengers, structure stabilizators and excess energy dissipators (Griffiths et al. 1955; Frank and Cogdell 1996; Polívka and Sundstrom 2004). GB has indeed been found to have a strong protective effect on the structure and function of the oxygen-evolving complex of PSII in vitro against multiple abiotic stresses (Mamedov et al., 1991, 1993; Papageorgiou et al., 1991; Papageorgiou and Murata, 1995; Allakhverdiev et al., 2003). Wang and colleagues (2010) found that drought stress can interfere with the state of the lipids in thylakoid membrane, which, if damaged, might cause PSII to be impaired. Concurrently, cell membrane stability depends on the absence of lipid peroxidation, caused by ROS accumulation. Moreover, they found that carotenoid concentrations in water stressed, GB treated plants were consistently higher compared to untreated plants. They also demonstrated that the ability of GB to decrease ROS levels is not direct, but rather indirect. These findings imply that GB acts as an elicitor to other scavenger molecules, thereby strongly supporting our findings on carotenoid concentration. Indeed, Wang and colleagues correlated GB accumulation to xanthophyll cycle dependent nonradiative energy dissipation, thereby drawing a strong connection between the protective action derived from GB overaccumulation and carotenoid synthesis. In addition, GB-synthetizing transgenic plants have been found to display higher activity of ROS-detoxifying enzymes (Ahmad et al., 2010; Yang et al., 2006). Supporting these arguments, Xu and colleagues (2018) hypothesized that GB may act both as an osmotic stress hardening molecule and as a signaling molecule in acclimation, rather than only via a direct action. Concerning energy supply, it is particularly interesting to notice the sharp accumulation of thiamine precursors, as thiamine plays a pivotal role in carbon metabolism and is essential for cell energy supply in all organisms. Moreover, it is essential in carbon fixation through the Calvin cycle and the non-mevalonate isoprenoid biosynthesis pathway, from which thousands of metabolites are derived including chlorophyll, phytols and carotenoids, as well as several phytohormones. (Noordally et al., 2020). Thiamine has also been linked to plant adaptation responses to persistent abiotic stress conditions, drought (Wong et al., 2006) and oxidative stress (Rapala et al., 2008; Tunc-Ozdemir et al., 2009) included. In addition, the over-accumulation of specific secondary metabolites suggests a stress priming activity induced by the biostimulant treatment: phytoalexins have been associated with increased drought tolerance in multiple species (Kuc, 1995; Hatmi et al., 2014; Vaughan et al., 2014); phenylpropanoid biosynthetic pathway is activated under abiotic stress conditions, resulting in stimulated biosynthesis of phenolic compounds with strong antioxidative potential (Sharma et al., 2019). Lastly, the over-accumulation of lipids and specifically of cutin synthase metabolites, supports the maximization of stomatal transpiration (Kerstiens, 1997, 2006). All things considered, the positive effect of GB on water stress resistance could be attributed both to the delayed onset of stress, and consequently the enhanced natural response of tomato plants, and the elicitation of stress priming through the induction of H2O2-mediated antioxidant mechanisms, as Park and colleagues suggested (2004; 2006), and molecules with strong antioxidant potential (such as xanthophylls).

In conclusion, the study demonstrated the potential of GAMM method to describe and discriminate biostimulant action (GB, in this case) to improve photosynthetic performance under water stress conditions. In this, the GAMM method was crucial in extracting the effect of biostimulant treatment under dynamic gas exchange acquisition. GAMM analysis effectively improved the interpretation of time series data, enabling both the description of the dynamics of water stress onset and the isolation of the effect related to the biostimulant treatment. Moreover, compared to the other treatments, water stressed biostimulant treated plants displayed a starkly different and stress tolerance related metabolic profile, in agreement with the findings on photosynthetic performance. The metabolites accumulated suggest a priming effect for stress tolerance, via detoxification and stabilization of the photosynthetic machinery. The duration and dynamics of the positive effect of the biostimulant treatment under water stress differed for photosynthesis, transpiration and WUE, the last two being limited in time. This could depend on an increased transpiration efficiency, translating into maximized soil water-use for transpiration, or effective use of water (EUW) for transpiration. Higher epicuticular wax deposition, and, therefore, minimization of leaf permeability is one of the main strategies plants employ to maximize soil water capture while diverting water to stomatal transpiration. Indeed, the metabolic profile findings support the increased EUW through over-accumulation of lipids and cutine synthase metabolites. The present research brought further evidence that GB protective action on photosystem II is not only direct but also strongly connected to the production of other scavenger molecules (e.g. carotenoids, phytoalexins), making the case that GB acts both as an osmotic stress hardening molecule and as a signaling molecule in acclimation. Nonetheless, further research is needed to deepen the connection between exogenous GB treatment and metabolic response.

2.5 Author Contributions

GA performed all analyses, wrote the first draft of the manuscript and interpreted the data. GA and MC designed and performed the statistical analysis of gas exchange measurements. BM-M performed statistical analysis of the metabolome and data interpretation and wrote part of the manuscript on molecular analysis. GA and AF set up and performed the experiment. SA provided the intellectual input and corrected the manuscript.

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2.7 Supplementary Material

Supplementary material tables 1,2 and 3 are available as excel files at the GitHub address: https://github.com/G-Antonucci/Combined-Biostimulant-HTS-Under-Drought.

Supplementary Table 1. Untargeted metabolomics dataset containing the annotated compounds in both MS-only and MS/MS following the UHPLC-QTOF-MS analysis.

Supplementary Table 2. Discriminant metabolites identified by the VIP analysis in leaves following biostimulant application. Compounds were selected as discriminant by possessing a VIP score >1.3.

Supplementary Table3. Differential metabolites as derived from Volcano analysis (P-value < 0.01; FC > 2) and uploaded to the PlantCyc pathway Tool software for the subsequent analysis.

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Chapter III

High-Throughput Plant Phenotyping for Evaluating Biostimulants: Biophysical Variables Estimation Through PROSAIL Inversion

Antonucci Giulia[1]. Impollonia Giorgio[1], Croci Michele[1], Potenza Eleonora[1], Marcone Andrea[1], Amaducci Stefano[1]

[1] Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore (UCSC), Piacenza, Italy

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Abstract

Drought management largely depends on the availability of timely, accurate and integrated information about its characteristics. Concurrently, biostimulants could represent a sustainable measure to foster the resilience of cropping systems under water-limited conditions. Nevertheless, scientific recognition of the potential of biostimulants has not grown as fast as the interest from industry: therefore, there is an urgent need to investigate biostimulant action. In recent decades, remote sensing has been successfully applied to crop growth and stress monitoring. The use of radiative transfer models, often rooted in artificial intelligence, to estimate plant traits from remotely sensed data can be considered the link between the generalisation and the spatialisation of data, bringing field phenotyping and remote sensing closer together. In this framework, the present study was designed as a factorial combination of irrigation treatment (3 levels) and biostimulant treatment (3 levels) and conducted on processing tomato in open field during the summer of 2020. PROSAIL inversion was carried out to retrieve three major biophysical parameters (LAI, LCC, CCC). The parameters dynamics during the season were investigated through GAM modelling. The validation of the model was carried out with a positive outcome in terms of accuracy. The PROSAIL inversion enabled the efficient retrieval of LAI and LCC at rates comparable to those in literature, while performing worse than literature findings only for CCC, probably due to the characteristics of tomato canopy. At the same time, the effect of irrigation was detected both for yield and quality data and detected through the GAM modelisation of the parameters. However, no biostimulant effect could be detected. The internal variability per plot of the retrieved biophysical parameters was high. This, jointly with the uncertainty surrounding biostimulant testing and the magnitude of biostimulant effects, corroborated by the absence of results regarding biostimulant effect on yield and DM, lead to hypothesise that the bottleneck was linked to the biostimulant effect itself.

3.1 Introduction

Drought is recognised as one of the major negative factors affecting agricultural production. In this context, drought management represents a key strategic tool to achieve sustainable agriculture while minimising drought-related losses of crop plant productivity (Osmolovskaya et al., 2018). Its management largely depends on the availability of timely, accurate and integrated information about its characteristics (Buma and Sang-Il, 2019).

Biostimulants, as pointed out by Del Buono (2020), could represent a sustainable measure to foster the resilience of cropping systems under water-limited conditions. Plants' first response to drought is represented by stomatal closure, which then results in the inhibition of photosynthesis (Michaletti et al., 2018) and therefore leads to CO2 uptake and concentration reduction (Medrano et al., 2002), leading to a lower accumulation of biomass. Among biostimulant constituents, osmolytes targeting water stress resistance in plants, such as glycinebetaine (GB), are rising in application. GB acts in multiple ways in order to contrast water stress: as an osmoregulator; by stabilising structures and activities of enzymes and protein complexes via detoxification of reactive oxygen species (Papageorgiou et al., 1991; Papageorgiou and Murata, 1995); by maintaining the integrity of membranes against the damaging effects of excessive salt (Mbarki et al, 2018; Tang et al., 2014, Tian et al., 2017, Yang and Lu, 2005) , cold (Quan et al., 2004), heat (Allakhverdiev et al., 2007; Yang et al., 2006) and freezing (Razavi et al., 2018; Wang et al., 2019), as well as drought (Ma et al., 2006). The role of GB in plant resistance to abiotic stressed has been widely investigated and documented (Ashraaf and Foolad, 2007; Chen and Murata, 2011; Gorham, 1995; Huang et al., 2020; Sakamoto and Murata, 2002). Nevertheless, scientific recognition of the potential of biostimulants has not grown as fast as the interest from industry (Fleming et al., 2019): this has been caused by limited fundamental research on their modes of action and the high speed at which new multi-compound products have entered the market. Therefore, there is an urgent need to investigate biostimulant action. In this context, the necessity of accurate assessment of phenotypic variables is emerging as a critical issue (Rouphael et al., 2018). In this context, emerging digital technologies such as sensors, automatic image acquisition and the connected algorithms and models have seen an increasing adoption.

In recent decades, remote sensing has been successfully applied to crop growth and stress monitoring (Barbedo, 2019; Xie and Yang, 2020; Galieni et al., 2021). The use of unmanned aerial vehicles (UAVs) in particular has been on the rise: representing an economical and efficient method to meet the increasing requirements of spatial, temporal, and spectral resolutions (Yue et al., 2017; Zheng et al., 2018; Heinemann et al., 2020; Qiao et al., 2020), they are able to provide flexible high information resolution.

As of today, thermal UAV is mainly employed in detecting drought stress: the most common drought stress signals in plants (i.e. temperature) are susceptible to detection by thermal camera technology, namely by exploiting the inverse linear relation between transpiration and surface temperature of leaves (Maes and Steppe, 2012; 2019).

Remote sensing represents a highly flexible and widely used instrument to assess various plant traits. Nevertheless, the assessment of complex traits such as the identification and quantification of abiotic and biotic stress is still regarded as challenging: there is a rising necessity to establish reliable retrieval techniques enabling the spatiotemporally explicit quantification of biophysical variables. Among others, precision agriculture (Zhang and Kovacs, 2012; Tao et al., 2020), crop phenotyping (Impollonia et al., 2022), monitoring of crop traits (Domingues Franceschini et al., 2017; Jay et al., 2017) and the improvement of yield prediction (Cilia et al., 2014; Goffart et al., 2008) all rely on the possibility to quantitatively estimate bio-physical/-chemical crop parameters accurately (Roosjen et al., 2018). Remote sensing in particular, and spectroscopy data at large, are suited for the cost-effective estimation of biophysical plant traits through the dense information content contained in few spectral bands, either narrow or broad (Verrelst et al., 2019).

While remote sensing approaches in the past have mainly involved hyperspectral cameras (Kanning et al., 2000; Duan et al., 2014; Kalisperakis et al., 2015; Li et al., 2015; Roosjen et al., 2018; Yue et al., 2018; Tao et al., 2020), crucial biophysical parameters such as leaf area index (LAI) have also successfully been retrieved through the use of multispectral cameras. Among multiple parameters that can be retrieved, leaf area index (LAI) is of particular interest: a key canopy structural variable, it is used in order to model crop growth (Zhao et al., 2013¸ Potgieter et al., 2017), yield variability (Mueller et al., 2012), monitor crop growth (Duveiller et al., 2011), predict the crop yield (Geipel et al., 2014), estimate the amount of aboveground biomass (Yue et al., 2017), and evaluate the effects of field management (Baez-Gonzales et al., 2005). At the same time, chlorophyll content, defined either at the leaf level (leaf chlorophyll content, LCC) or at the canopy level (canopy chlorophyll content, CCC) is used as a bioindicator of vegetation state, crop productivity and health status and photosynthetic capacity (Gitelson et al., 2006; Clevers et al., 2011; Hoeppner et al., 2020; Mutanga et al., 2004; Wu et al. 2008; Houborg et al., 2007). These variables of interest are also good proxies of the general health state of the crop and the use of remote sensing allows to estimate variations in these physical parameters at a relatively low cost compared to field measurements (Mutanga et al., 2004; Pu et al., 2014; Zhang et al., 2008; Wong and He, 2013).

In recent times, field phenotyping and remote sensing are undergoing a process of convergence. Formally, field phenotyping refers to a quantitative description of a plant's phenotype devoid of spatial effects and remote sensing refers to the site-specific observation of vegetation by a remote device and the retrieval of its qualitative or quantitative properties. It is therefore clear that, traditionally, both disciplines concentrate on the interactions of plant growth with the surrounding environment with two different scopes: the first to generalise it, the second to explore site-specific interactions and describe spatially explicit traits (Machwitz et al., 2021).

The use of radiative transfer models, often rooted in artificial intelligence, to estimate plant traits from remotely sensed data can be considered the link between the generalisation and the spatialisation of data. The aim of radiative transfer models, which describe the radiation transfer and interactions of plant canopies based on physical laws, is to generalise empirical results and improve the robustness of vegetation parameter retrieval. Therefore, they are generally applicable in different situations (Houborg et al., 2007; Jacquemoud et al. 2009). When used in an inversion, the main bottleneck of radiative transfer models is the necessity to avoid ill-posed problems: to achieve this, prior information in order to reduce the number of possible variable retrieval options (combinations of canopy parameters) are required, also in order to improve the inversion accuracy (Combal et al., 2002; Verger et al., 2011; Liang et al., 2015). Among radiative transfer models, the PROSAIL (PROSPECT (leaf optical properties spectra) + SAIL (Scattering from Arbitrarily Inclined Leaves) is widely used due to its simplicity, accuracy, and availability. Its robustness has been extensively tested through different sensors and platforms via ground, airborne, and spaceborne data sets (Jacquemoud et al., 2009).

The objective of this work was to identify whether UAV assisted image acquisition was a feasible means of detecting biostimulant treatment under water stress. The hypothesis tested here was whether retrieving LAI, LCC and CCC through PROSAIL inversion could efficiently detect the effect of a biostimulant (GB-based) treatment in open field on water stressed tomato and whether modelling this data dynamically through GAM could identify the windows of time where the biostimulant significantly impacted relevant traits. In order to achieve this, the PROSAIL was first validated through ground data, then inverted and applied to retrieve LAI, LCC and CCC and finally the dynamics of the effect on these parameters were investigated through GAM modelling. Additionally, the effect of the treatment on yield and quality parameters at harvest were tested.

3.2 Material and Methods

3.2.1 Experimental Site and Set-up

Field experiments were conducted in the north-west of Italy, in Piacenza province, where the climate is continental with an average annual rainfall of 827 mm (1961 – 1990) and rainfall peaks in autumn and spring. The experiment was located in San Polo (44°59'14.6"N, 9°44'29.3"E) (Tab.1).

Table 1. Characteristics of the experimental location and weather data from 2020. Weather data from the closest climatic stations were retrieved with the web app of the Regional Environmental Protection Agency (www.arpae.it accessed on 20 December 2021), Dext3r (https://simc.arpae.it/dext3r/ accessed on 20 December 2021).

Parameter	
Location	San Polo, Piacenza
Latitude	44°59'14.6"N
Longitude	9°44'29.3"E
Elevation (m a.s.l.)	80
Texture class	silty clay loam
Yearly precipitation (mm)	520.8
Yearly maximum temperature (°C)	36.4
Yearly average temperature (°C)	15.5
Yearly minimum temperature (°C)	-5.6

The experimental layout (Fig. 2.1) is a randomised block design, with four blocks and single plot size of 75 m2 (1.5 × 10 x 5 m). The experiment was designed as a factorial combination of irrigation treatment (3 levels) and biostimulant treatment (3 levels). The levels of irrigation treatment were determined based on plant available water (PAW) ranges: i) $28.8<x<57.6$, ii) $57.6<x<86.4$ and iii) $86.4<x<144$ in order to reach the respective thresholds of i) 25% PAW, ii)50%

PAW and iii)100% PAW. The irrigation rate to maintain the three PAW levels was calculated based on the daily evapotranspiration (ET_c) reported in the web service IRRINET (Rossi et al., 2004; Mannini et al., 2013). Concerning biostimulant treatment, the levels were three: untreated (control) (T0), treated twice at an interval of 2 weeks (T1), treated 3 times at intervals of 2 weeks (T2).

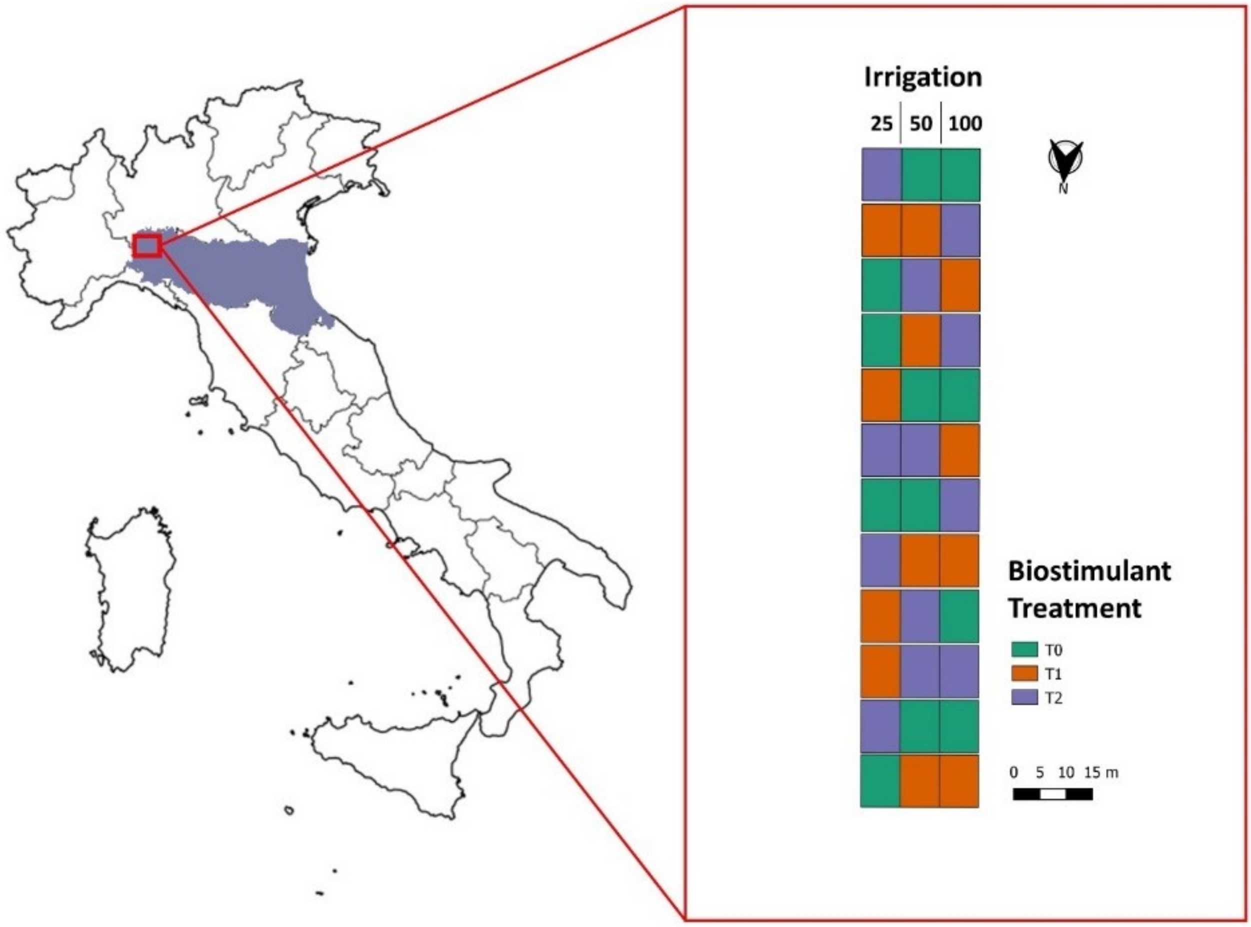

Figure 7. Location of San Polo experimental site and overview of the experimental design and plots. Different colours represent the different biostimulant treatment levels. The three levels of irrigation are 25%, 50%, 100% PAW. Plots are representative of 15 m^2. Biostimulant treatments are indicated as follows: T0 = control treatment; T1 = treated twice; T2 =treated three times.

The experiment was conducted on tomato plants (*Lycopersicon escolentum, L.,* 3406 Heinz), which were transplanted on 17th of May 2020. Planting density and interrow spacing were the following: 5 m^{-2}, 0.2 m. Mineral fertilisation was carried out in two doses (200 kg N ha^{-1}) on 30/05/2020 and on

11/06/2020. Organic fertilisation was carried out on 4/06/2020 at a rate of 131 kg ha^{-1}. Fertilisation and weed/pest control have been performed using local agronomic recommendations. Drip irrigation was spaced as follows: 0.3 m (on row), 1.5 m (between rows), with a flow rate of 0.7 l h^{-1}. Irrigation was managed at the intervals provided by the irrigation cooperative by fitting drip irrigation valves on every drip line and managing the quantity of water provided based on the duration of opening.

3.2.1.1 Biostimulant Characteristics and Treatment

The treatment with the biostimulant started on 23rd of June 2020 (37 DAT), at the start of flowering (Fig.2).

Biostimulant was sprayed at a rate of 6 kg ha^{-1}, with a dilution of 300 l ha^{-1} to a total of 10 g/plant. Dose and dilution were chosen based on commercial use of the product (Vegetal B60®, ED&F MAN Liquid Products Italia, Bologna, Italy). Vegetal B60® is an organic product extracted exclusively from sugar beet without any added chemical additives. It contains 30% of GB and 5% of L–amino acids, 5% of total organic nitrogen, and 12% of organic carbon. The following treatments were carried out at two-week intervals: the second treatment was carried out on 52 DAT and the third at 65 DAT.

3.2.2 Field Data Collection

3.2.2.1 Model Parameter Acquisition

In situ leaf mass per unit leaf area (C_m) and leaf water per unit area (C_w) were calculated based on samples collected in an area of the field outside of the biostimulant trial (akin to an untreated plot). The leaves were separated from the stems and transferred to -18 °C fridge. The leaf surface was determined by scanning the leaves, then the leaves were oven dried at 65 °C. After drying, the leaves were weighted. The C_m (g cm^{-2}) and C_w (g cm^{-2}) were calculated as the ratio of the dry weight (C_m) or of the water weight (C_w) of the leaves and their surface. Destructive sampling from the biomass harvest was conducted over a total of 24 complete plants selected from 1 m^2 picketed plots over six samplings (Fig.2). Lastly, leaf area index was estimated using a ACCUPAR LP-80 PAR/LAI ceptometer (METER Group) on 5 dates across the season (18/06, 26/06, 10/07, 20/07, 06/08), The LP-80 measures photosynthetically active radiation (PAR, 400- to 700-nm). Jointly with C_m and C_w measurements, leaf samples for leaf chlorophyll content (LCC) determination were collected, immediately stored in ice, and transferred to a refrigerator (-20°C) for later analyses.

Chlorophyll was evaluated in two phases:

- Extraction (protocol adapted from Ritchie, 2006): samples were grinded in liquid nitrogen. Subsequently 0.1 g were weighted in 15 ml extraction tubes and 10 ml of absolute ethanol (99.8%) were added. Samples were homogenised by an Ultra-Turrax (Model T18) for 1 min. Thereafter samples were centrifuged at 4000 rpm at 4°C for 10 min. Concurrently, a subsample was put to dry at 105°C for 24 h in order to determine sample water content.
- Microplate Quantification: Microplate measurements were made by pipetting 250 µl of sample (or blank) in a 96-well white plate which was then read with a monochromator-based microplate reader (Synergy HT, BioTek, Winooski, USA) based on Warren (2008). The samples were read at 649 nm and 665 nm. Chlorophyll content was then calculated as follows (Ritchie, 2006):

$$Chl_a\ (\mu g\ ml^{1}) = -5{,}2007 \times A_{649} + 13{,}5275 \times A_{665}$$

$$Chl_b\ (\mu g\ ml^{-1}) = 22{,}4327 \times A_{649} + (-7{,}0741 \times A_{665})$$

3.2.2.2 Unmanned Aerial Vehicle Multispectral Image Acquisition

The UAV used in the experiment was a four-rotator DJI Matrice 210 RTK (SZ DJI Technology Co., Shenzhen, Guangzhou, China) combined with a RTK (Real Time Kinematic) GPS positioning system. At each sampling event a UAV multispectral data acquisition was performed, in addition seven supplementary flight missions were carried out on each PS trial to increase the frequency of senescence tracking. Starting 27th May 2020, one multispectral UAV flight observation was conducted circa every week. Fourteen flights in total were performed across the season, five before the onset of treatments (Fig.2). The drone was equipped with MicaSense RedEdge-Mx multispectral camera (MicaSense, Seattle, WA, USA). RedEdge-Mx camera can acquire 5 different spectral bands images: blue (475 nm centre, 32 nm bandwidth), green (560 nm centre, 27 nm bandwidth), red (668 nm centre, 14 nm bandwidth), red edge (717 nm centre, 12 nm bandwidth) and near-infrared (840 nm centre, 57 nm bandwidth). All the flights were performed between 11.00 and 15.00. The flight altitude above ground level (AGL) was 50 m. The forward overlap was set at 80% and lateral overlap was set at 75% of the images. The flight speed was set at 3 m/s. The ground sampling distance (GSD) was 2.78 cm. The flight was performed in automatic mode with waypoints routes as the presence of a GPS navigation system enables a more accurate image acquisition. The DJI Pilot software (SZ DJI Technology Co., Shenzhen, Guangzhou, China) was used for flight planning and automatic mission control. For the radiometric calibration of the images, the reflectance of a spectral panel (MicaSense, Seattle, WA, USA) with reflectance values provided by MicaSense, was captured before each flight. In addition, a light sensor that automatically adjusts the readings to ambient light was mounted at the top of the UAV to minimise error during image capture. The radiometric calibration, image mosaicking and orthomosaic generation were done using the Pix4D mapper (Pix4D, S.A., Lausanne, Switzerland). To extract the spectral information of each experimental plot, the polygons of the experimental design were drafted in AutoCAD (Autodesk, San Rafael, California, USA) and georeferenced based on the UAV multispectral images by using QGIS software (QGIS Development Team, 2021).

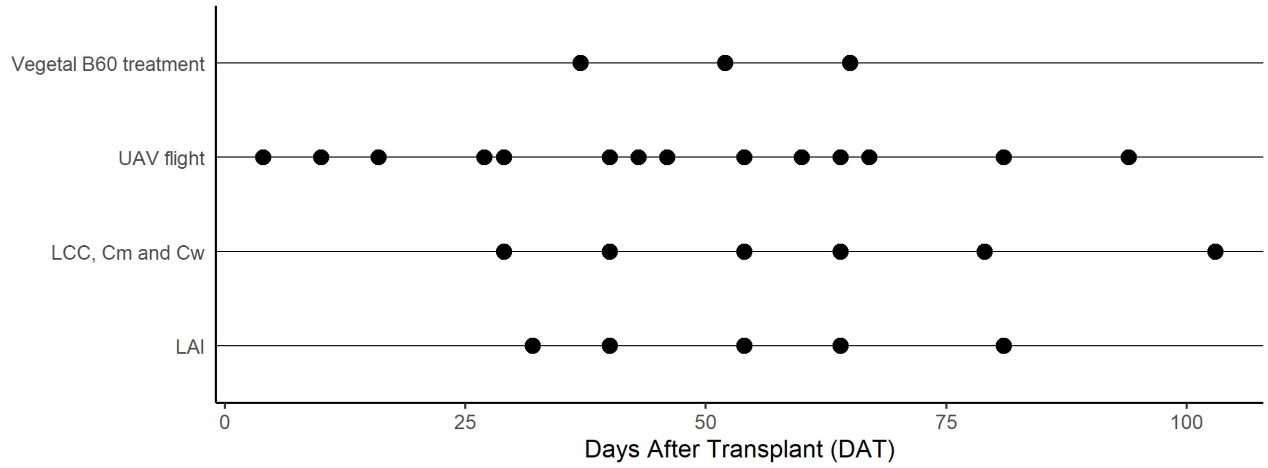

Figure 8. Vegetal B60 treatment, UAV flight, sampling (LCC, Cm and Cw) and leaf area index (LAI) ground measurements seasonal calendar.

3.2.3 Final Yield and Post-Harvest Analyses

The final harvest was carried out between 25th of August 2020 and 28th of August 2020. The same theses were harvested across blocks to minimise between-block variability. The harvested area was 1 m^2 repeated three times for each plot, for a total of 3 m^2 per plot. The harvest was divided between the biomass portion (leaves and stems) and the fruit portion, further classified in green, rotten and red fraction. A subsample for biomass and red fruits was oven dried at 105°C to determine their dry matter (DM) content.

Total soluble solids (TSS) and pH were measured on circa 200 g of homogenised fresh fruit subsamples from the total red fruits harvested. Every measurement was repeated thrice. TSS were measured via a digital refractometer (RX-5000 ATAGO, U.S.A., Bellevue, WA). The pH of the samples was measured via a digital pH meter (HI 5522, HANNA, USA).

3.2.3 Radiative Tranfer Model: PROSAIL

Leaf area index (LAI), leaf chlorophyll content (LCC) and canopy chlorophyll content (CCC) were retrieved though the inversion of the PROSAIL model (Jacquemoud et al., 2009). In PROSAIL data are derived from combining the PROSPECT leaf optical properties model and the SAIL canopy bidirectional reflectance model. The PROSPECT (Feret et al., 2008) model simulates the leaf reflectance and transmittance in the range from 400 to 2500 nm with four parameters: leaf structure parameter (N), leaf chlorophyll content (LCC), leaf dry matter content (C_m), and leaf equivalent water thickness (C_w).

The SAIL model is a radiative transfer model used to simulate the bidirectional reflectance of a canopy. The PROSAIL requires fourteen parameters in order to run: N, LCC, C_w, C_m, LAI, , hot-spot parameter (hot spot), observer zenith angle (tto), sun zenith angle (tts), relative azimuth angle (psi) , and average leaf angle (ALA). The PROSAIL inputs (parameter combinations) and outputs (spectral reflectance) were used to generate a database. In the database generation, 48,600 parameter combinations were generated following the ranges (minimum and maximum) and the step of the parameters summarised in Table 2. The ranges were optimised based on data collected in the field (see chapter 2.2): this is an efficient way of solving the ill-posed problem as suggested by Meroni et al. (2004). The spectral reflectance simulated (outputs) were resampled based on UAV cameras characteristics.

Table 2. LUT generation parameters ranges.

Parameter		**Abbreviation**	**Unit**	**Values**
Leaf	Structure parameter	N	Unitless	1.25
	Chlorophyll content	LCC	$\mu g\ cm^{-2}$	30 – 72 (step = 3)
	Equivalent water thickness	C_w	$g\ cm^{-2}$	0.022 – 0.028 (step = 0.003)
	Dry matter content	C_m	$g\ cm^{-2}$	0.003 – 0.004 (step = 0.0005)
Canopy	Leaf area index	LAI	$m^2\ m^{-2}$	0.1 – 6 (step = 0.3)
	Average leaf angle	ALA	deg	50 – 70 (step = 10)

	Hotspot parameter	hot	m m^{-1}	0.1
	Sun zenith angle	tts	deg	20 – 30 (step = 5)
	Observer zenith angle	tto	deg	10
	Relative azimuth angle	psi	deg	190 – 195

3.2.4 GPR Based PROSAIL inversion

Rooted in large dataset modelling, machine learning (ML) algorithms enable more accurate data mining by learning from training data to model, classify, and predict the variables in the whole datasets (Jagtap et al., 2021).

GPR is a vigorous, non-parametric Bayesian method used for solving regression problems and unknown modelling functions (Schulz et al., 2018; Gershman & Blei, 2012). Its main feature is capturing the different relationships between inputs and output variables by applying a hypothetically infinite number of parameters and allowing the dataset to determine the level of complexity via Bayesian inference (Williams, 1998). The parametrization of the Gaussian process is made using a kernel, the choice of which is flexible. Moreover, it is possible to combine the different kernels to perform the regression (Schulz et al., 2018). In this study, GPR radial basis function kernel was used to invert the PROSAIL database to retrieve LAI, LCC and CCC data by using the “caret” package (Kuhn, 2008; Alexandros et al., 2019). The structural hyperparameters of the GPR models were optimized by grid-searching method using cross-validation. These biophysical retrieved parameters were proxies to discriminate between irrigation and biostimulant treatments and their combination.

The coefficient of determination (R^2) (1) and Root Mean Square Error as such (RMSE) (2) and normalised against the mean (nRMSE) (3) were used to quantify the amount of variation explained, as well as the accuracy, of those relationships. The performance of the model was estimated by comparing differences in the R^2 and RMSE. The closer R^2 scored to 1 and the lower RMSE scored, the higher were the precision and accuracy of the inversion, and vice versa.

$$R^2 = \frac{\left(\sum_{i=1}^{n}(p_i - \bar{p}_i)(f_i - \bar{f}_i)\right)^2}{\sum_{i=1}^{n}(p_i - \bar{p}_i)^2 \sum_{i=1}^{n}(f_i - \bar{f}_i)^2} \qquad (1)$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(p - f_i)^2} \qquad (2)$$

$$nRMSE = \frac{RMSE}{\bar{p}_i} \qquad (3)$$

where n (i=1, 2, …, n) is the number of samples used to test ML model, p_i is the observed parameter (LAI, LCC or CCC), $\bar{p}_i$ is the corresponding mean value, f_i is the predicted parameter and $\bar{f}_i$ is the corresponding mean value.

3.2.5 Statistical Analyses

Statistical analyses were performed using RStudio, R version 4.0.4 (R Core Team, 2020).

3.2.5.1 ANOVA

Crop yield data (both fresh and dry matter) were analysed through two-way ANOVA to determine the significance of irrigation and biostimulant treatments and their interaction with the following model: lm(yield ~ irrigation × biostimulant treatment) followed by a pairwise comparison with the formula: emmeans(model, specs= irrigation × biostimulant treatment, adjust='sidak'), with the "emmeans" v.1.5.4 R package.

3.2.5.2 GAM For Phenotyping Tomato Water Stress Dynamic

To phenotype the dynamics of LAI, CCC and LCC and identify differences among biostimulant and irrigation treatments, statistical analysis of the traits time series was carried out via a generalised additive model (GAM). GAM are non-parametric regression models, which allow the integration of non-parametric smoothing functions and non-linear fitting of the variables. GAM models were fitted in R package "*itsadug*" (van Rij et al., 2020). The fitted model used biostimulant treatment and irrigation treatment as fixed factors and a smooth for DAT, while considering the block as a random error.

3.2.6 Biostimulant Effect Evaluation Workflow

Agronomic (fresh fruit yield, fruit dry matter) and model parameter data (C_m, LCC, LAI) were collected in the field. Agronomic data output was then analysed through two-way ANOVA (detailed in section 2.5.1) while model parameter data were first used to narrow the range of the database, and then to validate the PROSAIL model. The PROSAIL database generated was then used to train the GPR model to retrieve leaf area index (LAI), leaf chlorophyll content (LCC) and canopy chlorophyll content (CCC) of the crop from multispectral images. Finally, the data thus generated were analysed through generalised additive modelling (GAM, described in further detail in section 2.5.2) in order to evaluate the effect of the biostimulant application. The process is summarised in Figure 3.

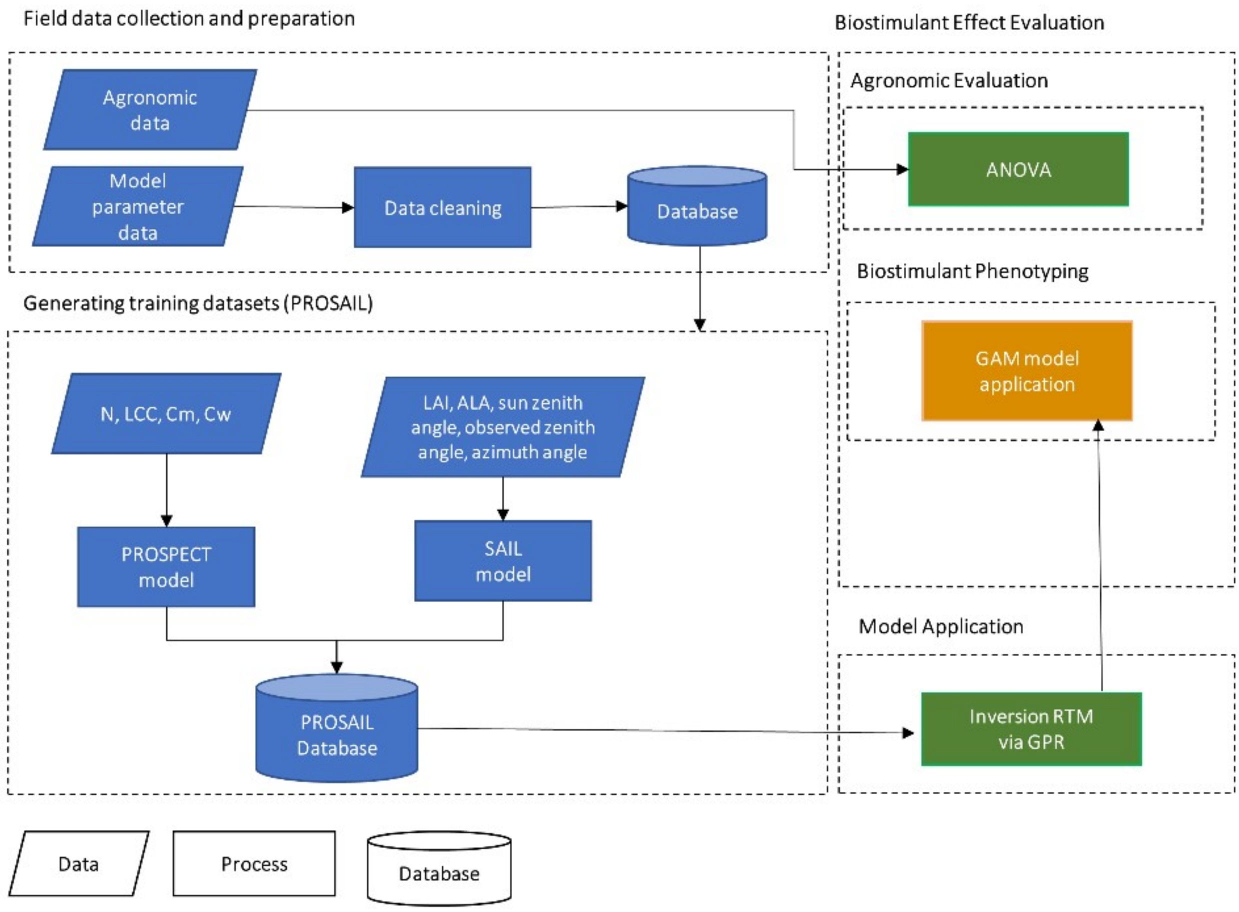

Figure 9. Overview of the objectives of this study and the implemented workflow.

3.3 Results

3.3.1 Crop Yield and quality parameters

Red fruit yield, both determined as fresh weight (Fig. 4) and dry weight (Fig. 5), was statistically affected by irrigation treatments but not by the biostimulant treatments.

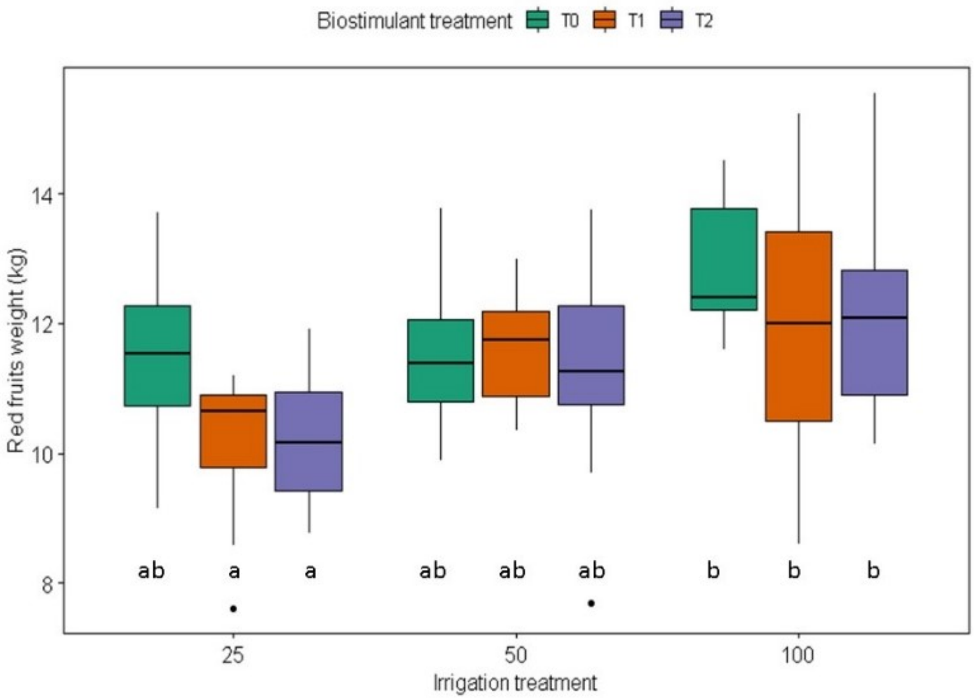

Figure 10. Agronomic response of tomato in terms of red fruit yield (kg) to three levels of irrigation (25%, 50%, 100% PAW). Data are representative of 12 m^2 per each combination of treatment. Biostimulant treatments are indicated as follows: T0 = control treatment; T1 = treated twice; T2 =treated three times. Different letters indicate statistically significant differences according to Sidak-test.

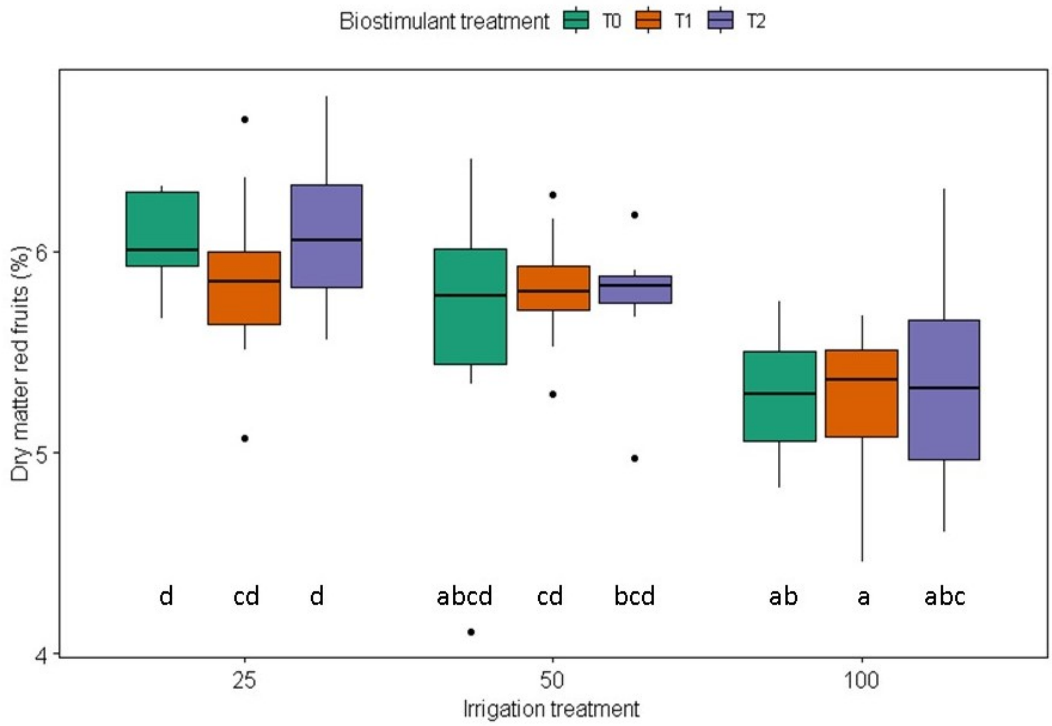

Figure 11. Agronomic response of tomato to three levels of irrigation (25%, 50%, 100% PAW). Data are representative of 12 m^2 per each combination of treatment. Biostimulant treatments are indicated as follows: T0 = control treatment; T1 = treated twice; T2 =treated three times. Different letters indicate statistically significant differences according to Sidak-test.

The analysis of total soluble solids (TSS) resulted in a significant difference in the sugar content among the two lower irrigation treatments and the control (100% PAW) treatment (Fig. 6). The two lower-level treatments (25 and 50% PAW) did not display any significant difference among them. Also in this case the effect of the biostimulant treatment, was not significant.

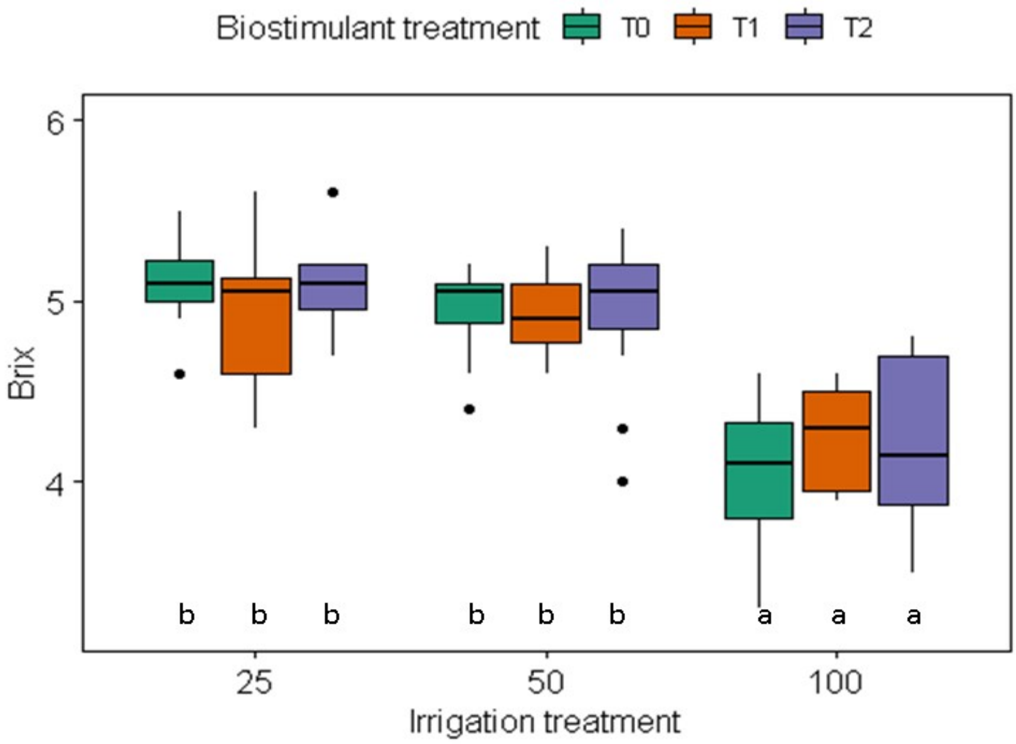

Figure 12. Total soluble solids (°Brix) content under three levels of irrigation (25%, 50%, 100% PAW) and three biostimulant treatments. Data are representative of 12 m^2 per each combination of treatment. Biostimulant treatments are indicated as follows: T0 = control treatment; T1 = treated twice; T2 =treated three times. Different letters indicate statistically significant differences according to Sidak-test.

3.3.2 PROSAIL Validation

The accuracy of LAI, LCC and CCC retrieved from the inversion of PROSAIL was evaluated against the independent dataset obtained from *in-situ* measurements. The RMSE value for LAI estimates was 0.53 (Fig. 7a). The RMSE value for LCC scored 6.72(Fig. 7b). Finally, the RMSE value for CCC (Fig. 7c) was the lowest (0.4). Nevertheless, in terms of nRMSE score, the best results were obtained for LCC (14.2) when compared to LAI (22.4) and CCC (32.5) (Fig. 6).

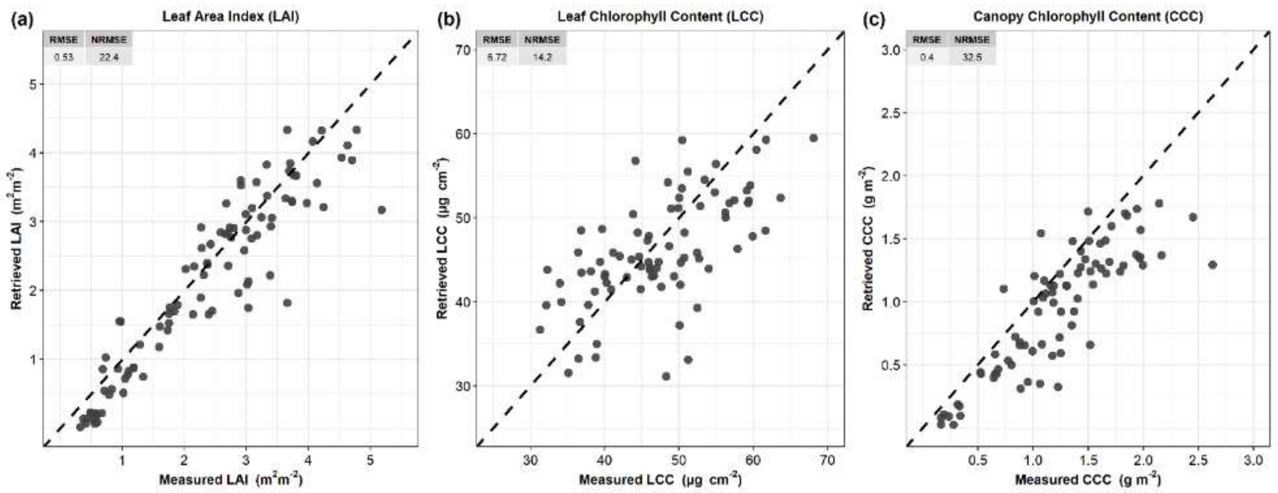

Figure 13. Comparison between the in-situ LAI measurements and the estimated LAI. Comparison between the canopy chlorophyll content (LCC) calculated based on measurements from the in-situ sampling and the estimated canopy chlorophyll content. Comparison between the leaf chlorophyll content (LCC) measurements from the in-situ sampling and the estimated leaf chlorophyll content.

3.3.3 GAM analysis of ANN inversion for LAI, LCC and CCC retrieval

After the PROSAIL model validation, LAI, LCC and CCC data were examined through generalised additive modelling (GAM) to evaluate the fitness of the combination of these techniques for biostimulant testing. GAM analysis was used to model dynamic seasonal data in order to evaluate the changes induced by biostimulant treatment, irrigation treatment and their interaction on the biophysical parameters. GAMs are non-linear regression models which are characterised by a sum of smooth functions.

The GAM model implemented had the following structure:

gam (CCC ~ Biostimulant Treatment * Irrigation Treatment + s(DAT, by = Irrigation Treatment, k= 5) + s(DAT, by = Treat, k=5) + s(Block, bs = "random error").

Where s stands for single effect smooths, k is the number of kernels, namely the dimension of the basis used to represent the smooth term, and bs indicates the penalised smoothing basis, in this case the random error. The R^2 and explained deviance of LAI, LCC and CCC models scores showed that the models could explain large amounts of variance (Tab. 3).

Table 3. Characteristics of models for LAI and CCC. Dev. Expl. (%) = deviance explained.

Model	R^2	Dev. Expl (%)
LAI	0.844	85.1
LCC	0.562	58.1
CCC	0.840	84.7

3.3.3.1 Irrigation Treatments Analysed via GAM

Irrigation treatments were efficiently discriminated across the season via GAM analysis. Concerning LAI, the comparison between irrigation levels highlighted a statistically significant difference between the 100% versus 50% and the 100% versus 25% PAW (Fig. 8A).

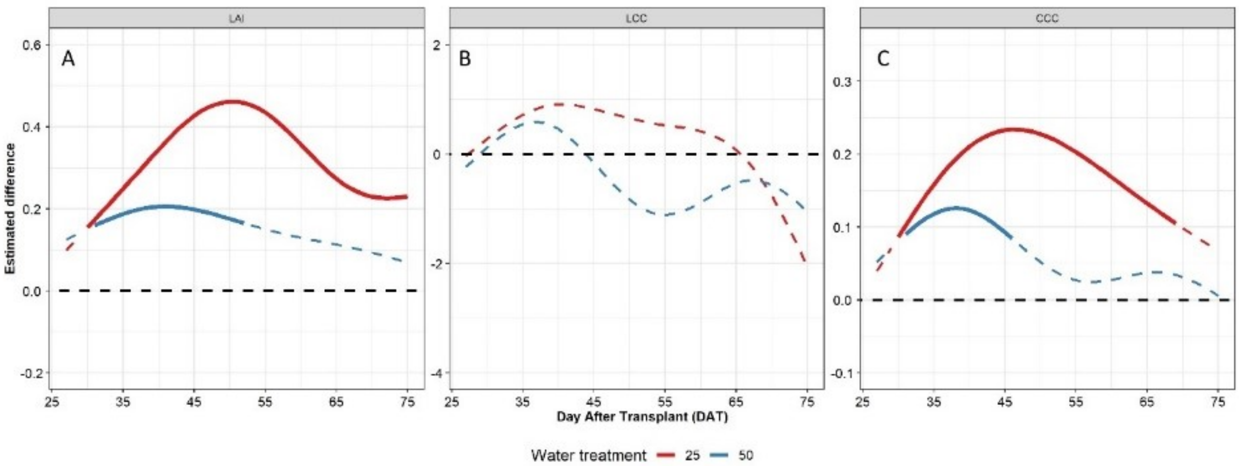

Figure 8. Difference between irrigation treatments for the three parameters (LAI, LCC, CCC) analysed via GAM. The dashed horizontal baseline represents treatment 100% PAW. When lines are full, the difference between that treatment and 100% PAW is significant (95% c.i.). Conversely, when lines are dashed the difference is not significant.

The significant difference between 100% and 50% started on 32 DAT and ended on 48 DAT, while the significant difference between 100% and 25% treatments started on 30 DAT until 81 DAT. In both cases 100% PAW treatment performed better than the other two theses, ranging from +0.1 to +0.45 in estimated difference. LCC comparison between irrigation levels highlighted no statistically significant difference among treatments (Fig. 8B). Concerning CCC, the comparison between irrigation levels highlighted a statistically significant difference among the 100% versus 50% and the 100% versus 25% PAW (Fig. 8C). The significance of the 100% versus 25% treatments started on DAT 31 until 66 DAT, while the significant difference between 100% and 50% started on 33 DAT and ended on 44 DAT. In both cases 100% PAW treatment performed better than the other two theses in a range from +0.05 to +0.25 in estimated difference.

3.3.3.2 Biostimulant Treatments Analysed via GAM

Biostimulant treatment was evaluated with the same statistical approach as irrigation treatment. Concerning LAI (Fig. 9A), the comparison between biostimulant treatments resulted in a statistically significant difference among the control treatment (T0) versus T2 (treated twice) treatment early in the season (27 DAT) until 53 DAT: the highest difference was detected around 25 DAT (-0.2), where T2 was higher.

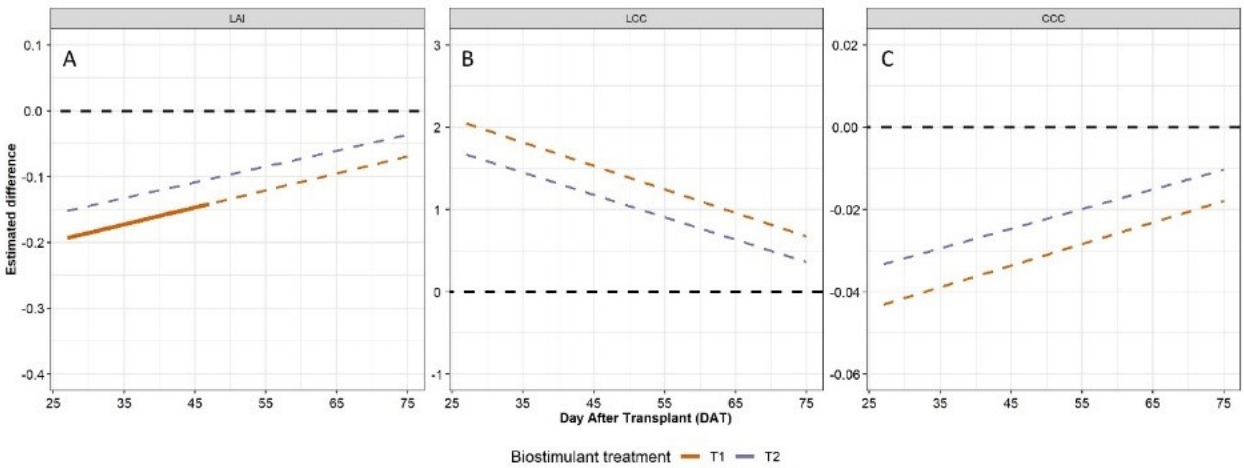

Figure 9. Difference between biostimulant treatments for the three parameters (LAI, LCC, CCC) analysed via GAM. The dashed horizontal baseline represents control treatment (T0). When lines are full, the difference between that treatment and T0 is significant (95% c.i.). Conversely, when lines are dashed the difference is not significant. T1 = treated twice, T2 = treated three times.

Nevertheless, when this data was matched with the treatment calendar, that is the DAT at which the biostimulant treatments were performed (tab. 2), the significant difference between T0 and T2 started before the first biostimulant treatment was applied (37 DAT).

Concerning LCC (Fig. 9B) and CCC (Fig. 9C), the comparison between biostimulant treatments across the season resulted in no statistically significant difference.

3.3.3.3 Irrigation and Biostimulant Treatments Analysed via GAM

When displayed as combination of irrigation and biostimulant treatment, no significant difference could be detected. The comparisons were carried out for each irrigation treatment across biostimulant treatments (e.g. treatment 1 versus treatment 2 at 25% PAW) for LAI, LCC and CCC. Only the control (T0) and T 1 (twice) and T0 (control) and T2 (thrice) under 25% PAW irrigation displayed significant differences. As for biostimulant treatment alone, when this data was matched with the treatment calendar, that is the DAT at which the biostimulant treatments were performed (tab. 2), the significant differences started before the first biostimulant treatment was applied (37 DAT).

3.4 Discussion

Spectral data from UAV are commonly used to monitor crop stress response (Barbedo, 2019; Xie and Yang, 2020; Galieni et al., 2021). Nevertheless, remote sensing applied to agriculture still faces a challenge: understanding how the collected data can be effectively utilised to characterise biophysical properties of the crop canopy (Thorp et al., 2012).

With the support of big data analysis, acquired spectral data enable the estimation of crop biophysical properties. This is particularly interesting in contexts where experimental replicates are limited (because of practical or experimental design related constraints and costs) while the main goal would be still to generalise experimental findings to the field scale and above. In this context, the PROSAIL model has been widely used to assess crop traits, such as the LCC, C_w, C_m and LAI

(Jacquemoud et al., 2009). In this sense, multiple approaches are being investigated: among these, machine learning (ML)-assisted radiative transfer model inversion is gaining importance as a means to enhance the robustness and transferability of biophysical traits retrieval.

Water deficiency directly affects primary metabolism (Lawlor & Cornic, 2002), resulting in variations in crop yield and dry matter (DM) content. At the same time, in tomato, water stress increases TSS content (Ozbahce & Tari, 2010). This was confirmed in the present experiment: red fruits yield was lower at the lowest irrigation treatment (25% PAW) than at the highest one (100% PAW). The same result was found for DM, despite it displaying a more scattered behaviour compared to fresh yield. While no significant difference could be detected for LCC and CCC related to biostimulant treatments, these data are in agreement with those retrieved from the PROSAIL inversion, corroborating its reliability. PROSAIL validation was carried out on multispectral data and, when considering LAI, yielded RMSE (0.53 m^2/m^2) results comparable to validations carried out on hyperspectral data based on smaller datasets (Li et al., 2015) or achieved a better accuracy (Duan et al., 2014). On the other hand, relationships between simulated and measured LCC indicated a better relationship in terms of nRMSE, compared to LAI and CCC, between sampled and simulated data. Moreover, the RMSE value (6.72 $\mu g\ cm^{-2}$) obtained for LCC was consistent with those found by Botha and colleagues (2007) on potato and the multispectral data retrieved by Wan and colleagues (2021) for rice and rapeseed (5.40 $\mu g\ cm^{-2}$), but considerably better than what found by Li and colleagues, (2015) for hyperspectral-base retrievals of LCC on winter wheat (13.36 and 9.35 $\mu g\ cm^{-2}$). CCC retrieval was the best in terms of RMSE (0.4 $\mu g\ cm^{-2}$), probably due to the strong influence of LAI in the relation: CCC represents the canopy-integrated chlorophyll content (LAI × leaf chlorophyll content), which is strongly influenced by the variability in LAI, especially when chlorophyll content is stable (Darvishzadeh et al., 2008). This was also modelled by generalised additive model (GAM) graphs, which displayed trends very similar to LAI GAM graphs. On the other hand, PROSAIL model is most appropriate for homogeneous canopies:

the turbid medium assumptions at its core negatively influence its capability to model non-uniform canopies such as crop-row canopies (Jiao et al., 2021; Sun et al., 2021). This represents a bottleneck that hampers the efficient implementation of PROSAIL on processing tomato: the nRMSE for CCC retrieval was 32.5, the highest among the three parameters, probably due to the characteristics of processing tomato canopy. This is further confirmed by the findings of Roojen and colleagues (2018), whose results on nadir LAI and LCC, were consistent with the findings of this study while performed much better when considering multi-angular simulations. Thanks to the sensitivity of multi-angular measurements to vegetation structure, these simulations take into account information on the structural properties of the canopy (Chen et al., 2003, Widlowski et al., 2004).

LAI, LCC and CCC were later analysed via GAM. The influence of irrigation and biostimulant treatments were evaluated both separately and jointly. When considering irrigation alone, the comparison between irrigation levels highlighted a statistically significant difference between the 100% versus 50% and the 100% versus 25% PAW. LCC, in turn displayed no significant difference between the two deficit irrigation treatments and the control treatment.

In terms of biostimulant action evaluation alone, the effects highlighted via GAM analysis were later matched with the treatment calendar, that is the DAT at which the biostimulant treatments were performed: the significant difference between T0 and T2 started before the first biostimulant treatment was applied (37 DAT). Moreover, the magnitude of the effect (-0.2 maximum) for LAI was negligible when compared to the RMSE error from the PROSAIL validation (0.53). These two aspects do not support the validity of the differences retrieved via GAM.
When both parameters (irrigation x biostimulant treatment) were included in the pairwise comparisons for each irrigation level, none of the biophysical parameters displayed a significant difference.

The properties of glycinebetaine (Ashraf and Foolad, 2007; Alia et al., 1998; Chen & Murata, 2002; Sakamoto & Murata, 2002; Papageorgiou & Murata, 1995; Allakhverdiev et al., 1996; Ma et al., 2006; Yang et al., 2005) indicate the possibility that GB-based biostimulants stabilise chlorophyll content across the season when the crop is undergoing water stress. At the same time, the maintenance of photosynthetic activity related to the protection of photosystems would indicate the possibility of achieving a higher LAI across the season compared to the untreated stressed crop. However, the present research was not able to observe any significant result of GB treatment on LCC and CCC and the effects observed on LAI were inconclusive. Generalised additive models were chosen in order better analyse the dynamic time-series dataset. The goal of the GAM analysis was to discriminate the effect of treatments (i.e., irrigation and biostimulant application) on the patterns (i.e., wiggliness) of LAI, LCC and CCC. Despite the successful application of GAM to model dynamic data on biostimulant action in a previous experiment on processing tomato in greenhouse (Antonucci et al., 2021), the results from the current experiment did not enable an effective detection and modelling of the biostimulant effect. In the comprehensive review by Wozniak and colleagues (2020) on the effects of biostimulants at whole-plant level amino acid-based and protein hydrolysates as a class were reported to increase the production of a limited number of crops (Al Majathoub, 2004; Grabowska et al., 2012; Kunicki et al., 2010), while at the same time reporting no influence on others (Colla et al., 2013; Koukounaras et al., 2013). This leads to think that the effects of biostimulant application in the field could be much more difficult to pin down than those of other products (such as fertilisers or pesticides) or even inconsistent, especially when considering the work of Mäkelä and colleagues (1998) who, contrary to this work, found that exogenous glycinebetaine applied to salt or heat stressed tomato crops during flowering resulted in an increase in fruit yield up to 39%. On-field research on biostimulant effects has been on the rise in the last few years, but the results on most parameters are elusive: this is further confirmed, for example, by the work from Francesca and colleagues (2020), who tested the efficacy of a biostimulant treatment on water deficient tomato crops in open field, only found significant effects on stomatal conductance and pollen viability.

3.5 Conclusions

In the framework of biostimulant testing, this was the first attempt at utilising biophysical parameters retrieved from PROSAIL inversion in order to quantify the effect of a biostimulant. The validation of the model was carried out with a positive outcome regarding accuracy. However, the internal variability per plot of the retrieved biophysical parameters was high. This, jointly with the uncertainty surrounding biostimulant testing and the magnitude of biostimulant effects, corroborated by the absence of results regarding biostimulant effect on yield and DM, leads to hypothesise that the bottleneck does not lie at the PROSAIL inversion stage, but is rather linked to the biostimulant effect itself. The PROSAIL inversion enabled the efficient retrieval of LAI and LCC at rates comparable to those in literature, while performing worse than literature findings only for CCC, probably due to the characteristics of tomato canopy.

3.6 Author Contributions

Giulia Antonucci: Conceptualization, Data Curation, Methodology, Investigation, Formal Analysis, Writing- Original draft preparation. **Giorgio Impollonia:** Conceptualization, Data Curation, Methodology, Investigation, Software, Writing - review & editing. **Michele Croci:** Conceptualization, Data Curation, Investigation, Software, Writing - review & editing. **Eleonora Potenza**: Investigation, Data Curation. **Andrea Marcone**: Investigation. **Stefano Amaducci**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

3.7 Acknowledgments

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3.8 Competing Interests

The authors declare no competing interest.

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Chapter IV

Synthesis

Adaptation to climate change is becoming central to the conversation about water management for agriculture (Iglesias and Garrote, 2015). In this context, drought management represents an essential tool to achieve sustainable agriculture while minimising drought-related losses of crop plant productivity (Osmolovskaya et al., 2018). To achieve this, a technology applicable to multiple crops in multiple locations would represent a desirable alternative. Del Buono (2020) pointed out that plant biostimulants (PBs) could represent a sustainable measure to foster the resilience of cropping systems under climate change-related environmental stress. Due to the vast range of molecules and possible combinations, and since the legislation requiring PB claims to be validated is recent and has yet to be enforced, there is no consensus over the best ways to test biostimulant products. At the same time, emerging digital technologies such as sensors, automatic image acquisition, and connected algorithms and models have seen increasing adoption, resulting in increasing volumes and complexity of data. The main challenge of these techniques lies in data management: the massive amount of data generated needs to be handled both at the acquisition and analysis stage through appropriate, often custom, tools (Coppens et al., 2017).

In this framework, the main objective of this thesis was to respond to the necessity to develop comprehensive, reliable, and accurate technical instruments to test biostimulants, particularly under drought conditions. In order to do this, different combinations of techniques, both in greenhouse and in open field conditions, were investigated. In this chapter, each research question will be addressed based on the main findings as presented in chapters 2-3.

1.Are high-throughput analytical procedures capable of efficiently detecting biostimulant effects at later plant development stages? Which are more suited?

This research question was addressed in both chapter II and III. Biostimulant effects are mainly investigated through screenings conducted at the early stages of plant development, especially those contrasting abiotic stresses. Promising results were obtained through the present research using continuous gas exchange acquisition, metabolomics and UAV imaging. In chapter II, the combination of continuous gas exchange acquisition and metabolomics was instrumental in describing the mode of action of the glycine betaine-based biostimulant. The differences in the duration and dynamics of the positive effect of the biostimulant treatment under water stress were efficiently modelled for photosynthesis, transpiration and WUE, the last two being limited in time. The differences were linked to an increased transpiration efficiency, translating into maximised soil water use for transpiration or effective use of water (EUW) for transpiration. Moreover, compared to the other treatments, water-stressed biostimulant treated plants displayed a starkly different, stress tolerance related metabolic profile, in agreement with the findings on photosynthetic performance. The metabolites accumulated suggest a priming effect for stress tolerance via detoxification and stabilisation of the photosynthetic machinery.

In Chapter III, UAV imaging-based PROSAIL inversion enabled the efficient retrieval of LAI and LCC at rates comparable to those in literature while performing worse than literature findings only for CCC, probably due to the characteristics of tomato canopy. On the other hand, while the effects of irrigation were successfully modelled, the effects of biostimulants were not. This result is further discussed under research question 3.

2. Which statistical instrument is most fit for the modelling of dynamic biostimulant effects?

This research question was addressed in both chapter II and III. Biostimulant effects are dynamic over time, especially when considering progressive abiotic stresses such as drought. Tailored statistical instruments are needed to model their effect, taking into account the dynamic characteristics of their action. In this thesis, the search for the appropriate statistical method that would adequately consider both this dynamic nature and the high-throughput characteristics of the collected data resulted in the choice of generalised additive models (GAM), which allow for modelling non-linear regressions. The rationale behind the choice of GAM was the multiple advantages of this approach, among them: the possibility to handle the complexity of the data without oversimplifying them, the determination of the relationship between the dependent variable and the predictors as a function of the algorithm- which can be but is not necessarily linear-, the inclusion of multiple numeric predictors and the possibility to include an autoregressive AR(1) error model for Gaussian models in order to handle the autocorrelation component of the error. The inclusion of autocorrelation is particularly relevant in time-series datasets, where each data point is clearly correlated to (and therefore dependent on) the previous and the following data points. Therefore, this analysis is particularly useful to datasets characterised by dynamic and time-series data. The study demonstrated the potential of the GAMM method to describe and discriminate biostimulant action (GB, in this case) to improve photosynthetic performance under water stress conditions. Moreover, GAMM analysis effectively improved the interpretation of time series data, enabling both the description of the dynamics of water stress onset and the isolation of the effect related to the biostimulant treatment. As a whole, Chapter II brought further evidence that GB protective action on photosystem II is not only direct but also strongly connected to the production of other scavenger molecules (e.g. carotenoids, phytoalexins), making the case that GB acts both as an osmotic stress hardening molecule and as a signalling molecule in acclimation.

3.Are biostimulant effects detectable via UAV imaging?

This research question was answered in Chapter III. UAV imaging is being widely used for stress detection and growth monitoring. Therefore, the question was whether it is suitable to detect biostimulant effects under abiotic stress conditions. While the inversion of PROSAIL actually enabled the efficient retrieval of LAI and LCC at rates comparable to those in literature, performing worse than literature findings only for CCC, no effects of biostimulant treatment could be detected. In such a framework, this was the first attempt at utilising biophysical parameters retrieved from PROSAIL inversion to quantify a biostimulant's effect. The validation of the model was carried out with a positive outcome regarding accuracy. However, the internal variability per plot of the retrieved biophysical parameters was high. This, together with the uncertainty surrounding biostimulant testing and the magnitude of biostimulant effects, corroborated by the absence of results regarding biostimulant effect on yield and DM, leads to hypothesise that the bottleneck does not lie at the PROSAIL inversion stage but is linked instead to the biostimulant effect itself. This result leads us to think that the effects of biostimulant application in the field could be much more difficult to pin down than those of other products (such as fertilisers or pesticides) or even inconsistent, especially considering the work of Mäkelä and colleagues (1998). Contrary to the present work, they found that exogenous glycine betaine applied to salt or heat-stressed tomato crops during flowering resulted in an increase in fruit yield up to 39%. On-field research on biostimulant effects has been on the rise in the last few years. However, the results on most parameters are elusive: this is further confirmed, for example, by the work from Francesca and colleagues (2020), who tested the efficacy of a biostimulant treatment on water-deficient tomato crops in the open field, only found significant effects on stomatal conductance and pollen viability.

The research performed in this thesis was motivated by the need for high quality, comprehensive, reliable and accurate technical instruments to test biostimulants.

The results presented in this thesis contribute to the following topics:

1. Insights on the fitness of continuous gas exchange acquisition and snapshot metabolomic profiling for the detection of biostimulant effects in a crop at advanced phenological stages (flowering, in this case) as demonstrated in chapter II.
2. Innovative statistical analyses strategies (GAM) to evaluate dynamic biostimulant effects as demonstrated in chapters II and III.
3. Potential of PROSAIL retrieved biophysical parameters in chapter III.

PBs are valuable for increasing crop performance under stress and reducing excessive water consumption. While in chapter II, such characteristics of PBs were demonstrated through continuous gas exchange measurement and metabolomics in greenhouse conditions, in chapter III, it was not possible to transfer such findings to the open field via UAV imaging. This might be due to intrinsic characteristics of the biostimulant tested, such as their activity threshold being below detectability in the field and their dose or stage of application not being optimal. At the same time, it must be noted that, while the experiment from chapter II featured a severe and sudden onset of stress, chapter III featured the imposition of progressive water stress across the season. These two types of stress might result in different efficacy of biostimulant treatments, although preliminary results for extreme water stress imposition in the open field (which are not discussed here) did not yield any results either. On these grounds, future research endeavours shall concentrate on furthering the knowledge on biostimulant activity thresholds and biostimulant mechanisms of action to better identify the instruments to investigate biostimulant effects and the related acquisition timeframes. Overall, this study serves as a stepping stone to explore biostimulant evaluation techniques.

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List of publications

Peer reviewed publications

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Under review

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Short biography

Giulia Antonucci was born on 2^{nd} of January 1994 in Como, Italy. Giulia pursued her B.Sc. degree in Agricultural Technologies at the University of Bologna from 2012 to 2015 and obtained a joint M.Sc. degree from the University of Bologna and the Free University of Bolzano in International Horticultural Sciences. During her master she was an Erasmus student at the BOKU University in Wien. She is passionate about plant physiology, bioeconomy, the water-food-energy nexus and the social aspects of agriculture. Her interests led her to participate in the RAUN (Regional Academy on the United Nations) fellowship program in 2019-2020, where she worked on the application of bioeconomy in sub-Saharan Africa. She also took part in the Emilia-Romagna ARTER Open Innovation initiative, where she collaborated on open innovation issues together with industrial stakeholders. She believes that **Laziness Does Not Exist**.