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Laboratory of Growth Regulators

Doctoral Thesis

Characterizing the mode of action of small molecule-based biostimulants

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Abstract: The biostimulants (BS) are an emerging trend that can alleviate the negative effects of climate change on crops and help the transition to greener agriculture. Among the different types of BS, the small molecule-based BS (smbBS), including polyamines (PAs), is an exciting option because of its simple formulation. This work aimed to understand the PA mechanism/mode of action using different omics, especially phenomics performed on the Olophen phenotyping platforms. Firstly, the suitability and accuracy of the platforms using different plant species were evaluated. Secondly, this work was focused on characterizing the application of putrescine (Put) and spermidine (Spd) to understand their mechanism/mode of action. Drenching with Put and Spd improved the yield quantity and quality in maize under a water restriction but Put, and Spd showed different mechanisms of action. Moreover, they affected the mineral composition of the kernels, raising an interesting question of using BS for crop biofortification. An additional experiment using *in vitro* Arabidopsis plants primed with Put, ornithine (Orn) as its precursor, and 1,3-diaminopropane as a compound of PA terminal catabolism was also performed. Almost all improved the growth of Arabidopsis *in vitro* seedlings under stress. The metabolic analysis revealed the implication of the N-acetylOrn and Orn and PA conjugation as the leading player regulating growth and development under control and stress conditions. To further understand the Orn involvement in regulating plant stress tolerance, and to evaluate the biological translation from Arabidopsis to a crop with economic value, an experiment on barley (*Hordeum vulgare* L. cv. Wildtype; WT) and a sensitive mutant (AZ34; AZ) was carried out, using Orn as a foliar application. As a preliminary result, we observed that Orn altered the physiology and metabolism of barley plants differently according to the genotype, pointing to this metabolite as an essential regulator of polyamine metabolism and endogenous abscisic acid and, hence, plants' water stress response.

Key Words:	biostimulants, drought, phenotyping, polyamines, priming, salt stress
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DECLARATION

Hereby I declare that this thesis summarizes original results obtained during my Ph.D. under the supervision of Ing. Nuria De Diego Sanchez, Ph.D., using the literature sources listed in the References section.

In Olomouc,

Ing. Alba Esteban Hernándiz

" Després de certes coses s'ha de tornar a casa"

V. Andrés Estellés

A ma mare.

Al meu germà.

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LIST OF PAPERS

This thesis is mainly based on the following publications, referred to in the text by corresponding Supplement N° I-IV attached in Supplement Section.

I.   Hormopriming to mitigate abiotic stress effects: a case study of N^9-substituted cytokinin derivatives with a fluorinated carbohydrate moiety

Bryksová Magdaléna, Hybenová Andrea, Hernándiz Alba E., Novák Ondřej, Pěnčík Aleš, Spíchal Lukáš, De Diego Nuria, Doležal Karel. 2020

Frontiers in Plant Science, 11, 2020, ISSN=1664-462X
https://www.frontiersin.org/article/10.3389/fpls.2020.599228

II.   Phloem exudate metabolic content reflects the response to water-deficit stress in pea plants (*Pisum sativum* L.)

Blicharz, S., Beemster, G.T., Ragni, L., De Diego, N., Spíchal, L., Hernándiz, A.E., Marczak, Ł., Olszak, M., Perlikowski, D., Kosmala, A. and Malinowski, R. 2021

The Plant Journal, 2021, 106: 1338-1355. https://doi.org/10.1111/tpj.15240

III.   Applying Biostimulants to Combat Water Deficit in Crop Plants: Research and Debate.

Jiménez-Arias D, Hernándiz AE, Morales-Sierra S, García-García AL, García-Machado FJ, Luis JC, Borges AA.

Agronomy. 2022 ; 12(3):571. https://doi.org/10.3390/agronomy12030571

IV.   Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario

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V.   Priming with Small Molecule-Based Biostimulants to Improve Abiotic Stress Tolerance in Arabidopsis thaliana

Hernándiz, Alba E., Carlos E. Aucique-Perez, Sanja Ćavar Zeljković, Nikola Štefelová, Sara Salcedo Sarmiento, Lukáš Spíchal, and Nuria De Diego. 2022

Plants 11, no. 10: 1287. https://doi.org/10.3390/plants11101287

CONTRIBUTIONS

I.   Supplement I: As co-author of this publication, AEH participated in this work on executing the *in vitro* protocol and operating the phenotyping platform and sampling.

II.   Supplement II: As co-author of this publication, AEH was in charge of implementing the existing protocol for phenotyping pea plants under drought conditions. AEH also daily operated the phenotyping planform and irrigated the plants. At the end of the experiment, AEH performed the sampling and processed and analyzed the obtained phenotyping data.

III.   Supplement III: As co-author of this work, AEH was involved in the nursery tasks, transplanting to the field, controlling the water status of the soil, collecting samples, harvesting, processing the maize cobs, and reviewing/correcting the manuscript.

IV.   Supplement IV: As the first author of this publication, AEH designed and performed the experiments related to the work, collected the samples, harvested the plant production, and analyzed the yield quality and quantity. AEH also wrote the manuscript.

V.   Supplement V: As the first author of this publication, AEH was fully involved in all the phases of the work. AEH designed and performed the experiments, operated the phenotyping platform, carried out the final sampling of the plants, analyzed the data, and wrote the manuscript.

ABBREVIATIONS LIST

A	Assimilation rate
aa	Aminoacids
ABA	Abscisic acid
AcOrn	N- acetylornithine
AcPut	N- acetylputrescine
Agm	Agmatine
AGR	Average growth ratio
APX	Ascorbate peroxidase
Asn	Asparagine
Asp	Aspartic acid
AUC	Area under the curve
AZ	Natural barley mutant AZ34
BABA	β- aminobutiric acid
BOP	Block Buffer Optimization Pack
BSs	Biostimulants
CAT	Catalase
CH	Carbohydrates
Chl a	Chlorophyll a content
Chl a/ Chl b	Ratio between chlorophyll a and chlorophyll b
Chl b	Chlorophyll b content
Ci	Internal carbon concentration
Cis	Cystine
CO2	Carbon dioxide
DAP	1,3- diaminopropane
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
DNA	Deoxyribonucleic acid
DW	Dry weight
E	Transpiration
ECc	Electrical conductivity of the saturated extract
ETc	Crop evapotranspiration
ETo	Reference evapotranspiration

ETR	Electron transport rate
FAO	Food and agriculture organisation
FC	Field capacity
FG	Final rosette area
FW	Fresh weight
GABA	γ- aminobutyric acid
GB	Glycine betaine
GC-Slope	Slope of the growth curve
GLI	Green leaf index
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GR	Glutathione reductase
gs	Stomatal conductance
H202	Hydrogen peroxide
HI	Harvest index
HomoSpd	Homospermidine
HTP	High throughput platforms
HTPPs	High throughput phenotyping platforms
IR	Infrared
L/W	Flag leaf ratio between length and width
MS	Murashige and Skoog medium
MSB	Menadione sodium bisulfite
NGRDI	Normalized green red difference index
NorSpd	Norspermidine
Orn	Ornithine
PA	Polyamine
PAs	Polyamines
PBCI	Plant biostimulant characterization index
PCA	Principal component analysis
PEG	Polyethylene glycol
PG	L-pyroglutamic
POD	Peroxidase
Pro	Proline

PSII   Photosystem II

Put   Putrescine

RGB   Red Green blue

RGR   Relative growth ratio

ROS   Reactive oxygen species

RWC   Relative water content

SDGs   Sustainable development goals

Ser   Serine

smbBS   Small molecule-based biostimulants

SOD   Superoxide dismutase

Spd   Spermidine

Spm   Spermine

ThSpm   Thermospermine

Total Chl   Total chlorophyll content

Trp   Tryptophan

TW   Turgid weight

Tyra   Tyramine

VARI   Visible atmospherically resistant index

WD   Water deficient

WT   Wild type

WUE   Water use efficiency

WUEinst   Instantaneous water use efficiency

WUEint   Intrinsic water use efficiency

WUEp   Production water use efficiency

WW   Well watered

ΦNPQ   Non photochemical quenching

ΦP   Coefficient of photochemical capacity/quenching

ΦPSII   Light-adapted maximum quantum yield of the photosystem II

Ψ_w Plant leaf water potential

Ψ_π Plant leaf osmotic potential

INTRODUCTION

Agriculture is the activity that provides the more considerable amount of the food and raw materials which humanity needs. The current production is mainly based on long-term unsustainable agricultural practices such as extensive fertilizers and pesticides and not rational irrigation management. This lousy management is causing the degradation and pollution of the natural resources, and this phenomenon is being worsened by the climate change-derived consequences. In order to help the crops to cope with adverse situations, environmentally friendly alternatives are appearing in the last years. Biostimulants are a promising tool that enables plants to "be prepared" for the stress conditions and perform better. In addition, the European Union legislation supports their use to move towards a "greener" agriculture. However, a big part of the already commercialized biostimulants is based on complex mixtures from seaweed extracts and protein hydrolysates, recycled from by-products of the agri-food industry. These products present two main issues: the difficulty of identifying the active substances that induce better plant performance and the possible lack of homogeneity amongst batches. The small molecule-based biostimulants are an excellent alternative to complex mixtures. Since they are pure substances, the mentioned problems are irrelevant because studying their mode/mechanism of action is straightforward. Besides, a better understanding of the biostimulant effects on the plant opens the door for studying the combined action of two or more pure substances, given their single effects. Additionally, they can help characterize the complex substance as positive controls.

The simultaneous analysis of many traits as possible along the crop life cycle can make more accessible the characterization of the biostimulant mode/mechanism of action. It can also be relevant to study the induced response at certain critical moments or under adverse conditions. In this scope, phenotyping approaches, specifically high throughput phenotyping (HTP) platforms, can be beneficial tools. These platforms are equipped with one or more devices monitoring the crops in canopy or single plants. Plant breeding is already exploiting these devices to relieve the time-consuming traditional phenotyping tasks, and recently, biostimulant testing assays are also using them.

This doctoral thesis evaluates the effect of certain small molecule-based biostimulants on plants to explain the mode/mechanism of action.

AIM AND SCOPES

This thesis aims to use phenotyping approaches to determine, characterize, and describe the tolerance mechanisms of different plant species grown under abiotic stress to identify the main metabolite pathways and, consequently, specific molecules, such as small molecule-based biostimulants, that enhance their performance under adverse growth conditions. To achieve this goal, we proposed the following partial objectives:

Objective n°1: To evaluate the Olophen platform for studying different plant species grown under abiotic stresses.

Objective n°2: To evaluate the effect of the polyamines putrescine and spermidine on the production, yield, and quality yield parameters of maize subjected to water deficit. This work was performed as the following study of the positive results obtained by Ugena, 2019, in which the seed priming with small molecule-based biostimulants improved maize emergence under optimal and stress conditions.

Objective n°3: To characterize the mode of action of the selected metabolites, the non-proteinogenic amino acid ornithine (Orn) as a precursor of the polyamine (PA) biosynthesis, putrescine (Put) as the most effective PA improving plant growth and stress tolerance, and 1,3- diaminopropane (Dap) as a product of the final PA catabolism of the polyamines, using Arabidopsis (*Arabidopsis thaliana* L.) as a model plant.

Objective n°4: To evaluate Ornithine as a possible small molecule-based biostimulant in crops of agricultural interest such as barley (*Hordeum vulgare* L.) under water restriction conditions.

LITERATURE REVIEW

1. Agriculture current challenges

Agriculture provides the more significant part of the world's food and fabrics and other materials such as paper and wood for construction. Agricultural land contributes, directly or indirectly, to approximately 90% of food calories (Cassidy et al., 2013) and to 80% of protein and fats (through livestock production) (Steinfeld et al., 2006), which are consumed by the world's population. For these reasons, it is a key element for food security, as well as to accomplish the Sustainable Development Goals (SDGs) set by the United Nations (UN), specifically the SDG-2: Zero Hunger (Viana et al., 2022). The world's population is expected to reach 10 billion by 2050, increasing the food requirements by 50%, especially in the developing countries, and by that date, the rate of undernourished people will increase up to 2 billion (FAO, 2017). The food production to nourish an increasing society is a challenge that agriculture has partially met by increasing the arable land (Boserup, 2014). However, this practice is no longer sustainable and, instead, the increment of the agricultural efficiency and enhancement of the yields should be boosted. The former significant increment of productivity in crops was accomplished after the Industrial Revolution, with the supply of fertilizers, pesticides, and freshwater through the irrigation systems. It was also improved by better management of the fields using fossil-fueled products and farm machinery (Yu and Li, 2022). The Green Revolution shacked the global agriculture system by increasing 250% world grain production.

Nevertheless, the energy input also paired with that increase by becoming 50 times bigger (Giampietro and Pimentel, 1993). Nowadays, the arable land and groundwater resources are already "impoverished" (Fróna et al., 2019). The use of agrochemicals induced by an excessive presence in the soil of N, P, and persistent pesticides deteriorate soil quality and contaminate groundwater resources, which poses a threat to human health since it is the primary source of drinking water (Srivastav, 2020). Another element that obstructs and affects food production is climate change. This phenomenon constitutes one of the biggest problems to address nowadays. It implies the increment of the atmospheric concentration of greenhouse gases, mainly carbon dioxide and (CO_2) and methane (Pachauri, R.K and Reisinger, 2007), affecting agricultural production worldwide. The maize and wheat global crop yield reduction caused by climate change reached 3.8 and 5.5%, respectively, and further declines in productivity are expected as temperature changes will exceed critical physiological thresholds (Lobell et al., 2011). Figure 1 reflects the changes in the global production of four major crops under the increment of the temperature of 2°C. The rising of the temperatures is not the only negative effect derived from the climate change; there is an evident change in the rainfall pattern, causing extreme meteorological phenomena such as the amplification of drought events and their frequency, duration, and intensity (Bouras et al., 2019; Tramblay et al., 2020). The lack of precipitation combined with the increment of the evapotranspiration, driven by high temperatures, leads to so-called agricultural drought (Maracchi, 2000). Drought is considered multidimensional stress affecting plants at various developmental stages, especially critical for seedling emergence and establishment (Blum, 1996). In addition, a drought at later phenological stages might also cause a reduction in plant size, leaf

area, and plant production (Anyia and Herzog, 2004; Rosales-Serna et al., 2004; Fazeli et al., 2007; Barnabás et al., 2008).

Another consequence of extended drought conditions is the significant increase in salinity in the root-zone soil, which would most likely occur in irrigated areas, but not exclusively (Corwin, 2021). Salinity represents a harmful and widespread source of stress, and it is a major environmental constraint to crop productivity throughout the arid and semi-arid regions (Carpici et al., 2009). Saline stress can also obstruct and/or delay seed germination and seedling emergence. Soil salinity has a detrimental effect on plants, often observed in two stages: the osmotic phase, characterized by a rapid response to the elevated osmotic pressure; and the so-called ionic phase, represented as a slower response due to the accumulation of Na^+ in leaves (Munns and Tester, 2008).
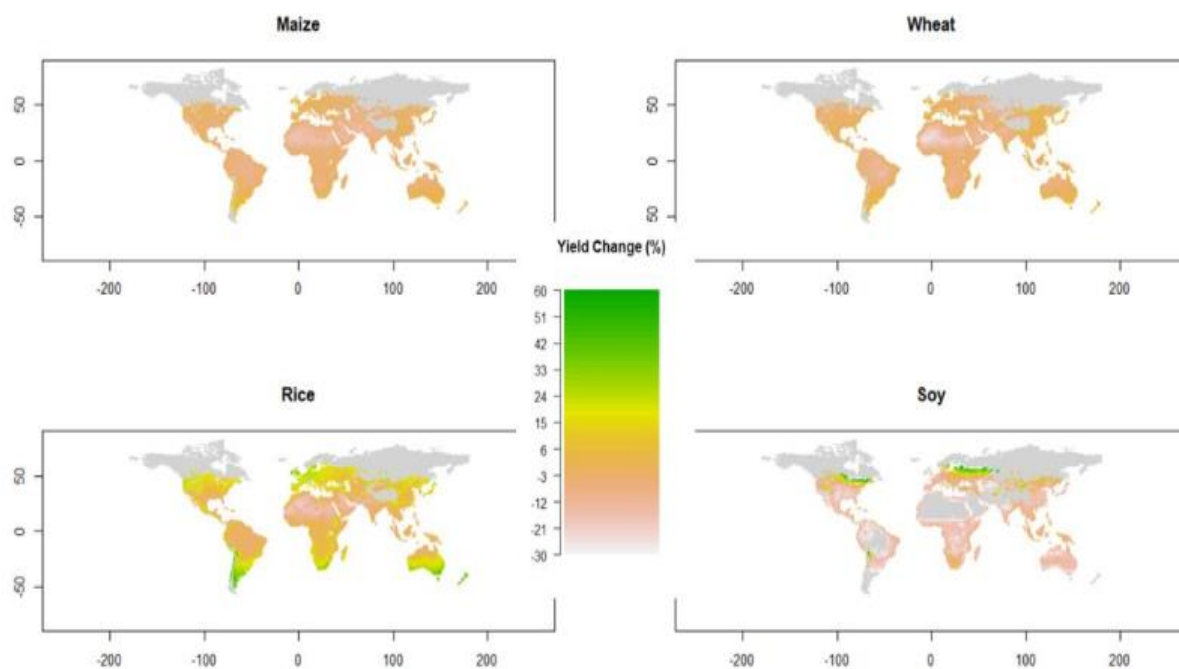

Figure 1. Forecast of the variation of the yield of maize, wheat, rice, and soybean in a 2°C increment scenario (Hertel, 2018); adapted from (Moore et al., 2017).

2.   Plant responses to stress

Through evolution, plants have developed a wide range of complex and efficient mechanisms to detect, react, and adapt to many environmental changes in response to the unfavorable situations they are exposed to throughout their life cycle. Environmental factors directly influence the physiological and morphological processes and hence, plant growth and yield. The unfavorable environmental factors such as drought reduce plant growth and yield, but when it is prolonged or very intensive can cause damage to the plant structures and even death (Sofia et al., 2013; Mosa et al., 2016). The concept of stress has been discussed amongst biologists since the 1930 (Levitt, 1985); and although many definitions have been proposed (Lichtenthaler, 1998; Gaspar et al., 2002; Souza and Mendes Cardoso, 2003), there are two common points in all of them: the description of an alteration in the environment,

which affect the plant; and the plant response to this change, as well as, the level of damage generated. According to the intensity and length of exposure to the stress, the plant might undergo four phases (Mosa et al., 2016), as described in Table 1.:

Table 1. Summary of the four phases of a plant under stress, according to its duration and severity.

Phase	Plant Status	Description	References
Response	**Alarm**	Alteration of the plants normal physiological condition and activation of the stress alleviation mechanisms, after sensing the change in the optimal growing conditions	(Lichtenthaler, 1998)
Restitution	**Resistance**	Adaptation process while the stressors still affect the plant development; establishment of new physiological thresholds and achievement of the maximum resistance level.	(Lichtenthaler, 1998)
End	**Exhaustion**	The presence of the stressor overcomes the maximum resistance level; reduction of the physiological functions, possible damage, and/or death.	(Lichtenthaler, 1998)
Regeneration	**Partial/ Full recovery**	Resume of normal physiological functions after the stressor disappears, survival of the plant if the senescence process is not predominant.	(Lichtenthaler, 1998) (Kranner et al., 2010)

The different stress categories are classified according to the following factors:

Table 2. Classification of the plant stressors according to their origin, period of exposure, and effect on plants.

Classification	Name	Description	Reference
Stressor origin	**Biotic**	The stress is a result of the interaction between the plant and another living organism	(Kranner et al., 2010)
	Abiotic	The stress is caused by non-living factors, such as environmental or nutritional conditions	(Mosa et al., 2016) (Andjelkovic, 2018)
Exposure period	**Short term**	Might be overcome by plants	(Lichtenthaler, 1996)
	Long term	Might cause significant and irreversible injuries in plants	(Kranner et al., 2010)
Effect on plants	**Eustress**	Positive or stimulating effect on the plant development	(Lichtenthaler, 1998)
	Distress	Negative effect on the plant development	(Kranner et al., 2010)

2.1.   Plant responses to abiotic stresses derived from climate change

Climate change boosted by human activities is a process much more accelerated than natural global climate change. Due to that fact, most plants will struggle to adapt to a fast-changing environment. According to Reynolds and Ortiz (2010), the major abiotic stresses expected to increase in response to climate change are drought, heat, salinity, and floods. Drought is expected to significantly influence the reduction of crop productivity linked to global warming. By the end of the twentieth century, 30% of the land will be exposed to extreme drought (Burke et al., 2006). One of the impacts of extended drought conditions is the significant increase in salinity in the root-zone soil, which would most likely occur in irrigated areas, but not exclusively (Corwin, 2021). The evident link between drought and salinity stress and its relevance for agricultural production turned them into an obvious target for breeding programs and research aimed to provide knowledge to enhance plant tolerance.

2.2.   Drought

Drought is generally understood as a period without rainfall (Shao et al., 2008; Jaleel et al., 2009). Nevertheless, this definition has been adapted according to different contexts; for example, from the agricultural and physiological point of view, drought stress occurs when the soil moisture is reduced over a certain level from which the available water for plants is scarce (Shamsi, 2010; Dai, 2013). Besides, water stress, understood as a deficiency, occurs when the transpiration rate from leaf surfaces is higher than the water uptake from roots. Due to the sessile nature of the plants, they developed various mechanisms, made possible by physiological, morphological, phenological, biochemical, and molecular responses, to cope with the stress to a greater or lesser extent. The response of the plants to the stress can range from molecular to a whole plant level, and they originate the strategies which plants can benefit from to overcome stress periods:

I.   **Escape.** The shortened life cycle or seasonal growth allows the plant to develop early flowers and reproduce before the water scarcity onset, although the yield is generally reduced (Bray, 2007; Khan et al., 2011). The phenological development of the plant matches periods with water availability.

II.   **Avoidance**. The control of transpiration by stomatal movement and the maintenance of the water uptake from soil thanks to better development of the root system are the key factors for this strategy (Bray, 2007; Farooq et al., 2009a).

III.   **Tolerance.** The reduction of the number and area of leaves (Bray, 2007), as well as the development of xeromorphic traits such as hairy leaves, trichomes (Farooq et al., 2009a; Khan et al., 2011), and essentially a great and dense root system (Farooq et al., 2009a) are characteristic features of this strategy. In addition, the osmotic adjustment, induction of antioxidant system, alteration in metabolic pathways, root/shoot ratio increment, and closure of stomata are also mechanisms participating in the tolerance strategy.

Water is the major component of the plant fresh biomass and plays a vital role in many physiological processes related to plant growth, development, and metabolism (Brodersen et al., 2019). Although the drought-induced symptoms vary according to species, developmental stage, the intensity of the stress, and other multitudes of factors, it can induce leaf loss of turgor, wilting, etiolation, yellowing, and premature leaf falling (Bernacchia and Furini, 2004; Farooq et al., 2009a; Jaleel et al., 2009; Bhargava and Sawant, 2013; Sapeta et al., 2013). One of the earliest effects of the drought on plants is reducing the relative water content (RWC) (Farooq et al., 2009a). Dehydration reduces the plant water potential and turgor, affecting the cell normal function, including expansion and division (Shamsi, 2010; Rahdari et al., 2012), essential processes for the plant growth and establishment (Bhargava and Sawant, 2013). Drought also negatively affects the quantity and quality of biomass and yield in crops (Jaleel et al., 2009; Zlatev and Lidon, 2012; Nezhadahmadi et al., 2013).

The water limitation could reduce the number of leaves and changes their anatomy, decreasing the stomata number and inducing the stomata closure, or modifying the cell wall thickening, cutinization of surfaces, the number of large conductive vessels, submersion of stomata (succulent and xerophyte),

leaf rolling (cereals), among others. These responses negatively affect net photosynthesis (Shao et al., 2008; Ding et al., 2013), reducing plant size. The stomata closure also limits transpiration, increasing the leaf temperature. High temperatures in leaves might cause protein denaturation, affecting the enzymes and changes in membrane flexibility. Besides, drought-induced leaf area reduction, stomatal closure, and elevated temperature negatively affect plant photosynthesis. Reduced CO_2 due to stomatal limitations also decreases photosynthetic electron transport components and, as a consequence, limits the molecular oxygen and produces reactive oxygen species (ROS) and H_2O_2, which can cause oxidative damage in the chloroplasts (Shao et al., 2008; Zlatev and Lidon, 2012; Bhargava and Sawant, 2013; Nezhadahmadi et al., 2013). On the other hand, reduced CO_2 uptake is the main element reducing the assimilation rate due to a decrease in the enzymatic reactions involved in CO_2 reduction. This fact produces an imbalance between the light and dark photosynthesis reactions and enhances the ROS accumulation in chloroplasts (Farooq et al., 2009a; Bhargava and Sawant, 2013; Nezhadahmadi et al., 2013). Finally, depending on the duration and severity of the stress, the plants can end with a decrease in the chlorophyll contents, conditioning light-harvesting capacity (Shamsi, 2010; Sapeta et al., 2013).

Drought also affects the radicular system. A water limitation can reduce ion uptake, mineral nutrition, and the synthesis of macromolecules (Zlatev and Lidon, 2012; Bhargava and Sawant, 2013; Rana et al., 2013; Sapeta et al., 2013). However, certain species can expand the root system to increase the capacity for water uptake (Shao et al., 2008; Farooq et al., 2009a; Franco, 2011; Bhargava and Sawant, 2013). When drought reduces the mineral nutrition, the cells lose ion homeostasis (Bray, 2007; Amin Kheradmand et al., 2014). Water deficit induces the N accumulation and reduces P and Ca levels (Shao et al., 2008; Farooq et al., 2009b; Bhargava and Sawant, 2013).

Water limitation also reduces the plants' protein content due to a reduced synthesis or degradation under severe stress conditions. Only the proteins, enzymes, and transcription factors involved in plant stress response increase under drought stress (Farooq et al., 2009b; Xoconostle-Cázares et al., 2010; Rahdari et al., 2012; Zlatev and Lidon, 2012; Ding et al., 2013; Nezhadahmadi et al., 2013; Labudda and Azam, 2014). There are different stress responses to drought. However, the processes involved in plant tolerance are the ones that take the greater attention of the scientific communities. For example, one of the most studied processes is the biosynthesis of compatible solutes or osmoprotectants in plants under drought because they participate in the osmotic adjustment mechanism, which is essential for plant tolerance to drought and salinity (Rao et al., 2006; Koyro et al., 2009; Labudda and Azam, 2014). The compatible solutes accumulate into the cells without causing any damage (Xoconostle-Cázares et al., 2010; Rahdari et al., 2012) and contribute to the increment of the cellular osmotic pressure to facilitate the water uptake from the soil. In addition, they also regulate the osmotic balance between vacuole and cytosol, maintain cell turgor pressure, prevent water loss, and protect the macromolecules from destabilization (Rao et al., 2006; Koyro et al., 2009).

Plants also developed specific mechanisms to reduce the drought-induced alterations between membrane lipids and proteins, which decreases the transport capacity of the bilayer (Farooq et al., 2009a; Amin Kheradmand et al., 2014) and ends with lipid peroxidation due to the oxidative damage

(Farooq et al., 2009a; Mafakheri et al., 2010; Rahdari et al., 2012). As a response to the oxidative stress induced by ROS accumulation, known as secondary stress plants, plants activate their antioxidant defense system via enzymatic (SOD, CAT, POD, APX, GR) (Bray, 2007; Jaleel et al., 2009; Zlatev and Lidon, 2012) and non-enzymatic (Glutathione, ascorbic acid, carotenoids, α- tocopherol) (Farooq et al., 2009a; Jaleel et al., 2009; Bhargava and Sawant, 2013) components which contribute to the ROS scavenging.

2.3.   Salinity

Salinity is defined as the concentration of dissolved mineral salts in the soil solution or water (Hu and Schmidhalter, 2004). The problem with the saline soils is that their salt content in the root zone is high enough to disturb plant growth and development. The extent of the damage depends on the plant species, variety, growth stage, environmental factors, and nature of the salts. Thus, the thresholds to define saline soils are challenging to set (Yadav et al., 2011). In response to this issue, the USDA Salinity Laboratory (Bernstein et al., 1954) established that soils with an electrical conductivity of the saturated extract (ECc) of 4dS m^{-1} or more are saline soils. The FAO has accepted this definition and specified that soils with an ECc > 15dS m^{-1} are considered strongly saline (FAO, 1996). Although the saline soils can exist naturally, the climatic factors, water management, and, more importantly, the anthropogenic actions can accelerate the salinization (Yadav et al., 2011). The non-rational use of fertilizers and irrigation is increasing this problematic issue, and it is estimated that by the end of this century, it will affect up to 50% of agricultural land (Shahid et al., 2018).

High salt concentration alters the morphological, physiological, biochemical, and molecular normal functioning of the plants, preventing the proper plant growth and development (Rahneshan et al., 2018). Traditionally, plants have been classified according to their tolerance to the salt in the soil; the "halophytes" are plants that can complete their life cycle in high salt concentrations, whereas the "glycophytes" cannot tolerate such concentrations of salt (Flowers et al., 1977). The response of the plants to salinity is generally explained in two phases:

I.   **Ion-independent shoot response**; occurs in a range from minutes to days and is probably related to the Na$^+$ sensing and signaling (Gilroy et al., 2014; Roy et al., 2014). In this first phase, the salinity can affect the water status, inducing stomatal closure and leaf rolling (Munns and Termaat, 1986), rapidly decreasing the plant growth (Munns et al., 1995).

II.   **Ion-dependent response**; develops in a period from days to weeks and is linked to the accumulation of ions, up to toxic concentrations, in the shoot (Munns and Tester, 2008). This accumulation is evident in old leaves, where it induces senescence. It can also affect the yield and cause plant death (Munns and Tester, 2008). It is a much slower process than the previous phase (Munns et al., 1995).

The main salt tolerance mechanisms were proposed by Munns and Tester (2008) as follows:

I.   **Ion exclusion**; exclusion of the toxic ions from the shoot.

II.   **Tissue tolerance**; compartmentalization of toxic ions into specific tissues, cells, and organelles.

III.   **Shoot ion-independent tolerance**; maintenance of growth and water uptake regardless of the accumulation of Na^+ in the shoot.

Most of the existing plants, including economic crops, are glycophites. Therefore, their growth and development are affected by salinity due to the low (negative) osmotic potential of the water in the soil, which induces water stress, nutritional imbalance, specific ion effect (salt stress), or the combination of these three factors (Ashraf, 1994). The presence of salt in high concentrations lowers the soil matric potential, reducing the water available for the plants. As a response, the plant leaf water (Ψw) and osmotic potential ($\Psi \pi$) reach more negative values, disturbing the plant water relation (Arif et al., 2020). It can finish with the turgor loss in the cells. Salinity also induces ROS that has fatal consequences in the plant, including the damage of unsaturated fatty acids (Ahmad et al., 2019), the protein alteration by site-specific amino acids modifications, the fragmentation of the peptide, the increased susceptibility to proteolysis, and the DNA damage by provoking deletions, mutations and other fatal effects (Tuteja et al., 2009). To deal with the water loss and ROS increase, plants accumulate compatible solutes to maintain the cell turgor and regulate stomatal conductance (Kosar et al., 2019). Many molecules are compatible solutes, including carbohydrates, organic acids, free amino acids, and polyamines. The amino acid proline is considered a stress related-molecule and the most versatile osmolytes because of its antioxidative capacities upregulating the synthesis of membrane proteins involved in water uptake, mitigating the ion toxicity, and controlling the cellular homeostasis (Chourasia et al., 2022). The non-proteinogenic amino acid γ-aminobutyric acid (GABA) also accumulates in stress events and acts as a ROS scavenger. GABA synthesis also provides CO_2 to the plant when the net photosynthesis is limited due to the stress, allowing the plant to maintain its metabolism (De Diego et al., 2013). Besides, after a severe stress situation, GABA has been proved to help the plant recover (Carillo, 2018).

Salinity severely affects the root development (Otsuka et al., 2021) by decreasing its length and surface, the number of lateral roots, root hairs, and dry matter content (Shannon and Grieve, 1998), but also by disrupting the cell membrane and ionic homeostasis, as a result of the osmotic stress. The reduction of the root growth results from the inhibition of the cell division and expansion induced by the stress. It also affects the absorption of essential nutrients (e.i. K^+, Ca^{2+}, and NO_3^-) negatively (Shaterian et al., 2005) and enhances the accumulation of Na^+ and Cl^- ions happens, which are toxic for plants and disturb physiological and biochemical processes such as photosynthesis, protein synthesis and damage cell organelles (Zörb et al., 2019).

The salt concentration also affects shoot growth by reducing cell expansion and lateral bud development (Munns and Tester, 2008). Therefore, it is commonly observed the reduction of the shoot length, total leaf area, leaf area index, and the number of branches (Läuchli and Grattan, 2007). The accumulation of Cl^- ions also can accelerate the leaf abscission due to the ethylene biosynthesis (Acosta-Motos et al., 2017), ending in lower shoot growth and biomass production. In the leaves, the photosynthetic assimilation can also be reduced by salt stress, starting with reduced transpiration and an induced stomatal closure, directly affecting the atmospheric CO_2 income (Kitayama et al., 2020).

Besides, the mineral uptake is also reduced, so it conditions the content of N and Mg, two essential elements for the chlorophyll synthesis (Kaya et al., 2009), which ends with an apparent reduction of the chlorophyll a and b content in salt-stressed plants (Zhu et al., 2020). In addition, the PSII reaction centers are disrupted, and the plastoquinone acceptor activity is restricted (Sobhanian et al., 2011).

Salinity has also been reported to affect the vascular system, reducing and increasing the thickness of the cortex and xylem, respectively (Dolatabadian et al., 2011). It has been reported that Na^+ ions come to the shoot by the xylem and it accumulates there, whereas the K^+ content decreases (Gibberd et al., 2002). Na^+ also accumulates in the phloem, causing a reduction in the flux volume of these vessels (Aliche et al., 2020; Chourasia et al., 2022). Consequently, the delivery of photo assimilates from the sink to the source can also be altered by salinity.

Although plants are more sensitive to salt stress in the early seedling and reproductive phases, proper functioning of the plant metabolism is required to maintain the yield under saline stress conditions (Chourasia et al., 2022). Thus, the plant's susceptibility to salinity at any developmental stage will be translated into a penalty in the final production. Altogether, it is crucial to identify technologies and varieties that can reduce the salinity-induced yield penalty.

3. Biostimulants as a tool to alleviate the stress symptoms

Biostimulants (BSs) are considered an innovative agronomic tool, there is much research focused on these substances, and their market is in constant expansion (Povero et al., 2016). Scientist, researchers, and agronomists have widely discussed their definition; Rouphael and Colla (2020) collected some of the most important definition attempts. Nevertheless, the most accepted was the one proposed by du Jardin (2015), who established that "A plant biostimulant is any substance or microorganism applied to plants to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content." BSs have been regulated according to national rules and included in the category of fertilizers until the approval of the European Fertilizer Regulation (EU) 2019/1009 (Regulation (EU) 2019/1009), which will apply from July 2022. This fact converts the European Union into the first governing organism to recognize the plant BSs as a different category of agricultural inputs. In this regulation, a new harmonized definition is introduced, where BSs are considered "a product stimulating plant nutrition processes independently of the products 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, and availability of confined nutrients in soil or rhizosphere." BSs comprise a wide range of substances, and despite many classification proposals (Yakhin et al., 2017), the widely accepted is the seven categories proposed by du Jardin (2015): humic and fulvic acids, protein hydrolysates and N-containing compounds, seaweed and botanical extracts, chitosan and other biopolymers, inorganic compounds, beneficial fungi, and beneficial bacteria. The active compounds found in those substances can range from phytohormones, free amino acids, free polyamines, carbohydrates, betaines, minerals, and others (Fuell et al., 2010; Sharma et al., 2014; Pacifici et al., 2015; Colla et al., 2017). An essential source of BSs is the waste and by-products from the food and agricultural industry; recycling organic waste to constitute a bio-

fertilizer is a traditional practice (Xu and Geelen, 2018). Moreover, the current "Circular economy" concept emphasizes converting the waste and used products into new sources that have reached the end of their life cycle. The European Union aimed to boost the use of biological wastes as new types of fertilizers [European Commission (2016b); Circular Economy: New Regulation to Boost the Use of Organic and Waste-Based Fertilisers. Brussels: European Commission]. Thus, the development of BSs from by-products was promoted as an innovative and environmentally friendly method to reduce waste production. This fact favored the popularity of the protein hydrolysates since they can be easily obtained from by-products of the agri-food industry (Colla et al., 2015).

Other BSs with interesting use are the seaweed extracts, for their content of complex polysaccharides, fatty acids, vitamins, phytohormones, and mineral nutrients (Battacharyya et al., 2015). While combining different compounds with different modes of action might synergistically enhance the BS's beneficial effect (Bulgari et al., 2019), there are some considerations regarding such complex mixtures. To begin with, the complexity of these substances makes it difficult to understand their effect on the plant physiology and metabolism, unveiling their mechanism of action (Baltazar et al., 2021). This is partially due to the problem of identifying and isolating the active substances present in small amounts and becomes costly in terms of time and resources (García-García et al., 2020). For seaweed extracts, protein hydrolysates, and humic and fulvic acids, the complete composition of all compounds is often unknown (Sible et al., 2021).

Moreover, the homogeneity of the batches can be compromised since plants and animal origin by-products, and seaweeds might have a different composition according to their developmental stage, seasonality, environmental conditions, and interaction with other organisms (Yakhin et al., 2017). Some authors have already stated the importance of selecting the optimal moment to obtain the raw materials to yield the most desired active substances (Parys et al., 2009; Apostolidis et al., 2011). While this practice can be applied to the harvest of seaweed and plant by-products, it is not so easily applicable to animal origin sources. Although an effort is being made to collect and process the BS sources under controlled conditions and analyze the final products, it is hard to guarantee the standardized production protocol and the total identification of the active principles, especially if they are scarce or partially unknown (Yakhin et al., 2017). As a possible solution to these inconveniences the use of small molecule-based BSs might ease the description of the physiological effect and the mode and mechanism of action and simplify the formulation, registration, and certification process (Yakhin et al., 2017; García-García et al., 2020). For these reasons, companies are putting much effort into developing new BS based on promising active substances (Bulgari et al., 2019) and their characterization, which might trigger the desired physiological responses in plants (Povero et al., 2016). This approach has been used to reveal the potential use of different molecules such as melatonin, γ-aminobutyric acid (GABA), α- tocopherol, and menadione sodium bisulfite (MSB) as plant stress alleviator (Borges et al., 2014; Li et al., 2017; Ali et al., 2019; Arnao and Hernández-Ruiz, 2019). As stated by García-García et al. (2020), BS products based on small pure substances could fight the negative consequences of abiotic stresses and enhance the nutritional and organoleptic qualities of the yields, increasing their value. In the light of these facts, the use of single or combined pure compounds is a new and convenient

niche of research that will participate in the improvement of the BS formulations and production and provide a better understanding of the underlying effects on the plants. Our research group is focused on the study and use of this compounds as BS (Ugena et al., 2018; Podlešáková et al., 2019). Moreover, the understanding and characterizing their mode of action would allow the use of those substances as positive controls to complement the evaluation of more complex BS.

4. Phenotyping approaches to understand the mode and mechanism of action of biostimulants.

The word "phenotype" comes from the Greek *phainein* (show) and *typos* (type) and stands for the external shape or appearance of the organism, which can be described by direct inspection or more refined techniques, and serves as a tool to distinguish the type of organisms (Johannsen, 1911). The study of the crop phenotype to select desirable traits is one of the keys to plant breeding activities (Sandhu et al., 2021). Traditional phenotyping approaches are very time-consuming and constitute the bottleneck of this activity, which cannot keep up with the advances in genetics. High throughput phenotyping (HTP) approaches have gained much attention recently since they allow the automatic and non-invasive monitoring of multiple morpho-physiological traits along the time, so the progression of the growth, plant performance, and stress response can be followed. Besides, they contribute to reducing the labor and time for the determinations. These factors successfully integrated HTP into the breeding programs (Araus and Cairns, 2014; Tardieu et al., 2017). The phenotyping activities aim to increment the accuracy and precision of the phenotype assessment to interpret it at the whole plant level better. At the same time, plant phenotyping based on non-invasive approaches reduces the labor cost through automation and remote-sensing technologies and allows data integration and experimental design (Omari et al., 2020). The concept of plant phenomics stands for "the high-throughput, accurate acquisition and analysis of multidimensional phenotypes along the crop growing stages at the organism level, including the cell, tissue, organ, individual plant, plot, and field levels" (Yang et al., 2020), has become an asset for both researchers and private companies. Nowadays, many HTP platforms have been developed and tested, from stationary devices to self-propelled ones, including unmanned aerial vehicles (UAV) that can carry the sensors. Other HTP platforms consist of fully automated facilities with precise environment control, which hosts a robot with the sensing equipment. Regardless of the platform type, the goal is to automatically obtain and collect plant images and other data that might be relevant for quantifying the genotype-environment interactions called "phenotype" (Fahlgren et al., 2015). In this regard, the following table 3 summarizes the most common sensors and their applications:

Table 3. Most common imaging techniques used for high-throughput plant phenotyping.

Imaging technique	Application	Reference
Visible, RGB	Computation of structural or morphological phenotypes, biomass, and plant growth	(Li et al., 2014; Yang et al., 2014)
Fluorescence, near-infrared and infrared	Analysis of chlorophyll, photosynthetic function, water content, reactive oxygen species signal, and temperature	(Li et al., 2014; Fichman et al., 2019)
Thermal infrared	Analysis of the plant temperature as a result of changes in the stomatal aperture and transpiration rate	(Zarco-Tejada et al., 2012; Li et al., 2014)
Hyperspectral	Provides insights into the functional properties of the plants as leaf tissue structure, pigments, water content, and stress resistance methods	(Mahlein et al., 2012; Wahabzada et al., 2016)

3D scanning techniques based on images (Fang et al., 2016) or laser scanning (Paulus et al., 2014) are also popular since they provide 3D models that calculate spatial and volumetric traits. Nowadays, the trend is to combine multiple techniques to benefit from the advantages they can provide (Yang et al., 2020). Multi-source data can better explain biological phenomena such as biotic and abiotic stress resistance (Song et al., 2021). Some works performed during the Ph.D. studies are presented as examples. In planta, the monitoring of pea plants (*Pisum sativum* L.) subjected to drought stress was found to have a decreased biomass production which correlated with a reduction in the photosynthetic functions, represented by a reduction in the light-adapted maximum quantum yield of PSII and the photochemical quenching, and an increment of the non-photochemical quenching (Blicharz et al., 2021; Supplement II). Interestingly, the most sensitive parameter was the leaf temperature, which revealed the difference between the irrigation treatments from the onset of the water restriction. Marchetti et al., (2019) developed a method for screening young barley populations under drought stress to identify biomarkers for future faster screenings. In addition, they were able to report the different sensitivities of mutant plants to the water deficit. Other studies were performed using *in vitro* experiments on specific platforms (e.i. XYZ Plant Screening™ system in Olophen, http://www.plant-phenotyping.org/db_infrastructure#/tool/57). These studies are based on the rosette growth of *Arabidopsis thaliana* L. seedlings grown under different stress conditions using a simple RGB camera (De Diego et al., 2017).

Considering the proven sensitivity and suitability of the HTP platforms to monitor large amounts of plants and provide many physiological traits, these devices are suitable for biostimulant screenings. Several works have validated our HTP screening based on Arabidopsis rosette growth for studying the mechanism of action of simple and complex substances [Bryksová et al., 2020 (Supplement I); Sorrentino et al., 2021]. The outcome of the analysis allows to categorize diverse substances into plant growth promotors/inhibitors or stress alleviators and describe their mechanism of action. The possibility of evaluating the biostimulants through *in vivo* experiments is also possible (Ugena, 2019) demonstrated that the polyamines putrescine (Put) and specially spermidine (Spd) used as seed priming agents can enhance the synchronicity and other emergence related parameters in maize under salt stress. In summary, the HTP technologies are key factors in developing faster and more efficient methods to characterize the biostimulants.

MATERIAL AND METHODS

1. Biological materials and growing protocol

Seeds of *Arabidopsis thaliana* (L.) Heynh. (Col-0 ecotype) were used for Supplement I and V. Seeds were surface sterilized with 70% Ethanol plus 0.01% Triton X-100 following the protocol described in (Ugena et al., 2018). The sterilized seeds were distributed homogeneously with the help of toothpicks autoclaved on a sheet of autoclaved filter paper moistened with sterile water under laminar flux chamber conditions. After 3-4 min, the filter paper containing the seeds was transferred into a square plate (120 × 120 mm, P-Lab, Ref. 212358.2) filled with sucrose-free half- strength solid Murashige and Skoog (Phytotechlab M519) medium (germination medium). The priming agents evaluated in Supplement II and IV were added to the germination medium. After the seed sowing, the square plates were sealed with micro-pore tape and kept in the dark at 4°C for 4 days. Then, the plates were positioned vertically in the growth chamber under controlled conditions: a 16/8h (light/dark) regime, a temperature of 22°C, an RH of 60%, and a light intensity of 120 µmol photons $s^{-1} m^{-2}$ (De Diego et al., 2017) for five days. Three days after germination, seedlings of similar size were manually transferred, under laminar flux chamber conditions, into 48 and 24-well plates (Jetbiofil, Guangzhou, China), one plant per well. Each of the 48 and 24-well plates was previously filled with 1× MS growth medium (pH 5.7; supplemented with 0.6% Phytagel). After the transfer, the well plates were sealed with transparent film manually perforated to allow the water and gas exchange and avoid water condensation that might make the image analysis difficult. Finally, the sealed well plates with plants were transferred to the OloPhen platform (http://www.plant-phenotyping.org/db_infrastructure#/tool/57), consisting of the PlantScreen™ XYZ system, where the growth conditions were set to simulate a long day (a 16/8 h light/dark cycle) with a temperature of 20/22 °C (day/night), an RH of 60% and an irradiance of 120 µmol photons $s^{-1} m^{-2}$.

Seeds from green pea (*Pisum sativum* L.) cv. "Walor" (PlantiCo Zielonki Sp. z o.o., https://plantico.pl), developed for fresh consumption or processed canned pea production, was used for the physiological characterization of the plants under drought stress included in Supplement II. Seeds were kept in autoclaved distilled water for 16 h, then transferred to a tray containing damp vermiculite closed with a transparent lid, and kept in a growing chamber until seeds germinated after two days. After that, germinated seeds were transferred to pots (11 x 11 x 12 cm, 1.5 L) and carefully placed at 2.5 cm deep. Pots were filled with the substrate Klasmann No. 11 soil (pH 6.3), and after the seeds were transferred, they were watered to achieve a soil moisture tension of pF 2.8 (Kirkham, 2014). Plants were grown under control conditions, with a 16/8 h light/dark regime, 25/23°C day/night temperature, a light intensity of 300 µmol $m^{-2} s^{-1}$, and relative humidity (RH) of 40%. The optimal irrigation was maintained for 5 days, and then, to achieve the drought condition, pots were left to dry gradually until pf 4.2, which was considered the stress threshold, and maintained for 7 days. After the drought, pots were irrigated to reach the optimal conditions and kept in the rewatering phase for 3 days. For the physiological characterization of pea responses under drought stress, the plants were monitored with the OloPhen platform for 10 days, including the drought and recovery period.

The material employed for Supplement III and IV was a local maize variety from Gran Canaria Island (*Zea mays* L. c.v. Lechucilla), provided by a local nursery. Seedlings were acquired in a 150-socket nursery tray one week after sowing. Then, plants were placed in a growth chamber under controlled conditions with a temperature of 22 °C, a photoperiod of 16/8 h light/dark, an RH of 60–70%, and a light intensity of 300–400 µmol m^{-2} s^{-1}. Plants in the V1 stage, when the lowermost leaf has a visible leaf collar (Zhao et al., 2012), were used for dose optimization experiments; and for the greenhouse experiments, the plants were transplanted to the soil 15 days after sowing. For Supplement III, an additional dosage optimization in hydroponic conditions was performed. The treatment solution was applied by submerging the roots for 24 h. After that, the plants were placed again in the hydroponic system. Once the plants were located in the hydroponic system, the stressor (20% PEG) was applied, and they were kept in those conditions for a week (Jiménez-Arias et al., 2019b). The greenhouse was located at the Escuela de Capacitación Agraria de Tacoronte (Tenerife), Canary Islands (28°29'47.0''N 16°25'12.0'' W), and the experiment was performed from June to August 2021. For this period, the average maximum and minimum temperatures were 30 and 22°C, respectively, and the RH average was about 80%. The soil is clay-loam (35% clay, 27% silt, 38% sand). The experiment was distributed in random blocks of 20 m^2 with three replicates, each block containing 80 plants.

A final experiment was performed using barley seeds (*Hordeum vulgare* L.) from the cultivar Steptoe as wild-type (WT) and an induced mutant line (AZ34) partially deficient in the abscisic acid (ABA) synthesis. This experiment was executed in a sequence of assays: first, transpiration curves were recorded for excised leaves that were kept in optimal conditions (water) or simulated osmotic stress (PEG) with or without 1 mM Orn, slightly modified from (Ceciliato et al., 2019). Second, individual plants were grown in pots in the PlantScreen™ Compact System under controlled conditions (16/8 h light/dark regime, 22/20 °C day/night, HR of 40%, and 320 µmol m^{-2} s^{-1}). Half of the plants were well-watered (60% of the pots field capacity), and the other half were subjected to controlled water deficit (30% of the pots field capacity). Additionally, two foliar treatments with 1 mM Orn were applied on half of the plants of each group, while the rest were sprayed with distilled water as a control treatment. This experiment was based on the canopy assay developed by Marchetti et al., 2019, which allows comparing 50 plants per variant to represent micro-populations. The plant morphology (growth-related parameters) and physiology (chlorophyll fluorescence and canopy temperature) were monitored using the PlantScreen™ Compact System from the OloPhen platform.

2. Chemicals

The four compounds (N⁹-substituted CK Derivatives With a Fluorinated Carbohydrate Moiety) were synthesized by a slightly modified one-step synthesis (Wan et al., 2005) of 9-(2´-deoxy-2´- fluoro-*β*-D-arabinofuranosyl) hypoxanthine with benzylamine or isopentenylamine hydrochloride as appropriate in the presence of BOP and DIPEA in DMF by a chemist from our department (Bryksová, 2020; Bryksová et al., 2020) and tested as described in Supplement I. The compounds were used as a priming agent in the Arabidopsis seeds and added at four concentrations (from 10^{-7} to 10^{-4} M) into the previously described *in vitro* Arabidopsis germination medium.

L-pyroglutamic acid (PG) (CAs number: 98-79-3) and glycine betaine (GB) (CAs number: 590-46-5) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). In Supplement III, PG and GB were applied at 0.1, 1, 2.5, 5 and 10 mM for the hydroponic dosage optimization. For the nursery trays dosage optimization GB at 0.1 and 0.05 mM and PG at 2.5 and 1 mM were evaluated. The best performing concentrations (1 mM PG, and 0.1 mM GB) were then used in the field experiment.

For the experiment regarding small molecules-based BSs, three polyamines (PAs), putrescine (Put) (CAS number 333-93-7), spermidine (Spd) (CAS number 124-20-9), and 1,3-diaminopropane (DAP) (CAS number 109-76-02) and the non-proteinogenic amino acid ornithine (Orn) (CAS number 3184-13-12) were used for the studies presented in Supplement IV and V, all purchased by Aldrich Chemical Co. (St. Louis, MO, USA). In Supplement IV, Put and Spd were applied at 0.01, 0.1, 0.5, 1, and 2 mM as drenching in maize seedlings for the dosage optimization. After that, the best-performed concentrations (0.1 mM Put and 0.5 mM Spd) were used in maize plants for the greenhouse experiment. In Supplement V, the amino acid Orn and two polyamines, Put and DAP, at 0.1 and 1 mM concentrations, were added as a priming agent into the *in vitro* Arabidopsis germination medium.

3. Instrumentation

For studying the pea response to drought stress (Supplement II), the PlantScreen™ Compact System (Photon Systems Instruments, Brno, Czech Republic) from the OloPhen platform was used. It consists of a growth chamber with controlled conditions equipped with conveyor belts that transport the plants from the "growing area" to the "monitoring area". The "monitoring area" is composed of two spaces; the acclimation room, where it is possible to subject the plants to dark adaptation, and the sensors room, where the infrared (IR), hyperspectral, and chlorophyll fluorescence sensors and the top and side view RGB cameras are located. The images from each sensor were automatically stored in the database server. The images were then evaluated with the specific software PlantScreen Data Analyzer. This experiment also monitored the soil moisture by measuring the soil pF with a ProCheck dielectric water potential sensor (MPS-6; Decagon Devices, Pullman, USA).

The PlantScreen™ Compact System (Photon Systems Instruments, Brno, Czech Republic) was also used to study the effect of Orn as a small molecule-based BS to improve drought stress tolerance in barley. The CO_2 fixation curves and the gas exchange parameters were also measured using an Infrared gas exchange system (Ciras3) from PPSystems (Amesbury, USA).

The HTP screening studies based on Arabidopsis rosette growth (Supplement I and V) were performed in a chamber equipped with the PlantScreen™ XYZ system (Photon Systems Instruments, Brno, Czech Republic), one of the OloPhen platforms. The chamber has a fully controlled environment, cool-white LED, and far-red LED lighting (Photon Systems Instruments, Brno, Czech Republic). The PlantScreen™ XYZ system consists of a robotically driven arm holding an RGB camera with a customized lighting panel and growing tables with approximately 7 m^2 where it can be placed 572 multi-well plates with fixed positions for accurate measurement of every plate. The XYZ robotic arm moves automatically above the plates to take RGB images (resolution 2500 x 2000 pixels) of a single plate

with a file size of approximately 10 MB in the PNG compression format. The images are stored in a database on a server, using a filename containing information about the acquisition time and the (x, y) coordinates of the camera. The imaging of each 24 and 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days.

For the Supplement III and IV a multi-detection plate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) was used to determine carbohydrates at an absorbance of 490 nm. Finally, the mineral content of the samples in Supplement III was determined with the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Avio® 500 (Perkin Elmer, Waltham, USA).

For the targeted analysis (free polyamines and amino acids) performed in Supplement V, a sonic bath (Bandelin, Germany), centrifuge (Prism, Labnet, USA), and vacuum chamber (Centrivapm Labconco, USA) were used for the sample preparation. After that, the extracted samples were quantified using a UHPLC-MS/MS [Nexera X2 UHPLC (Shimadzu Handels GmbH, Kyoto, Japan) coupled with an MS-8050 (Shimadzu Handels GmbH, Kyoto, Japan)]. Chromatographic separation was performed on an Acquity UPLC BEH AMIDE (Waters, Milford, USA) (50 × 2.1 mm; 1.7 µm particle size) with the appropriate pre-column.

For the chlorophyll quantification performed for the Preliminary Results, the incubator ThermoMixer C (Eppendorf, Hamburg, Germany), centrifuge 5415R (Eppendorf, Hamburg, Germany), and the spectrophotometer Synergy H4 Hybrid Reader (Biotek, Winooski, USA) were used.

4.   Biometric determinations

4.1.   *Growth related parameters*

The determination of the area under the growth curve (AUC) for the growth of Arabidopsis rosettes (Supplement V) was calculated as follows:

$$AUC = \sum\left(\left(s_i - s_{(i-1)}\right)/2\right) \qquad\qquad \text{(Eq. 1)}$$

where s_i corresponds to the rosette size at the moment i, and $s_{(i-1)}$ the rosette size in the immediately previous measurement.

The relative growth ratio (RGR) was calculated as,

$$RGR = \left[\left(ln(s_2) - ln(s_1)\right)/(t_2 - t_1)\right] \qquad\qquad \text{(Eq. 2)}$$

where s_1 and s_2 correspond to the initial and final size, respectively, and t_1 and t_2 stand for the initial and final time, respectively (Supplement V). The same formula but replacing s_1 and s_2 with the initial and final dry weight was used in Supplement III and IV.

The absolute growth ratio (AGR) was calculated as,

$$AGR = \left[(s_2 - s_1)/(t_2 - t_1)\right] \qquad\qquad \text{(Eq. 3)}$$

where the components s and t correspond to the same variables described above (Supplement I)

For the Supplement III and IV, ten randomly selected plants per variant were used to determine morphometric parameters such as plant length (from insertion to the soil until the base of the flower) and width (at the middle of the stem length), and the length and width of the last fully developed leaf. The biomass production was reported through the dry mass of the plants (mass, g or mg), which was calculated after harvesting the plants and oven-dry them for 48 h at 85°C.

The water use efficiency (WUE) for maize plants (Supplement III and IV) was calculated as follows (Kuglitsch et al., 2008):

$$WUE = \ Plant\ Biomass\ (DW)/Total\ Irrigation, \qquad \text{(Eq. 4)}$$

using the plant dry weight (mg) as the biomass and the total irrigation (mL) provided.

4.2.   Colorimetric index

The color index green leaf area (GLI) was calculated in Arabidopsis seedlings (Supplement I and V) from the combination of the particular color channels red (R), green (G), and blue (B) extracted from each pixel within the plant mask according to the formula as described by Ugena et al., (2018):

$$GLI = [(2G - R - B)/(2G + R + B)], \qquad \text{(Eq. 5)}$$

The color indices visible atmospherically resistant (VARI) and normalized green red difference index (NGRDI) were also calculated for the Supplement I, according to the following formulas described by Ugena et al., (2018):

$$VARI = [(G - R)/(G + R - B)] \qquad \text{(Eq. 6)}$$

$$NGRDI = \ [(G - R)/(G + R)] \qquad \text{(Eq. 7)}$$

4.3.   Relative water content

The relative water content (RWC) was calculated in pea (Supplement II) and maize plants

(Supplement III) according to the formula:

$$RWC = \ (FW - DW)/(TW - DW), \qquad \text{(Eq. 8)}$$

where FW stands for fresh weight, DW stands for dry weight, and TW stands for turgid weight.

4.4.   Production parameters

The production water use efficiency (WUEp) was calculated in maize plants (Supplement III and IV) as follows (Kiziloglu et al., 2009):

$$WUE_p = Final\ yield/ET_c, \qquad \text{(Eq. 9)}$$

where the final yield represents the total production (kg) estimated for one hectare, and the ETc corresponds to the accumulated effective crop evapotranspiration for maize. This parameter is obtained

from the reference evapotranspiration (ETo) and the crop coefficient (Kc), according to (Allen, R G; Pereira, L S; Raes, D; Smith, 1998):

$$ET_c = ET_o * K_c \qquad \text{(Eq. 10)}$$

The estimated yield per hectare and harvest index (HI) of maize (Supplement III and IV) were calculated, respectively, as follows:

$$Yield/ha = \ Avg\ Kernel\ Weight/cob * Avg\ Cob\ n^{\underline{o}}/plant * Estimated\ Plants/ha \quad \text{(Eq. 7)}$$

$$HI = \ Total\ KW/Avg\ Cob\ FW \qquad \text{(Eq. 11)}$$

where Total KW corresponds to the weight of all the dry kernels per cob, and Avg Cob FW corresponds to the mean fresh weight of the cobs.

The number of adequately developed maize cobs per plant and variant was counted (Supplement III and IV). The cobs to evaluate production were weighed to record the fresh weight and then oven-dried at 65°C for one week to reduce the possible bias due to different moisture levels. The dry cob weight, the number and total weight of all kernels per cob, and the weight of 100 kernels (in triplicate from each cob) were registered. In addition, the length and circumference of the cob (number of kernels) and the cob diameter (cm) were registered.

5. HPLC method

The analysis of the free amino acids and total and free polyamines in Arabidopsis rosettes (Supplement V) was performed as described by (Abdelhakim et al., 2022). Briefly, for the analysis of free amino acids, pulverized plant material (3–5 mg) was mixed with 1 mL of 50% EtOH and sonicated for 10 min (Bandelin, Germany). After centrifugation (Prism, Labnet, USA) at 14,500 g, the supernatant was transferred into the new vial and kept at 20°C until analysis. A 200 µL of supernatant was evaporated to dryness at 40°C under a vacuum (Centrivapm Labconco, USA) and re-dissolved in the mobile phase. For the quantification, UHPLC-MS/MS analysis was performed on Nexera X2 UHPLC (Shimadzu Handels GmbH, Kyoto, Japan), coupled with MS-8050 (ShimadzuHandels GmbH, Kyoto, Japan). Chromatographic separation was performed on an Acquity UPLC BEH AMIDE (50 x 2.1 mm; 1.7-µm particle size) (Waters, Milford, USA) with an appropriate pre-column. All target amino acids were separated using a binary gradient consisting of 15 mM formic acid, pH 3 (component A), and 0.1 % formic acid in ACN (component B).

For free polyamines, 200 µL of 2 M NaOH was added to 200 µL of supernatant from the amino acid extraction, followed by 2.5 µL of benzoyl chloride (in MeOH, 50:50, v:v), and after vortexing for 5 s, the reaction mixture was stirred for 40 min at 25°C. About 500 µL of saturated NaCl was added, and benzoylated polyamines were extracted with 2 µL x 500 µL of diethyl ether. The solvent was evaporated under the vacuum at 40 °C, and dry samples were dissolved in 200 µL of the mobile phase and analyzed according to the method described before (Marchetti et al., 2019). For total polyamines, 200 µL of supernatant was acidified with 50 µL of concentrated HCl and shaken for 16 h at room temperature. The 200 µL of 6 M NaOH was added, and derivatization was performed as described above.

6. Nutritional composition of the flour

In Supplements III and IV, all kernels from the selected cobs were ground to a fine powder to evaluate quality parameters, including total protein and carbohydrate (CH) content (%) and mineral composition.

For the total protein content, the Kjeldahl method was applied (Kjeldahl, 1883; Kirk, 1950), and for the determination, 50 mg of flour from each sample was used. This protocol has three main steps: digestion, distillation, and titration. The digestion breaks all nitrogen bonds in the samples to turn all the organically bonded nitrogen into ammonium ions (NH_4^+). At the same time, organic carbon and hydrogen form carbon dioxide and water, whereas organic material is carbonized. This origin is a black foam, which decomposes and gives a clear liquid that indicates the endo of the reaction. For this step, the sample is mixed with sulfuric acid and heated up between 350°C and 380°C, and it is possible to add salts and catalysts to increase the reaction speed. When digestion is complete, the sample has to cool to room temperature and be diluted with water before transferring to the distillation unit. During the distillation, the addition of alkali (NaOH) allows the conversion of the ammonium ions (NH_4^+) into ammonia (NH_3). The ammonia is then transferred into the receiver vessel through steam distillation. This vessel is filled with an absorbing solution to capture the dissolved ammonia gas. Finally, the determination of the captured ammonium ions was determined by performing an acid-base titration using a standard solution of sulfuric acid as the indicator. Then the final protein content is obtained by multiplying the total nitrogen by 6.25; this conversion factor is based on the assumption that the available nitrogen content in food proteins is 16% and that all nitrogen in foods is protein-bound (Mæhre et al., 2018).

The total carbohydrate (CH) content was also determined. 100 mg of flour from each sample was used for the slightly modified Phenol Sulphuric Acid method in multi-well plates (Jiménez-Arias et al., 2019a). The sample was hydrolyzed with 5 ml of 2.5 N HCl for 3 h at 95°C and then cooled to room temperature and neutralized with solid sodium carbonate until the effervescence stopped, which means the end of the reaction. The resulting mixture was diluted at 50% with water. From that dilution, 50 µl was used for the Phenol Sulphuric Acid method (Dubois et al., 1956). The 50 µl of the diluted sample was placed in a 96 multi-well plate (Masuko et al., 2005) and mixed with 30 µl of phenol 5% and 150 µl of sulphuric acid 96%. Then, it was incubated at 90 °C for 5 min and cooled at room temperature for another 5 min. Finally, the absorbance was measured, and the total carbohydrate content was calculated on a fresh-weight basis using L-glucose as standard.

Finally, one gram of flour from each sample was used to analyze the mineral content (Ca, Mg, K, P, Na, Cu, Zn, and Fe). Each sample was kept in the oven at 105ºC for 5 h and then transferred to a desiccator to reduce the possible remaining water particles. From the dry sample, 500 mg were weighted and converted to ash prior to the mineralization in a muffle furnace at 480°C dried with hydrochloric acid 6 N. The mineral content was determined with the Avio® 500 ICP-OES (Perkin Elmer, Waltham, USA) using a standard curve method.

7. Chlorophyll quantification

Approximately 10 mg of lyophilized material from barley leaves was weighted and subjected to an ethanol extraction for the chlorophyll quantification. Three extractions were performed, two with ethanol 80% and the last with ethanol 50% (Cross et al., 2006). After adding ethanol, the samples were incubated for 20 min at 80°C (Thermomixer C, Eppendorf, Hamburg, Germany), then cooled down and centrifuged at 4ºC, 10,000 rpm for 10 min (Centrifuge 5415R, Eppendorf, Hamburg, Germany). Then, the supernatant was collected, and the pellet was subjected to the following extraction until finishing the process. After the three extractions, 10 µL of the collected supernatant were dissolved in 750 µL of 90% ethanol in each well. Four technical replicates were evaluated per each biological replicate. Then, the 96 well-plate was placed in the spectrophotometer (Synergy H4 Hybrid Reader, Biotek, Winooski, USA) and read at 645 and 665 nm. The chlorophyll content was determined following the formulas described by Porra et al., (1989).

8. Data analysis

To assess differences between treatments of the Supplement I (compound and concentration) values for each non-invasive trait extracted using image analysis, a non-parametric (Dunn's test after Kruskal–Wallis' test parametric) method and a parametric method (Tukey's HSD test after two-way ANOVA) was applied using the packages *multcomp*, *FSA,* and *agricolae* in RStudio (Version 1.1.463 – 2009-2018 RStudio, Inc.).

The statistical significance of differences among the well-watered and water-restricted pea plants (Supplement II) was determined by ANOVA and post-hoc Fisher's test with STATISTICA (Tibco, https://www.tibco.com). $P < 0.05$ was considered a significant change.

Different statistical approaches were used to evaluate the contribution of small molecules-based biostimulants to the vegetative and reproductive development of maize plants (Supplement III and IV). Two-way ANOVA ($p \leq 0.05$) followed by multiple comparisons with LSD and Tamhane post-hoc test were used for parametric data and Kruskal Wallis' test ($\alpha = 0.05$) for nonparametric data. For better visualization, multivariate statistical analyses with the yield-related parameters were also carried out. One principal component (PC- Dim) analysis and matrix correlation were constructed in RStudio V. 2021.09.1+ using the packages *factoextra*, *ggplot2,* and *corrplot*.

For Supplement IV, Kruskal–Wallis was used to determine the differences in the AUC of the plants' growth with the Infostat software. Multi-ANOVA was performed to determine the differences in the plant metabolites. Data were log-transformed to normalize them before the analysis. Duncan's test was used as a post-hoc test for the multiple comparisons between the variants. Principal component analysis (PCA) was conducted using singular value decomposition, and PCA biplots were constructed. Heatmaps with dendrograms were produced. Pearson correlations were computed and displayed. All analyses were performed in RStudio (R Software version 4.1.0).

SURVEY OF RESULTS

This thesis aimed to use phenotyping approaches to explain the mode and mechanism of action of small molecule-based biostimulants used on different plants grown under abiotic stress conditions. As the first objective, we evaluated the accuracy and reproducibility of different plant phenotyping protocols by performing experiments with two different plant species, Arabidopsis seedlings grown *in vitro* and pea plants grown *in vivo*, subjected to different abiotic stresses (Supplement I and II). The second objective was inspired by the good results of using Put and Spd as priming agents improving maize emergence under salt stress, previously performed in our research group (Ugena, 2019). They were good stress alleviators homogenizing maize seedling performance and accelerating their emergence. To analyze further the benefits of these compounds as stress alleviators in maize, we evaluated their effects on the quantity and quality production of plants grown in a greenhouse under well-watered (WW) and water restriction (WR) conditions (Supplement III). The third objective was to understand the mechanism of action of Put to improve the plant stress tolerance. With this aim, Orn, as a non-proteinogenic amino acid precursor of polyamine biosynthesis, and the polyamine DAP, as a final product of their terminal catabolism, and Put were evaluated as plant growth promotors and/or stress alleviators using our HTP screening based on Arabidopsis rosette growth. In addition, a complementary study of the use of Orn as a foliar spray on two barley (*Hordeum vulgare* L.) genotypes, a tolerant (WT) and a sensitive mutant (AZ34), was also performed to accomplish the fourth objective. From this experiment, some preliminary results are shown.

1. **Plant Phenotyping Systems to study abiotic stress tolerance in plants (Supplement I and II).**

In collaboration with the former Chemical Biology and Genetics department from the Centre of the Region Haná for Biotechnological and Agricultural Research (Faculty of Science, Palacký University, Olomouc), we used the PlantScreen™ XYZ System to test the efficiency of four new N^9-Substituted cytokinin derivatives with a fluorinated carbohydrate moiety (Supplement I). The four newly-developed chemicals were used as seed priming agents on *Arabidopsis thaliana* (L.) Heynh seeds and non-primed seeds were used as a control treatment. Besides, the cytokinin aromatic cytokinin benzylaminopurine (BAP) was used as a positive control because it is considered the most effective and cheapest. All seeds were germinated under optimal conditions and then transferred into 48 well-plates with 1× MS for optimal growing conditions or supplemented with 100 mM NaCl or 100 mM mannitol to induce salt or osmotic stress.

The seed priming positively affected the early seed establishment, except for compound 1. Each compound enhanced Arabidopsis growth at a different concentration (higher for compounds 2 and 3 and lower for 4). There was a significant interaction between compound- concentration and growth conditions (ANOVA) regulating Arabidopsis rosette growth under control and stress conditions (Supplement I, Supplementary Figure 2). Growth parameters as the slope of the growth curve (slope), relative growth ratio (RGR), absolute growth ratio (AGR), and final growth of the rosette (rosette area) were also calculated for each growth condition, treatment, and concentration, plus a positive control

(Supplement I, Figure 3 and Supplementary Figure 3). Under optimal conditions, all the N^9-substituted aromatic compounds had a similar effect on the seedlings, improving the early seedling establishment, slope, and AGR. Specifically, compound 2 at almost all concentrations had larger rosettes than the negative and positive controls and presented a more homogeneous population (Supplement I, Figures 3 and 4). Under salt and osmotic stress, the hormopriming improved the tolerance of the seedlings by increasing the values of the slope, RGR, AGR, and rosette area compared to the negative and positive controls (Supplement I, Figure 3). Again, compound 2 at almost all doses provided higher rosette areas. Interestingly, the best results were provided by compound 4 at the lowest concentration, which, on the other hand, inhibited plant growth when applied at the highest dose (Supplement I, Figure 3, and Supplementary Figures 1 and 2). The combination of the growth parameters studied was used to calculate the plant biostimulant characterization index (PBCI), which summarizes and simplifies the outcome of the priming effect to define the BS mechanism of action (Ugena et al., 2018). The PBCI revealed that all the compounds worked as plant growth regulators and stress alleviators, at some concentrations, in a higher proportion than the positive control. The greenness of the seedlings was also studied since chlorophyll degradation is a clear symptom of stress conditions. For this, three-color indices were calculated [normalized green red difference index (NGRDI), visible atmospherically resistant index (VARI), and green leaf index (GLI)], and there were significant differences in all three between the non-primed and primed seedlings under every growing condition (Supplement I, Figure 5). Especially for compound 2, the higher value under optimal conditions coincides with the most effective growth promotor. Besides that, the optimal doses were lower for salt and osmotic stress. Nevertheless, these results support the antisenescent effect properties found in cytokinin-like substances. Overall, the newly created N^6-substituted-2´-deoxy- 2´-fluoro-9-(β)-D-arabinofuranosylpurines positively affected the Arabidopsis growth and increased the tolerance to salt and osmotic stress. The phenotyping platform provided enough accurate data to determine the best performing concentrations as growth promotors and stress alleviators.

The PlantScreen™ Compact System was used to perform an integrative phenotyping study of pea (*Pisum sativum* L.) plants grown under water-limited conditions. The study was performed with a polish research group led by Dr. Robert Malinowski. The aim was to conduct a complementary and more detailed time-course experiment to assess the effect of the drought on commercial green pea plants (*Pisum sativum* L.). The phenotyping platform provided the required data to corroborate that the drought-induced biomass reduction was linked to changes in chlorophyll fluorescence parameters. The fraction of the PSII reactive centers in the open state, which is described by the light-adapted maximum quantum yield of PSII (ΦPSII), and the coefficient of photochemical capacity/quenching (ΦP) were significantly reduced by the water deficit (Supplement II, Figures 5a and b). At the same time, the non-photochemical quenching (ΦNPQ), involved in the heat dissipation, was significantly increased (Supplement II, Figure 5c). However, the most sensitive parameter was the minimum leaf temperature, recorded by the infrared camera. The foliar temperature reported significant differences since the beginning of the water restriction (Supplement II, Figures 5a and d). Moreover, there was a high correlation between the changes in the ΦPSII median value and the leaf temperature changes (Supplement II, Figure 5e), which was more evident in the afternoon measurements than in the morning,

so it showed how the drought-stressed plants were not able to regulate their temperature, thus decreasing the photosynthetic efficacy. Overall, the phenotyping platform was a valuable tool to understand better the pea plants' performance subjected to a water restriction. Moreover, the versatility of the protocols previously developed in our group was proven by adapting it from a monocot specie to a dicot, with a different development.

2. Characterization of small molecule-based biostimulants to the production and nutritional quality in maize (Supplement III and IV)

The results explaining this topic are contained in two supplements (III and IV). First, a study in collaboration with the IPNA, CSIC (Tenerife, Spain), was performed to optimize the experimental setup in maize plants (Supplement III). Here, the optimization of the dose for glycine betaine (GB) and L-pyroglutamic acid (PG) was performed under hydroponic conditions, according to the slightly modified protocol described in Jiménez-Arias et al., (2019b). The osmotic stress reduced the growth of the non-treated (Control) plants by 33% (Supplement III, Figure 1). The GB ameliorated the plant growth inhibition under osmotic stress in a concentration-dependent manner. The best performing dose for the PG was 1 mM, which increased 80% the plant growth. The translation of these results to the plant growing in soil was also evaluated. A second dosage optimization was performed on nursery trays. Reducing 50% of the irrigation reduced the maize seedlings' size by 14%. Applying 0.1 mM GB and 1 mM PG improved plant growth under water restriction and were the selected doses in the field trial (Supplement III, Figure 2). In the field experiment, 25% less irrigation than optimal conditions significantly reduced plant growth and production (Supplement III, Table 2). Compared to well-watered (WW) plants, the leaf length and width were reduced by 14 and 12%, respectively. In the application of GB, this difference was reduced up to 5% for both parameters, whereas PG application only by 1%. The same trend was observed in the yield parameters (Supplement III, Table 2), where plants under water restriction (WR) reduced the cob number, the weight of 100 grains, and total grain weight compared to the WW plants. Again, the application of GB reduced the differences with the WW plants, but PG was a less effective treatment, reducing only those differences up to less than 1% for all three parameters. The limited water availability also reduced the yield expressed as Kg of grain mass per hectare (50% approx.) (Supplement III, Table 3). The application of GB reduced this difference up to 11%, whereas PG had an almost similar yield in both irrigation conditions. Regarding WUE, GB in both conditions had the highest values, followed by the PG-treated plants (Supplement III, Table 3). Both treatments prevented the water losses by maintaining RWC (Supplement III, Figure 3). While the RWC of control plants dropped after the water limitation onset, the application of GB and PG was able to keep the WD plants in the same hydric status as the WW.

The water availability also affected the mineral composition of the kernels from control plants, significantly decreasing Ca and accumulating P and Mg (Supplement III, Table 4). Both treatments incremented the Mg content, especially PG under WD conditions. Fe, Cu and Zn did not significantly differ for the control plants. However, both treatments induced their accumulation. The carbohydrates

and protein content were negatively affected by the irrigation reduction. Nevertheless, both treatments prevented those reductions (Supplement III, Figure 4). Overall, the application of GB and PG as drench treatment proved to improve the WUE, preventing evapotranspiration losses and maintaining the nutritional benefits of the maize. However, for a higher yield, GB was more effective.

In a previous doctoral thesis carried out in our group, it was reported that the polyamines Put and Spd used as priming agents on maize seeds improved various aspects of their emergence, such as time lag and synchronicity under control and salt stress conditions (Ugena, 2019). Considering that the works studying the BS mechanism of action are focused on the vegetative stage of the crops, we aimed to investigate the role of these two PAs on the production of maize (Supplement IV). Firstly, to further understand Put and Spd mechanism of action, and secondly, to corroborate if the results obtained in studies performed at vegetative stages could be translated to the reproductive phases.

As a previous step to the field experiment, the dose optimization on nursery trays provided that Put at 0.1 mM was the more beneficial treatment, while Spd did not substantially benefit the water-restricted seedlings (Supplement III, Table 1 and Supplementary Table S2). In this case, the less prejudicial dose was selected. In the final experiment, 0.1 mM Put and 0.5 mM Spd were applied as drenching in maize plants grown in a greenhouse. After the second application, the water status of the plants was evaluated with the following results. The water restriction significantly reduced the RWC (%) of the non-treated plants in both studied time points (Supplement III, Figure 1). Both PAs applications were able to significantly enhance the water status of the plants at 15 (t_1) and 30 (t_2) days after the water restriction onset compared to controls (Supplement III, Figures 1B and C). Nevertheless, some singularities were observed on the different dates. At t_1 Put treatment enhanced the RWC in the WW plants but not under WD. Contrarily, Spd did not affect the status of the WW plants compared to the controls, but maintained better RWC under WD. Additionally, at t_2 the interaction between treatment and water condition was significant, and the slightly better water status of the WD treated plants could be observed compared to the corresponding WW.

The production of biomass was evaluated by the measurement of the stem diameter, the plant length (measured from the transition to the roots, up to the emission of the anthers), flag leaf length and width, and the ratio length/width (L/W) (Supplement III, Figure 2 and Supplementary Table S4). Put treated plants presented the thinnest stem, while Spd treatment did not affect this parameter. Besides, Put treatment slightly improved the plant height, leaf dimensions, and L/W in WW plants, while under WD plants, it ameliorated the leaf dimensions and L/W ratio up to the level of the WW control plants. Contrarily, Spd treatment negatively affected the leaf dimensions under WW and more under WD.

These differences are also observed in the contribution to the plants' production (Supplement III, Figure 3A). For WW conditions, Put increased the cobs production by 22.78%, whereas Spd reduced 25.32%. Spd treatment produced the same cobs regardless of the water condition, and this was translated into an increment of 62.86% more than the WD control plants. Under WD conditions, Put treatment was not able to level up the production of the WD control. From this data, we reported that

Put on WW conditions can significantly increase the productivity water use efficiency (WUE$_P$) (Supplement III, Figure 3B). Spd also increased WUE$_P$ under water regimes, especially under WD conditions, due to the increment in cobs (Supplement III, Figure 3B). Other parameters related to the cobs and kernel characteristics were determined (Supplement III, Supplementary Table S5). Both PA applications increased the fresh cob weight but the length under WW conditions. Under WD, Spd treatment produced longer cobs. Besides, the yield per ha of stressed plants treated with Put and Spd increased up to the WW condition level, and including the Put treated plants under WD reached a higher harvest index than the plants under WW condition. According to the irrigation regime, these parameters were integrated into two PBCI for better visualization (Supplement III, Figure 4). The PBCIs revealed a positive effect of the Put on both irrigation conditions, whereas Spd was only positive under WD conditions, harming under WW.

The quality of the maize flour was also affected by the PA application since the carbohydrates (CH), and protein content (%) was affected by the treatment and the water regime (ANOVA) (Supplement III, Figure 5 and Supplementary Table S6). The water restriction decreased the CH content and increased the protein content of the non-treated plants. The PA application induced the accumulation of CH of WD plants up to the WW levels (Supplement III, Figure 5A). Interestingly, the response for the protein content was different for each PA (Supplement III, Figure 5B). Put increased the protein content under WW conditions but reduced it in the plants under WD compared to the respective controls. Spd enhanced the protein content in the stressed plants but did not affect it under WW conditions. From the mineralogical analysis of the flour, we observed that Na, P, and Cu were the elements more responsive to the interaction between water regime and treatment, with significant interactions (Supplement III, Table 2, and Supplementary Table S7). Even though the flour composition has an evident alteration caused by the PA application, the growing conditions also contribute to this variation.

In summary, different mechanisms of action were observed between these two PAs (Supplement III, Figure 6). Under WR conditions, Put might improve plant growth by higher leaf biomass and the content of Zn and Fe in the flour, whereas Spd enhances production with longer cob length and higher fresh cob weight, and more Cu content in the flour. The PA application induced a strategy that conditioned the vegetative or reproductive biomass production and the quality of the flour.

3. **The use of Small Molecule – Based Biostimulants (smbBS) to improve Arabidopsis stress tolerance (Supplement V)**

To further understand the PA application's effect on plants, we performed a new experiment including Put as positive control and its precursor Orn and the final product DAP as priming agents to improve Arabidopsis growth under optimal growth conditions osmotic and salt stress (Supplement IV). Under optimal conditions, none of the priming at any concentrations affected the rosette growth (Supplement IV, Figures 1A, B, and C). The osmotic and salt stress reduced 70% and 78% of the growth of plants grown under optimal conditions, respectively. However, the seed priming modified the response, but the effect depended on the concentration and the stressor. The priming with 0.1 and 1

mM DAP improved the growth of plants under salt but not under osmotic stress (Supplement IV, Figure 1D and 1G). 0.1 mM Orn also improved the rosette growth under salt stress but not under osmotic stress (Supplement IV, Figures 1E and H). Contrarily, 1 mM Orn reduced the plants' growth for every assayed condition (Supplement IV, Figures 1B, E, and H). Priming with Put did not affect the plants under osmotic stress. However, both concentrations enhanced the growth under salt stress, although just the 1 mM dose was significant (Supplement IV, Figure 1F and I).

Besides the growth, other parameters were extracted from the analysis of the RGB images. The slope of the growth curve (GC-slope), area under the curve of the growth curve (AUC), relative growth ratio (RGR), final rosette area (FG), and the color index GLI were integrated into three independent PBCI to quickly determine the effect of the priming in each growing condition (Supplement IV, Figure 2). For the control conditions, only 0.1 mM DAP at enhanced the AUC and GLI, resulting in a global positive effect (Supplement IV, Figure 2A). The seed priming of plants grown under osmotic stress revealed a positive effect (Supplement IV, Figure 2B); the best performing 0.1 mM Put and Orn enhanced the GC-slope and RGR, respectively. 1 mM DAP also presented a general improvement of all the parameters except the GLI index. Finally, under salt stress, all the priming agents contributed to a better performance of the plants, except for the 1mM Orn (Supplement IV, Figure 1C). The more effective stress alleviators were 1 mM DAP, and 0.1 mM Put and Orn, and all of them contributed to the improvement of the AUC and FG significantly and, to a lesser extent, a higher GLI.

A targeted metabolomics assay was performed to quantify the plants' free amino acids (AAs) and total and free PAs since the selected priming are involved in their metabolism. The most abundant free amino acids for all treatments and growth conditions were Pro, aspartic acid (Asp), and glutamine (Gln), while cystine (Cis) appeared in the lowest concentration (Supplement IV, Figure 3 and Supplementary Table S2). The priming and growing conditions induced some changes in the metabolite content. All primed plants accumulated N-acetylornithine (AcOrn) under optimal and salt stress conditions, except for the 1 mM and 0.1 mM Put-treated plants under optimal or salt stress conditions, respectively. Met was significantly accumulated with 0.1 mM Orn and 1 mM DAP under optimal conditions. The latter priming and 0.1 mM Put significantly accumulated Pro under optimal and osmotic stress conditions, respectively, whereas 1mM Orn decreased it. 1 mM Put increased the Glu content, except under osmotic stress in which both Put doses significantly decreased it. Put also reduced Cis levels at optimal and osmotic stress. Asp levels were negatively affected by 0.1 mM DAP at and 1 mM Orn. On the other hand, the most abundant PAs, in the total and free forms, were Put, Spd, and agmatine (Agm), while tyramine (Tyra) was present in the lowest concentration (Supplement IV, Figure 3 and Supplementary Table S3). Under optimal conditions, seed priming with 1 mM and 0.1 mM Orn and both Put doses induced increments in the PA free forms; moreover, all primed plants presented higher total thermospermine (ThSpm) and spermine (Spm) compared to the control. This trend was also observed on 1 mM DAP primed plants under osmotic stress and optimal conditions. Homospermidine (HomoSpd) accumulated greatly in 1 mM Orn and Put primed plants under optimal conditions, and norspermidine (NorSpd) accumulated in 0.1 mM Put primed plants under optimal and osmotic stress conditions, and under 0.1 mM DAP, and 1 mM Orn and Put under salt stress.

A principal component analysis (PCA) was performed to understand better the correlation between morphology and the metabolic results for each growing condition. For the optimal conditions, the first two dimensions explained 60.68% of the total model variation (Supplement IV, Figure 4). Under optimal conditions, the control treatment was related to Cis, NorSpd, and dry biomass but opposite the acetylated compounds N-acetylputrescine (AcPut) and AcOrn. Put-primed plants positively correlated with final growth, Glu, and total PAs, especially NorSpd and Agm. Contrarily, DAP-treated plants correlated with almost all free amino acids and PAs, including Pro. The accumulation of Pro presented a negative correlation with the dry biomass, which, together with the final growth, positively correlated with the free ThSpm, Spm, and spermidine (Spd). Finally, the accumulation of the free amino acids Glu, serine (Ser), asparagine (Asn), Arg, and Gln was negatively correlated to the final growth. Altogether, the priming agents that induced the accumulation of free amino acids, especially Pro, and those related to Glu metabolism reduced free PAs levels like Spd and limited plant growth under optimal conditions.

Under osmotic conditions, the first two dimensions of the PCA explained 63,69% of the total model variation (Supplement IV, Figure 5). The metabolic profile differed from the one observed under optimal conditions. 0.1 mM Put-primed seeds accumulated the highest number of free amino acids, the free and total NorSpd, whereas 1 mM Put and DAP-treated plants showed higher total and free PAs and bigger final growth and dry biomass. The rest of the treatments were located close to the controls and positively correlated to Cis, tryptophan (Trp), Glu, and the total and free HomoSpd. Similar results were obtained in the correlation matrix; a strong correlation was observed between the final growth and the total Put, Spd, ThSpm, and Spm, free Put, and the free amino acids GABA and Asp. Dry biomass positively correlated with free PAs but negatively with Cit, Orn, Arg, Ser, and NorSpd.

Finally, for the last PCA computed for the salt stress, the first two dimensions explained 57.07% of the total model variation (Supplement IV, Figure 6). The controls correlated with GABA, methionine (Met), Cis, and AcPut. Oppositely, 1 mM Put primed seedlings accumulated higher levels Glu and Orn, free and total HomoSpd and NorSpm, the free Spm. Meanwhile, the DAP treatment was related to Pro, almost all the PA forms (free and total), including Spd and Cad, and the plant biomass. The correlation between high levels of Pro, Cad, and Spd with the final biomass was corroborated by the correlation matrix with a highly significant linear correlation. Contrarily, the accumulation of β-aminobutyric acid (BABA), Asn, isoleucine (Ile), phenylalanine (Phe), Met, and histidine (His) was opposite to the final growth. Overall, we reported that Glu metabolism, through Orn as a precursor of PA, conditions plant growth under all growth conditions. Therefore, it is clear that the effectiveness of the seed priming with Orn, Put, and DAP for improving plant growth and stress tolerance depends on the type of growth conditions. However, contrary to published information (Hussein et al., 2019), 1 mM Orn negatively affected the seedlings' growth.

4. The use of Ornithine as a foliar spray to enhance the tolerance to water deficit in sensitive barley (Preliminary results)

To understand better the role of Orn regulating plant growth under optimal and stress conditions, we studied the effect of its foliar application on two different barley lines of barley with different stress

tolerance; cultivar Steptoe as the wild type (WT) and tolerant genotype, and the mutant AZ34 (AZ) which is partially deficient in the ABA synthesis as sensitive. Firstly, transpiration curves were recorded from excised leaves that were kept in optimal conditions (water) or simulated osmotic stress (PEG) with or without 1 mM Orn (Figure 2). The different genotypes present several physiological differences concerning the gas exchange. While the assimilation rate (A) was similar for both genotypes, AZ34 plants showed slightly higher internal CO_2 concentration (C_i) as a result of the higher transpiration (E) and stomatal conductance (g_s) (Figure 2A-D). The Orn treatment incremented more A, E, and g_s and in less extent C_i in AZ plants. The light-adapted maximum quantum yield of PSII (ΦPSII) and electron transport rate (ETR) were slightly higher than the AZ plants without Orn. AZ plants with Orn showed higher values than the other variants for all the studied parameters during the time (40 min) they were analyzed under the optimal conditions (Figure 2E and F). Contrarily, most of the studied parameters in the WT plants under optimal conditions remained unaffected by the Orn treatments except the E, which was slightly incremented.

Once the 20% PEG was added to the solution to simulate osmotic stress, all variants experienced a notable drop in all parameters, especially in E and g_s (Figure 2). It is remarkable that for the A, the Orn treated variants showed the lowest values while keeping the highest values of C_i. Interestingly, the transpiration of the AZ treated with Orn dropped to similar values as the non-treated AZ plants, whereas the WT with Orn revealed lower E values than the non-treated WT plants.

Regarding the plants' water use efficiency, both intrinsic (WUE_{int}) and instantaneous (WUE_{inst}) followed more or less the same pattern (Figure 2G and H). The AZ plants had higher WUE_{inst} than WT, and the Orn application reduced it to the WT levels. Orn also reduced the WT values. Once the stressor is applied, the non-treated WT reached the WUE_{inst} values of non-treated AZ, whereas the Orn treated plants of both genotypes increased it and followed the same pattern described for the optimal conditions (Figure 2G). Similar results were observed for WUE_{int}, with Orn-treated plants having slightly higher values than the non-treated WT. On the other hand, the Orn treatment AZ plants increased the WUE_{int} in AZ plant during the stress that reached the non-treated WT and AZ values. However, the Orn application reduced this parameter in the treated WT plants (Figure 2H).

The second experiment performed in the PlantScreen™ Compact System provided a more integrative approach for understanding how the Orn affects the barley plants. Both genotypes were grown under two irrigation regimes, 60% of the field capacity (FC) as WW and 30% of the FC as water deficit (WD) treatment (Figure 3). The genotypes show a clear difference in growth, where the mutant was significantly smaller (25%) than the WT in WW conditions. Under WD, untreated WT and AZ decreased the plant height by 48% and 33.4%, respectively (Figure 3A). The foliar application with Orn did not affect the WT plants under WW conditions but enhanced the AZ growth up to the WT levels. On the other hand, the Orn treatment did not significantly affect the WD conditions, although it is visible that it slightly increased the plant height.
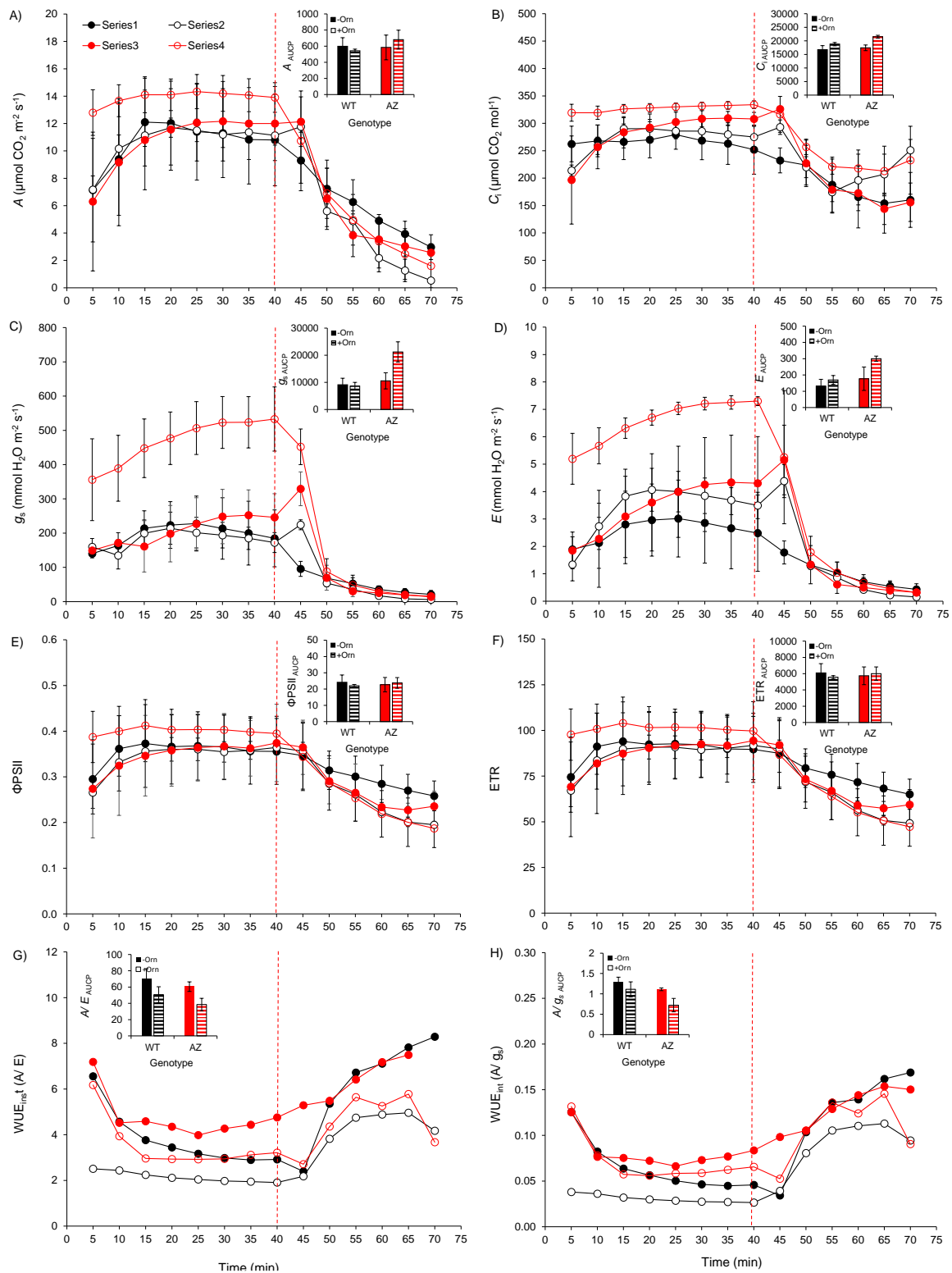
Figure 2. Transpiration curves performed on excised leaves kept in water (optimal conditions, -Orn) or in water with 1 mM Orn (optimal conditions, +Orn) for 40 min, and then, PEG 20% was added to simulate osmotic stress (period after the red dashed line). The represented parameters are A) net assimilation (A, $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), B) internal CO_2 concentration (C_i, $\mu mol\ CO_2\ mol^{-1}$), C) stomatal conductance (g_s, $mmol\ H_2O\ m^{-2}\ s^{-1}$), D) transpiration (E, $mmol\ H_2O\ m^{-2}\ s^{-1}$), E) the light-adapted maximum quantum yield of PSII (ΦPSII), F) the electron transport rate (ETR), G) instantaneous water use efficiency (WUE_{inst}, A/E) and H) intrinsic water use efficiency (WUE_{int}, A/g_s). There is a bar chart within each curve graph that represents the area under the curve (AUC) for the represented variants (genotype * treatment). The curves are the average value of 3 curves measured in different plants with the standard deviation values.

These findings are supported by the biomass analysis performed after a destructive sampling on the last day of the experiment (Table 4). The WT fresh weight (FW) remained unchanged after the Orn application under WW. However, Orn significantly increased the AZ biomass but did not reach the WT level. Under the WD condition, Orn induced an increment in FW regardless of the lack of significance.
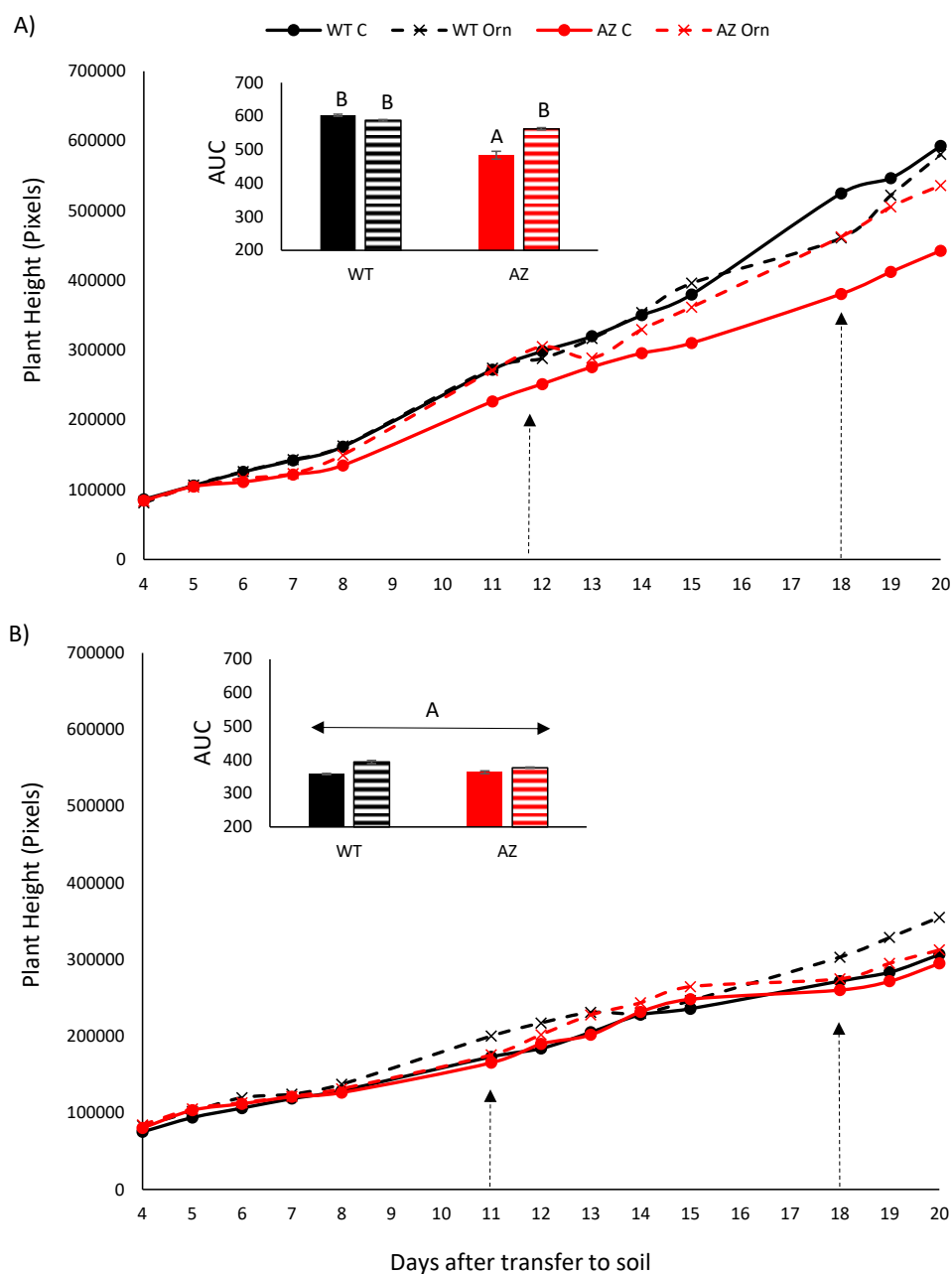
Figure 3. Plant height increment expressed in pixels along the experiment duration expressed as days after the seeds transfer to the soil. The pixel values are obtained from the RGB images for A) the well-watered condition and B) the water deficit condition. The arrows indicate the dates where the foliar Orn application was performed. The included bar charts summarize the information on the height increment of the plants by expressing the area under the curve (AUC) for each condition. For the AUC charts, different letters indicate significant differences (ANOVA p<0.05).

No differences were observed regarding the dry weight (DW) for the WT plants. Interestingly, Orn significantly increased the DW in AZ plants until the WT values were reached under WW conditions. No differences existed under WD conditions, even if the same trend observed before for the FW is observed. The same trend observed for the DW is reproduced by the relative water content (RWC) (Table 4). The Orn application did not affect the WT under the WW regime, although it significantly improved the RWC of the AZ plants up to the WT plants. Contrarily, no significant effect was observed on the WD conditions.

Table 4. Fresh weight (FW, g), dry weight (DW, g), and the content of chlorophyll a (Chl a, mg/ g DW), chlorophyll b (Chl b, mg/ g DW), chlorophyll a - chlorophyll b ratio (Chl a/ Chl b ratio), total chlorophyll (Total Chl, mg/ g DW), and the relative water content (RWC, %). Average value ± standard error. Different letters for each parameter within the same irrigation condition, well-watered (WW) and water deficit (WD), indicate significant differences (ANOVA p<0.05).

		WW		WD	
		Control	1 mM Orn	Control	1 mM Orn
FW (g)	WT	1.54 ± 0.07 **C**	1.37 ± 0.07 **BC**	0.64 ± 0.03 **AB**	0.74 ± 0.05 **B**
	AZ	0.92 ± 0.07 **A**	1.24 ± 0.04 **B**	0.59 ± 0.03 **A**	0.64 ± 0.04 **AB**
DW (g)	WT	0.17 ± 0.01 **B**	0.15 ± 0.01 **AB**	0.08 ± 0.00 **A**	0.09 ± 0.01 **A**
	AZ	0.10 ± 0.01 **A**	0.16 ± 0.03 **B**	0.07 ± 0.00 **A**	0.11 ± 0.03 **A**
Chl a (mg/g DW)	WT	6.04 ± 0.79 **B**	5.42 ± 0.14 **AB**	4.91 ± 0.15 **A**	5.32 ± 0.61 **A**
	AZ	4.53 ± 0.20 **A**	6.09 ± 0.21 **B**	4.64 ± 0.10 **A**	5.53 ± 0.22 **A**
Chl b (mg/g DW)	WT	2.79 ± 0.11 **A**	2.87 ± 0.18 **A**	3.46 ± 0.21 **B**	2.81 ± 0.08 **A**
	AZ	2.56 ± 0.12 **A**	2.92 ± 0.06 **A**	2.88 ± 0.11 **A**	3.08 ± 0.21 **AB**
Chl a / Chl b ratio	WT	2.14 ± 0.19 **B**	1.90 ± 0.07 **AB**	1.44 ± 0.11 **A**	1.89 ± 0.19 **A**
	AZ	1.77 ± 0.01 **A**	2.09 ± 0.11 **AB**	1.62 ± 0.09 **A**	1.83 ± 0.21 **A**
Total Chl (mg/g DW)	WT	8.84 ± 0.89 **B**	8.29 ± 0.32 **AB**	8.37 ± 0.24 **A**	8.13 ± 0.65 **A**
	AZ	7.09 ± 0.33 **A**	9.01 ± 0.15 **B**	7.52 ± 0.08 **A**	8.61 ± 0.11 **A**
RWC (%)	WT	84.17 ± 0.98 **B**	81.53 ± 1.02 **AB**	70.74 ± 0.95 **A**	63.15 ± 0.67 **A**
	AZ	75.70 ± 0.75 **A**	84.15 ± 1.05 **B**	73.04 ± 1.37 **A**	74.76 ± 1.15 **A**

The water regime significantly influenced the chlorophyll content (Preliminary Table 1). A significant interaction between genotype and treatment according to ANOVA (p=0.0241) was observed for chlorophyll a (Chl a) under WW condition, which resulted in a significant increment in the Orn treated AZ plants, which reached the WT plants levels. There was no significant effect on the WD conditions, although Orn treatment tends to increase the Chl a content. The chlorophyll b (Chl b) content behaved oppositely. While no significant differences were found under WW conditions (besides an interesting increment trend induced by the Orn), under WD conditions, the Orn application significantly reduced the Chl b content in WT (significant interaction genotype*treatment, p=0.213), but AZ plants remained unaffected. Regarding the Chla/Chl b ratio, Orn decreased it in the AZ line, behaving the opposite for the WT under WW conditions since there was a significant interaction genotype*treatment (ANOVA, p=0.033). No differences were found for the WD condition. However, the increment trend after the treatment is visible. Finally, the total Chl revealed that Orn treatment did not affect the WT but

significantly increased the Total Chl for the AZ plants under WT, but no differences were found for the WD conditions.

From these preliminary results, we observed that the foliar application of Orn elicitated the adverse stress effects by improving plant physiology, especially on photosynthesis. This effect was more visible in the sensitive barley mutant AZ than in WT under optimal growth conditions. These results pointed to the application of Orn as an excellent approach to improve the vigor of no-well performing genotypes. However, under water restriction, the Orn effect of Orn must be further studied with the help of other –omics approaches. So far, we could not report any beneficial effect, as it was already reported in sugar beet (*Beta vulgaris* var. saccharifera L.) (Hussein et al., 2019). Nevertheless, we expect that the integration of further analysis to the existent results will allow us to understand the Orn mode of action better.

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CONCLUSIONS AND FUTURE PERSPECTIVES

The present thesis pursues to confirm the adaptability and suitability of the protocols developed in our team based on high throughput phenotyping platforms (HTPPs). Moreover, it was intended to provide a deeper understanding of the mode of action of pure substances, which might be considered small molecule-based biostimulants (smbBS). The main conclusions of this work are:

- The suitability and accuracy of *in vitro* and *in vivo* protocols for PlantScreen™ XYZ System and PlantScreen™ Compact System have been proved. The protocols can be adapted to different plant species and are robust and versatile enough to find stress biomarkers as the minimum temperature, in the case of Supplement II, or to evaluate the potential stress alleviator effect of newly developed compounds, as in the case of the Supplement I.

- The beneficial effect of the smbBS was proved by applying different compounds to water-limited maize plants and studying their performance. The reported benefits of using polyamines (PAs) used as seed priming are not lost when the PAs are applied via drenching to the roots. We described the different effects of putrescine (Put) and spermidine (Spd) on the development of the vegetative and reproductive stages of the maize. Moreover, PAs, and in general smbBS treatments affect the composition of the kernels. Therefore, smbBS as a biofortification tool is an exciting area to investigate further.

- The use of smbBS with well-known PAs and uncommon ones such as 1,3-diamino propane (DAP) can be an efficient technological approach for plant hardening and improving plant stress tolerance. The results obtained with Orn were contradictory since the higher doses induced the growth inhibition of the plants, which, on the other hand, could be part of conservative response.

- As part of the preliminary results, we observed that the foliar application with Orn alters the physiology and metabolism of barley plants differently in WT (as control) and AZ34 (as sensitive), pointing to this metabolite as a vital regulator of PA metabolism and endogenous ABA, and hence, plants' water stress response.

Because of these results, the HTPPs are valuable tools for bioassays for any research that intends to provide a deeper understanding of the plant processes, either evaluating screening for stress markers or assessing the BS efficacy. The smbBS as a biofortification strategy could be considered to further studies involving more challenging growing conditions and testing multiple plant species. Finally, the results also revealed a vital role of Orn regulating PA metabolism and plant stress response, but further studies will be needed to understand its involvement deeper.

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SUPPLEMENT I:

Hormopriming to mitigate abiotic stress effects: a case study of N^9-substituted cytokinin derivatives with a fluorinated carbohydrate moiety

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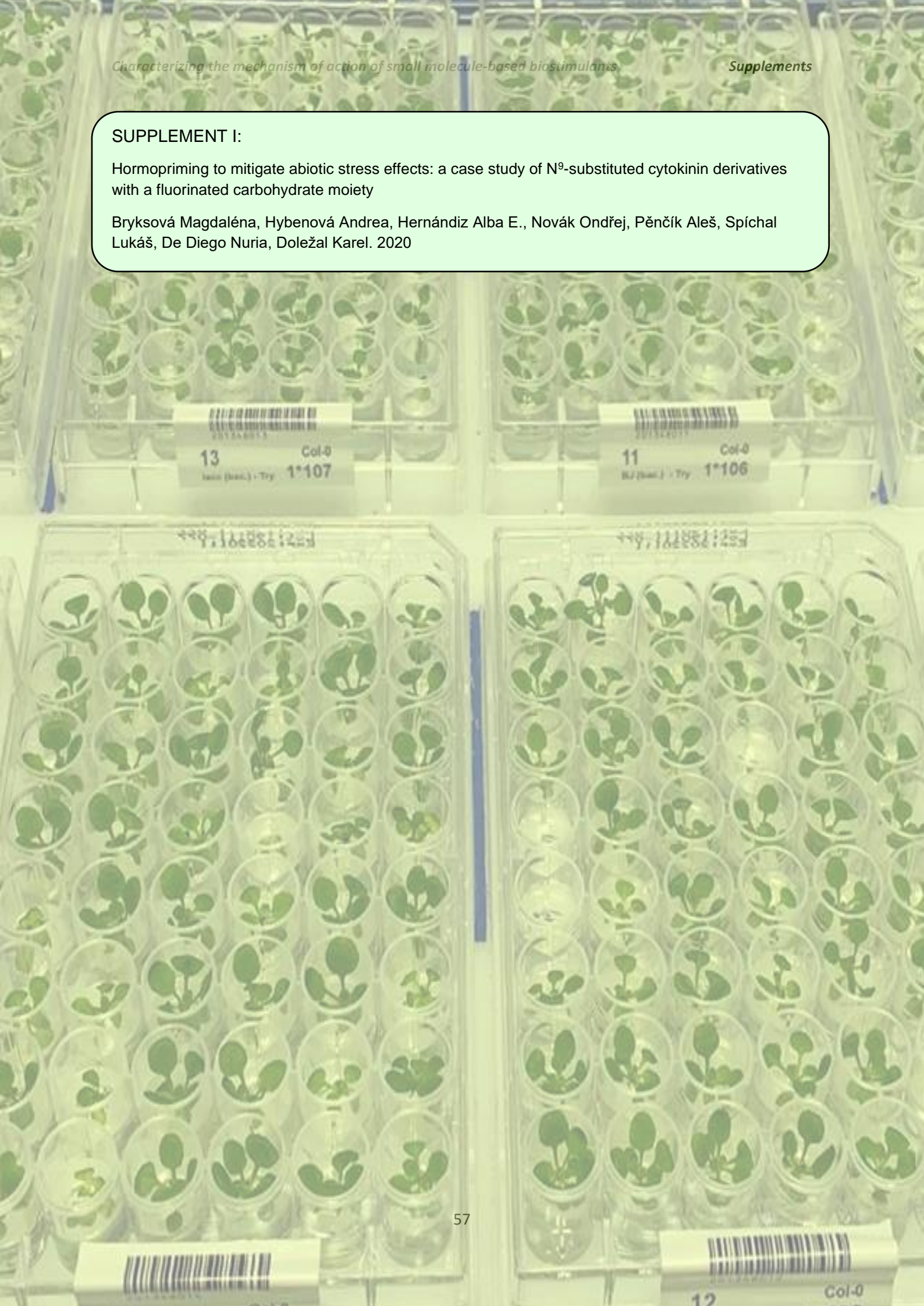
Hormopriming to Mitigate Abiotic Stress Effects: A Case Study of N^9-Substituted Cytokinin Derivatives With a Fluorinated Carbohydrate Moiety

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Drought and salinity reduce seed germination, seedling emergence, and early seedling establishment, affect plant metabolism, and hence, reduce crop yield. Development of technologies that can increase plant tolerance of these challenging growth conditions is a major current interest among plant scientists and breeders. Seed priming has become established as one of the practical approaches that can alleviate the negative impact of many environmental stresses and improve the germination and overall performance of crops. Hormopriming using different plant growth regulators has been widely demonstrated as effective, but information about using cytokinins (CKs) as priming agents is limited to only a few studies using kinetin or 6-benzylaminopurine (BAP). Moreover, the mode of action of these compounds in improving seed and plant fitness through priming has not yet been studied. For many years, BAP has been one of the CKs most commonly applied exogenously to plants to delay senescence and reduce the impact of stress. However, rapid endogenous N^9-glucosylation of BAP can result in negative effects. This can be suppressed by hydroxylation of the benzyl ring or by appropriate N^9 purine substitution. Replacement of the 2′ or 3′ hydroxyl groups of a nucleoside with a fluorine atom has shown promising results in drug research and biochemistry as a means of enhancing biological activity and increasing chemical or metabolic stability. Here, we show that the application of this chemical modification in four new N^9-substituted CK derivatives with a fluorinated carbohydrate moiety improved the antisenescence properties of CKs. Besides, detailed phenotypical analysis of the growth and development of Arabidopsis plants primed with the new CK analogs over a broad concentration range and under various environmental conditions revealed that they improve growth regulation and antistress activity. Seed priming with, for example, 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine promoted

plant growth under control conditions and alleviated the negative effects of the salt and osmotic stress. The mode of action of this hormopriming and its effect on plant metabolism were further analyzed through quantification of the endogenous levels of phytohormones such as CKs, auxins and abscisic acid, and the results are discussed.

Keywords: abiotic stress, antisenescence, Arabidopsis, cytokinin analogs, hormopriming, plant biostimulant characterization index

INTRODUCTION

Global climate change is increasing the severity of drought and soil salinity, with deleterious effects on already-stressed agricultural ecosystems. Moreover, predictions for the future indicate that the areas affected by these two types of stress are going to expand and as a consequence the productivity of many plant species will be reduced (Savvides et al., 2016; Uddin et al., 2016; Pavlů et al., 2018). The development of biotechnological approaches that increase plant tolerance and assure the maintenance of yield under these challenging growth conditions is therefore one of the main aims of plant scientists and breeders.

One of the technologies that attracts a high level of interest nowadays is "seed priming" (Savvides et al., 2016). Seed priming is an effective pre-sowing technology in which seeds are treated with small doses of certain agents just prior to germination. Unlike un-primed seeds, primed seeds are able to respond to very low levels of specific stimuli, which helps plants to prepare their metabolism for better defense responses to stress factors (Conrath, 2011; Paparella et al., 2015). Thus, priming can improve seed performance, ensure higher uniformity among the seeds, result in faster and better synchronized germination, and enhance plant growth (Gamir et al., 2014; Ibrahim, 2016; Lutts et al., 2016). Several methods of seed priming, including hydropriming, osmopriming, hormopriming, biopriming, and chemical priming, have been developed (Jisha et al., 2013; Paparella et al., 2015). Hormopriming consists in the exogenous application of plant growth regulators or phytohormones that can stimulate seed imbibition and modify seed metabolism. The plant growth regulators most often used in this way are abscisic acid (ABA), gibberellins, cytokinins (CKs), auxins, ethylene, and polyamines (reviewed by De Diego and Spíchal, 2020).

In plants, CKs are involved in many biological processes: regulating sink/source relationships (Roitsch and Ehneß, 2000; Werner et al., 2008), nutrient uptake (Sakakibara, 2006; Criado et al., 2009), leaf senescence (Jordi et al., 2000; Marchetti et al., 2018), and responses to abiotic stress (Bielach et al., 2017). Since the discovery of the first CK, kinetin, by Skoog, Miller, and associates in 1955 (Miller et al., 1955), the number of chemicals fitting the definition of CK has grown to include a large array of natural and synthetic compounds, among which are adenine and phenylurea derivatives (Mok and Mok, 2001). Depending on their chemical structure, natural CKs are adenine

derivatives with an isoprenoid or aromatic N^6-side chain (Mok and Mok, 2001). CKs are present in plants in the forms of free bases, glucosides, nucleosides and nucleotides, at very low concentrations [pmol g^{-1} fresh weight (FW)] (Strnad, 1997). The precursor nucleotides, namely N^9-riboside-5′- mono-, di-, and tri-phosphates, are endogenously synthesized *de novo* and converted to active free bases. The bases can be subsequently conjugated with glucose at positions N^3, N^7, and N^9 of the purine ring and at the hydroxyl group of the side chain, which can be also conjugated with xylose (Frébort et al., 2011). Addition of sugar moieties to the N^9 position of the purine ring can also form N^9-riboside-glucoside (George et al., 2008). While the O-glycosylated forms can be converted back into active CKs, N-glycosylation occurs primarily at positions N^7 or N^9 of the purine ring, and is thought to be irreversible (Brzobohatý et al., 1993), except in the case of the *tZ* forms (Hošek et al., 2020). Furthermore, it has been demonstrated that some of these conjugates may have significant CK activity, especially in the case of N^9-riboside analogs (Doležal et al., 2007).

The aromatic CK benzylaminopurine (BAP) is considered the most effective and the cheapest CK, which has led to its widespread use in biotechnology. However, many disadvantages associated with its applications have been reported (Werbrouck et al., 1995; Bairu et al., 2009). Negative effects can be caused by natural N^9-glucosylation of the applied purine based CK, leading to extensive accumulation of non-active CK glucosides (Werbrouck et al., 1995). Moreover, N^9-glucosides can activate ethylene production and the ethylene signaling pathway causing inhibition of root elongation (Podlešáková et al., 2012). One way of avoiding the negative effects of N^9-glucosylation is to suppress it by appropriate N^9 purine substitution in BAP or by hydroxylation of its benzyl ring (Plíhal et al., 2013).

Fluorination has a long tradition in nucleoside chemistry and the replacement of the 2′ or 3′ hydroxyl group of a nucleoside with a fluorine atom causes only a minor change in the overall structure, but significantly affects the stereoelectronic properties of the sugar moiety (Thibaudeau et al., 1998). It has been reported that important factors in the substitution of fluorine for hydrogen are the comparable size of the two atoms and the powerful electron withdrawing properties of fluorine relative to hydrogen, as well as the increased stability of the carbon-fluorine bond relative to the carbon-hydrogen bond. Hence, replacement of hydrogen by fluorine in a bioactive molecule is expected to cause minimal steric perturbations with respect to the molecule's mode of binding to receptors or enzymes (Pitzer, 1960). Moreover, replacement of the hydrogen by fluorine causes not only changes in biological activity, but also increases the chemical and metabolic stability of nucleosides. The conformation of the sugar

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Abbreviations: BAP, 6-benzylaminopurine, ABA, abscisic acid, CKs, cytokinins, IAA, indole-3-acetic acid, iP, N^6-isopentenyladenine, *cZ*, *cis*-zeatin, DHZ, dihydrozeatin, *tZ*, *trans*-zeatin, HTS, high-throughput screening, PBC, plant biostimulant characterization index.

moiety of these analogs is strongly affected by the presence of the fluorine substituent and is different from that of natural deoxynucleosides (Pankiewicz et al., 1992).

Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been widely used in biochemical research and therapeutic treatment (Meng and Qing, 2006; Hagmann, 2008; Kirk, 2008). However, to date only few fluorinated CK derivatives have been prepared and their biological activity tested in plants (Clemenceau et al., 1996; Doležal et al., 2007). Only recently, several 6-benzylaminopurines substituted with β-D-arabinose at the N^9-position with similar structures were synthesized in our laboratory, and subsequently patented as powerful antisenescence compounds compared with BAP, but the activity of these compounds were only tested in a detached wheat leaves senescence bioassay (Patent No. US 10,100,077 B2, 2018). Here, we present a new class of N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine derivatives which show not only high levels of antisenescence activity but also promise as seed priming agents due to their high efficiency as plant growth promotors and plant stress alleviators. Their mode of action as priming agents is also discussed.

MATERIALS AND METHODS

General Synthesis of N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurine Derivatives

All the compounds presented here were prepared by a slightly modified one-step synthesis (Wan et al., 2005) of 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine with benzylamine or isopentenylamine hydrochloride as appropriate in the presence of BOP and DIPEA in DMF (**Figure 2**). Firstly 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine (200 mg, 1 equiv.) and BOP (396 mg, 1.2 equiv.) were mixed together in DMF (4 mL) and subsequently DIPEA (194 μL, 1.5 equiv.) and benzylamine (1–3) or isopentenylamine hydrochloride (4) (1.2 equiv.) as the last component were added. Each reaction mixture was stirred under an argon atmosphere in an oil bath at a temperature of 55–60°C for 24 h and the effectiveness of the reaction was checked by Thin-layer chromatography (TLC) (CHCl$_3$/MeOH 4:1). The reaction mixture was evaporated using a vacuum rotary evaporator to give a specifically colored gel. The resulting residue was carefully purified by column chromatography (1 and 3) or by preparative HPLC (2 and 4) to give the desired product, which in some cases (1 and 3) could be crystallized from various solvents.

General Procedures

The chromatographic purity and mass spectra of the compounds described were characterized using the HPLC-PDA-MS method. Samples (10 μL of 3×10^{-5} M in 1% methanol) were injected onto a reverse-phased column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, United States) tempered at 25°C. Solvent (A) consisted of 15 mM ammonium

FIGURE 1 | Scheme for the preparation of substituted 6-benzylamino-2′-deoxy-2-′fluoro-9-(β)-D-arabinofuranosylpurine derivatives by a method using Castro's reagent and Hunig base.
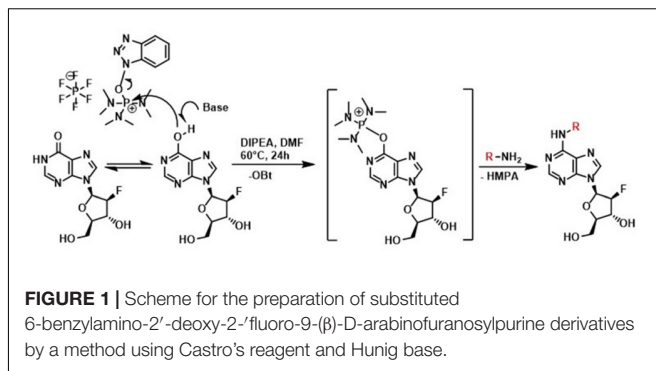
formate adjusted to pH 4.0 and solvent (B) consisted of methanol. The flow-rate was set to 200 μL min^{-1}. A binary gradient was used: 0 min, 10% of B; 24 min; 90% of B; 34 min; 90% of B; 45 min; 10% of B using a Waters Alliance 2695 Separations Module (Waters, Manchester, United Kingdom). Then the effluent was introduced to a Waters 2996 PDA detector (Waters, Manchester, United Kingdom) (scanning range 210–700 nm with 1.2 nm resolution) and a tandem mass analyzer Q-Tof micro Mass Spectrometer (Waters, Manchester, United Kingdom) with an electrospray. The cone voltage was set to 20 V. Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters, United Kingdom) operating in positive ion mode and recorded as $(M + H)^+$. Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected. ^{1}H NMR spectra were analyzed on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz. The samples were prepared by dissolving in DMSO-d$_6$. Tetramethylsilane (TMS) was used as an internal standard. TLC was carried out using silica gel 60 WF$_{254}$ plates (Merck). Purification *via* column chromatography was performed using silica gel Davisil R LC60A 40-63 micron.

HPLC-MS Purification

A preparative HPLC-MS chromatography machine (Agilent 1290 Infinity II) was used coupled to a UV-VIS detector with a mass LC/MSD detector (Agilent InfinityLab) and an Agilent Prep-C18 column (5 μm, 21.2 mm × 50 mm, Waters, Milford, MA, United States) to obtain the final products. Analyzed samples were dissolved in 50% MeOH before injection. The mobile phase was methanol (A):H$_2$O (B) with a flow rate of 20 mL min^{-1} and linear gradients (0 min, 10% B; 0–12 min; 90% B) were used.

HRMS Conditions

Samples (5 μL) were characterized using the HPLC-PDA-MS method. They were injected onto a reversed-phase column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, United States) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following linear gradient was used at a flow rate of 250 μL min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation temperature 550°C, capillary

voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas ($50 \, L \, h^{-1}$) and the desolvation gas ($1000 \, L \, h^{-1}$). Data acquisition was performed in full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in positive mode (ESI$^+$), therefore protonated molecules $(M + H)^+$ were collected in each MS spectrum. For exact mass determination experiments, external calibration was performed using lock spray technology and a mixture of leucine/encephalin ($50 \, pg \, \mu L^{-1}$) in an acetonitrile and water (1:1) solution with 0.1% formic acid as a reference. Accurate masses were calculated and used to determine the elemental composition of the analytes with a fidelity better than 1.0 ppm.

Cytokinin Bioassays

Cytokinin bioassays, including Amaranthus, tobacco callus and senescence bioassays, were carried out as previously described by Holub et al. (1998), using BAP as a positive control for all three classical CK bioassays. Results were recorded to define the highest activities of the four compounds prepared. All of them were dissolved in 0.5% DMSO and tested at five concentrations (from 10^{-8} to 10^{-4} M).

Plant Phenotyping – Rosette Growth of Seedlings From Arabidopsis Hormoprimed Seeds

The four compounds synthesized were tested as priming agents under optimal and two different stress conditions. Arabidopsis seeds (*Arabidopsis thaliana* accession Col-0) were sterilized and germinated as described by Ugena et al. (2018). During germination the compounds were added at four different concentrations (from 10^{-7} to 10^{-4} M) to germination medium containing $0.5\times$ MS (pH 5.7) supplemented with a gelling agent (0.6% Phytagel; Sigma-Aldrich, Germany). Three days after germination, seedlings of similar size were transferred under sterile conditions into 48-well plates (Jetbiofil, Guangzhou, China). One seedling was transferred to each well filled with 850 mL $1\times$ MS medium (pH 5.7; supplemented with 0.6% Phytagel), without stress treatment (optimal conditions) or containing 100 mM NaCl (as salt stress) or 100 mM mannitol (as osmotic stress), and the plates were sealed with perforated transparent foil allowing gas and water exchange. Hormopriming of 10^{-8} M BAP was also used as positive control for all tested growth conditions.

The 48-well plates containing the transferred Arabidopsis seedlings were placed in an OloPhen platform,[1] which uses the PlantScreen^{TM} XYZ system installed in a growth chamber with a controlled environment, and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czechia). The conditions were set to simulate a long day with a temperature regime of 22/20°C in a 16/8 h light/dark cycle, an irradiance of $120 \, \mu mol$ photons of PAR $m^2 \, s^{-1}$ and a relative humidity of 60%. The PlantScreen^{TM} XYZ system consists of a robotically driven arm holding an RGB camera with customized lighting panel

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[1] http://www.plant-phenotyping.org/db_infrastructure#/tool/57

and growing tables. The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. The imaging of each 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days as described in Ugena et al. (2018). As outcome, the individual image of 48 Arabidopsis seedlings per variant (treatment vs. growth condition) as biological replicates were used for the analyzed phenotyping traits.

Different traits were determined from the RGB images: Arabidopsis rosette growth curves [as changes in the green area (Pixels)], relative (RGR) and absolute (AGR) growth rate and final rosette size. All these traits were then used to define the mode of action of the compound under test. Using the traits, the plant biostimulant characterization (PBC) index was determined as described by Ugena et al. (2018). The PBC index was calculated as the sum of the values obtained from each phenotyping trait calculated as the differences (as the log2 of the ratio in each case) between the controls and treatment variants (compound and concentration) under the same growth conditions.

Determination of Arabidopsis Rosette Color Indices

To estimate the greenness of the Arabidopsis seedlings, and changes in leaf color, three vegetation indices (NGRDI, VARI, and GLI), which have been shown to be correlated with plant biomass, nutrient status, or tolerance to abiotic stress (Gitelson et al., 2002; Perry and Roberts, 2008; Hunt et al., 2013), were used. The images captured on the seventh day of an Arabidopsis rosette growth assay subjected to HTS were segmented for the extraction of leaf rosettes using software described in our previous report (De Diego et al., 2017). The values corresponding to particular color channels (red, R; green, G; and blue, B) were then extracted for each pixel within the plant mask, and the vegetation indices were calculated as described by Ugena et al. (2018). Subsequently, indices representing particular seedlings were determined by calculating the mean values for each plant mask. The mean and the standard error (SE) values for each 48-well plate were then calculated and represented in a graph.

Plant Hormone Quantification

Four independent biological replicates consisted in four individual pools from 12 Arabidopsis seedlings per variant were collected for the hormonal analysis. After purification and extraction, the concentration of each analyte was calculated using the standard isotope dilution method (Rittenberg and Foster, 1940). Briefly, as the first step a micro solid-phase extraction (µSPE) based on StageTip (STop And Go Extraction Tip) technology was used to purify the plant tissue samples. The µSPE protocol used in CK extraction and purification was applied as described by Svačinová et al. (2012), whereas auxins and ABA were isolated as described by Pěnčík et al. (2018). CKs were determined using ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (an Acquity UPLC

I-Class System coupled with a Xevo TQ-S MS, Waters). Quantification of auxins and ABA was performed and the concentration of each analyte was calculated using the standard isotope dilution method on a 1260 Infinity II system coupled with a 6495B Triple Quadrupole LC/MS system (Agilent Technologies).

Statistical Analysis

To assess differences between treatments (compound and concentration) values for each non-invasive trait extracted by means of image analysis, a non-parametric (Dunn's test after Kruskal–Wallis' test parametric) method and a parametric method (Tukey's HSD test after two-way ANOVA) were applied using the packages *multcomp, FSA,* and *agricolae* in RStudio (Version 1.1.463 – 2009-2018 RStudio, Inc.). Multivariate statistical analyses, including heatmap and principal component (PC) analysis, were also performed in RStudio using the packages *gplots, cluster, tidyverse, factoextra, heatmap.plus, ggpubr, factoextra, FactoMineR,* and *corrplot.*

RESULTS

Synthesis of Four N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety

In this work, a group of three N^9-substituted aromatic or one isoprenoid CK derivatives with a fluorinated carbohydrate moiety were synthesized (**Figure 1**) and their biological activity was investigated. The compounds prepared were characterized by ^{1}H NMR, elemental analysis, melting points, TLC and ESI + MS. The purity of the prepared derivatives was confirmed by high-performance liquid chromatography (HPLC-UV) (**Table 1**).

First, the synthesis of a 2-fluoropentose from a pentoside precursor followed by its conversion into 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine was performed as reported in 1969 (Wright et al., 1969). Subsequently, 3-deoxy-3-fluoro-D-glucose was synthesized and converted into 2-deoxy-2-fluoro-D-arabinose via oxidation by sodium metaperiodate as described by Reichman et al. (1975). The compound 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine (F-ara-A) has previously been prepared by condensation of 6-chloropurine with 2-deoxy-2-fluoro-D-arabinofuranosyl bromide followed by conversion of the purine into adenine, but the reaction produced a mixture of four isomers and only a very low yield of the desired isomer could be isolated (Marquez et al., 1990). Later, a three-step synthesis of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine was carried out via displacement of the 2′-hydroxyl group of 03′,05′,N^6-tritrityladenosine and 03′,05′-ditritylinosine with diethylaminosulfur trifluoride, as published by Pankiewicz et al. (1992). The importance of introducing a fluorine at the 2′(S)(ara) site of purine deoxynucleosides has been highlighted, since 2′-deoxy-2′-fluoroarabinosides have been found to be biologically active and chemically stable against hydrolysis catalyzed both chemically and by purine nucleoside phosphorylase (Chu et al., 1989).

TABLE 1 | Physico-chemical properties of four new synthesized compounds: elemental analysis, melting temperature, mass of positively charged molecular ions analyzes by HPLC-MS, purity, and HRMS mass analysis.

Compound	Elemental analysis calculated/found			Mp (°C)	ES-MS (M + H⁺)	HPLC (%)	HRMS			Elementary analysis (M + H⁺)
	% of C	% of H	% of N				Exact mass	Theoretical monoisotopic mass	Difference (ppm)	
1	56.82/56.18	5.05/5.02	19.49/19.28	179–180	360	99⁺	359.1393	359.1394	−0.23	C17H19FN5O3
2	54.40/53.61	4.83/5.00	18.66/18.23	101–103	376	99⁺	375.1341	375.1343	−0.48	C17H19FN5O4
3	55.52/56.05	5.18/4.86	17.99/19.24	212–213	390	99⁺	389.1502	389.1499	0.74	C18H21FN5O4
4	53.40/54.94	5.98/5.80	20.76/18.73		338	99⁺	337.1552	337.155	0.4	C15H21FN5O3

In the present study, all the aforementioned steps were followed and finally the synthesis of new compounds was performed as previously reported by Wan et al. (2005) with some modifications. Typically, the synthesis of purine nucleosides is based on the protection of hydroxyl groups, which prolongs this method to a four-step process with low yield. This transformation usually causes cleavage of the glycosyl bond, therefore only acid-labile protecting groups must be used. In our new simple one-step unprotected synthesis, BOP was used to activate the formation of a C-N bond. Subsequently, substitution by appropriate amines led to the formation of final products, after elimination of hexamethylphosphoramide (HMPA). However, the nucleophilic substitution of unprotected purine nucleosides with amines required longer reaction times compared with their protected counterparts (Wan et al., 2005).

CK-Like Activity of the New N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety in Cytokinin Bioassays

To evaluate the CK activities of the newly synthesized compounds, three classical *in vitro* CK bioassays were used. Despite the fact that all four of the new compounds are derived from CKs with known high levels of activity in all three bioassays, their 2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosyl purine derivatives showed decreases in activity in the Amaranthus and tobacco callus bioassays (**Supplementary Table 1**). On the other hand, high antisenescence activity was recorded in the bioassay based on evaluating the effect of the compound on retention of chlorophyll in excised wheat leaves kept in the dark (**Table 2**). The greatest ability to prevent chlorophyll degradation was shown by compounds 1 and 2, which reached, respectively, 277 and 267% of the values for the positive control BAP at concentrations of 10^{-4} M, followed by compound 3, which showed 179% of the BAP activity. Compound 4 had comparable activity to BAP (**Table 2**). Overall, these results showed that substitution at the N^9 position with a fluorinated carbohydrate moiety selectively influences the CK-like activity, specifically improving the antisenescence properties of CKs modified in this way. This suggests that such CK analogs could activate plant processes related to stress responses and would therefore have antistress properties when applied to plants.

Priming With N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety Improves the Growth of Arabidopsis Under Both Optimal and Stress Conditions

To corroborate the involvement of these compounds in plant stress tolerance and better define the mode of action of our four newly synthesized compounds, we tested their effects on Arabidopsis growth and development under optimal and stress conditions using a complex multi-trait high-throughput screening approach (Ugena et al., 2018). The four compounds

TABLE 2 | Relative CK activities of four new synthesized compounds in the senescence bioassay.

Compounds	Senescence bioassay	
	Optimal concentration (M)	Relative activity (%)
1	10^{-4}	277 (±9)
2	10^{-4}	267 (±17)
3	10^{-4}	179 (±3)
4	10^{-4}	95 (±6)

The optimal concentration for compounds 1–3 was compared with the activity of benzylaminopurine (BAP), where 100% means 10^{-4} M BAP. The optimal concentration for compound 4 was compared with the activity of isopentenyladenine (iP), where 100% means 10^{-4} M iP.

were used as seed priming agents at four concentrations (from 10^{-7} to 10^{-4} M). Non-primed and primed seeds were germinated under optimal conditions and then the seedlings were transferred into 48 well plates with 1× MS alone, or supplemented with 100 mM NaCl or 100 mM mannitol to induce salt or osmotic stress, respectively. First, we evaluated how the priming affected early seedling establishment. To do so, the rosette area of the seedlings transferred to control conditions (1× MS) at day 1 was determined. Here, we saw a clear interaction between compound and concentration affecting early seedling establishment (**Figure 3** and **Supplementary Figure 1**). The seedlings developed from seeds primed with all the compounds except compound 1 had increased rosette area. The largest rosettes were observed after priming with the highest concentrations of compound 2 and 3, or lower concentrations of compound 4. Interestingly, priming with the highest concentration (10^{-4} M) of compound 4 caused strong growth inhibition, leading to seedlings reaching only half the size of the control (MOCK) seedlings (**Figure 3** and **Supplementary Figure 1**).

The rosette areas of the seedlings were further analyzed twice a day for an additional 6 days to record a growth curve (**Supplementary Figure 2**). All four compounds improved Arabidopsis seedling growth under control and stress conditions at some of the concentrations tested and there was significant interaction between compound concentration and growth conditions according to ANOVA. On the other hand, the highest concentration of compound 4 (10^{-4} M) showed inhibitory activity under all three of the conditions tested and the rosette areas were significantly reduced to 20, 60, and 48% of those in the non-primed control (MOCK) seedlings under, respectively, control, salt, and osmotic stress (**Supplementary Figure 2**).

Other traits such as relative growth rate (RGR) and absolute growth rate (AGR) were also calculated. For better visualization, these traits together with early seedling establishment and final rosette area (at day 7, **Supplementary Figure 3**) are presented in a parallel coordinate plot shown in **Figure 3**. To construct this, the differences between the controls and variants (compound and concentration) under the same growth conditions were calculated as the log2 of the ratio. The value obtained for each trait is shown in twelve independent parallel coordinate plots, one per compound (a total of 4) for optimal conditions, salt stress,
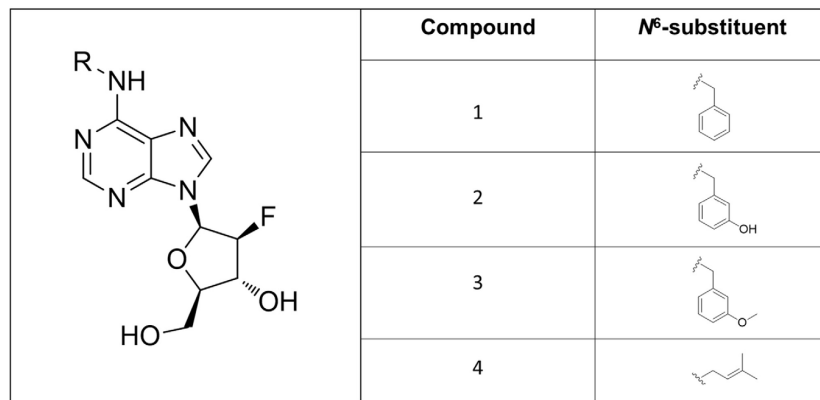
FIGURE 2 | Structures of the newly synthesized 6-benzylamino-2′-deoxy-2-′fluoro-9-(β)-D-arabinofuranosylpurine derivatives.
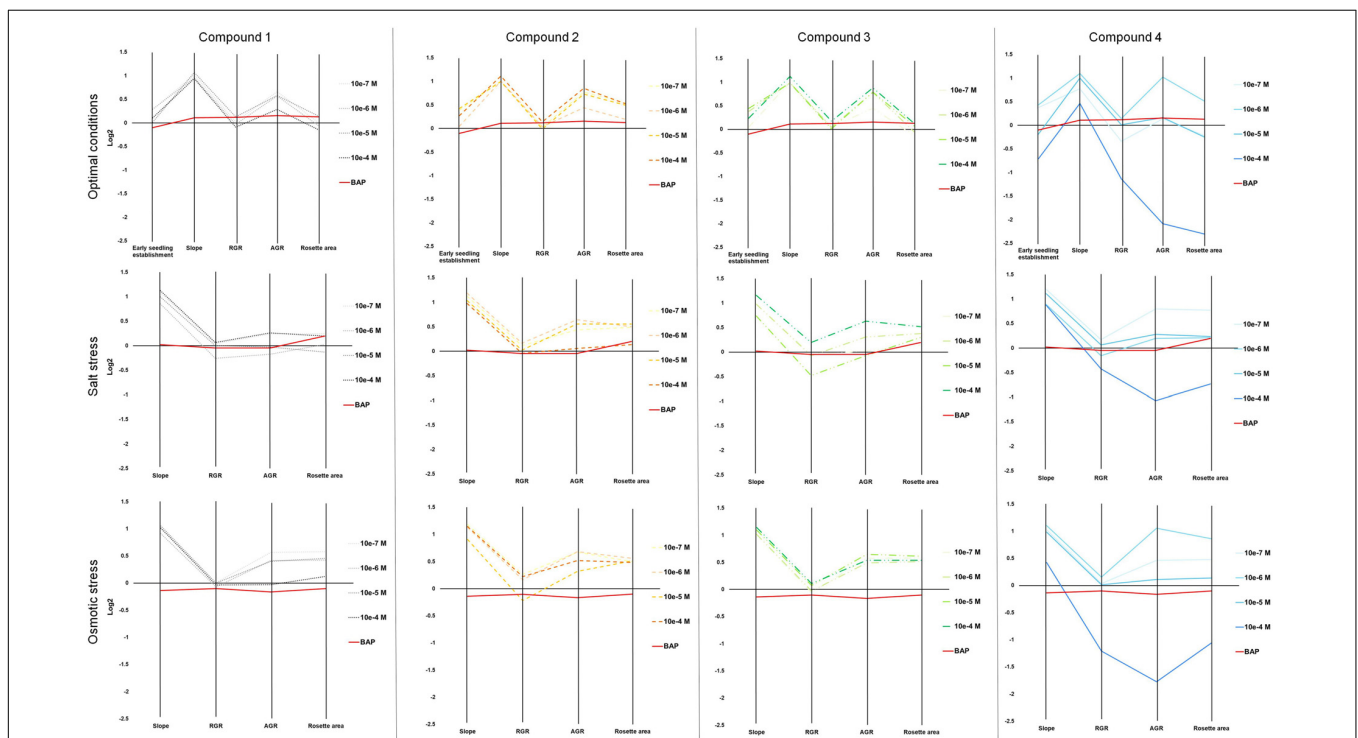

FIGURE 3 | Parallel coordinate plot of the traits (Early seedling establishment, slope of the growing curve, RGR, AGR and the final rosette area) obtained from multi-trait high-throughput screening of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) and grown under optimal (upper panels), or salt (100 mM NaCl, middle panels) or osmotic (100 mM mannitol, bottom panels) stress conditions (N = 48). BAP at 10^{-8} M was used as positive control.

and osmotic stress (**Figure 3**). Additionally, the priming effect of 10^{-8} BAP was evaluated as a positive control. Interestingly, in the parallel plot the three N^9-substituted aromatic had similar profile whereas isoprenoid CK derivative showed different response (**Figure 3**). Under optimal growth conditions, priming with the new CK analogs improved some growth related traits analyzed (early seedling establishment, the slope of the curve, and AGR). At the assay end-point, mainly the seedlings primed with almost all concentrations of compound 2 had larger rosettes compared

to the non-primed seedlings (MOCK) or those primed with the positive control (BAP) (**Figure 3**). These plants also presented higher homogeneity of the population (represented by coefficient of variance = standard deviation/Mean, %) compared with the MOCK variant (28.81 and 38.40, respectively) (**Figure 4**).

Importantly, hormopriming improved the tolerance of the Arabidopsis seedlings to salt and mannitol induced stress by increasing the values of the slope of the curve, RGR, ARG and final rosette size compared to the negative and positive
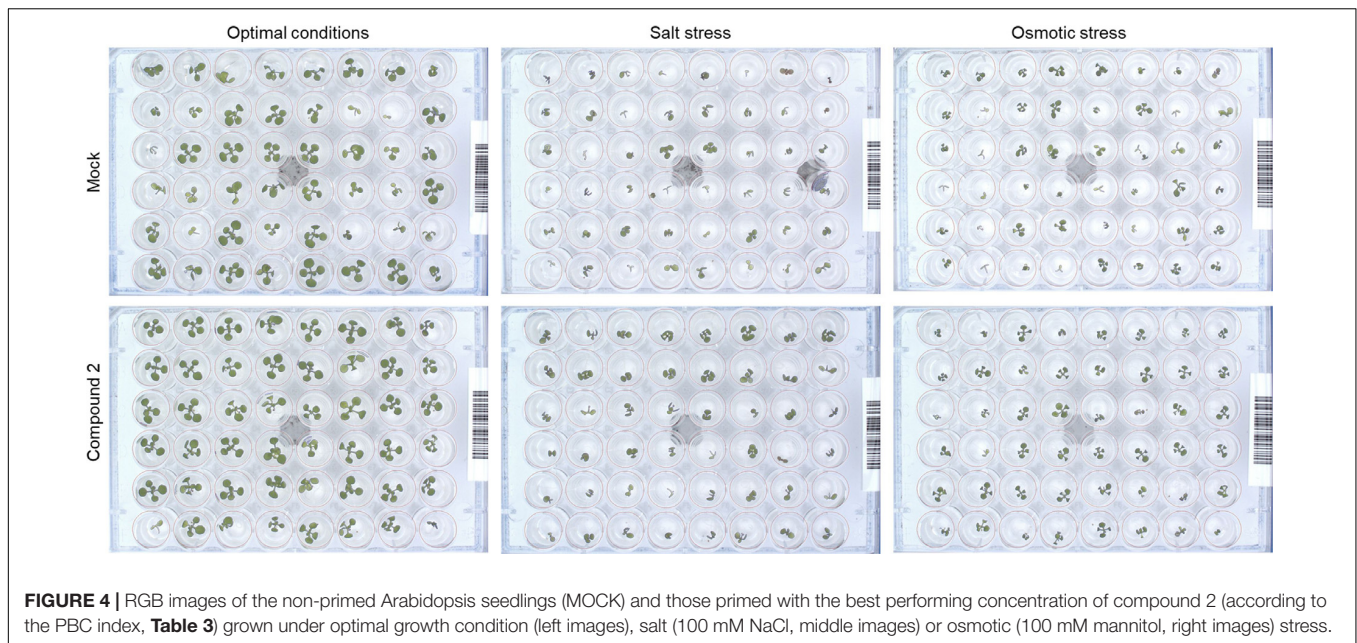
FIGURE 4 | RGB images of the non-primed Arabidopsis seedlings (MOCK) and those primed with the best performing concentration of compound 2 (according to the PBC index, **Table 3**) grown under optimal growth condition (left images), salt (100 mM NaCl, middle images) or osmotic (100 mM mannitol, right images) stress.

controls (**Figure 3**). In both cases, low concentrations of compound 4 (10^{-7} M for salt stress and 10^{-6} M for osmotic stress) resulted in the highest increases in the traits analyzed, whereas a concentration of 10^{-4} M inhibited plant growth under all growth conditions (**Figure 3** and **Supplementary Figures 1**, **2**). Conversely, plants primed with compound 2 showed improvements in all traits under both control and stress conditions (**Figure 4**). All these results were then combined to calculate the PBC index, which helps to simplify and sum up the overall outcomes in order to define the mode of action of a biostimulant (Ugena et al., 2018). As listed in **Table 3**, all compounds worked as plant growth promotors and stress alleviators at some of the concentrations tested, all with higher efficiency than the control CK BAP. The most efficient plant growth promotor was compound 4 followed by compound 2. However, whereas compound 2 improved growth at all concentrations tested and growth conditions (working as strong plant growth promotor and stress alleviator), compound 4 was highly toxic at the highest concentration (10^{-4} M), at which it showed a growth inhibitory effect (**Table 3**). Overall, we conclude that priming with the newly prepared N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines had positive effects on Arabidopsis growth and, importantly, improved tolerance to salt and osmotic stress, with a stronger effect in the latter case (**Figures 3**, **4** and **Table 3**).

Hormopriming With N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurines Maintains Seedling Greenness

To gain a further understanding of priming with compound 2 (N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines), changes in seedling color after 7 days

under different growth conditions (optimal, salt, or osmotic stress) were determined. The degradation of chlorophyll, manifested as a change in Arabidopsis rosette color, represents one of the most important symptoms of stress (Ugena et al., 2018). Three different indices (NGRDI, VARI, and GLI) were calculated and presented in **Figure 5**. Significant differences were observed between seedlings from non-primed and primed seeds, especially regarding NGRDI and VARI indices under all growth conditions (**Figure 4**). Under optimal conditions, the highest values were obtained when the compound 2 was applied at 10^{-4} M, a concentration that also resulted in the highest PBC index (**Table 3**). However, under salt and osmotic stress, the highest NGRDI and VARI indices were observed when 10^{-5} M and 10^{-6} M were used (**Figure 5**). Taken together, these results corroborated the aforementioned antisenescence effect of this compound observed in the CK-like bioassays (**Table 2**).
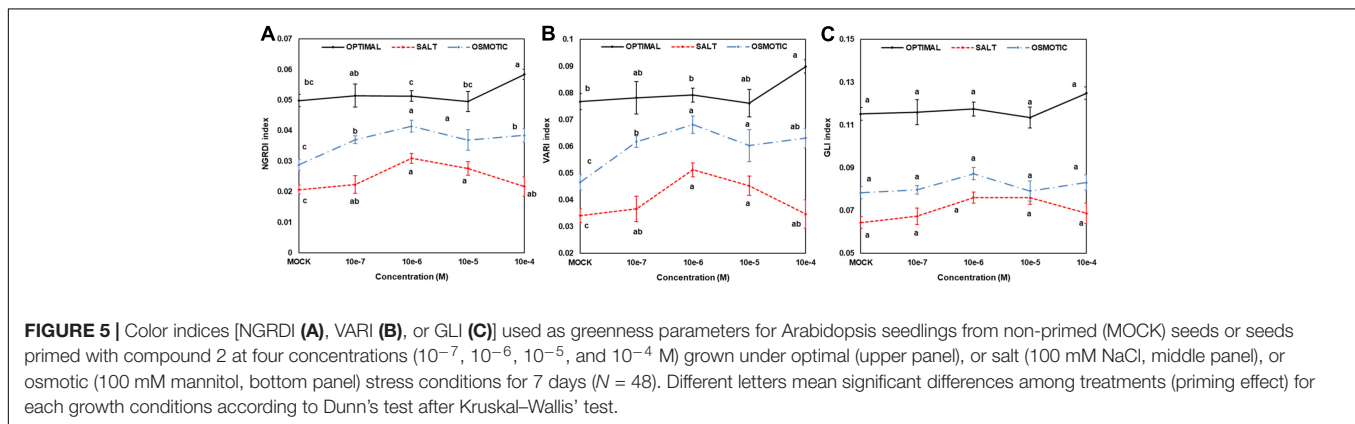
Hormopriming With N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurines Improves Arabidopsis Growth and Stress Tolerance by Altering the Hormonal Profile

To understand the molecular nature of the mode of action of priming by the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines, the hormonal profile of Arabidopsis seedlings primed with the best performing compound 2 was analyzed at the end of the phenotyping experiment. The endogenous levels of CKs (**Table 4**), some auxins and ABA were quantified using LC/MS (**Supplementary Table 2**). For better visualization and interpretation, all metabolites were analyzed together using a heatmap (**Figure 6A**). The results separated the variants (treatments and growth conditions) into two clusters

TABLE 3 | Plant biostimulant characterization (PBC) index calculated by summing the relative changes (log2) obtained for the parallel coordinate plot (**Figure 4**) for each synthetized compound (four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety) at four concentration (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) and growth condition; optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) (N = 48).

	Optimal growth condition				100 mM NaCl				100 mM mannitol			
	Concentration (M)				Concentration (M)				Concentration (M)			
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Comp_1	2.00	1.73	1.95	1.11	0.53	−0.40	−0.16	0.52	1.16	0.77	0.86	0.06
Comp_2	2.73	1.73	2.64	2.90	1.04	1.31	1.13	0.15	1.43	1.40	0.62	1.22
Comp_3	1.40	2.14	2.29	2.54	0.12	0.62	−0.25	1.35	1.09	0.98	1.33	1.19
Comp_4	0.74	3.22	0.75	−5.77	1.74	0.26	0.58	−2.22	0.97	2.07	0.26	−4.04
BAP	0.41				0.10				−0.37			
Comp_1	**Strong growth promotor**				Weak stress alleviator				Medium stress alleviator			
Comp_2	**Strong growth promotor**				**Strong stress alleviator**				**Strong stress alleviator**			
Comp_3	**Strong growth promotor**				Medium growth promotor				**Strong stress alleviator**			
Comp_4	Growth promotor and inhibitor				Stress alleviator and inhibitor				Stress alleviator and inhibitor			
BAP	Weak growth promotor				Weak stress alleviator				Stressor			

BAP at 10^{-8} M was used as positive control. Bold terms indicate the best performing compounds at the indicated growth conditions. Blue shades indicates positive effect, white no effect and red shade indicates negative effect.

FIGURE 5 | Color indices [NGRDI **(A)**, VARI **(B)**, or GLI **(C)**] used as greenness parameters for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (upper panel), or salt (100 mM NaCl, middle panel), or osmotic (100 mM mannitol, bottom panel) stress conditions for 7 days (N = 48). Different letters mean significant differences among treatments (priming effect) for each growth conditions according to Dunn's test after Kruskal–Wallis' test.

using the Spearman correlation as the distance method: one for the plants grown under salt stress and the control for osmotic stress, and a second cluster for the rest. Additionally, the first group was separated into two subclusters in which all plants grown under salt stress showed, in general, a reduction in content of the CK nucleotides, total auxin, and ABA (**Figure 6A**). On the other hand, the variants represented in the second cluster showed increased levels of these metabolites and of some N- and O-glucosides (DHZ7G, DHZ9G, tZ7G, and tZ9G), and IAA conjugated with glucose (IAAGlu). On the other hand, they reduced content of total CKs, bases, and ribosides, especially in the case of the hormoprimed seedlings under optimal and osmotic stress conditions (**Figure 6A**). Similar results were obtained when the distance among variants was determined (**Supplementary Figure 4**), in which the hormopriming seedlings grown under optimal and osmotic stress were separated by a short distance (close to 0; similar behavior), but the distance was longer for the primed plants grown under salt stress conditions.

To extend the analysis, a PC analysis was also performed (**Figure 6B**). The components PC1 and PC2 accounted for 60.5% of the total variance of the model. In PC1, there was

clear evidence of contrasting behavior between all plants grown under optimal conditions and hormoprimed seedlings under salinity stress (blue ellipses). Thus, whereas the first group was positively correlated with the phenotyping traits and the CK nucleotides (synthesized de novo), which also showed a strong relationship (**Supplementary Figure 5**), the second group had a higher content of total CKs due to an increase in ribosides and N-glucosides (iP7G and iP9G) and O-Glucosides (cZOG and cZROG) (**Figure 6B**). Interestingly, hormopriming with the highest concentration (10^{-4} M) of compound 2 (as shown by PC2, red ellipses) induced similar contents of total auxins and the degradation form 2-oxindole-3-acetic acid (oxIAA) independent of growth conditions (**Figure 6B**), a pattern opposite to that in the MOCK variant under salt and osmotic stress. Overall, we demonstrated that in general hormopriming with the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines presented here induced changes in the hormonal content of Arabidopsis seedlings, thus conditioning the final phenotype, with the changes depending on the concentration of the compound and on growth conditions.

TABLE 4 | Changes in CK levels (pmol g^{-1} FW) of 10-day-old *Arabidopsis thaliana* seedlings from non-primed seeds or seeds hormoprimed with compound 2 grown at four different concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) under optimal conditions, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) for 7 days.

Conditions	Cytokinins	Optimal conditions				
		MOCK	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Optimal conditions	Total CKs	104.73 ± 18.27	108.33 ± 14.54	97.85 ± 14.04	114 ± 21.56	114.54 ± 16.29
	Bases	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.018
	Ribosides	18.65 ± 3.53	19.96 ± 4.08	16.98 ± 4.72	20.1 ± 5.28	16.77 ± 2.88
	Nucleotides	15.35 ± 5.90	12.25 ± 0.97	12.98 ± 1.44	14.73 ± 2.41	18.51 ± 3.29
	O-glucosides	7.53 ± 1.30	7.94 ± 1.21	6.65 ± 1.56	7.63 ± 2.02	6.49 ± 0.91
	N-glucosides	63.12 ± 9.19	68.11 ± 9.45	61.15 ± 7.43	71.46 ± 14.07	72.7 ± 9.93
	iP-types	47.40 ± 8.85	47.29 ± 5.40	45.92 ± 6.3	54.05 ± 9.58	56.09 ± 8.43
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	11.92 ± 2.64	12.3 ± 2.99	10.81 ± 3.08	13.3 ± 3.71	11.49 ± 2.23
	iPRMP	6.43 ± 1.43	6.05 ± 1.04	7.01 ± 0.62	7.23 ± 1.42	10.22 ± 1.72
	iP7G	25.02 ± 4.29	25.48 ± 3.05	24.99 ± 2.85	29.69 ± 5.79	29.84 ± 4.42
	iP9G	4.03 ± 0.68	3.46 ± 0.59	3.12 ± 0.39	3.83 ± 0.67	4.54 ± 0.49
	*t*Z-types	25.41 ± 4.52	32.79 ± 6.04	28.94 ± 3.01	32.33 ± 6.57	30.42 ± 6.23
	*t*Z	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.023	0.07 ± 0.016	0.07 ± 0.018
	*t*ZR	2.27 ± 0.33	4.41 ± 1.32	4.07 ± 1.03	4.09 ± 0.89	3.06 ± 0.61
	*t*ZRMP	1.45 ± 0.25	2.1 ± 0.47	2.26 ± 0.22	2.12 ± 0.39	2.76 ± 0.65
	*t*ZOG	1.89 ± 0.33	2.92 ± 0.42	2.37 ± 0.54	2.94 ± 0.66	2.17 ± 0.37
	*t*ZROG	0.73 ± 0.20	0.32 ± 0.05	0.2 ± 0.03	0.35 ± 0.09	0.23 ± 0.02
	*t*Z7G	7.79 ± 1.82	10.17 ± 0.97	12.45 ± 2.59	13.53 ± 2.30	10.68 ± 2.86
	*t*Z9G	3.13 ± 0.61	9.79 ± 1.07	10.32 ± 2.66	8.6 ± 2.44	5.99 ± 1.37
	DHZ-types	2.96 ± 0.51	4.36 ± 0.74	3.46 ± 0.94	4.09 ± 0.90	4.42 ± 0.53
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.15 ± 0.04	0.17 ± 0.07	0.12 ± 0.04	0.15 ± 0.05	0.09 ± 0.01
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	1.5 ± 0.37	3.20 ± 0.90	3.80 ± 0.83	4.18 ± 0.48	1.49 ± 0.44
	DHZ9G	0.07 ± 0.02	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.07 ± 0.02
	*c*Z-types	28.97 ± 7.73	23.89 ± 3.33	19.53 ± 4.8	23.52 ± 4.90	23.61 ± 2.97
	*c*Z	<LOD	<LOD	<LOD	<LOD	<LOD
	*c*ZR	4.33 ± 1.24	3.07 ± 0.47	1.98 ± 0.64	2.57 ± 0.66	2.16 ± 0.42
	*c*ZRMP	7.48 ± 5.00	4.1 ± 1.36	3.71 ± 1.04	5.37 ± 0.67	5.53 ± 1.02
	*c*ZOG	1.02 ± 0.22	0.58 ± 0.11	0.52 ± 0.1	0.59 ± 0.14	0.55 ± 0.13
	*c*ZROG	3.90 ± 0.86	4.12 ± 0.69	3.57 ± 1.02	3.76 ± 1.21	3.54 ± 0.77
	*c*Z7G	14.46 ± 3.81	9.16 ± 2.50	10.69 ± 2.64	11.32 ± 1.35	13.79 ± 3.38
	*c*Z9G	0.57 ± 0.14	0.59 ± 0.16	0.55 ± 0.14	0.51 ± 0.09	0.64 ± 0.20
Salt stress (100 mM NaCl)	Total CKs	157.34 ± 35.63	173.13 ± 27.9	208.79 ± 19.85	244.29 ± 61.86	164.18 ± 22.6
	Bases	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06
	Ribosides	75.35 ± 17.82	53.94 ± 16.41	76.5 ± 16.27	95.05 ± 28.28	44.51 ± 11.92
	Nucleotides	4.51 ± 1.06	4.25 ± 1.43	6.8 ± 0.96	9.36 ± 5.12	8.35 ± 2.36
	O-glucosides	8.48 ± 1.47	14.33 ± 3.63	15.66 ± 2.17	16.32 ± 3.80	14.5 ± 3.38
	N-glucosides	68.90 ± 17.88	100.11 ± 7.04	109.36 ± 13.89	123.37 ± 25.39	96.63 ± 18.40
	iP-types	67.33 ± 15.565	73.08 ± 7.08	95.7 ± 11.8	117.44 ± 25.00	80.28 ± 8.09
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	30.56 ± 5.83	27.47 ± 8.71	36.77 ± 10.13	47.19 ± 13.93	26.68 ± 8.57
	iPRMP	1.88 ± 0.49	1.7 ± 0.47	2.59 ± 0.56	3.12 ± 0.82	4.32 ± 0.51
	iP7G	30.49 ± 8.87	39.03 ± 5.18	51.16 ± 10.73	61.20 ± 9.55	45.16 ± 8.81
	iP9G	4.41 ± 1.09	4.88 ± 1.29	5.18 ± 0.19	5.93 ± 1.33	5.2 ± 1.42

(Continued)

TABLE 4 | Continued

Conditions	Cytokinins	Optimal conditions				
		MOCK	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
	tZ-types	24.3 ± 6.30	57.99 ± 10.36	42.58 ± 9.15	41.62 ± 11.39	19.86 ± 4.25
	tZ	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06
	tZR	4.19 ± 1.10	15.19 ± 4.67	7.14 ± 1.73	7.93 ± 2.54	2.25 ± 0.66
	tZRMP	0.50 ± 0.12	0.43 ± 0.08	1.18 ± 0.26	1.73 ± 0.53	0.78 ± 0.18
	tZOG	1.72 ± 0.51	5.5 ± 1.38	3.48 ± 0.97	3.65 ± 1.13	1.58 ± 0.44
	tZROG	0.77 ± 0.21	0.7 ± 0.18	0.65 ± 0.13	0.68 ± 0.17	0.32 ± 0.11
	tZ7G	10.87 ± 2.14	19.13 ± 4.16	17.41 ± 4.46	9.97 ± 1.76	10.52 ± 2.24
	tZ9G	6.04 ± 1.36	10.54 ± 2.45	10.03 ± 2.59	4.76 ± 1.54	6.81 ± 1.89
	DHZ-types	2.04 ± 0.51	3.46 ± 0.81	4.06 ± 0.69	4.33 ± 1.06	2.22 ± 0.5
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.58 ± 0.11	0.57 ± 0.12	1.02 ± 0.26	0.93 ± 0.25	0.32 ± 0.09
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	2.59 ± 0.54	2.9 ± 0.54	3.27 ± 0.84	1.76 ± 0.52	2.65 ± 0.66
	DHZ9G	0.12 ± 0.01	0.14 ± 0.02	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.04
	cZ-types	63.67 ± 15.175	38.6 ± 10.84	66.44 ± 9.76	80.9 ± 25.69	61.83 ± 10.99
	cZ	<LOD	<LOD	<LOD	<LOD	<LOD
	cZR	40.02 ± 12.19	10.72 ± 3.1	31.58 ± 6.52	39 ± 12.4	15.25 ± 3.84
	cZRMP	2.13 ± 0.55	2.12 ± 1.02	3.03 ± 0.57	4.51 ± 3.81	4.34 ± 1.22
	cZOG	1.90 ± 0.59	2.02 ± 0.53	1.87 ± 0.19	3.08 ± 0.76	2.43 ± 0.75
	cZROG	4.10 ± 0.90	6.12 ± 1.58	9.67 ± 1.38	8.91 ± 1.95	10.17 ± 2.52
	cZ7G	14.49 ± 2.53	19.28 ± 2.58	24.63 ± 7.6	28.43 ± 8.26	16.72 ± 4.28
	cZ9G	0.73 ± 0.17	1.03 ± 0.16	0.78 ± 0.25	1.21 ± 0.39	0.86 ± 0.28
Osmotic stress (100 mM mannitol)	Total CKs	83.87 ± 18.50	112.08 ± 8.93	103.89 ± 9.47	148.80 ± 15.57	138.51 ± 10.90
	Bases	0.056 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.03	0.23 ± 0.08
	Ribosides	16.83 ± 3.28	15.71 ± 2.19	13.91 ± 3.03	21.62 ± 2.88	25.41 ± 4.07
	Nucleotides	5.64 ± 1.52	10.41 ± 1.25	9.43 ± 1.44	15.15 ± 2.53	16.63 ± 0.98
	O-glucosides	6.44 ± 1.27	11.41 ± 1.34	8.71 ± 1.13	11.30 ± 1.15	12.15 ± 1.75
	N-glucosides	54.92 ± 14.04	74.45 ± 6.65	71.77 ± 6.45	100.63 ± 10.93	84.09 ± 10.49
	iP-types	41.23 ± 9.84	49.60 ± 2.20	46.33 ± 3.91	70.72 ± 6.57	61.58 ± 6.30
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	10.83 ± 2.35	11.25 ± 1.62	9.02 ± 1.97	15.12 ± 1.64	16.54 ± 2.72
	iPRMP	2.10 ± 0.63	4.93 ± 1.25	3.8 ± 0.75	6.81 ± 1.36	6.04 ± 1.26
	iP7G	25.38 ± 7.08	30.05 ± 1.13	30.24 ± 1.85	44.51 ± 4.67	35.41 ± 5.77
	iP9G	2.94 ± 0.82	3.38 ± 0.53	3.28 ± 0.72	4.28 ± 0.74	3.58 ± 0.33
	tZ-types	16.33 ± 3.34	28.25 ± 3.95	24.96 ± 2.68	31.01 ± 6.53	28.95 ± 3.19
	tZ	0.056 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.026	0.23 ± 0.08
	tZR	2.04 ± 0.46	2.78 ± 0.61	2.29 ± 0.32	3.21 ± 0.69	4.15 ± 0.26
	tZRMP	0.565 ± 0.15	1.47 ± 0.41	1.08 ± 0.27	1.66 ± 0.28	1.96 ± 0.38
	tZOG	1.595 ± 0.31	2.69 ± 0.52	2.25 ± 0.44	3.18 ± 0.56	2.58 ± 0.36
	tZROG	0.32 ± 0.05	0.41 ± 0.06	0.37 ± 0.07	0.33 ± 0.08	0.41 ± 0.11
	tZ7G	11.815 ± 2.56	11.59 ± 2.08	13.41 ± 2.63	11.21 ± 1.53	4.01 ± 1.07
	tZ9G	6.26 ± 1.51	7.3 ± 1.15	9.12 ± 2.94	8.42 ± 1.37	2.19 ± 0.65
	DHZ-types	2.08 ± 0.50	3.14 ± 0.24	3.32 ± 0.79	4.33 ± 1.08	4.58 ± 0.66
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.15 ± 0.04	0.11 ± 0.03	0.07 ± 0	0.15 ± 0.05	0.22 ± 0.07
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD

(Continued)

TABLE 4 | Continued

Conditions	Cytokinins	Optimal conditions				
		MOCK	**10^{-7} M**	**10^{-6} M**	**10^{-5} M**	**10^{-4} M**
	DHZ7G	1.92 ± 0.33	3.17 ± 0.76	4 ± 1.01	4.2 ± 0.67	1.06 ± 0.34
	DHZ9G	0.11 ± 0.03	0.11 ± 0.02	0.17 ± 0.05	0.17 ± 0.03	0.05 ± 0.01
	*c*Z-types	24.23 ± 5.10	31.09 ± 2.9	29.28 ± 2.93	42.74 ± 6.71	43.4 ± 1.98
	*c*Z	<LOD	<LOD	<LOD	<LOD	<LOD
	*c*ZR	3.83 ± 0.97	1.57 ± 0.17	2.56 ± 0.75	3.14 ± 0.91	4.5 ± 1.23
	*c*ZRMP	2.98 ± 0.935	4.01 ± 0.52	4.55 ± 0.63	6.68 ± 1.68	8.63 ± 1.23
	*c*ZOG	0.87 ± 0.19	1.4 ± 0.26	0.75 ± 0.16	0.96 ± 0.3	1.12 ± 0.25
	*c*ZROG	3.67 ± 0.835	6.91 ± 0.72	5.34 ± 0.99	6.84 ± 1.37	8.04 ± 1.15
	*c*Z7G	14.20 ± 2.68	15.53 ± 2.02	24.35 ± 4.67	20.14 ± 2.4	7.87 ± 2.07
	*c*Z9G	0.67 ± 0.18	0.55 ± 0.16	0.79 ± 0.23	0.96 ± 0.14	0.34 ± 0.07

Mean and SD.

DISCUSSION

In this work, a group of four N^9-substituted CK derivatives with a fluorinated carbohydrate moiety (three with an aromatic and one with isoprenoid N^6 side chain) were synthesized (Wan et al., 2005) by a slightly modified one-step reaction of 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine with the appropriate amine or amine hydrochloride in the presence of BOP and DIPEA in DMF (**Figure 1** and **Table 1**). Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been used successfully in many biochemical research studies and therapeutic treatments. As an example, the ability of 9-(2-deoxy-β-D-arabinofuranosyl)adenine to completely inhibit the protozoan parasite *Trichomonas vaginalis* (Shokar et al., 2012), as well as its antibacterial (Gao et al., 2015) and antitrypanosomal (Ranjbarian et al., 2017) effect, have been reported. Significant antiviral activity was also confirmed for their dideoxy analogs. Montgomery et al. (1992) proved that 2-fluoro-9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)adenine had anti-HIV properties, and 9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)adenine was identified as an anti-HBV agent (Maruyama et al., 1999). However, their effects on plant species have never been investigated. Only few works presenting positive bioassay results for kinetin and isopentenyladenine analogs with N^9-substituted with short aliphatic chains been published (Mik et al., 2011a,b). Similar to these results, our newly synthesized compounds (especially compound 2) also showed high levels of antisenescence activity. Moreover, the low activity of the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine derivatives in the callus bioassay confirms that fluorination of the sugar moiety prevents its hydrolysis to free bases and makes these compounds metabolically stable. Due to the strong effect of the CK analogs with a fluorinated carbohydrate moiety on the retention of chlorophyll in excised wheat leaves in the dark (**Table 2**), we hypothesized that the compounds would have antistress properties and analyzed their mode of action and their potential use as priming agents.

For many years, it has been shown that seed priming with certain hormones or other compounds improves seed germination and fitness in many plants (Van Hulten et al., 2006; Hussain et al., 2016). Seed priming improves stress tolerance through "priming memory," which is established during priming and can be recruited later when seeds are exposed to stresses during germination (Chen and Arora, 2013). The beneficial effect of seed priming with CKs has been previously described under a range of growth conditions for many plant species, such as spring wheat (*Triticum aestivum* L.) (Iqbal and Ashraf, 2005; Iqbal et al., 2006) or basil (*Ocimum basilicum* L) (Bagheri et al., 2014). Despite this, there is not always a clear positive effect of priming, and it may also have a negative effect (Miyoshi and Sato, 1997; Sneideris et al., 2015; Willams et al., 2016), depending on the type of compound, the concentration used for priming, or the plant species and cultivars tested (reviewed by De Diego and Spíchal, 2020). In this work, hormopriming with the new N^9-substituted CK derivatives improved early seed establishment and plant growth in *A. thaliana* under optimal and stress growth conditions (**Figure 3**), mainly by making the population more homogeneous, maintaining plant greenness (less chlorophyll degradation) and better nutrient status as defined by higher color indices (**Figure 5**). However, this response was concentration dependent. The best-performing compound was 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine, which was a good growth promotor under optimal growth conditions and a stress alleviator under both salt and osmotic stress at almost all concentrations tested, according to the PBC index (**Table 3**). As an exception, compound 4 at 10^{-4} M showed a strong growth inhibitory (toxic) effect. However, lower concentrations (10^{-7} or 10^{-6} M) improved plant growth under different growth conditions (**Table 3**). This underlines the importance of testing chemicals over broad concentration ranges and under different growth conditions. This is possible through initial high-throughput approaches using model plants such as Arabidopsis, followed by studies in the targeted species and specific growth conditions (Rouphael et al., 2018).

To understand better how these new compounds modify plant metabolism when they are used as priming agents, the endogenous levels of some plant hormones (CK, auxins, and ABA) were quantified. It was clear that hormopriming

FIGURE 6 | Heatmap **(A)** representing the changes in CKs, auxins, and ABA for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (black line), or salt (100 mM NaCl, red line) or osmotic (100 mM mannitol, blue line) stress conditions for 7 days. Principal component analysis (Dimension, Dim) **(B)** of the same Arabidopsis seedlings.

with compound 2 disrupted the plants' hormonal homeostasis (**Figure 6B**). However, the changes varied depending on the conditions under which the plants were grown. Thus, different behaviors were observed between hormoprimed seedlings under optimal and osmotic stress conditions, and those under salt stress (**Supplementary Figure 4**). For example, under optimal conditions, primed Arabidopsis seedlings accumulated higher levels of ribotides (precursors), which were positively correlated

with phenotypic traits such as AGR, RGR, slope of the growing curve and final rosette area (**Supplementary Figure 5A**). However, primed plants grown under salt stress conditions elevated their total CK content by increasing the amounts of conjugated forms including ribosides (iPR. DHZR and *c*ZR), *O*-glucosides (*c*ZOG and *c*ZROG), and *N*-glucosides (iP7G and iP9G). It has been reported that riboside accumulation under stress conditions can be a defense mechanism, helping plants to

deal with stress (Veerasamy et al., 2007; Man et al., 2011; De Diego et al., 2015). This may be because they play a crucial role in CK-mediated leaf longevity, and hence senescence, through phosphorylation of the CK response regulator ARR2 (reviewed by Hönig et al., 2018). For several years the cZ-type CKs and the base cZ were considered to be low-activity forms. However, in recent years, it has been proved that cZ-type CKs play important roles during plant development and in environmental interactions (Schäfer et al., 2015; Lacuesta et al., 2018). Thus, in primed Arabidopsis plants high levels of accumulation of $cZOG$ and $cZROG$ could be a strategy for maintaining plant growth under salt stress conditions. In support of this, it has been reported that the content of cZ-type CKs changes rapidly during maize seedling growth, and that cZ catabolism and glycosylation by cZ O-glucosyl transferases work synergistically to fine-tune cZ levels during plant development (Zalabák et al., 2014). Finally, in these primed plants there was also considerable accumulation of iP7G and iP9G. These two iP derivates are the terminal products of iP metabolism (Hošek et al., 2020). The iP metabolites including iP-N9G are the least active CKs, which seem not to be hydrolyzed and simply accumulate in the tissue (if not degraded by CKX) with no physiological effects (Hoyerová and Hošek, 2020). Overall, it is clear that priming with CK analogs modifies CK metabolism, but these changes are dependent on plant growth conditions. The results also pointed to the iP-type and cZ-type CKs as the main metabolites regulating the alleviation of salt stress in primed Arabidopsis seedlings.

Regarding auxins, levels of oxIAA mainly increased when compound 2 was applied at a high concentration (10^{-4} M) (**Figure 6** and **Supplementary Table 2**). In recent years it has been proved that oxidizing IAA into oxIAA is of major physiological significance in the regulation of plant growth and development (Stepanova and Alonso, 2016). However, changes in other auxin-related metabolites did not show any correlation with the phenotypical changes in plants primed with compound 2.

CONCLUSION

In summary, in this case study we showed that hormopriming with N^9-substituted CK derivatives with a fluorinated carbohydrate moiety seems to be a promising biotechnological approach for improving early seedling establishment and plant growth under both control and stress conditions. This is due to changes in plant hormone metabolism (especially of CKs and auxins) that differs according to growth conditions. Moreover, we believe that we have shown here that a complex approach is needed for selection of suitable compounds, by employing strategies allowing simultaneous testing of a broad range of concentrations and different growth conditions to define the conditions in which they are most efficient as priming agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MB and KD synthesized the compounds. AH, AEH, LS, and ND designed and performed the phenotyping experiments. AH, AP, and ON carried out the metabolite quantification. AH and ND performed the data analysis. MB, AH, LS, KD, and ND wrote the manuscript. All authors discussed the results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.599228/full#supplementary-material

Supplementary Figure 1 | Early seedling establishment of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown under control conditions ($N = 48$). Mean ± SE. Different letters mean significant differences among variants according to Tukey's HSD test after ANOVA.

Supplementary Figure 2 | Growth curves for Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions ($N = 48$). Mean ± SE.

Supplementary Figure 3 | Maximum rosette size of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions ($N = 48$). Mean ± SE. Different letters mean significant differences among variants according to Tukey's HSD test after ANOVA.

Supplementary Figure 4 | Distance between Arabidopsis seedlings non-primed (MOCK) or primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal (C), salt stress (100 mM NaCl, S) or osmotic stress (100 mM mannitol, O) conditions.

Supplementary Figure 5 | Correlation matrix **(A)** and contribution of the loadings to each PC (Dim) **(B)** according to multivariate statistical analyses of traits and metabolites in Arabidopsis seedlings non-primed (MOCK) or primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions ($N = 48$).

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SUPPLEMENT II:

Phloem exudate metabolic content reflects the response to water-deficit stress in pea plants (*Pisum sativum* L.)

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Phloem exudate metabolic content reflects the response to water-deficit stress in pea plants (*Pisum sativum* L.)

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SUMMARY

Drought stress impacts the quality and yield of *Pisum sativum*. Here, we show how short periods of limited water availability during the vegetative stage of pea alters phloem sap content and how these changes are connected to strategies used by plants to cope with water deficit. We have investigated the metabolic content of phloem sap exudates and explored how this reflects *P. sativum* physiological and developmental responses to drought. Our data show that drought is accompanied by phloem-mediated redirection of the components that are necessary for cellular respiration and the proper maintenance of carbon/nitrogen balance during stress. The metabolic content of phloem sap reveals a shift from anabolic to catabolic processes as well as the developmental plasticity of *P. sativum* plants subjected to drought. Our study underlines the importance of phloem-mediated transport for plant adaptation to unfavourable environmental conditions. We also show that phloem exudate analysis can be used as a useful proxy to study stress responses in plants. We propose that the decrease in oleic acid content within phloem sap could be considered as a potential marker of early signalling events mediating drought response.

Keywords: abiotic stress, developmental plasticity, drought, phloem, oleic acid, *Pisum sativum*.

INTRODUCTION

Water deficit has a large impact on the yield of *Pisum sativum* (pea) crops. Even mild and brief periods of drought stress affect important aspects, such as growth and internal trophic relations. As in other multicellular organisms, including humans, responses in particular organs have to be coordinated via the vascular system. The monitoring of vascular samples (blood) has long been established as an effective method to diagnose a wide range of conditions in human health (Coller, 2015). In plants this approach awaits further popularization as we lack effective tools and tests to determine plant physiological status based on phloem or xylem exudate

content. The composition of phloem exudates is not constant and depends on the plant species, its development stage (Pate and Atkins, 1983) and the nutritional conditions (Tilsner *et al.*, 2005). Phloem sap contains carbohydrates, sugar alcohols (polyols), amino acids, organic acids (Canarini *et al.*, 2016), ions (Alfocea *et al.*, 2000), phytohormones (Regnault *et al.*, 2015), secondary metabolites associated with the stress response (Gowan *et al.*, 1995), as well as macromolecules such as proteins (Malter and Wolf, 2011), RNA (Gamboa-Tuz *et al.*, 2018) and fatty acids (Barbaglia and Hoffmann-Benning, 2016). The main form of sugars transported by phloem is sucrose (Lalonde *et al.*, 2003), but other non-reducing

sugars are also present, such as raffinose, stachyose, verbascose, ajugose or sugar alcohols, such as mannitol and sorbitol (Canarini *et al.*, 2016; Dinant *et al.*, 2010). The possibility that phloem could be involved in the transport of reducing sugars had for a long time been discounted; however, in 2008 it was shown that glucose or fructose can be present in the phloem exudates of some plant species and constitute over 80% of the transported carbohydrates (van Bel and Hess, 2008). In addition to carbohydrates, the composition of phloem exudates also includes amino acids such as histidine, arginine, asparagine, glutamine, threonine, glutamic acid, proline, valine, methionine, isoleucine, leucine, phenylalanine or tryptophan (Canarini *et al.*, 2016). Phloem exudates may also contain carboxylic acids that are Krebs cycle intermediates, that is, citric, isocitric, succinic, malic, fumaric and oxaloacetic acids (Canarini *et al.*, 2016). Vascular exudate analyses have been applied successfully to study long-distance coordination in plants at the RNA level (Kehr and Kragler, 2018) or to study movement of the RNA–protein complexes (Kehr and Kragler, 2018; Pahlow *et al.*, 2018). The diversity of molecules representing core metabolic processes in phloem sap suggests that analysis of its composition may reveal a great deal of information concerning plant responses to stress. So far, the analysis of the compounds with ^{13}C labelling in exudates has been useful in determining water-use efficiency (WUE) and CO_2 binding (Keitel *et al.*, 2006). Moreover, it has been shown that leaf gas exchange measurements are very well correlated with carbon content in the phloem exudates (Merchant, 2012). If phloem sap composition reflects both the status of photosynthesis and cellular respiration it could be used to study stress adaptation mechanisms in plants. At present, however, there is a need to understand how changes in vascular sap reflect particular responses, therefore we have combined exudate metabolic studies with the monitoring of physiological and biometric parameters. Based on this approach, we have tried to elucidate how pea plants maintain their carbon and nitrogen balance during mild water deficit stress and to determine the contribution of vascular transport in the redistribution of these compounds. In addition, we have analysed phloem morphodynamics under these conditions; since drought triggers changes in the viscosity and water status of phloem sap, plants have to adopt certain strategies to avoid the scenario where stress completely compromises vascular transport (Sevanto, 2014). To understand how plants protect their conduits to maintain long-distance coordination during abiotic stress, we have analysed phloem composition at the cellular level.

Our results show that plant responses to drought correlate with changes in phloem sap content, therefore we suggest that the exudate analysis method could be used as a diagnostic tool to search for drought-tolerant genotypes.

RESULTS

Pea plants quickly adapt their growth and anatomy to water deficit conditions

Drought has the most significant impact on the yield of pea when it occurs at the flowering stage (Andersen and Aremu, 1991). It has been found, however, that during the early vegetative stage drought can affect the morphology of the plant (in particular the position of the pod on a particular node), and therefore also the productivity of the plant (Klimek-Kopyra *et al.*, 2017). To gain more insight into the potential consequences of spring drought stress on peas, we have analysed changes in phloem sap content and evaluated their use as an indicator of physiological status. Five-day-old seedlings were subjected to a gradual decrease in water availability until the soil reached a pF status of 4.2, which was maintained for 7 days, followed by 10 days of a return to the optimal watering regime (Figure 1a). Morphological changes were monitored on the first and seventh day of pF 4.2 status (drought) and 10 days after rewatering (pF 2.8), and then compared with optimally watered controls (pF 2.8). We found that severe changes in the morphology of the above-ground parts of plants can be observed already after 7 days of mild drought and plants could not recover their growth even 10 days after rewatering (Figure 1b). The relative water content (RWC) of fully expanded leaves decreased by 3.55% after 1 day and by 7.13% after 7 days of drought, compared with appropriate controls (Figure 1c). At 10 days after rewatering the RWC status increased to 7.44% compared with control plants of the same age (Figure 1c). The expression of a drought response marker gene *PsRD29* (Yamaguchi-Shinozaki and Shinozaki, 1993) was highly upregulated (46.85-fold increase, $P < 0.001$) at 7 days after drought stress (Figure 1d). Additional phenotypical characterization using spectral imaging with the same soil pF values, light intensity and temperature showed that shoot growth represented as the green area (Figure S1) and canopy perimeter (Figure S2) both decreased significantly. After rewatering, plants slightly increased their perimeter; however, they did not recover their green area size.

Organ growth reduction can be considered as an evolutionarily conserved mechanism used by plants to avoid unnecessary energy investment during adverse conditions. Modulation of leaf growth and leaf rolling mechanisms help to avoid water losses through evaporation (Avramova *et al.*, 2015; Cal *et al.*, 2019; Clauw *et al.*, 2016). We found that even short periods of water deficit resulted in a decrease in leaf canopy. To develop a proper understanding of this response, we analysed the cellular composition of the abaxial epidermis in the second leaflet of leaves 4 and 8 following 1 and 7 days of drought. The choice of leaves was dictated by the lack (leaf 4) or presence (leaf 8) of meristematic activity within the epidermis at the time of
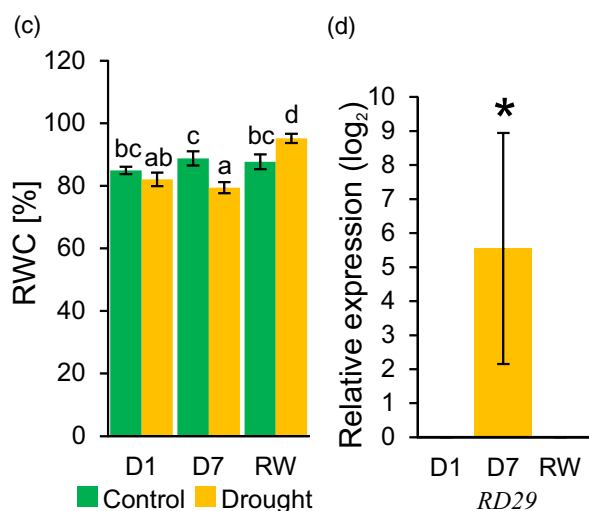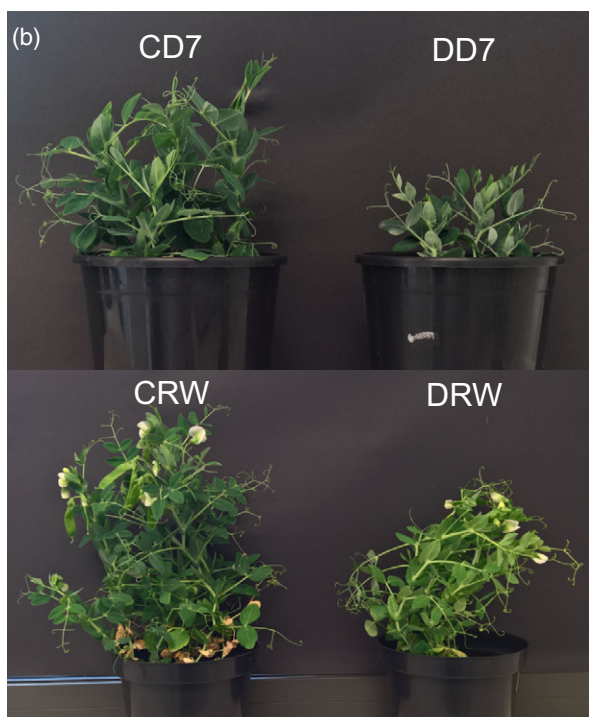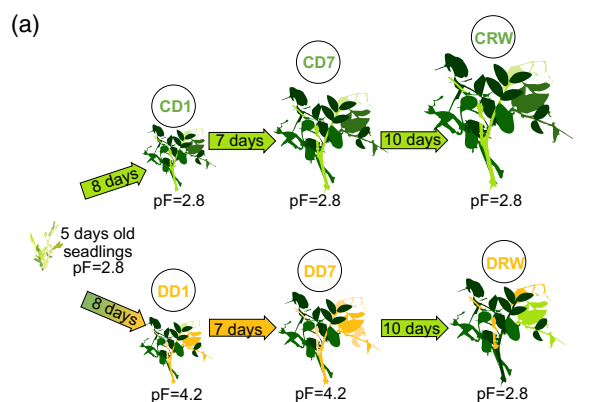
(a)

(b)

(c)   (d)

Figure 1. Experimental design and overall plant reaction to drought.
Plants were subjected to stress by lowering the soil pF to 4.2 and appropriate samples were collected after 1 and 7 days of drought (DD1 and DD7) and 10 days after rewatering and adjustment of soil pF to 2.8 (DRW). Control samples were taken from plants cultivated under an optimal water regime of pF = 2.8 (CD1, CD7 and CRW).
(a) Illustration of experimental design.
(b) Photographs of representative plants showing the restriction of growth induced by drought.
(c) RWC in fully expanded leaves; different letters indicate significant differences, as determined by analysis of variance (ANOVA) with Fisher's post-hoc testing ($P < 0.05$). Error bars indicate the SEs ($n = 7$).
(d) Expression of *PsRD29* in droughted leaves relative to controls. The asterisk indicates a significant difference (pairwise fixed reallocation randomization test, $P < 0.001$); error bars represent SEs ($n = 3$).

drought application (Figure 2a). Samples for epidermal growth analysis were collected 10 days after rewatering when both leaves 4 and 8 have already completed their growth. After tissue clearing, we took pictures of the abaxial epidermis (Figure 2b) and analysed organ growth changes. We found that the leaflet area decreased upon drought in both leaves studied (Figure 2c). Cellular morphometry revealed that the observed changes had a different cellular basis. In leaf 4 a trend towards decreases in cell area and cell number, compared with controls, was observed but did not cross the threshold for significance ($P = 0.06$ and 0.07, respectively; Figure 2d,e). In contrast, in leaf 8 the cell number decreased significantly (Figure 2e), while the cell size was not affected (Figure 2d). On the other hand, the stomatal index decreased in leaf 4, whereas the observed decreases for leaf 8 were smaller and not statistically significant (Figure 2f). In conclusion, even mild and short drought stress induced leaf size reduction; however, particular changes in epidermal anatomy depend on the developmental context of cells at the time of stress application, with both cell division and expansion proving sensitive.

Morphometric analysis revealed that the applied drought conditions did not affect phloem proliferation in the stems, petioles and midvein, with the exception of phloem parenchyma (PP), the number of which was reduced in the midvein of leaf 4 upon stress treatment. However, our analysis identified that drought triggers a whole spectrum of growth decreases in phloem cells (scored as the cell area, cell perimeter and cell eccentricity) (Figures 3, S3 and S4). These changes led to a decrease in the phloem area per bundle only in the midveins of leaf 4 and stem regions located below these organs (Figure 3d). PP are the most responsive cells in all studied organs, and cell area and cell perimeters are the cellular features that were most reduced by drought. It is interesting to note that PP area and perimeter increased in the first and second order of bundles of stems located below leaf 8, whereas the same parameter has decreased in bundles of stems located below leaf 4. Knowing that, at the time of drought stress

Figure 2. Anatomical changes in pea leaves depend on their meristematic status at the time of drought stress application.

(a) Cell divisions in epidermal cells of leaf 4 and leaf 8 at day 1 (D1) of drought application, indicated by Aniline Blue staining marking callose deposition in newly created cell walls.

(b) Abaxial epidermis of pea leaves at the indicated times. Pictures taken on cleared objects under Nomarski contrast. Scale bars for (a) and (b): 50 μm.

Right panel represents the colour-coded cell outlines used for further quantitative analysis: (c) leaflet area, (d) average cell area, (e) cell number, (f) stomatal index. Error bars represent SEs (*n* = 10). Statistically significant changes determined by ANOVA and a post-hoc Fisher's test (average cell area and stomatal index) and Kruskal–Wallis test (cell number) are indicated with asterisks.
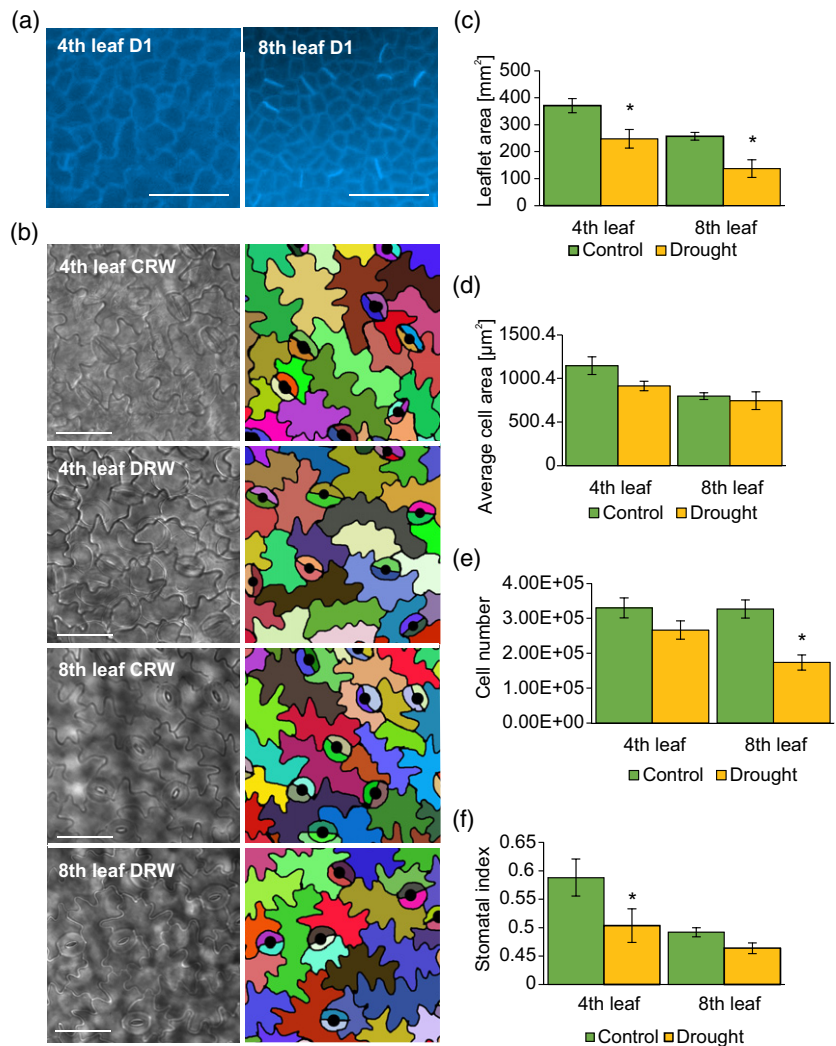
application, leaf 8 is an actively dividing organ and a potential physiological sink, the observed changes may reflect an anatomical plasticity that facilitates nutrient redirection. It is also remarkable that phloem cell shape and growth, but not phloem cell proliferation, is affected by drought. Our biometric analysis has revealed another level of complexity that is related to vascular tissue differentiation. Namely, we observed a decreased number of bundles in petioles of leaf 8 subjected to drought, whereas in leaf 4 this phenomenon was not present. Moreover, the number of bundles in the stem section located below leaf 8 increased upon drought (Figure S5). We believe that this may be an important mechanism that helps to adjust the capacity of the vascular system to particular needs in times of energy restriction caused by drought.

Pea plants modulate photosynthesis and respiration in response to mild drought

To understand how our experimental conditions affected the physiology of pea plants, we measured parameters relating to changes in solar energy conversion and subsequent carbon and nitrogen metabolism in plants. On the first day of drought plants already decreased their stomatal conductance (Figure 4a). This was further reflected by lower evaporation and photosynthesis rates (Figure 4b,c). Interestingly, these changes were not accompanied by a reduction of intercellular CO_2 concentration (Figure 4d). Measurements of photosystem II (PSII) activity (Figure 4e, f) showed that its maximum photochemical efficacy (F_v/F_m) decreased after 7 days of drought. On the first day of stress PSII trapped more light energy relative trapped energy flux per leaf cross section (TR_0/Cs_{rel}); however, it did not influence the PSII electron transport chain relative electron transport flux per leaf cross section (ET_0/Cs_{rel}) and the collected energy was dissipated as heat relative dissipated energy flux per leaf cross section (DI_0/Cs_{rel}). The impact of drought on PSII was more pronounced on day 7 because a decrease in reaction centre density (RC/Cs_{rel}) had occurred. Moreover, despite the fact that TR_0/Cs_{rel} efficacy of light energy harvesting did not differ from the well-watered

control, the leaves were unable to sequester the energy productively and so it dissipated in the form of heat (DI_0/Cs_{rel}). After rewatering, all PSII parameters recovered (Figure 4g).

(a)

(b)

(c)

Midvein

	SE 4th	SE 8th	CC 4th	CC 8th	PP 4th	PP 8th
Avg cell area		decrease			decrease	
No. of cells					decrease	
Perimeter	decrease	decrease	decrease	decrease		
Eccentricity						increase

no difference = white
significant decrease = blue
significant increase = red

Petiole - 1st order

	SE 4th	SE 8th	CC 4th	CC 8th	PP 4th	PP 8th
Avg cell area						decrease
No. of cells						
Perimeter		decrease			decrease	
Eccentricity		decrease		decrease	decrease	

Petiole - 2nd order

	SE 4th	SE 8th	CC 4th	CC 8th	PP 4th	PP 8th
Avg cell area					decrease	decrease
No. of cells						
Perimeter	decrease	decrease			decrease	
Eccentricity	increase			decrease		

Stem - 1st order

	SE 4th	SE 8th	CC 4th	CC 8th	PP 4th	PP 8th
Avg cell area	decrease				decrease	increase
No. of cells						
Perimeter	decrease				decrease	increase
Eccentricity		decrease	decrease	decrease	increase	

Stem - 2nd order

	SE 4th	SE 8th	CC 4th	CC 8th	PP 4th	PP 8th
Avg cell area	decrease				decrease	increase
No. of cells						
Perimeter	decrease				decrease	increase
Eccentricity	decrease	decrease	decrease	decrease		

(d)

More detailed time-course experiments carried out in the phenotyping system corroborated that the drought-induced growth reduction was largely related to changes in chlorophyll fluorescence parameters. The light-adapted maximum quantum yield of PSII (Φ_{PSII}) and the coefficient of photochemical capacity/quenching (Φ_P), describing the actual fraction of reactive centres being in the open state, were significantly reduced by drought stress (Figure 5a,b). At the same time, the Φ_{NPQ} parameter, describing non-photochemical quenching that helps to dissipate heat, increased significantly (Figure 5c). The determination of leaf temperature by infrared (IR) imaging was even more sensitive, and significant differences were observed from the first point of the drought stress (Figure 5a,d). We also observed that changes in the median value of the fluorescence parameter Φ_{PSII} correlated with fluctuations in leaf temperature (Figure 5e). This was particularly apparent in the afternoon when the drought-stressed plants were not able to cool down, thereby decreasing their fluorescence efficacy.

Together with biometric data these measurements were our reference for a greater understanding of changes in the metabolite content of phloem exudates.

The content of pea phloem sap reveals drought-induced responses and a tolerance that develops over the period of stress duration

To identify metabolic changes underlying the reduction in plant growth observed upon water limitation, we have collected phloem sap exudates from excised leaves on the first and seventh day of drought and 10 days after rewatering, along with appropriate control combinations from plants growing under optimal water conditions. Samples were lyophilized and subjected to further GC/MS analysis and metabolite identification. To summarize the changes in phloem composition, we performed principal component (PC) analysis and projected the results onto a biplot

Figure 3. Drought affects the growth of phloem cells in *Pisum sativum* (pea).
(a) Representative transverse section of pea vascular bundle stained with Toluidine Blue.
(b) Grayscale picture with colour coding of particular phloem cell fractions used for scoring developmental plasticity within veins with LITHOGRAPHIX: plum, companion cells (CC); yellow, sieve elements (SE); and cyan, phloem parenchyma (PP). Scale bars: 20 μm.
(c) Summary of quantitative changes in phloem cell anatomy. White indicates no change, red indicates a significant increase compared with controls and blue indicates a significant decrease under drought ($P < 0.05$, determined by ANOVA and a post-hoc Fisher's test).
(d) Phloem area in conducting bundles in midvein (MV), petiole (PE) and stem (ST), calculated as the total area of all types of phloem cells. Error bars indicate SEs ($n = 3$, with five randomly chosen sections for each biological repeat).

Figure 4. Changes in photosynthesis and gas exchange occurring in pea plants subjected to drought.

Stomatal conductance (a), evaporation rate (b), photosynthesis rate (c) and intercellular CO_2 concentration (d). Error bars indicate SEs ($n = 6$). Drought-induced changes in electron transport were estimated by chlorophyll fluorescence measurements ($n = 10$; Pi_{abs}, performance index for energy conservation from photons absorbed by PSII antenna in the reduction of quinone; RC/Cs_{rel}, relative density of active reaction centres per leaf cross section; ABS/Cs_{rel}, absorption flux per leaf cross section; TR_0/Cs_{rel}, relative trapped energy flux per leaf cross section; DI_0/Cs_{rel}, relative dissipated energy flux per leaf cross section; ET_0/Cs_{rel}, relative electron transport flux per leaf cross section; F_v/F_m, maximum quantum efficiency of PSII photochemistry) at day 1 (D1) (e), day 7 (D7) (f) and rewatering (RW) (g), presented as radar charts. The means and SEs of 10 replicates were calculated using ANOVA and post-hoc Fisher's test. Different letters indicate a significant difference between means.
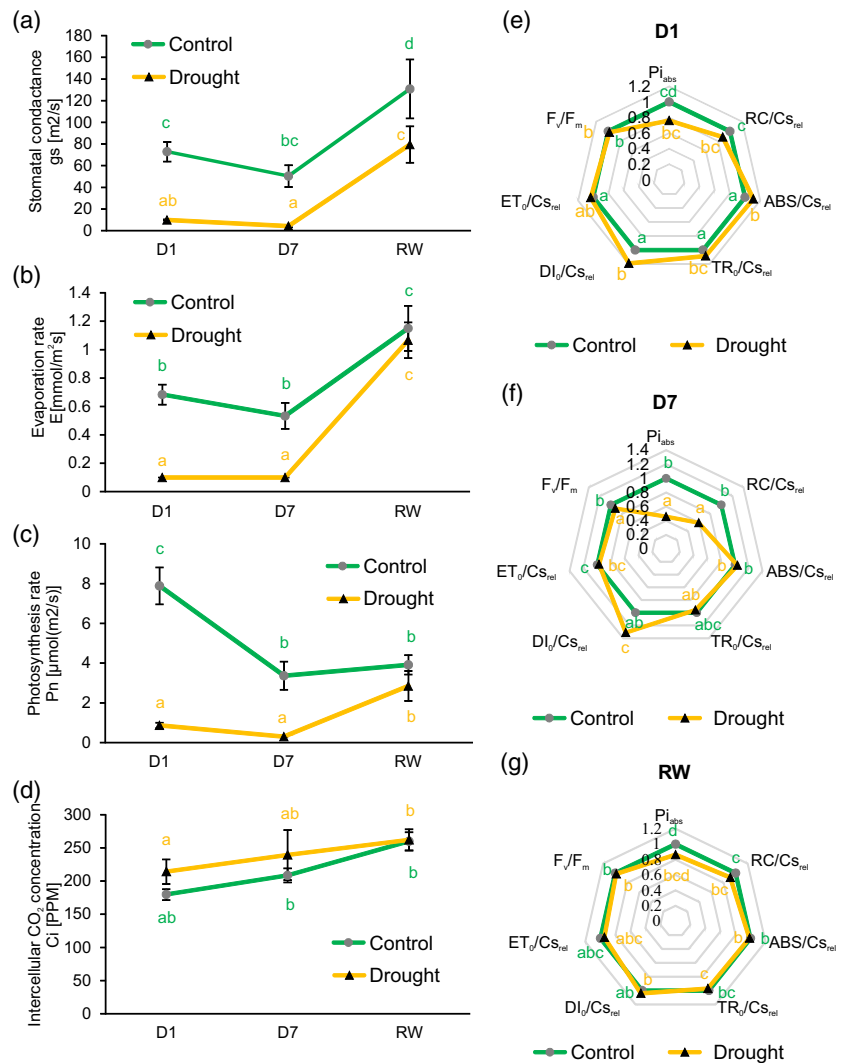
representing the scores (variants as treatment and days) and loadings (metabolites). The first two PCs together captured 71.5% of the variance. PC1 (Dim1), which accounted for 55.2% of the total variation, separated the drought-stressed plants from the controls and rewatered plants. Furthermore, PC2 (Dim2) separated the drought variants into two groups: (i) the plants on the first day of drought (DD1) were located with the metabolites (loadings) related to them; and (ii) the plants on the seventh day of drought (DD7) were located with their related metabolites (Figure S6). Upon drought and in response to rewatering (RW), 63 compounds exhibited differential accumulation in at least one time points (D1, D7 or RW) (Figure 6; Table S3).

We observed a statistically higher accumulation of 32 metabolites at D1 and 29 metabolites at D7 after drought. The accumulation of only a few components decreased upon drought (at D1, oleic acid content decreased to non-

detectable levels, whereas at D7 the levels of lyxopyranose and sucrose decreased 3.12- and 6.61-fold, respectively). The dynamics of oleate content change was further investigated in a separate experiment by targeted metabolomics in a time course spanning the period of water content decrease, leading up to the soil reaching the pF value of 4.2. Changes in this targeted experiment did not exactly match the picture obtained in non-targeted experiments; however, again a very rapid decrease of oleate content in response to the reduction in water availability was observed (Figure 7).

After rewatering the majority of significantly differentially accumulating compounds decreased in the phloem sap of plants recovering from drought, compared with controls (46 compounds), whereas only one component increased (Figure 6; Table S3). Analyses of metabolite accumulation show that drought-stressed time points are more alike than rewatered plants (Figure 6). The class of compounds predominantly accumulating in drought-

(a)

(b)

(c)

(d)

(e)
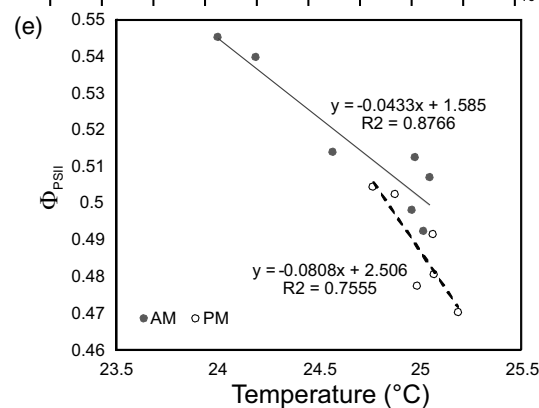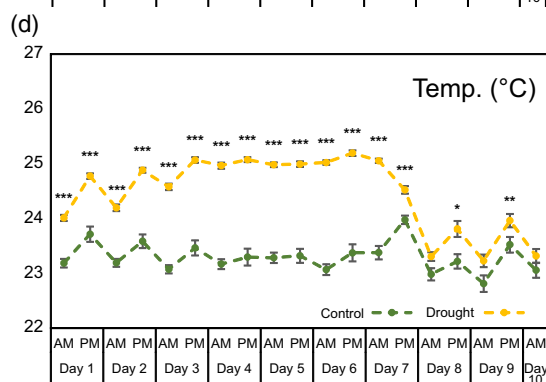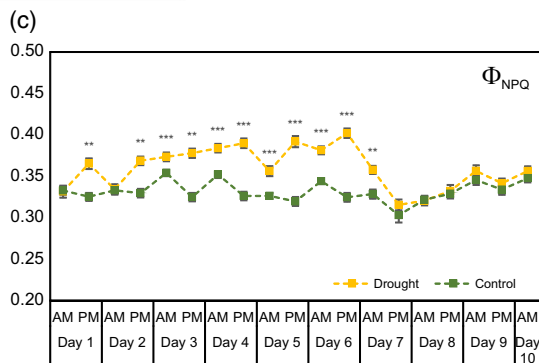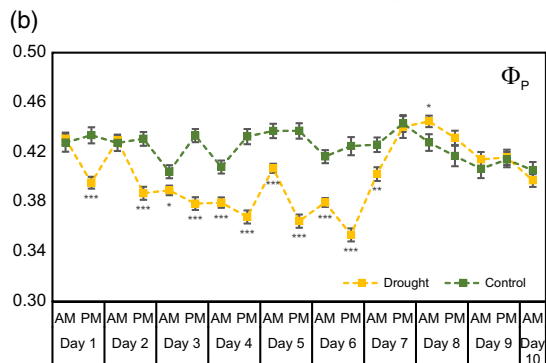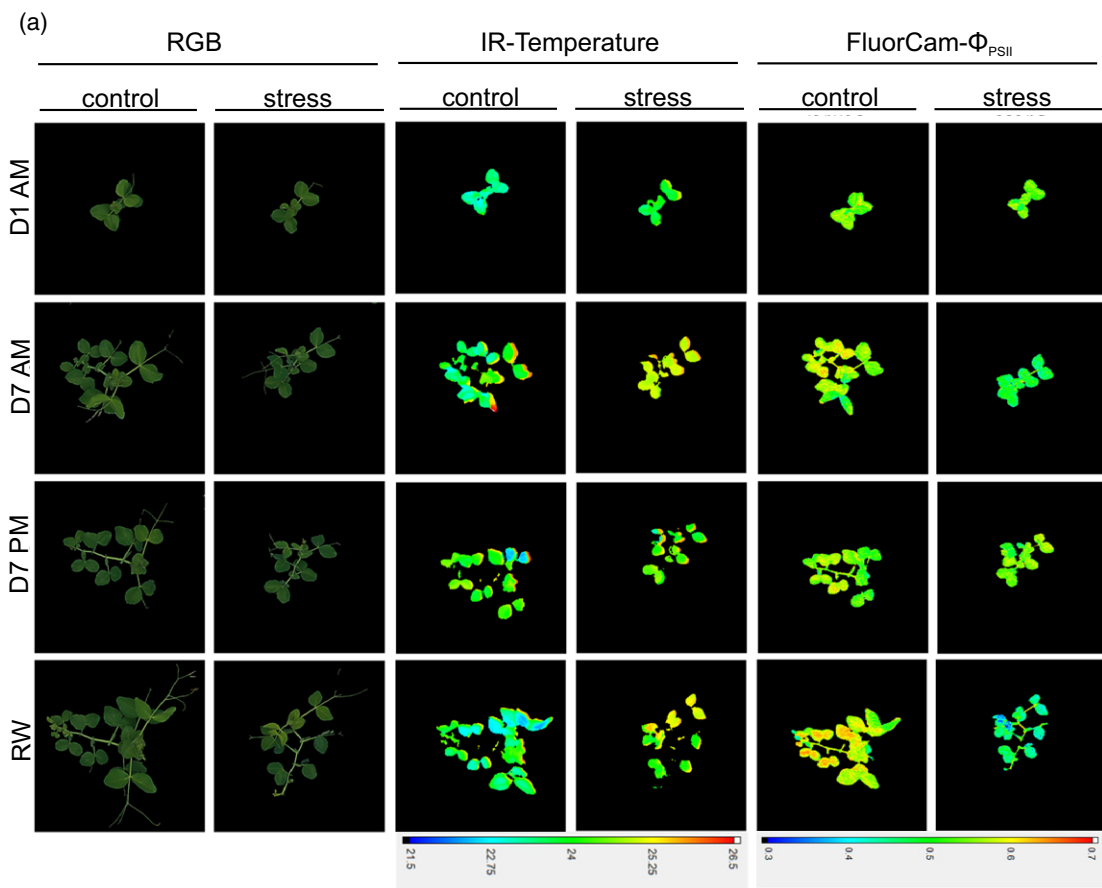
Figure 5. Drought-induced changes in growth, heat dissipation and chlorophyll fluorescence in pea leaves monitored by RGB, IR and FluorCam imaging (a). Changes in the coefficient of photochemical capacity/quenching Φ_P (b), non-photochemical quenching Φ_{NPQ} (c) and leaf temperature (d) upon drought stress. Values are means $\pm$ SEs ($n = 18$ for control and $n = 30$ for drought). Statistically significant changes determined by ANOVA and a post-hoc Fisher's test are indicated with asterisks: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. Correlations of median parameters indicated light-adapted maximum quantum yield of PSII (Φ_{PSII}) and fluctuations in leaf temperature (e).

stressed plants were amino acids, followed by carbohydrates and organic acids. At D1 of drought, the accumulation of nine amino acids increased significantly: proline (30.53-fold), ornithine (13.00-fold), alanine (4.03-fold), threonine (3.99-fold), γ-aminobutyrate (GABA; 3.31-fold), tyrosine (3.09-fold), aspartate (2.79-fold), pyroglutamate (2.37-fold) and glycine (2.21-fold). At D7 of drought the phloem exudates had elevated accumulations of six amino acids: proline (12.18-fold), glutamate (8.34-fold), GABA (5.01-fold), aspartate (4.46-fold), alanine (3.33-fold) and threonine (3.09-fold). The carbohydrates and derivatives increasing in abundance at D1 of drought were: xylulose (19.77-fold), L-arabinopyranose (6.89-fold), D-arabitol (5.24-fold), D-glucose (5.23-fold), maltose 1 (5.05-fold), aucubin (4.88-fold), gluconate (4.50-fold), glyceryl glycoside (3.61-fold), maltose 2 (3.52-fold), L-arabitol (3.23-fold), galacturonate (2.88-fold) and mannopyranose (2.21-fold). The longer period of stress (D7) resulted in an increase of 13 components from this group, namely: galactose phosphate (24.22-fold), D-pinitol (7.31-fold), xylulose (5.58-fold), D-galacturonate (4.43-fold), D-glucose (4.33-fold), DL-arabinose (3.95-fold), maltose 1 (3.91-fold), D-mannitol (2.79-fold), glyceryl glycoside (2.73 fold), L-arabitol (2.49-fold), D-rybofuranose 1 (2.16-fold) and levoglucosan (1.81-fold). Conversely, the amount of D-lyxopyranose and sucrose in phloem exudates decreased after D7 of drought, by 3.12- and 6.61-fold, respectively. The 11 organic acids that accumulated in phloem exudates at significantly higher levels on D1 of drought, compared with controls, were: aconitate (8.17-fold), deoxytetronate (7.37-fold), hexanoate (5.97-fold), tartarate (5.06-fold), fumarate (4.67-fold), threonate (4.59-fold), ribonate (4.23-fold), malate (2.38-fold), methyl succinate (2.13-fold), maleate (1.69-fold) and 4-hydroxybenzoate (1.29-fold). Interestingly, at D1 of drought the content of oleate droped down to non-detectable levels and was significantly lower than in control sap. After D7 of drought higher accumulations of 10 carboxylic acids were observed, namely deoxytetronate (8.95-fold), 2-oxoglutarate (6.81-fold), threonate (4.45-fold), hexanoate (3.80-fold), fumarate (3.56-fold), succinate (3.56-fold), ribonate (3.10-fold), malate (2.56-fold), maleate (1.71-fold) and 4-hydroxybenzoate (1.31-fold).

Different accumulation patterns were observed between D1 and D7, with some components increasing only at one time point, whereas others exhibited quantitative differences at both time points (Figure 6). For example, only at D1 was there a significant increase in glycine, ornithine, aconitate, D-arabitol or aucubin, whereas at D7

higher amounts of the components pinitol, mannitol and glutamate are present in phloem sap. It was also interesting to see a higher accumulation of pyroglutamate at D1 versus increased glutamate at D7. Another interesting point is that in exudates collected at D7 after drought we can observe a significant decrease in sucrose levels, which is accompanied by increases in glucose and pinitol. These differences may in fact reflect a shift in the plant physiological status that occurs over time during the response to drought.

Technical considerations related to metabolic studies of phloem sap

Drought stress treatment leads to a decrease in water content, and therefore we can expect that some changes that we observed are the consequence of an increase in phloem sap viscosity. To some extent this phenomenon together with higher osmotic potential is an important mechanism that helps to retain water and to preserve phloem sap flow during times of water deficit (Sevanto, 2014). To determine how different viscosities affect the exudation velocity, we have performed a study in which samples were collected after 0, 0.5, 1, 2, 3 and 6 h of exudation and the sucrose content was quantified. We found that differences in exudation velocity between stressed and non-stressed samples occurred within the first 3 h, but 6 h of exudation led to the collection of the majority of sucrose (Figure 8a,b). We can also conclude that the increased viscosity of phloem sap does not compromise sucrose exudation.

On the other hand, longer exudation times may also create a problem where some components degrade over time. Such a phenomenon was previously noticed in the case of sucrose stability (Tetyuk *et al.*, 2013). Apparently, we also faced this scenario as higher levels of sucrose at 7 days after drought compared with representative non-stressed controls were observed when exudation collection was performed over short periods (Figure 8c). We also re-analysed glucose content in these the same exudates. We found that mean values for this component were higher in the phloem sap of drought-treated plants; however, with the large differences between biological repeats, these changes were not statistically significant (Figure S7). These findings prompted us to perform an additional study that could at least partially address the origin of carbohydrates in the phloem sap of stressed plants. For this analysis, we used the $^{13}CO_2$ isotope and traced sucrose with incorporated ^{13}C. We found that in stressed plants a lower
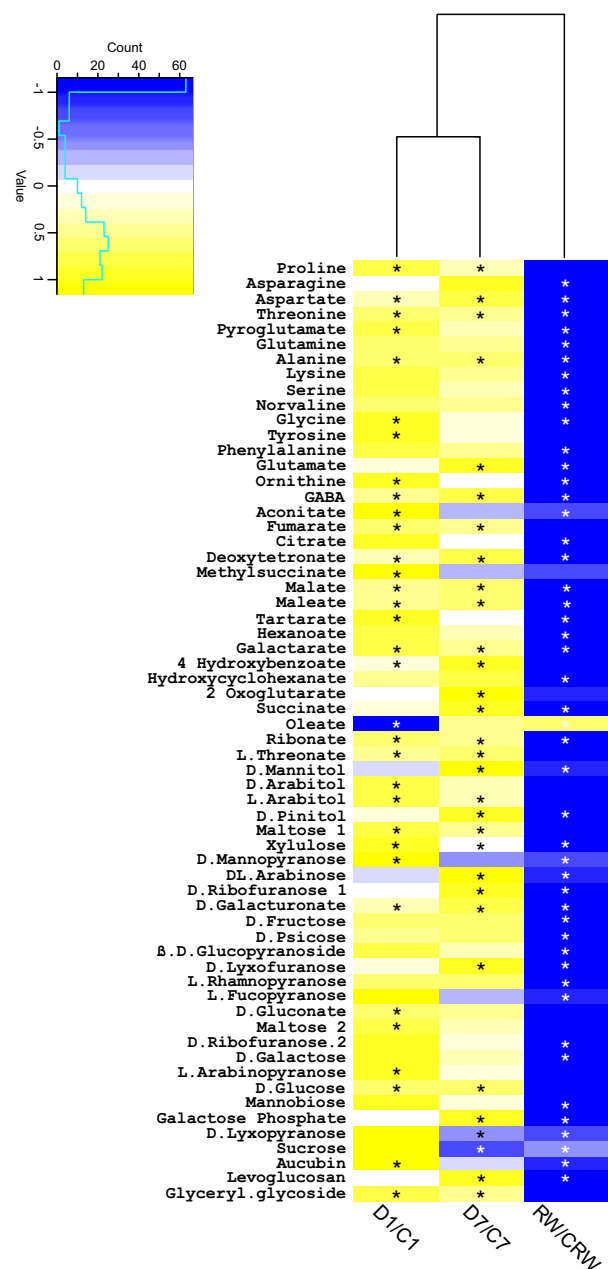
Figure 6. Differential accumulation of metabolites in phloem sap of pea plants upon drought and subsequent rewatering.

The heat map presents the mean (log base 10) ratio between drought stress and controls, averaged over three biological replicates, with yellow representing increased abundance upon drought, blue representing decreased abundance upon drought and white representing no change. The stepped light-blue line within the colour scale inset indicates the number of metabolites counted per level. Statistically significant changes marked with asterisks were calculated based on an analysis of variance (ANOVA) with subsequent Fisher's post-hoc test at $P < 0.05$ ($n = 3$).

Figure 7. The decrease in the oleic acid content of phloem exudate occurs before soil reaches the wilting point.

After water withdrawal phloem sap fractions were sampled when soil pF reached 3.85, 3.95 and 4.2 (critical wilting point). For each of these stages control exudates from optimally watered plants were also collected. (a) Illustration of the experimental design. The experiment was carried out in three independent biological repeats ($n = 3$); each consisted of exudates collected from four plants (with three randomly chosen fully developed leaves for each plant, which gives 12 leaves for each repeat). (b) Results of targeted analysis of oleic acid content. Statistical significance was determined by ANOVA, and post-hoc Tukey's honestly significant difference (HSD) test; asterisks represent statistically significant differences between optimal watering and water depletion combinations within the same time points ($\alpha = 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

proportion of labelled to non-labelled sucrose was present (Figure 8d). This is in agreement with the photosynthesis decreases observed in physiological measurements (Figure 4).

Another issue with the analysis of phloem metabolite content is the possibility of phloem sap contamination by xylem content. This is very difficult to estimate; however, it can be at least partially ruled out when exudates from the petioles are analysed. Based on our knowledge on the hydrodynamics of the vascular system, we can expect that when phloem exudates are collected from whole leaves, transpiration prevents leakage from the xylem, whereas exudates from petioles should also contain xylem components (Stroock *et al.*, 2014). We have performed such a study and the metabolic petiole-specific fingerprints did not correlate with the patterns observed in leaf phloem exudates. Figure S8 shows the distinct clustering, with

Figure 8. Detailed study of changes in sucrose content in the phloem sap of *Pisum sativum* (pea) plants subjected to drought.

Velocity of sucrose exudation at day 1 (D1) (a) and day 7 (D7) (b) carried out for 0, 0.5, 1, 2, 3 and 6 h. Data are presented as the average values of normalized peak area reads characteristic for sucrose; error bars show SEs. The formula describing the exudation velocity is indicated by colour for each combination.

(c) The sum of sucrose content in phloem sap collected as separate fractions in short intervals (0, 0.5, 1, 2, 3 and 6 h).

(d) Proportion of labelled (^{13}C) to non-labelled (^{12}C) sucrose based on peak area. Statistical differences were calculated by unpaired Student's *t*-test. Scale bars indicate SEs ($n = 3$; $\alpha = 0.05$; *$P \leq 0.05$). Each biological repeat consisted of exudates collected from four plants (with three randomly chosen fully developed leaves for each plant, which gives 12 leaves for each repeat).
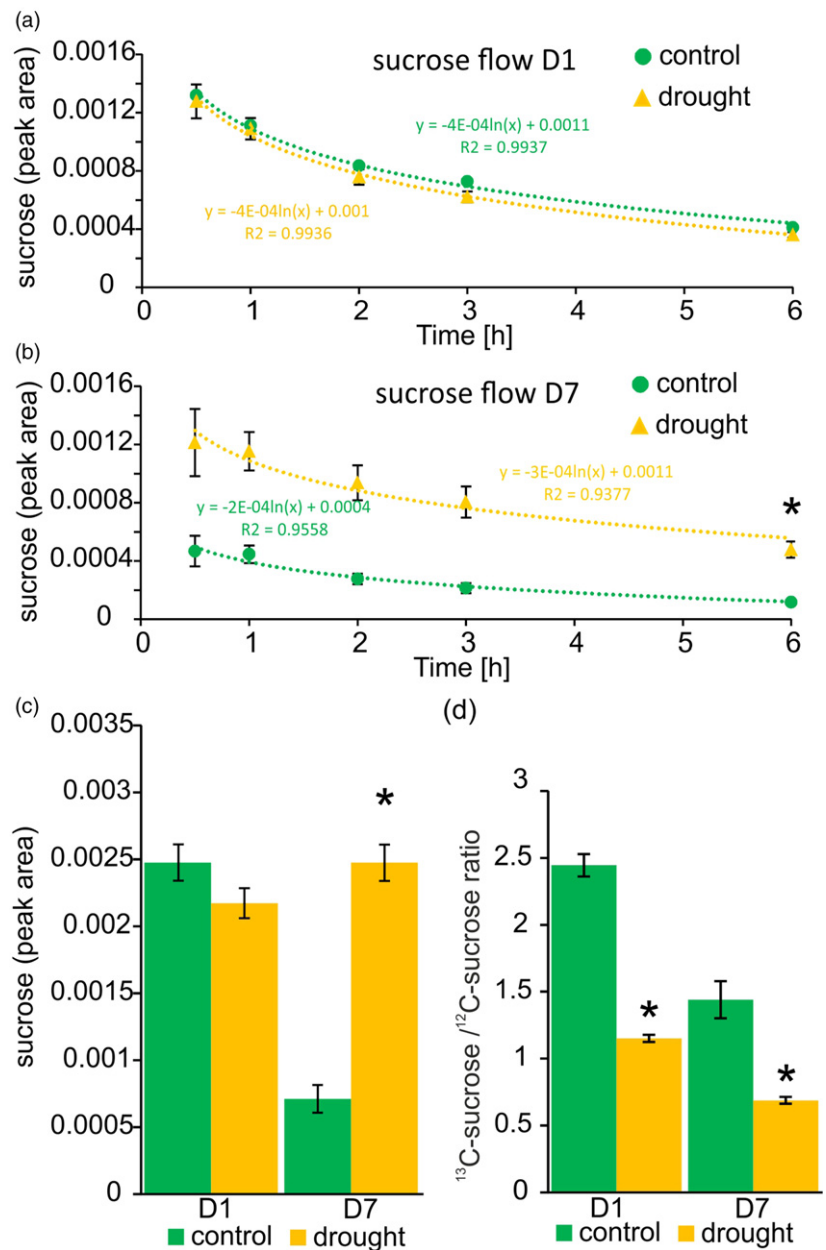
dim1 accounting for 59.3% of the variance and dim2 accounting for 6.5% of the variance. We cannot rule out the possibility of contamination; however, this test does prove that the resulting metabolic fingerprints are based on changes in phloem sap.

Based on our velocity flow test, we can assume that longer exudation periods are more appropriate, especially when physiological stresses affecting phloem sap viscosity are the subject of study. The analyses presented, however, have shown that for some components (such as sucrose) additional inhibitors preventing degradation should be added. This is of course difficult when a non-targeted analysis of the whole metabolome is performed, as numerous

components are expected to be analysed. We found that sucrose degraded during exudation, and therefore we have to revise our previous observation and contend that the decrease in sucrose is an artefact related to the longer duration of sample collection.

DISCUSSION

Relationship between the drought-triggered decrease in above-ground growth and metabolic changes

Cell expansion in plants is driven by internal turgor pressure (Braidwood *et al.*, 2014), therefore it is not surprising that limited water availability results in a decrease of

growth. This developmental strategy, however, is not just a physical outcome of cellular water content but also a complex and finely adapted mechanism that enables plants to limit the loss of energy during difficult times. Cellular processes determining expansion (cell wall synthesis) or proliferation (cell cycle machinery) consume energy that will be scarce during times of stress. Confronted with drought stress, plants may deploy energy reserves to expand their root systems in search of new water resources or limit energy expenditure, and hence water use, to survive a temporary scarcity. Frequently, growth investments during a period of drought happen underground, whereas the upper part of the plant reduces expansion and its role is mainly to support the underground organs. Even a slight drop in the RWC of plants triggers long-distance signal transduction (Kollist *et al.*, 2019). In agreement with the central role of the phloem in the long-distance coordination of numerous responses in plants (Takahashi and Shinozaki, 2019), our metabolomic studies showed that already after 1 day of drought the levels of aconitate, aucubin, ornithine, glycine, pyroglutamate, proline and GABA were increased. We speculate that this in turn triggers physiological responses aimed at conserving energy and the redistribution of carbon- and nitrogen-containing components. We found that already by the first day of drought, stomatal conductance, the photosynthesis rate and the evaporation rate declined. This was not immediately reflected by a reduction in above-ground growth, as the first significant decreases in plant canopy perimeter could only be seen after 5 days of drought (Figure S2). The observed physiological changes were the outcome of stomatal closure, which is a well-known reaction to drought (Pirasteh-Anosheh *et al.*, 2016). It is intriguing that closing stomata did not lead to a decrease in intercellular CO_2 concentrations. Partially this may be explained by the observed drop in the photosynthesis rate, which would diminish CO_2 consumption; however, the higher production of metabolic CO_2 during stress should also be considered. This suggests a scenario where plants decrease their water losses by limiting transpiration and try to cope with lower energy production (decrease in photosynthesis) through increased catabolism and carbon redistribution. This has been reflected by the higher accumulation of amino acids in phloem sap, perhaps being remobilized to physiological sinks like young leaves or roots from older leaves to compensate for the limitation in assimilate export. We have also found higher levels of glutamate, which plays a double role as a molecule that, after perception by glutamate receptor-like ion channels, triggers the calcium ion signalling cascade, propagated over long distances through the phloem (Toyota *et al.*, 2018), and (together with ornithine, proline and GABA) is a central component of nitrogen metabolism in plants (Majumdar *et al.*, 2016). It is also worth mentioning that the so-called 'GABA shunt' is a key pathway regulating carbon–nitrogen balance in plants (Michaeli and Fromm, 2015; Podlešáková *et al.*, 2019). Furthermore, the increased accumulation of tricarboxylic acid cycle (TCA)/Krebs cycle components within the phloem of drought-exposed plants suggests that pea plants facing water limitations may redirect metabolites that can be used to support primary metabolism in organs that are in need. In particular, we have found elevated levels of amino acids like proline (Pro), aspartate (Asp), threonine (Thr), alanine (Ala), glycine (Gly) and tyrosine (Tyr) that could be redirected towards actively dividing young organs during stress conditions to provide the components of the TCA cycle (Hildebrandt *et al.*, 2015). We have also seen increases in other core components of the TCA pathway and interconnecting biochemical shunts allowing for alternative routes for the passage of carbon and nitrogen (Figure S8). These include *trans*-aconitate, oxoglutarate, succinate, fumarate and malate (Rocha *et al.*, 2010). Depending on the demand and oxygen availability, some of these components (e.g. oxoglutarate and succinate) can be incorporated into the main TCA cycle or, after conversion, used to provide ATP in the cells of actively growing organs. As droughted plants gradually decrease their growth, some changes observed in phloem sap at D7 after drought treatment may also arise from lower carbon consumption. Our biometric studies performed after rewatering showed that both the expansion and the proliferation of cells within leaves is affected by drought. During drought, numerous metabolites are redirected towards young and actively dividing leaves (Mundim and Pringle, 2018). This process however is not sufficient to support proliferation over longer periods of drought, therefore a decrease in cell number occurs. Such changes cannot be compensated for after rewatering as by that time meristematic activities cease. It has been shown that the decrease in the shoot/root ratio during abiotic stress is modulated over long distances by Pro synthesis or turnover (Sharma *et al.*, 2011) and we found high levels of this amino acid in exudates from stressed plants. The higher levels of galactose phosphate detected in exudates after 7 days of drought also accords with the observed decrease in cell and organ size, as this component has been previously linked with the inhibition of auxin-induced growth (Yamamoto *et al.*, 1988). That change is also accompanied by a significant decrease in sucrose in phloem sap, which is known to regulate plant organ growth at multiple levels, including long-distance coordination (Kircher and Schopfer, 2012). After verification, we found increased levels of sucrose in the exudates from stressed plants (Figure 8c). However, our further studies with $^{13}CO_2$ supplementation have shown that a larger fraction of sucrose accumulated in stressed plants has no ^{13}C incorporated (Figure 8d). That suggests the situation when carbohydrate reserves are remobilised in response to water deficit and decrease in

leaf photosynthetic and gas exchange activity. In this case, even the higher levels of sucrose accumulating in phloem sap will not be directly translated into higher organ growth. A decrease in lamina size has obvious advantages because it reduces surface evaporation and helps to avoid light-induced damage to photosystems. Frequently drought conditions coincide with light excess; however, in our experiments, conducted in controlled conditions, the light energy was the same for control and stressed treatments. Despite this fact, we could observe that drought stress itself induced non-photochemical quenching to dissipate heat. Most probably this was not enough to fully protect the photosynthetic machinery and stressed plants were dissipating the energy by external heat emission. Larger leaf lamina and overall canopy size would additionally increase the impact of drought on the first phase of photosynthesis and make the maintenance and protection of photosystems more difficult. Our study shows that long-distance coordination and phloem-mediated change in the allocation or recycling of components that facilitate energy remobilization contribute to a drought-avoidance strategy based on decreased above-ground growth. The overview on changes in primary carbon and nitrogen metabolism observed in pea phloem exudates is presented in Figure S9.

Phloem transport integrity during limited water availability

Vascular tissue in plants shows a wide range of developmental plasticity upon environmental stress (Figure 3). Changes in the vasculature affect the efficacy of phloem and xylem transport. As xylem plays a major role in water uptake and distribution, numerous studies describing the correlation between its particular anatomical features and parameters like hydraulic conductance and the likelihood of embolism formation have been conducted (Sack and Scoffoni, 2013). Phloem tissue is connected with xylem and therefore in response to changes in water potential triggered by drought, plants launch actions that aim to maintain the osmotic balance between these two vascular tissues (Sevanto, 2018). Plants intensify sugar loading to phloem that, along with the regulation of stomatal closure, is an important mechanism in regulating internal water relations. Sugar metabolism is very sensitive to stress conditions (Lemoine *et al.*, 2013), and therefore it is not surprising that we have observed significant change in the abundance of particular carbohydrates within the phloem sap of pea plants subjected to drought (Figure 6; Table S3). Carbohydrates are tightly linked to basic cellular processes like photosynthesis and respiration, which are usually influenced by stress conditions (Figure S9). Initially we found that at D7 the sucrose content was decreased; however, this observation has been re-evaluated after further targeted study and time-course collection. We found that

the amount of sucrose in the phloem increases, but that the long duration of phloem sap collection resulted in sucrose degradation (Figure 8b). This observation is in agreement with previous experiments performed by Tetyuk, Benning and Hoffmann-Benning (2013), where similar effects of exudation time on sucrose stability have been observed. After the completion of all experiments, we can state that drought leads to the increased accumulation of sucrose, glucose and sugar alcohol (pinitol, arabitol and mannitol) in the phloem sap of pea plants. This elevates phloem sap viscosity and helps to maintain the high osmotic potential within the vasculature that prevents water loss and transport discontinuity during periods of low water availability. In addition, the content of Pro, which efficiently increases osmotic strength, was also elevated.

Plants have also adapted their phloem anatomy (Figure 3), possibly to avoid the scenario where prolonged drought increases phloem sap viscosity and disrupts the osmotic balance (Sevanto *et al.*, 2014). To prevent decreases in the turgor pressure of phloem cells and potential blockages of assimilate transport, the sieve elements (SEs) and companion cells (CCs) are surrounded by parenchymatic cells that can stabilize and buffer turgor changes. Our detailed inspection of phloem anatomical changes triggered by drought has shown that, similar to tissues in other organs, cells in the vasculature grow less in response to water deficits. It is worth noting that the PP cells most strongly responded to changes in water availability. This is in agreement with their potential role in the optimization of water balance to protect vascular flow. Intriguingly, we have noticed that PP cells in stem regions located below the axis of leaf 4 and leaf 8 differentially responded to drought. The average PP cell area and perimeter increased in phloem bundles located in stems below the eighth leaf axis, whereas in PP cells of the same region below leaf 4 these same parameters were decreased. Aniline blue staining shows that, at the time of drought stress application, leaf 8 is an actively dividing organ. Cell proliferation requires a high-energy investment that comes from major metabolic processes, including photosynthesis, mitochondrial respiration and protein or carbohydrate turnover (Siqueira *et al.*, 2018). Our phloem sap analysis shows that there is an intensive redirection of metabolites related to the TCA cycle as well as carbohydrates, which can act as a source of energy. In plants, nutrients are delivered to rapidly growing organs from roots and older leaves (López-Salmerón *et al.*, 2019), therefore efficient carbohydrate transport via the phloem as well as the exchange between xylem and phloem of some amino acids (e.g. Arg, Asp or Glu), providing nitrogen to meristematically active tissues, must be secured. The observed developmental responses of PP cells to drought may be related to a particular physiological situation,

where the plant needs to adapt phloem anatomy to facilitate lateral exchange between xylem and phloem and to maintain the flow of nutrients (especially nitrates) from the roots to rapidly growing organs (Aubry *et al.*, 2019). Savage *et al.* (2013) have shown that newly developed phloem tissues mainly act to facilitate carbohydrate transport towards leaf primordia and young proliferating leaves requiring large energy investments, and moreover that the changes in carbon transport via phloem reflected the transition of true leaves from sink to source. These observations indicate that phloem-driven carbohydrate transport is heterologous and depends on the developmental stage of the leaves. At present, we do not know the exact role of the observed differences in the drought-induced developmental plasticity of PP cells in the stem regions underneath the proliferating leaf 8 and meristematically inactive leaf 4. At least partially it may arise from the different nutrient requirements of these organs. However, further understanding of the observed phenomenon needs more detailed experiments with labelled root-derived nitrogen compounds or photosynthesis products of leaves.

Vascular exudates as indicators of physiological responses in plants

The collection of molecules that move over long distances via vascular tissues provides a valuable insight into understanding how plants cope with adverse conditions. Phloem sap contains physiologically essential components like carbohydrates, amino acids, proteins or RNA molecules, and therefore changes in its composition may reflect particular plant responses. As mentioned by Dinant and Suárez-López (2012), despite the potential usefulness of phloem sap collection methods they are artefact prone. The content of phloem exudates can be affected by phloem sap viscosity changes, degradation that occurs during exudation or possible contamination from xylem. On the other hand, phloem exudate collection approaches seem to be plausible for high-throughput analysis of the long-distance coordination of plant responses or for monitoring plant physiological status. In such studies, however, emerging patterns should be analysed rather than the change of a single component. In our work we used an ethylenediaminetetraacetic acid (EDTA)-assisted method that helps to overcome the blocking of sieve tubes at the excision site by callose, thereby assuring efficient exudation (Tetyuk *et al.*, 2013). We studied changes in the metabolic content of phloem sap, collected this way, that occur upon drought. Differential levels of some metabolites can be used in the future as potential indicators of the stress response. We have found that oleic acid content decreases in phloem rapidly upon water withdrawal. It has been shown that low levels of oleic acid promote nitric oxide (NO) synthesis and correlate with the upregulation of genes involved in NO-mediated signalling (Mandal *et al.*,

2012). We propose that the decrease in oleic acid within phloem sap could be considered as a potential marker of early signalling events that occur when pea plants are subjected to drought. Our study also shows that amino acid content as well as the accumulation of sugars and sugar alcohols in phloem sap reflects responses of pea plants to drought. The profiling of metabolic changes in phloem sap does not provide information establishing the origin and final destination of particular compounds. Such data can be obtained when isotope-based approaches are applied (Gessler *et al.*, 2004; Merchant, 2012). Our additional experiments where drought was applied in the presence of $^{13}CO_2$ have shown that the proportion of labelled to non-labelled sucrose was lower in stressed plants than in non-stressed controls. This is in accord with observed growth reductions as well as physiological data showing that pea plants recalibrate various catabolic processes to cope with decreased photosynthesis and limited gas exchange. Our non-targeted metabolomic studies provide an overall picture of the long-distance mediation of drought responses in pea; this has proven to be very informative when combined with physiological measurements. Additional studies of the phloem content of other molecules like phytohormones, RNA or proteins may bring more detailed information regarding long-distance signalling during stress response transduction (Buhtz *et al.*, 2008; Giavalisco *et al.*, 2006; Zhong *et al.*, 1996). Based on our results, however, we conclude that changes in phloem sap amino acid metabolic profiles, as well as those of carbohydrates, can be used to evaluate the overall physiological reactions of plants to abiotic stress. We also propose that in the future this concept can be further explored for the development of diagnostic markers of stress responses in plants. Recent work presenting the use of a handheld near-infrared (NIR) sensor for the determination of amino acid and lipid content included some aspects where oleic acid has been measured (Aykas *et al.*, 2020). A properly developed sensor that allows for the rapid and non-destructive estimation of oleic acid content in vasculature could be a valuable tool for breeders and commercial growers. This could also be an important component of future intelligent, water-efficient, plant growth systems.

EXPERIMENTAL PROCEDURES

Biological material and growth conditions

Our experimental object was the green pea (*P. sativum* L.) cultivar 'Walor', developed for fresh consumption or processed canned pea production (PlantiCo Zielonki Sp. z o.o., https://plantico.pl). Five plants per pot were grown in pots filled with 0.5 kg of Klasmann No. 11 soil (pH 6.3) and were watered to achieve a soil moisture tension of pF 2.8, which has been proposed as an optimal water regime for pea (Kirkham, 2014). Plants were grown with 25°C days, 23°C nights in a 16-h day/8-h night cycle with 300 µmol m^{-2} sec^{-1} light intensity and a relative humidity of 40%.

Optimal conditions were maintained for 5 days, then for the drought treatment watering was suspended until the soil moisture tension had reached pF 4.2, a critical wilting point for plants (Kirkham, 2014). The measurement of soil pF was performed with a ProCheck dielectric water potential sensor (MPS-6; Decagon Devices, http://ictinternational.com). Drought conditions were maintained for 7 days with the soil moisture tension kept at the pF 4.2 value. After 7 days the droughted plants were returned to optimal conditions and supplied with water to maintain pF 2.8, this rewatering phase was carried out for 10 days. For control plants appropriate watering for pF 2.8 was maintained during the whole experiment. The time points selected for measurements and analyses carried out in this work were the first and seventh day of drought (D1 and D7, pF 4.2) and 10 days after rewatering (RW, pF 2.8). All appropriate control combinations were sampled at the D1, D7 and RW time points for plants grown under optimal water conditions throughout the whole experiment (Figure 1a).

For a deeper physiological characterization of pea responses under drought stress, plants were profiled with the OloPhen platform (http://www.plant-phenotyping.org/db_infrastructure#/tool/57), equipped with integrated multiple sensors – top-view red green blue (RGB) and thermal infrared (IR) camera and chlorophyll fluorescence imaging (FluorCam) – for non-invasive analysis of plant physiological and morphological features. The experiment was performed following the same design as described above with minor modifications. For improved image analysis, one individual plant was grown per pot with at least 18 plants per treatment for a drought period of 7 days and a rewatering period of 3 days.

Physiological measurements

Measurements of relative water content (RWC), gas exchange (GE) and various parameters of PSII activity were carried out on a fully expanded leaf (leaf 2) for the D1 and D7 time points. As a result of the fact that after rewatering leaf 2 was already beginning to senesce, the fully expanded leaf 4 was measured at this time point. For RWC, freshly cut leaves were weighed and subsequently incubated in water for 24 h in the dark, the weight of leaves at their maximal turgor was determined and the leaves were then desiccated for 48 h at 65°C to obtain dry weights. Then the RWC factor was calculated:

$$RWC(\%) = \frac{FM - DM}{TM - DM} * 100\%,$$

where FM is the fresh mass of the leaf, DM is the dry mass of the leaf and TM is the turgid mass of the leaf.

Measurements were conducted on seven individual plants per treatment and sampling point. Gas exchange was measured at the D1, D7 and RW time points with the Ciras 2 portable photosynthesis system (PP Systems, https://ppsystems.com) for six control and six stressed plants growing in separate pots. Photosynthesis (Pn), evaporation (E), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were determined. Measurements were performed under artificial light with an intensity of 150 μm of PAR m^{-2} sec^{-1}, 25°C temperature, 380 ppm CO_2 concentration, 100% humidity and a flow speed in the measuring chamber of 220 ml min^{-1}. PSII photosystem activity was tested using a Pocket PEA device (Hansatech Instruments, http://www.hansatech-instruments.com). For each combination and time point 10 measurements were performed. The parameters calculated are described in detail in Table S1.

The statistical significance of differences was determined by ANOVA and post-hoc Fisher's test with STATISTICA (Tibco, https://

www.tibco.com). $P < 0.05$ was considered as a significant change. Image acquisition in the automatic phenotyping platform was carried out twice per day during the drought period and following rewatering using the RGB, IR and FluorCam sensors. The RGB image allowed the evaluation of shoot biomass growth as the change in number of green pixels (area). The kinetic changes in growth (canopy area and perimeter) were determined and the relative growth rate (RGR) related to area was calculated daily using the morning measurements:

$$RGR = \left[\ln(\text{green area})_{ti} - \ln(\text{green area})_{ti-1} \right] / ti - (ti - 1),$$

where ti is time i (days).

The top-view IR camera captures the heat signature of the plants, providing an indirect measure of stomatal conductance and transpiration (Sirault *et al.*, 2009). Chlorophyll (Chl) fluorescence parameters were recorded using the top-view FluorCam. A standard protocol was used for the measurement of Chl fluorescence quenching using the Chl fluorescence imaging (CFIM) protocols of the PlantScreen platform as described by Marchetti *et al.* (2019). Chl fluorescence parameters were calculated using FLUORCAM 7 (Photon Systems Instruments, https://psi.cz). The data obtained describe the quantum yield of PSII photochemistry in the light-adapted state (Φ_P), the quantum yield of regulatory light-induced non-photochemical quenching (Φ_{NPQ}) and the maximal quantum yield of the PSII photochemistry for a light-adapted state ($\Phi_{PSII} = (F_M' - F_0')/F_M'$).

Biometric analyses

Images of cellular growth of the abaxial leaf epidermis were measured 10 days after rewatering on the second leaflet of leaf 4 and leaf 8. Cell images were acquired using a Zeiss Axio Scope A1 microscope (Zeiss, https://www.zeiss.com) under Nomarski's differential interference contrast (DIC) contrast. Approximately 150–350 epidermal cells per leaf were outlined using a drawing tablet (Wacom, https://www.wacom.com) and IMAGEJ (Schneider *et al.*, 2012). Individual cell areas were quantified with the use of the image analysis algorithm developed by Andriankaja *et al.* (2012). Average cell area was determined for each leaf analysed and the number of cells per leaf was estimated by dividing leaf blade area by average cell area. Stomatal index was calculated as a fraction of stomata in the total number of epidermal cells. For each experimental combination 10 leaves from randomly chosen plants were used. ANOVA and the post-hoc Fisher's test were conducted to determine the statistical significance of changes in cell area and stomatal index, whereas cell number data were assessed with the Kruskal–Wallis test.

Cell divisions in the abaxial epidermis of leaf-4 and leaf-8 primordia at D1 of drought application were visualized with Aniline Blue staining performed as described by Kuwabara *et al.* (2011). Images were acquired under an M2 motorized Zeiss microscope equipped with a Colibri LED system, where fluorescence was excited with a 365-nm LED and 4′,6-diamidino-2-phenylindole (DAPI) Filter Set No. 49 was used to observe the Aniline Blue emission signals.

Phloem anatomy was determined for five randomly chosen sections from three biological repeats. Changes in midveins were scored for the second leaflets of leaves 4 and 8, the petioles and the stem regions located just below the axis of the analysed leaves from control and stressed plants 10 days after rewatering. Collected tissues were fixed in 4:1 98.8% EtOH/glacial acetic acid, embedded in Technovit 7100 (Kulzer, https://www.kulzer.com) and sectioned on a Leica RM2235 microtome (Leica Biosystems, https://www.leicabiosystems.com). For each area studied, three

10-μm sections spaced 1 mm from each other were mounted on microscopy slides and stained with 0.05% Toluidine blue re-suspended in a buffer containing 0.1 M Na_2HPO_4 and 0.05 M $Na_3C_6H_5O_7$. Images were acquired under an AXIO Image M2 microscope (Zeiss) equipped with a motorized stage and AxioCamICc5 camera. The morphodynamics of phloem were quantified using LITHOGRAPHIX (de Reuille and Ragni, 2017; Sankar *et al.*, 2014; Wunderling *et al.*, 2016) and parameters such as number, area, perimeter and the eccentricity of cells were scored for the different phloem cell types (CC, PP and SE). The statistical significance of cell number change was evaluated with a Kruskal–Wallis test, whereas other parameters were assessed with ANOVA followed by post-hoc Fisher's test. $P < 0.05$ was considered as significant.

Quantitative gene expression

The *PsRD29* transcript sequence homologous to the Arabidopsis *RD29a* and *RD29b* genes (Yamaguchi-Shinozaki and Shinozaki, 1993) was retrieved from the Pea RNA-Seq gene atlas (Alves-Carvalho *et al.*, 2015), where it has been designated as *PsCam023410*. A 2-μg portion of RNA isolated from leaf 2 at D1 and D7 and from leaf 4 at RW from stressed and control plants was used for the first-strand cDNA synthesis performed with the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific, https://www.thermofisher.com). A 1-μl volume of a fivefold dilution of this cDNA was used as a template for quantitative real-time polymerase chain reaction (qRT-PCR), carried out using the LightCycler 480 instrument (Roche, https://www.roche.com) and SensiMix SYBR No-ROX Kit (Bioline, https://www.bioline.com). Primers were designed using QUANTPRIME (https://quantprime.mpimp-golm.mpg.de/) (Table S2). Expression has been scored for three biological repeats and the levels obtained were normalized relative to the *PsUBC21* (*UBIQUITIN CONJUGATE 21*) gene (*PsCam043751*). Normalization was performed with REST-384 2 (Pfaffl *et al.*, 2002) and statistical significance was scored by the pairwise fixed reallocation randomization test.

Phloem exudate isolation and metabolite analysis

Phloem sap was collected on the first and seventh day of drought and at 10 days after rewatering from whole leaves of stressed and control plants using the EDTA-assisted method described by Tetyuk *et al.* (2013). Additionally, we performed control exudation from excised petioles on the first day. The experiment was carried out for three independent biological repeats and for each time point and treatment combination, exudates were collected from 12 randomly chosen fully developed leaves (three leaves from four plants for D1; four leaves from three plants for D7 and RW) that were placed in an Eppendorf tube containing 20 mM K_2EDTA solution for 1 h in the darkness. After that solution was discarded and replaced with sterile water the exudate collection was carried out in the same conditions for 6 h, and samples were subsequently frozen in liquid nitrogen and stored at −80°C for further analysis.

Samples were lyophilized and subjected to derivatization with the *N*-methyl-*N*(trimethylsilyl) trifluoroacetamide (MSTFA). The GC/MS analysis was performed using an Agilent 7890A gas chromatograph (Agilent, https://www.agilent.com) connected to the Pegasus® 4D GCxGC-TOFMS two-dimensional gas chromatography time-of-flight mass spectrometer (Ouaked *et al.*, 2003). A DB-5 bonded-phase fused-silica capillary column (30 m in length, 0.25 mm inner diameter, 0.25 μm film thickness) (J&W Scientific Co., now Agilent) was used for separation. The GC oven temperature programme was as follows: 2 min at 70°C, raised by 10°C min⁻¹ to 300°C and held for 10 min at 300°C. The total time

of GC analysis was 36 min. Helium was used as the carrier gas at a flow rate of 1 ml min⁻¹. One microliter of each sample was injected in splitless mode. The initial injector temperature was 40°C for 0.1 min and after that time raised by 600°C min⁻¹ to 350°C. The septum purge flow rate was 3 ml min⁻¹ and the purge was turned on after 60 s. The transfer line and ion source temperatures were set to 250°C. In-source fragmentation was performed with 70 eV energy. Mass spectra were recorded in the mass range 35–850 *m/z*.

Data acquisition, automatic peak detection, mass spectrum deconvolution, retention index calculation and library search were performed using CHROMATOF 4.51.6.0 (LECO, https://www.leco.com). To eliminate retention time (R_t) shift and to determine the retention indexes (RIs) for each compound, the alkane series mixture (from C-10 to C-36) was injected into the GC/MS system. The metabolites were automatically identified by library search in Fiehn and National Institute of Standards and Technology (NIST) libraries (Kind *et al.*, 2009; Yang *et al.*, 2017). The analytes were considered to be identified when they passed a quality threshold: that is, similarity index (SI) above 700 and matching retention index ± 10. Artefacts (alkanes, column bleed, plasticizers, MSTFA and reagents) were identified analogously, and then excluded from further analyses. To obtain accurate peak areas for the deconvoluted components, unique quantification masses for each component were specified and the samples were reprocessed. All identified compounds were aligned using the STATISTICAL COMPARE module of the CHROMATOF package and then exported for further calculations. The profiles obtained were normalized against the sum of the chromatographic peak area using the total ion chromatogram (TIC) approach (Noonan *et al.*, 2018).

The metabolomic data obtained were analysed using ANOVA in STATISTICA. For visualization, the data were also analysed using multivariate statistical analysis in R STUDIO 1.1.463 (R Studio, https://www.rstudio.com) and for the heat map, the mean (log base 10) ratio between drought stress and controls was calculated.

To determine the potential involvement of oleic acid in early drought responses, phloem exudates were isolated from whole leaves of plants growing under control (pF 2.8) and progressive drought-stress conditions (pF 3.85 and pF 3.95). Oleic acid content was estimated in a targeted analysis where chromatograms for selected ions of analysed compounds were plotted and their areas under the peaks were measured. These values were related to the area of the peaks plotted for the oleic acid standard (Supelco No. 75090) and analysed in known concentrations in their given range. This allowed for drawing calibration curves for these analytes and then absolute concentrations of compounds were calculated based on the least-squares method.

¹³C labelling

To establish the origin of phloem-located sucrose, $^{13}CO_2$ feeding studies were performed in control and drought conditions. Microcentrifuge tubes containing 250 mg of $NaH^{13}CO_3$ (99 atom % ^{13}C; Sigma-Aldrich, https://www.sigmaaldrich.com) were attached to pots that were subsequently placed in plastic zip bags (with five plants in a single pot per bag) and additionally sealed with tape. Release of $^{13}CO_2$ was triggered by the injection of 500 μl of saturated citric acid solution to the tubes. For each combination, samples were kept in this condition for 30 min followed by leaf excision and subsequent phloem sap collection across a time course of 0, 0.5, 1, 2, 3 and 6 h). Fractions of phloem sap were collected by moving detached leaves to fresh tubes containing water and immediately frozen in liquid nitrogen. After lyophilization and derivatization with MSTFA, samples were analysed by GC/MS as

described above. The total level of sucrose was calculated by summing the intensities for all fractions, and the ratio of unlabelled to ^{13}C-labelled sucrose was determined (where sucrose molecules incorporating at least a single ^{13}C were taken into account).

From this experiment we also calculated the sucrose velocity flow and presented it as the ratio of intensity observed in a particular measurement point to the time that passed from the start of the whole experiment.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

AUTHOR CONTRIBUTIONS

SB individually or in cooperation with other co-authors performed all the experiments and she wrote the first draft of the manuscript. GB supervised and directed the work on leaf epidermis microscopy. LR supervised and directed the work on phloem anatomy. NDD, LS and AEH performed phenomics studies. ŁM performed the MS analysis of metabolites. MO assisted with some physiological measurements and helped to maintain plant growth. DP and NDD performed statistical data mining. AK helped to plan drought experiments and interpret the physiological data. RM designed and supervised the whole work, was involved in the data interpretation and wrote the final version of the article. The outcome of this work has been discussed by all the co-authors and they all contributed in the preparation of the article.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Above-ground growth determined as change in green pixel area in RGB imaging. Error bars represent SE ($n = 18$ for control and $n = 30$ for drought). Statistically significant changes

determined by ANOVA and a post-hoc Fisher test are indicated with asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

Figure S2. Above-ground growth determined as green pixel-based change in the perimeter. Error bars represent SE ($n = 18$ for control and $n = 30$ for drought). Statistically significant changes determined by ANOVA and a post-hoc Fisher test are indicated with asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

Figure S3. Phloem anatomy in plants subjected to stress and appropriate controls after rewatering.

Figure S4. Quantitative changes in phloem cell anatomy in response to drought. Error bars indicate SE ($n = 3$; 5 randomly chosen sections for each biological repeat). Statistically significant changes determined by ANOVA and a post-hoc Fisher test (area, perimeter, eccentricity) and Kruskal-Wallis test (cell No. / bundle) are indicated with asterisks.

Figure S5. Change in bundle number that occurs after rewatering in comparison with a representative control.

Figure S6. Principal component analysis showing a scatter plot for differentially accumulated metabolites ($n = 3$) at each time point on principal component 1 (Dim1) and principal component 2 (Dim2).

Figure S7. The sum of glucose content in phloem sap collected as separate fractions over short intervals (0, 0.5, 1, 2, 3 and 6 h).

Figure S8. Principal component analysis ($n = 3$) showing a scatter-plot comparison of metabolite fingerprints obtained for phloem exudates from leaves (black) and from excised petioles (red).

Figure S9. Visualization of changes in selected metabolites of primary carbon and nitrogen metabolism in phloem exudates of drought-treated pea plants at the represented times.

Table S1. Physiological parameters measured with Ciras 2.

Table S2. Sequences of nucleotide primers used for qRT-PCR.

Table S3. Metabolite identification based on the Fiehn and NIST libraries (Kind et al., 2009; Yang et al., 2017).

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SUPPLEMENT III:

Applying Biostimulants to Combat Water Deficit in Crop Plants: Research and Debate.

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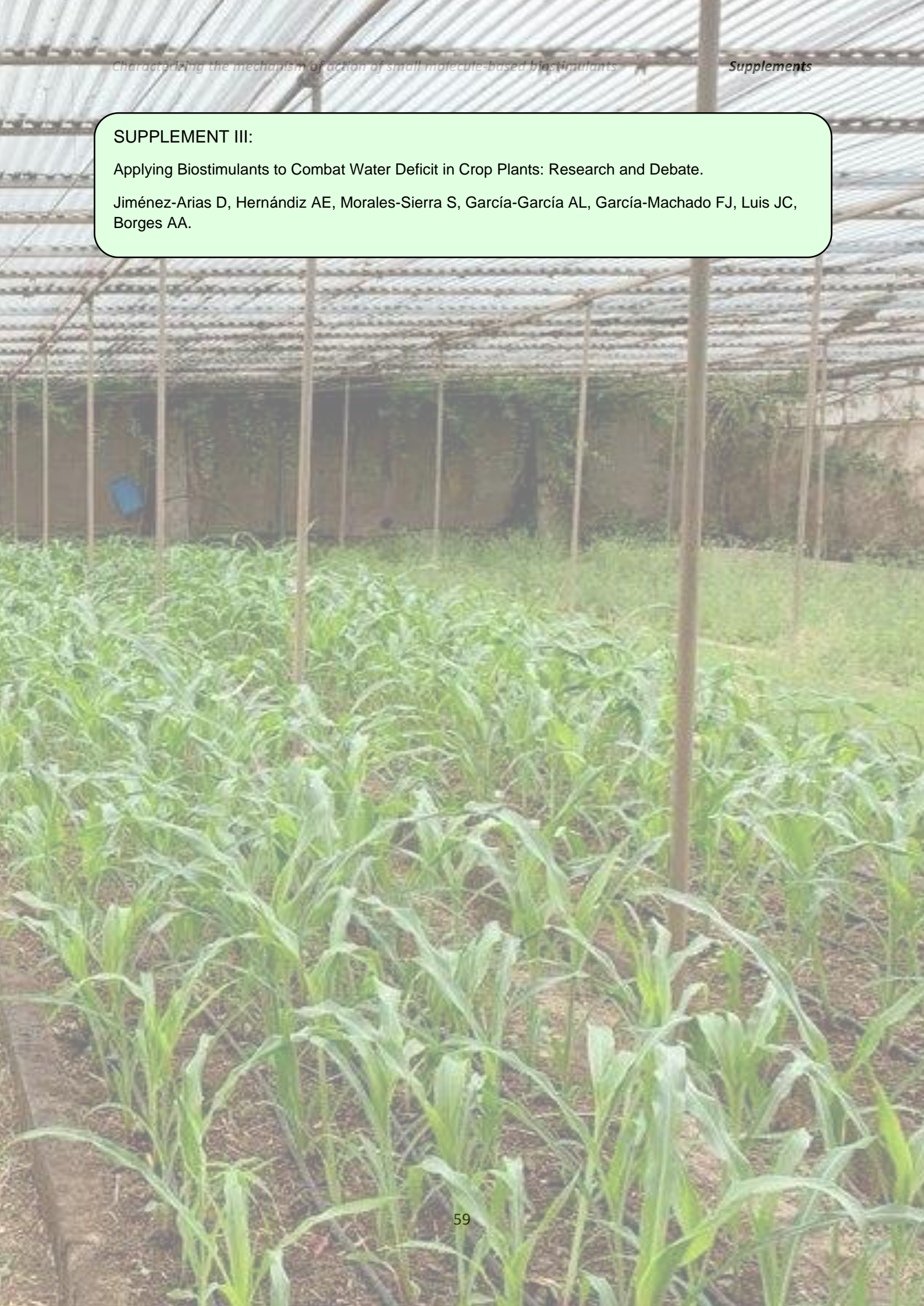
Applying Biostimulants to Combat Water Deficit in Crop Plants: Research and Debate

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Abstract: Climate change has increased the severity of drought episodes by further reducing precipitation in vulnerable zones. Drought induces a substantial decrease in agricultural water, reducing crop yields. Consequently, addressing water consumption can increase farmers' profits. This work describes lab-to-field research in *Zea mays*, using two biostimulants: glycine betaine (GB) and L-pyroglutamic acid (PG). The biostimulant optimal dosages were selected using a hydroponic system with 20% polyethylene glycol and nursery experiments under water-deficit irrigation. The established dosages were evaluated in field trials in which irrigation was reduced by 20%. Laboratory biostimulant optimisation showed in stressed treated seedlings (GB 0.1 mM; PG 1 mM) an increased dry weight, relative growth rate and water use efficiency, reducing seedling growth loss between 65 and 85%, respectively. Field trials using a GB-optimised dosage showed an increase in plants' growth, grain yield and flour Ca content. In addition, grain flour carbohydrate content and protein remained similar to control well-watered plants. Finally, the economic aspects of biostimulant treatments, water consumption, water sources (ground vs. desalinated) and grain biomass were addressed. Overall, GB treatment demonstrated to be a valuable tool to reduce water consumption and improve farmers' earnings.

Keywords: water deficit; biostimulants; pyroglutamic acid; glycine betaine; climate change

1. Introduction

Drought is considered the greatest threat to farmers growing field crops, the frequency and severity of which has increased worldwide [1]. Agriculture and water resources in an overwarming scenario have been the subject of research [2,3], with direct losses suffered by 1.5 billion people being estimated at US $124 billion from 1998 to 2017 [4]. Therefore, water scarcity is a critical concern in agriculture due to its direct impact on crop yield, which directly affects the worldwide economy [5]. Water supply can be achieved using new technologies—for example, desalination facilities [6], particularly in the Mediterranean region [7]. Beyond some other disadvantages amplified by the public, environmentalists and opinion [8], water acquisition via desalination technology is twice as expensive as from groundwater [9]. This higher cost is an impediment for farmers, who resultantly cannot

gain their deserved profits from the activity. Crop water management is therefore one of the most important objectives to achieve in the present high drought-risk scenario.

Biostimulants are presented as a potential new way to aid in water management [10–12], reducing field productivity losses [13]. They are compatible with the European eco-friendly philosophy [14] because most of the compounds used are from natural sources [15] and easily degraded in soil [16]. This has doubtlessly encouraged interest from the agrochemical industry in the biostimulants field. Indeed, it is an important investment hotspot with tremendous economic potential as is evident from a global market estimation expected to reach US \$4.14 billion by 2025 [17]. The application of biostimulants in stress mitigation is widely reviewed in the literature, but their effects on production figures are not usually assayed quantitatively [10].

Glycine betaine (GB) is a compatible solute that is accumulated in many plants. It is normally used to increase tolerance against abiotic stresses, such as freezing, salinity and drought stress [18]. Foliar application of GB results in rapid uptake, translocating to different plant organs [18], enhancing antioxidant defence [19,20], leaf gas-exchange attributes [21,22] and growth under stress conditions [22]. A recent publication shows how a foliar application of 11.5 g L^{-1} can increase yield [23]. L-pyroglutamic acid (PG) is another interesting biostimulant. It is a non-proteinogenic amino acid [24], and this group has recently shown potential as a source of new biostimulants [10]. To date, PG has been scarcely studied as a biostimulant but is at least able to alleviate a water deficit in lettuce using a root treatment [13].

The focus of the present study is to evaluate the suitability of using PG and GB to reduce economic water deficit losses in maize. Doses were optimised for root treatment and physiological and productive traits analysed. After testing if biostimulants can be used to reduce water consumption, the suitability of applying them as a tool is discussed to thus improve farmers' earnings from their harvest.

2. Materials and Methods

2.1. Plant Material

A local forage variety of maize from Gran Canaria Island (*Zea mays* L. c.v. Lechucilla) was provided by a local plant nursery in a 150-socket nursery tray. One week after sowing, plants were placed in a growth chamber in controlled conditions at 22 °C, 16 h light (300–400 µmol m^{-1} s^{-1}) and 60–70% relative humidity. Plants in the V1 stage were used for dose optimisation experiments, and those in the V2 stage were transplanted out for field experiments [25].

2.2. Dosage Optimisation

L-pyroglutamic acid (CAs number: 98-79-3) and glycine betaine (CAs number: 590-46-5) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). Doses of GB and PG were optimised using two approaches—hydroponic culture and direct application under two watering regimes in the nursery. Both experiments were repeated twice.

Hydroponic culture was employed as previously described but using maize with 20% polyethylene glycol (PEG) as a stressor instead of tomato with salt [26]. The treatment was applied for 24 h directly to the roots using 0.1, 1, 2.5, 5 and 10 mM GB or PG dissolved in distilled water. Then, the plants were placed again in the hydroponic buckets for the next 24 h. After this time, the medium was changed, and by adding 20% PEG, the onset of the stress was triggered. A control group without any treatment or PEG was used as a reference of normal maize growth. Ten plants were weighed at the beginning of the experiment to set up T_0, and the others submitted to different conditions were weighed after one week of growth. Plants were oven-dried at 65 °C for two days and used to calculate the RGR [27]. The concentrations that achieved the best result for this parameter and the consecutive lower doses were used in the followed experiment.

The nursery experiment ensured the suitability of the plant for root treatment. Using the nursery mentioned above, 20 plants were used for each condition. Treatments were

applied directly to the roots of V1 plants and consisted of 5 mL of a half-concentration Hoagland solution [28] containing the biostimulants at two concentrations: GB at 0.1 and 0.05 mM and PG at 2.5 and 1 mM. The water-deficit experiment consisted of watering all treated plants and a control without biostimulants with half the amount of water necessary to reach field soil capacity. To compare normal growth rates, another 20 untreated plants were watered to full soil capacity. The parameters measured were as follows. (1) Dried plant weight after 48 h in the oven. (2) Relative growth rate (RGR) [27], RGR = (ln DW 2 − ln DW 1)/(t2 − t1), where DW 1 and DW 2 were seedling dry weights at times t1 and t2 (t1 was the beginning of water deficit and t2 the end of the water deficit). Plant water-use efficiency (WUE) considering all the water used over the experiment timespan, WUE = plant biomass/water used [29] and the weight reduction with respect to control were each calculated using the well-watered untreated plants.

2.3. Field Experiment

Maize plants at the V2 stage were used in this study. The field trial was conducted at Escuela de Capacitación Agraria de Tacoronte, Tenerife, Canary Islands (28°29′47.0″ N 16°25′12.0″ W) during the months of June to August. The hydroponic experiment was in a greenhouse sectored into blocks equipped with a drip irrigation system. During the experimental periods, average daily maximum and minimum temperatures were 30 °C and 22 °C, respectively, with an average relative humidity of 80%. The soil at the site was classified as clay-loam (35% clay, 27% silt, 38% sand). The experiments were performed in randomised 20 m^2 blocks with three replications, with each block containing 80 plants. Irrigation volumes were calculated according to the FAO [30], taking into account the evapotranspiration rate (ET$_o$) provided by a nearby meteorological station, property of the island council, Cabildo de Tenerife. Soil humidity was measured near the roots of the plants within the wet bulb (TEROS 12 sensor from PESSL INSTRUMENTS GmbH, Weiz, Austria). Two irrigation regimes were established 30 days after transplanting: control (WW, 100% field capacity) and deficit irrigation (WD, 20% less than the control) and separated into two different blocks. Treatments consisted of 20mL of 1 mM PG (CAS number: 149-87-1) or 20 mL of 0.05 mM GB (CAS number: 590-46-5) purchased from Aldrich Chemical Co. (St. Louis, MO, USA), applied directly to the root system (Table 1). The treatment was two weeks after transplanting and was repeated two weeks later at the start of the water deficit regime. Irrigation restriction was continued until harvesting the cobs 45 days later.

Table 1. Treatment and water conditions summary.

Treatment	Water Regime	
	100%	80%
None	WW	WD
L-pyroglutamic acid 1 mM	WW-PG	WD-PG
Glycine betaine 0.1 mM	WW-GB	WD-GB

2.4. Growth, Yield and Water Status Measures

After forty-five days, the number of maize cobs per plant was counted for each condition; ten random plants from each condition were selected to measure the length and width of the last fully developed leaf. From those plants, the cobs were selected and harvested for yield measurements and dried in an oven at 65 °C for 1 week in order to avoid bias due to different moisture contents. Yield parameters measured were cob weight, weights of 100 grains (in triplicate from each cob) and weight of all grains. The average weight of grains per cob and the average number of cobs (Table 2) were used to calculate the total grain mass per ha, using as a reference 50,000 maize plants per hectare (Table 3). The result obtained was used to calculate the grain water-use efficiency (WUEg) as the ratio of the mass of grain produced to the water use throughout the growing period [31].

Table 2. Growth and yield parameters in greenhouse experiment.

Treatment	Leaf Lenght (cm)	Leaf Width (cm)	Grain Number	100 Grain Weight (g)	All Grain Weight (g)
WW	77.6 ± 19.6 [a]	7.8 ± 1.1 [a]	1.2 ± 0.4 [a]	10.3 ± 0.5 [a]	37.9 ± 5.5 [a]
WD	66.4 ± 17.3 [b]	6.9 ± 1.2 [b]	0.8 ± 0.6 [b]	8.3 ± 1.4 [b]	29.2 ± 8.7 [b]
WW-GB	73.1 ± 13.1 [ab]	7.9 ± 1.2 [a]	1.2 ± 0.3 [a]	11.2 ± 1.3 [c]	42.2 ± 8.5 [a]
WD-GB	69.2 ± 11.8 [ab]	7.5 ± 1 [a]	1.1 ± 0.1 [a]	10.5 ± 1.1 [ac]	37.9 ± 11.2 [a]
WW-PG	70.2 ± 13.5 [ab]	7.5 ± 1.3 [a]	1.1 ± 0.3 [a]	9.8 ± 1 [a]	35.2 ± 2.1 [a]
WD-PG	69.6 ± 13.3 [ab]	7.2 ± 1.1 [ab]	1.1 ± 0.1 [a]	9.7 ± 1 [a]	35.1 ± 2.7 [a]

Values followed by the same letter means no significant differences at $p < 0.05$.

Table 3. Yield and grain water use efficiency.

Treatment	Grain Mass (kg/ha)	WUEg (kg ha/m^3)
WW	2274	0.82
WD	1168	0.52
WW-GB	2321	0.91
WD-GB	2084	0.94
WW-PG	1936	0.70
WD-PG	1930	0.87

After fifteen and thirty days of the water regime, relative water content (RWC) [32] was calculated from twenty excised 1 cm diameter discs for each treatment. We weighed all leaf discs immediately to provide a measure of fresh mass (Wf), then soaked them 24 h in deionised water and re-weighed the resultant turgid mass (Wt). After drying at 85 °C, discs were again weighed to establish the dry-mass (Wd). RWC for each leaf was calculated according to: RWC = (Wf − Wd)/(Wt − Wd).

2.5. Protein, Carbohydrate, and Mineral Determinations from the Maize Flour

Total protein content was determined from total nitrogen by the Kjeldahl Method [33] multiplying by the coefficient 6.25. Total carbohydrates were quantified by the Phenol Sulphuric Acid method using a multiplate protocol as described in [13]. All measurements were repeated four times, and the mean plus the standard deviation was the value used.

From the selected cobs, the grains were ground to a fine powder for the mineral analysis (Ca, Mg, K, P, Na, Cu, Zn and Fe). One gram of this maize flour was taken from each sample, converted to ash in a muffle stove at 480 °C and mineralised by the dry method with 6 N HCl. This extract was determined by ICP OES Avio 500 (Perkin Elmer, Waltham, MA, USA). All measurements were done in triplicate.

2.6. Statistical Procedures

Statistical analyses for growth experiments were performed by a one-way ANOVA ($\alpha = 0.05$). The significance of differences between experimental groups was calculated using a Tamhane post-hoc test.

3. Results

3.1. Glycine Betaine and L-Pyroglutamic Acid Improve Drought Tolerance under Water Deficit Stress

Doses were optimised using two different approaches—first of all using hydroponic culture plus 20% PEG. As shown in Figure 1, plants subjected to stress decreased significantly in growth by 33%. However, a clear dose-dependent reduction in the difference from the control plants was detected after GB treatment, the best treatment concentration being 0.1 mM, which increased tolerance to water deficit by 77%. In contrast, PG needed higher doses to increase tolerance by 80%, with its best treatment at 1 mM.
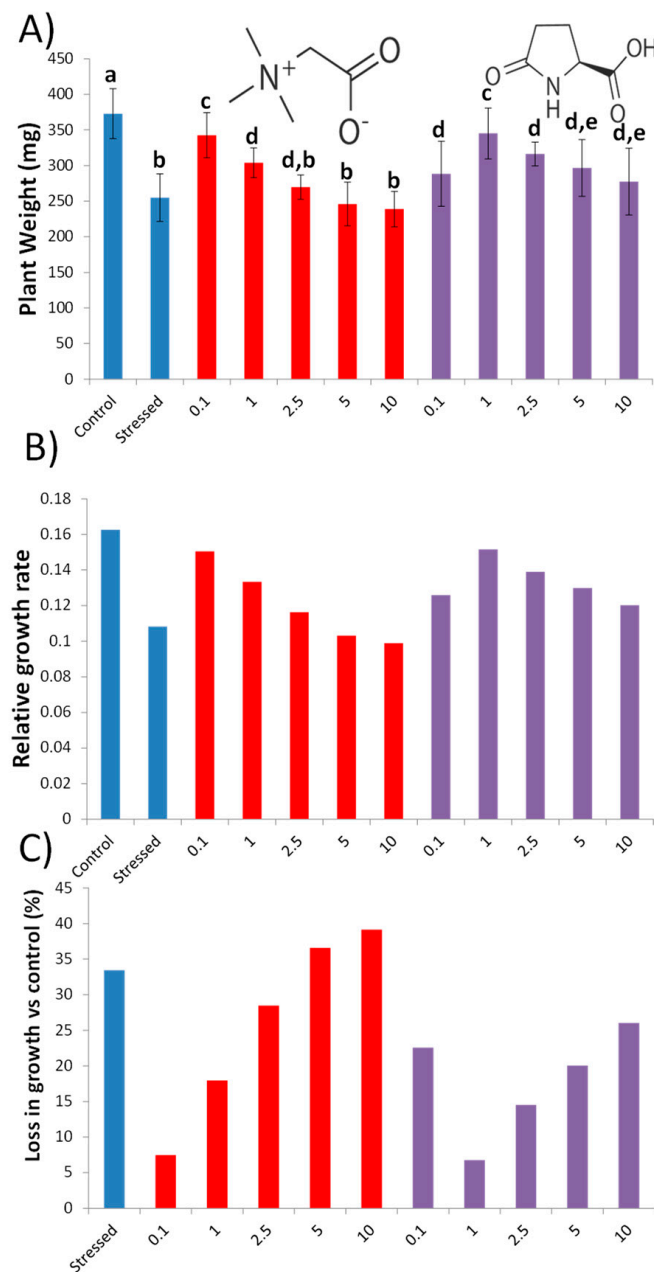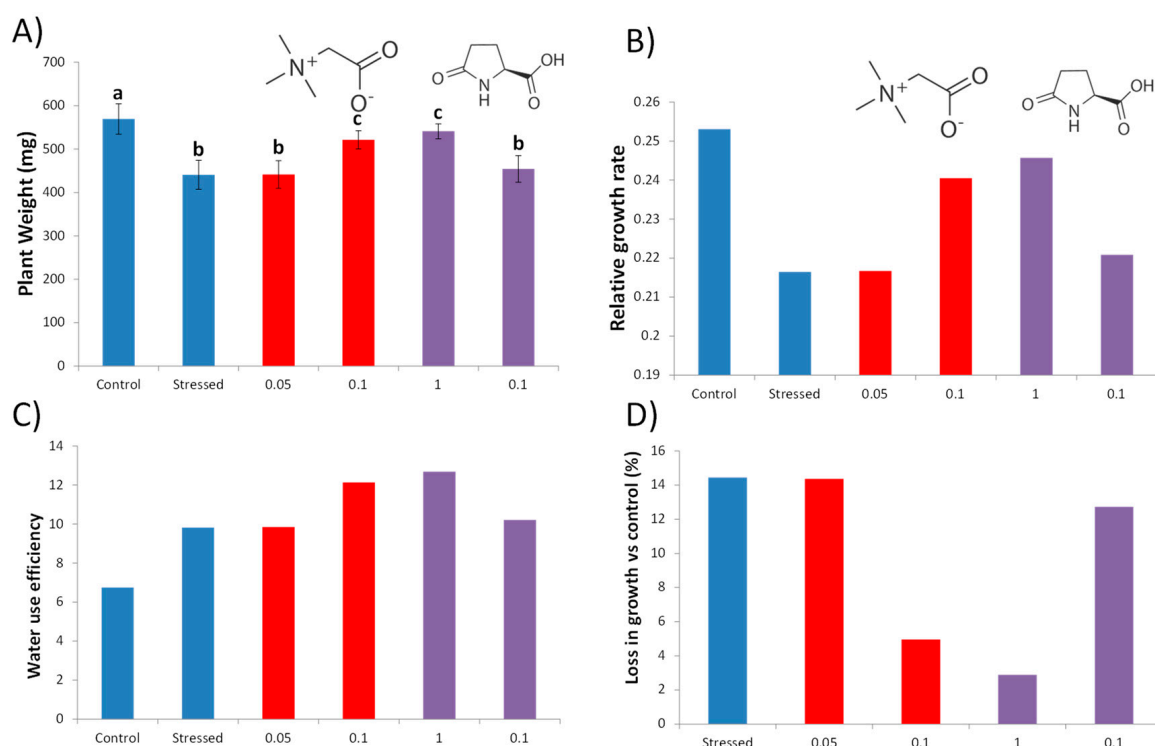
Figure 1. Hydroponic dosage evaluation using PEG 20% as a stressor. Red and violet correspond to GB and PG treatments, respectively. (**A**) Plant weight in mg. (**B**) Relative growth rate. (**C**) Percentage growth loss against control. Bars labelled with the same letter showed no significant differences at $p < 0.05$.

To ensure absorption after root treatment, we applied the compound directly to the soil in nursery trials. Watering with 50% less water for one week decreased maize growth by 14% (Figure 2). This situation was prevented by applying GB and PG, which increased tolerance by 65 and 85% at 0.1 and 1 mM, respectively. Water use efficiency was higher in all plants subjected to this slight water stress. The biostimulants achieved better results, showing more effectiveness in stress adaptation (Figure 2C).

Figure 2. Soil dosage evaluation using 50% less water as a stressor. Red and violet correspond to GB and PG treatment, respectively. Plant weight in mg. (**A**) Relative growth rate. (**B**) Water use efficiency. (**C**) Percentage growth loss against control. (**D**) Bars labelled with the same letter did not show significant differences at $p < 0.05$.

3.2. Biostimulants Are Capable of Ameliorating Negative Effects Caused by 20% Less Watering under Field Conditions

Plants submitted to a watering regime of 20% less than maize plants' optimum water necessities showed significant negative effects in growth and production, as seen in Table 2. With GB treatment, leaf weight and width decreased by 14 and 12%, respectively. These differences were reduced in both parameters by only 5%. Pyroglutamic acid further reduced variations due to the different water regime to less than 1%. Yield parameters showed how the regimen significantly affected the cob number, the weight of 100 grains and total grain weight by reducing them by 33, 19 and 23%, respectively. Again, these differences between well-watered and water-stressed plants were reduced using GB treatment to 8, 6 and 10%, respectively, and further reduced by the use of PG. However, it is worth highlighting that GB increased the grain weight, reaching significantly higher values in the 100 grain parameter and higher, but not statistically different, values in total grain weight.

Using the data obtained in Table 2, we calculated the grain mass in kg/ha and grain water use efficiency (Table 3). Untreated plants stressed by water deprivation had a yield reduction of 1106 kg per hectare in comparison to well-watered. This difference became less with the use of biostimulants: 236 kg with GB but just 6 kg for PG. Water-use efficiency was lower in the water-deficit plants without treatment; this did not happen if the biostimulants were applied, reaching even higher WUE levels with respect to well-watered plants.

Treatments with biostimulants prevented growth losses, as indicated by the RWC value (Figure 3A). Untreated plants subjected to water deficit showed drops in RWC after fifteen days, and this trend continued after 30 days of the two watering regimes; both biostimulant treatments prevented this. The results are consistent with soil water content (Figure 3B); the soil dried to 80% of the humidity level only after 3 days of stress onset. Then, it was more or less stable throughout the experiment, with two periods of more intense water deficit, reaching 65% of the moisture level, which was translated into a lower RWC except for the plants treated with biostimulants.
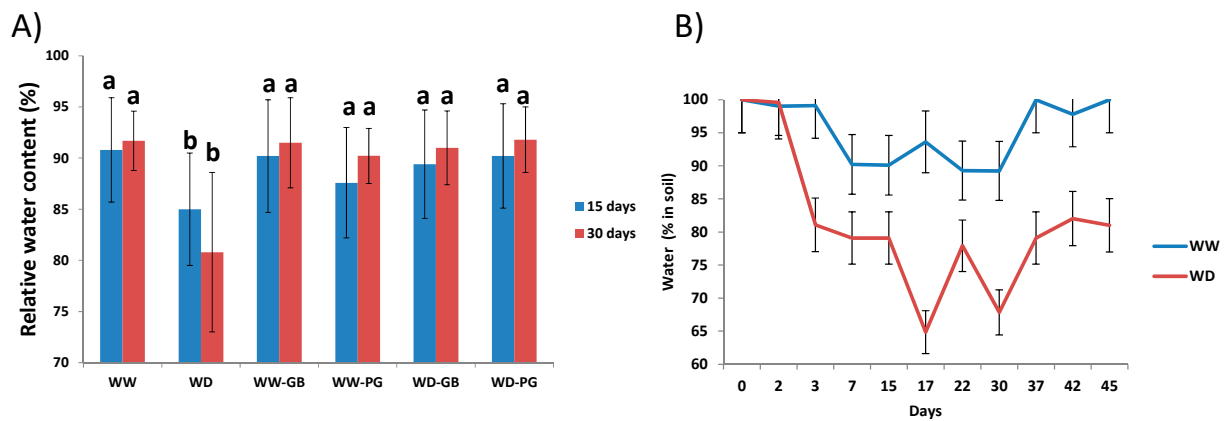
Figure 3. Plant water relations. (**A**) Relative water content. (**B**) Soil water content. Bars labelled with the same letter did not show significant differences at $p < 0.05$.

3.3. Nutritional Quality of Maize Flour Using Biostimulants

Mineral determination revealed how the water deficit significantly decreased the amount of calcium in flour, which was prevented by applying the biostimulants. Moreover, flour from the treated plants contained significantly more calcium than from untreated ones (Table 4). Interestingly, the P and Mg content significantly increased after water deprivation. Biostimulants showed the same trend for P. For Mg, however, all mineral contents were significantly higher in comparison to the untreated well-watered plants, especially in PG-treated plants under water deprivation, which accounted for the significantly higher magnesium levels. Potassium showed similar behaviour; only the latter group showed significantly higher concentrations. Iron, Cu and Zn did not show any difference for both untreated plants; however, plants treated with biostimulants had higher levels of Fe and Cu, and PG significantly increased all three.

Table 4. Flour mineral contents.

Treatment	Mineralogical Compositionin mg/100g Flour						
	Ca	**P**	**Mg**	**K**	**Fe**	**Cu**	**Zn**
WW	6.1 ± 0.3 [a]	143.9 ± 13 [a]	54.7 ± 8.6 [a]	309.6 ± 60.7 [a]	0.6 ± 0.2 [a]	0.3 ± 0.1 [a]	0.3 ± 0.1 [a]
WD	5.0 ± 0.3 [b]	195.5 ± 11.7 [b]	86.9 ± 9.7 [b]	356.4 ± 43.2 [a]	0.5 ± 0.1 [a]	0.4 ± 0.4 [a]	0.4 ± 0.1 [a]
WW-GB	9.5 ± 1.4 [c]	147.7 ± 20.4 [a]	71.1 ± 4.7 [b]	325.7 ± 40.7 [a]	0.8 ± 0.2 [ab]	0.2 ± 0.1 [a]	0.5 ± 0.1 [ab]
WD-GB	12.8 ± 1.7 [c]	185.7 ± 6.3 [b]	71.5 ± 88.4 [b]	335.5 ± 41.3 [a]	1.1 ± 0.1 [b]	0.3 ± 0.1 [a]	0.8 ± 0.1 [b]
WW-PG	10.5 ± 2 [c]	131.8 ± 5.8 [a]	71.4 ± 5.4 [b]	317.0 ± 64.3 [a]	0.8 ± 0.1 [ab]	1.2 ± 0.4 [b]	0.5 ± 0.1 [ab]
WD-PG	12.4 ± 1.7 [c]	189.8 ± 15.1 [b]	100.7 ± 4.6 [c]	482.1 ± 37.4 [b]	1.2 ± 0.2 [b]	1.4 ± 0.1 [b]	1.4 ± 0.1 [c]

Same letter means no significant differences at $p < 0.05$.

Total carbohydrate percentage (Figure 4A) was significantly reduced by the reduced irrigation and was prevented with the addition of GB and PG. The same correlation was shown for the protein percentage; the water deficit significantly affected only the plants without treatment and subjected to stress (Figure 4B).
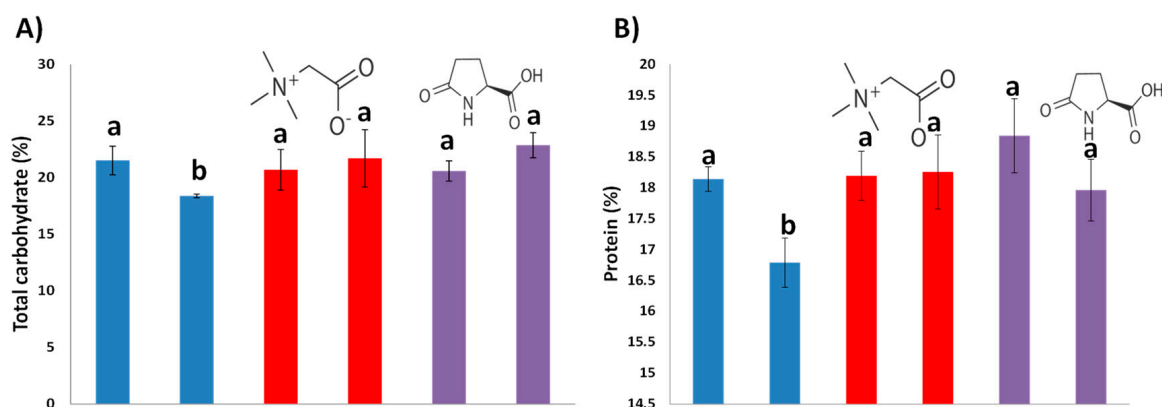
Figure 4. Carbohydrate and protein determination. Red and violet correspond to GB and PG treatment, respectively. Total carbohydrate determination % (**A**); protein % (**B**). Bars labelled with the same letter did not show significant differences at $p < 0.05$.

4. Discussion

Drought periods are expected to become more frequent and intense, especially in the Mediterranean area and western Europe. Moreover, the whole European continent will be affected by more frequent and severe extreme drought episodes [34]. The direct consequence, beyond the climatic and environmental implications, will be increased water scarcity [35] and the resultant difficulty to obtain it. Indeed, it is expected that by 2050, 6 billion people will lack access to a clean water source [36]. In agriculture, water demand will likely be 60% higher [36]. This would result in a rise in water prices for crop irrigation, so that farmers may have to use non-conventional water sources that increase the final production cost [37]. Proper water management is an obvious and promisingly achievable tool to maintain food supply and farmers' earnings, especially when faced with the coming greater uncertainty about its supply [38].

Water deficit stress can be prevented using biostimulants [12], which seems to be both attractive and eco-friendly to prevent losses caused by water shortage [38]. Indeed, the use of these kinds of substances to cope with stress is a prolific field of research that issues a huge number of publications per year. However, yield is not usually evaluated; in [10], an interesting list of biostimulants used against abiotic stress is assessed, but only 14% take it into account. It is possible to find some promising biostimulant applications—for example: a foliar application of mixed chitosan derivatives [39] increased grain yield by 35%. Other interesting work with maize [23] used foliar treatments of GB and salicylic acid to cope with drought stress, increasing grain yield by 40.52% and 60.49%, respectively. Here, we studied the possibility to apply two biostimulants, GB and PG, by fertirrigation, which is probably the easiest and most economical method for farmers to use such treatments [26]. The experiments show how plants subjected to water deprivation had a yield reduced by over 48% (Table 3). Application of the biostimulant GB reduces this difference by 10% and PG by less than 1%. The results are consistent with a previous study [23], although they were obtained through foliar treatment at a much higher concentration (100 mM) than used in our trials. In the case of PG, we here present the first data about the potential of this amino acid for use in maize to prevent losses due to drought, with it being previously reported only in lettuce [13].

Water use efficiency (WUE) is mostly recognised as a key constraint on crop production [31]. The results in Table 3 indicate that plants without biostimulant treatment had lower WUEg values, which indicate that plants are not able to acclimate adequately to the imposed stress [40]. However, the increase in WUEg induced by these biostimulants in water-deprived plants points to an enhanced ability to tolerate this imposed stress. They maintained the yield reached by well-watered plants, maximising acquisition of the available water [41]. Foliar GB application was also demonstrated to be capable of improving yield and water-use efficiency [42], as presented in our results. Leaf relative water content is

correlated with maize tolerance against drought [43] and is also correlated with WUE [44]. It is therefore not surprising that plants treated with biostimulants reached higher levels under water deficit (Figure 3), as shown in other studies using GB [42]. In these, treated plants decreased their transpiration rate, which was translated into a better, more efficient yield under stress conditions.

Water deprivation itself has interesting effects on nutritional mineral composition of maize flour and therefore nutrient acquisition by the plants. Phosphorus and Mg increased in concentration, whereas Ca showed a significantly lower concentration under the low-watering regimen, consistent with previous published results [45]. Biostimulants maintain this positive behaviour of the grain in its accumulation of these elements, especially the WD-PG, which reached statistically the highest Mg concentration. This result is very interesting since nutritional health specialists warn us about hypomagnesaemia [46], owing to the fact that two-thirds of the world population does not consume a sufficient amount of Mg daily [47]. One of the reasons is because the concentration of these ions is decreasing in food crops over time [48], with drought events being one of the causes of this phenomenon [49]. Pyroglutamic acid treatment can thus be an interesting way to increase the amount of this nutrient in a food crop. The losses in Ca are prevented by both biostimulants, reaching amounts against WW control plants that are consistent with previous results in lettuce using PG [13]. Glycine betaine seems to behave the same, with it being an interesting point of study regarding biostimulant applications. Water deficit did not significantly change the amounts of the micronutrients studied. However, GB significantly increased the amount of Fe and Zn in plants subjected to stress, while PG increased the concentrations in the mentioned elements and also Cu (Table 4).

It is well-known that drought stress causes changes in cereal grain composition, such as an important decrease in carbohydrate accumulation and an increment in protein content [49,50]. However, our results show a decrease in both parameters (Figure 4). The difference in protein concentration may be due to most maize hybrids being selected according to their drought tolerance, while grain protein content is one of the common selected parameters [51]. Our variety was a local forage variety of maize and not a commercial hybrid. Biostimulant application under drought conditions seems to be conducive to increased total carbohydrate amounts [23,39]. Total carbohydrates can be used as compatible osmolytes by maize [52]. Moreover, for some varieties, the total carbohydrate content can be correlated with a higher tolerance against drought stress [53]. Our results are consistent with such observations—treatments with GB and PG prevented the growth losses caused by drought (Figure 4), in our opinion, by helping to reach the osmotic balance. Again, previous results using PG in lettuce are consistent with carbohydrate loss prevention, which could be because these biostimulants can maintain high rates of photosynthesis in plants undergoing water deficit stress [13]. There are similar results with GB utilisation in the relevant literature [23].

Such biostimulants may result to be an excellent option for farmers, as both GB and PG are able to increase tolerance, and treated plants show no negative differences from untreated well-watered plants. However, is the treatment economically profitable? Using Table 3, the water used in the study, the price in € of a metric ton of maize grains [54], water prices [9] and the biostimulants' price per kg using Sigma Aldrich as the provider (PG @256 € per 500 g and GB @153 € per 1 kg), we calculated Table 5. At a first look, without considering water prices, we can show with our data that the WW-GB treatment could increase profit by 72 € in comparison with WW plants; however, WW-PG decreased it by 272 €. This huge difference between GB- and PG-derived profits is caused by their cost, which is 4.3 € and 171 € per hectare, respectively.

Using the price of conventional water (0.3 € per m^3 [9]), economic losses caused by water deprivation in the WD plants reached 272 €. The WD-GB treatment caused a 1€ profit loss in comparison with WW plants, whereas WD-PG increased profit by 56€ in comparison with WD plants. Using the price of desalinated water (0.6 € per m^3 [8]), profits decrease three-fold in WW; so, under WD or using the PG treatment in both water regimes, the crop

remains profitable. However, WW-GB increases profit by 72 € compared to WW, despite a 20% reduction in watering.

Table 5. Economic study of the selected biostimulant application.

Treatment	Grain Mass (kg/ha)	Water Consumption m³/ha	Profit Using Ground Water €/ha	Profit Using Desalinated Water €/ha
WW	2274		389.5	99.2
WW-GB *	2321	967.9	462.4	172.8
WW-PG+	1936		117.4	−172.9
WD	1168		116.9	−115.4
WD-GB *	2084	774.3	386.7	154.4
WD-PG+	1930		173.9	−58.5

* GB application costs 4.3 €/ha, whereas PG costs 171 €/ha.

5. Conclusions

Climate change is a concerning situation for agriculture and farmers. Beyond the natural disasters and plagues caused by a rapidly changing environment, water scarcity due to lower precipitation in vulnerable zones is a serious threat to overcome in order to derive a profit from crops. One of the most studied and promising solutions is the application of biostimulants.

Foliar biostimulant treatments are usually applied in field trials; here, we demonstrate that GB and PG can be administered as root treatments. This adds or injects them into the fertirrigation system, reducing operational costs. Furthermore, we optimise the doses, demonstrating that 0.1 mM GB and 1 mM PG reduced yield losses in a situation where 20% less water was provided. In addition, both treatments improve water use efficiency, preventing evapotranspiration losses and maintaining the nutritional benefits of the maize.

Nevertheless, considering the price of the treatment and the yield obtained in this assay, together with the price of water, only GB can be proposed as a viable biostimulant to cultivate maize in a water-deprivation regimen. Its extra cost is 4.3 € per hectare, reaching an additional profit ranging from 154.4 to 386.7 € under those conditions.

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SUPPLEMENT IV:

Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario

Alba E. Hernandiz, David Jiménez-Arias, Sarai Morales-Sierra, Andres A. Borges, Nuria De Diego.

Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario

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Conflict of interest statement

Author contribution statement

A.E.H, D.J-A., A.A.B., and N.D.D designed the idea of the project. A.E.H, D.J-A., and S.M-S performed the experiments. A.E.H and N.D.D analyzed the data and wrote the manuscript. All authors agreed with the last version of the manuscript.

Keywords

Drenching, Mineral nutrition, Polyamines, yield, Zea mays. (Min.5-Max. 8

Abstract

Word count: 282

Biostimulants have become an asset for agriculture since they are a greener alternative to traditionally used plant protection products. Also, they have gained the farmers' acceptance due to their effect on enhancing the plant's natural defense system against abiotic stresses. Besides commercially available complex products, the small molecule-based biostimulants are very useful for industry and research purposes. Amongst them, polyamines (PAs) are very well-studied nature compounds that can elicit numerous positive responses in drought-stressed plants. However, the studies are merely focused on the vegetative development of the plant. Therefore, we aimed to evaluate how drenching with putrescine (Put) and spermidine (Spd) modified maize production and the yield quality parameters. First, a dosage optimization was performed, and then the best PA concentrations were applied by drenching the maize plants grown under well-watered (WW) conditions or water restrictions (WR). Different mechanisms of action were observed for Put and Spd regarding maize production, including when both PAs similarly improved the water balance of the plants. The application of Put enhanced the quality and quantity of the yield under WW and Spd under WD. Regarding the nutritional quality of the grains, both PAs increased the carbohydrates content, whereas the contribution to the protein content changes by the interaction between compound and growth conditions. The mineral content of the grains was also greatly affected by the water condition and the PA application, with the most relevant results observed when Spd was applied, ending with flour richer in Zn, Cu, and Ca, minerals considered very important for human health. We showed that the exogenous PA application could be a highly efficient biofortification approach. Our findings open a new exciting use to be deeper studied in the biostimulant research.

Contribution to the field

Dear Editor, Here we submit a manuscript entitled "Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario" by Hernandiz et al., to be considered for publication as a research article in Frontiers in Plant Science to be part of the research topic "Biostimulants in Agriculture II: Towards a Sustainable Future." This work uncovered the benefits of using the polyamines putrescine (Put) and spermine (Spd) as small molecule-based biostimulants. The study was performed on maize plants in the greenhouse under two water regimes, well-watered and water restriction. The drenching with the two polyamines was mainly evaluated based on the quality and quantity of the final yield. To our knowledge, there are not many studies determining the biostimulant mechanism of action on crop production. Different mechanisms of action were observed for Put and Spd. Whereas the application with Put was more effective under optimal growth conditions, Spd was under water deficit. The flour mineral nutrition of the treated plant was also improved, especially with Spd. The flour's nutritional and mineralogical modifications provide exciting insights about biostimulant use for crop biofortification. Our paper manuscript presents new findings with conclusions thoroughly supported by critical experimental evidence and can bring a new point of view concerning the use of biostimulants. We believe that our study fulfills the journal's scope. For this reason, we are convinced that our work will be of broad interest to Frontier in Plant Science readers. Sincerely, Nuria De Diego

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Ethics statements

Studies involving animal subjects
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Studies involving human subjects
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Inclusion of identifiable human data
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Data availability statement

Generated Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario

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Keywords: drenching, mineral nutrition, polyamines, yield, *Zea mays*. (Min.5-Max. 8)

Abstract

Biostimulants have become an asset for agriculture since they are a greener alternative to traditionally used plant protection products. Also, they have gained the farmers' acceptance due to their effect on enhancing the plant's natural defense system against abiotic stresses. Besides commercially available complex products, the small molecule-based biostimulants are very useful for industry and research purposes. Amongst them, polyamines (PAs) are very well-studied nature compounds that can elicit numerous positive responses in drought-stressed plants. However, the studies are merely focused on the vegetative development of the plant. Therefore, we aimed to evaluate how drenching with putrescine (Put) and spermidine (Spd) modified maize production and the yield quality parameters. First, a dosage optimization was performed, and then the best PA concentrations were applied by drenching the maize plants grown under well-watered (WW) conditions or water restrictions (WR). Different mechanisms of action were observed for Put and Spd regarding maize production, including

36 when both PAs similarly improved the water balance of the plants. The application of Put enhanced
37 the quality and quantity of the yield under WW and Spd under WD.

38 Regarding the nutritional quality of the grains, both PAs increased the carbohydrates content, whereas
39 the contribution to the protein content changes by the interaction between compound and growth
40 conditions. The mineral content of the grains was also greatly affected by the water condition and the
41 PA application, with the most relevant results observed when Spd was applied, ending with flour richer
42 in Zn, Cu, and Ca, minerals considered very important for human health. We showed that the
43 exogenous PA application could be a highly efficient biofortification approach. Our findings open a
44 new exciting use to be deeper studied in the biostimulant research.

45

46 **1   Introduction**

47 Plants are sessile organisms exposed to a rapidly changing environment. They respond to external
48 stimuli, which might result in plant acclimation to specific growing conditions. When this is
49 impossible, growth becomes inhibited and, later on, may die. Abiotic stresses are the principal cause
50 of severe yield losses of 50–80%, depending on the crop and geographical location (Zhang et al., 2018).
51 The global climate change projections forecast an increase in extreme weather events' occurrence,
52 frequency, and severity (FAO, 2018). The incidence of abiotic stresses such as drought will raise and
53 compromise the yield of the crops, especially in arid and semiarid areas. Drought is multidimensional
54 stress affecting plants at various developmental stages, including the plant's production (Blum, 1996).
55 One of the most promising methods to cope with the inevitable abiotic stresses is the application of
56 biostimulants to enhance plant resilience to environmental perturbations (Van Oosten et al., 2017).
57 Their action relies on the "preparation" effect (priming or hardening) that their application exert on the
58 plants (Gebremedhn and Berhanu, 2013; Maiti and Pramanik, 2013; Savvides et al., 2016).
59 Biostimulants have been proved to improve plant growth and photosynthesis efficiency by modifying
60 plant metabolism under abiotic stress conditions (Paul et al., 2019; Sorrentino et al., 2021). Moreover,
61 the recent recognition of biostimulants as an independent group of agricultural inputs by the European
62 Union and their contribution to more sustainable agricultural practices forecasts a growing interest in
63 these substances (Ben Mrid et al., 2021).

64 Typically, biostimulants are a mixture of several substances, such as protein hydrolysates or seaweed
65 extracts; this has been seen as a great opportunity by some companies to join the circular economy
66 trend since they can give a second life to waste and by-products (Xu and Geelen, 2018). However,
67 these products present the disadvantage of lack of uniformity between batches and the problematic
68 identification of the active substances (Yakhin et al., 2017). Studying pure organic active compounds
69 as biostimulants will lead to better standardization, quality control of formulation, and understanding
70 their mode and mechanism of action (García-García et al., 2020). While the effectiveness of
71 biostimulants has been widely researched and summarized in extensive reviews (Battacharyya et al.,
72 2015; Bulgari et al., 2015, 2019), there is limited information available on how their application might
73 affect the final yield and quality.

74 Maize (*Zea mays* L.) is one of the most important cereal crops for human nutrition in large parts of the
75 world (Huma et al., 2019) and an essential grain used as livestock feed in the world (Loy and Lundy,
76 2018). Drought negatively affects plant growth, dry biomass content, and yield (Anjum et al., 2017;
77 Kim et al., 2019). The water deficit during maturation might cause problems in the grain filling stage
78 (Zhang et al., 2019) and reduce the quality parameters such as starch, proteins (Barutcular et al., 2016),

79 or the mineral content (Aqaei et al., 2020). Maize kernel mainly comprises carbohydrates, protein, and
80 oil (Chaudhary et al., 2014). Starch accounts for approximately 70% of the kernel weight (Orhun et al.,
81 2013). According to the release and absorption of glucose in the intestines, the starch is divided into
82 three categories: rapidly and slowly digestively starch and resistant starch; the last category has
83 particular importance for human nutrition since its consumption has been linked to a decrease in the
84 risk of developing type II diabetes and colon cancer (Hoebler et al., 1999). Protein content varies from
85 8 to 11% of the kernel weight on common varieties of maize (Landry and Moureaux, 1980). However,
86 the nutritional value is poor for monogastric and human consumption due to the low content of essential
87 amino acids such as lysine, tryptophan, and threonine (Li and Vasal, 2015). The discovery of the
88 opaque-2 gene and its link with higher lysine and tryptophan was the beginning of the Quality Protein
89 Maize (QPM) research, which produced hybrids whose kernels had a significant increment on the
90 mentioned amino acids. Nevertheless, the yield was reduced in these hybrids, and their agronomical
91 performance was deficient (Chaudhary et al., 2014). The kernel mineral composition is strongly
92 affected by environmental factors, soil moisture, and fertility, but it is mainly genotype-dependent,
93 with the varietal differences as the most significant contributor to the reported variance (Feil et al.,
94 2005; Menkir, 2008).

95 The development of nutritionally enhanced food crops using traditional breeding practices and modern
96 biotechnology approaches is known as "biofortification" (Chaudhary et al., 2014; Garg et al., 2018).
97 The first main achievements in this field were the lysine and tryptophan enriched maizes or vitamin A-
98 rich orange sweet potato; much effort is put into transgenic research, although traditional breeding
99 practices are best accepted (Garg et al., 2018). Given the exposed facts, we decided to test biostimulants
100 as an alternative approach for maize biofortification due to their effect on plant metabolism and stress
101 tolerance. With this aim, we applied two polyamines (PAs) with proven efficiency as stress alleviators,
102 putrescine (Put) and spermidine (Spd) (Li et al., 2015, 2018; Marcińska et al., 2020; Islam et al., 2022),
103 although the dose was optimized to the specific cultivar of maize used. The maize plants were treated
104 with the two PAs via drenching, which has been proven to have positive results towards drought stress
105 (Jiménez-Arias et al., 2019) because drenching provides a more extended period of continuous uptake
106 of the supplemented substance than the foliar application (Parkunan et al., 2011). Some reports also
107 revealed that the drench application improves soil fertility by increasing the availability of the minerals,
108 the soil aeration, and water holding capacity and promotes the development of essential microbes
109 (Battacharyya et al., 2015; du Jardin, 2015). We expect that applying PAs by drenching will improve
110 maize's biofortification and stress tolerance.

111 **2   Material and Methods**

112 **4.1. Plant material, growth conditions and dose optimization**

113 A local plant nursery provided a local forage variety of maize from Gran Canaria Island (*Zea mays* L.
114 c.v. Lechucilla) in a 150-socket nursery tray. One week after sowing, plants were placed in a growth
115 chamber under controlled conditions with a temperature of 22 °C, a photoperiod of 16/8 h light/dark
116 with a light intensity of 300–400 μmol m^{-2} s^{-1}), and relative humidity around 60–70%. Plants in the V1
117 stage, when the lowermost leaf has a visible leaf collar (Zhao et al., 2012), were used for dose
118 optimization experiments.

119 Putrescine and spermidine were purchased from Sigma Aldrich (Put CAS number 333-93-7; Spd CAS
120 number 124-20-9). In the direct application-optimized doses of both chemicals under two water
121 regimes in the nursery tray, 20 plants were used as biological replicates per variant (treatment x growth

122 condition), as described in (Jiménez-Arias et al., 2022). The nursery experiment ensured the suitability
123 of the plant for root treatment. Treatments were applied directly to the roots of V1 plants, consisting
124 of 5 mL of a half-strength Hoagland solution (Hoagland and Arnon, 1938) for controls or containing
125 the tested substances at five concentrations: 0.01, 0.1, 0.5, 1, 2 mM for the treated plants. Twenty plants
126 per variant were grown under a water limitation of 50% of the field capacity for a week. Two harvesting
127 times were performed; at the beginning and the end of the dry period. The whole plant, including the
128 aerial part and roots, was harvested. The dried weight (DW, mg) of the twenty plants was recorded
129 after being oven-dried at 85°C for 48 h to calculate the relative growth ratio (RGR) (Hoffmann and
130 Poorter, 2002),

131 $RGR = (lnDW_2 - lnDW_1)/(t_2 - t_1),$　　　　　　(Eq. 1)

132 where DW_1 and DW_2 corresponded to the dry weights of maize seedlings at times t_1 and t_2 (beginning
133 and end of the water deficit).

134 Plant water use efficiency (WUE, mg mL^{-1}) was calculated considering all the water provided during
135 the experiment timespan (Kuglitsch et al., 2008);

136 $WUE = Plant\ Biomass/Total\ Irrigation$　　　　(Eq. 2)

137 **4.2 Greenhouse experiment**

138 　　　Once the data was evaluated for the dosage optimization, an additional study with maize variety
139 was conducted at a greenhouse property of the Escuela de Capacitación Agraria de Tacoronte
140 (Tenerife), Canary Islands (28°29'47.0"N 16°25'12.0" W) from June to August 2021. The ambient
141 conditions were recorded daily (Supplementary Table S1). The average maximum and minimum
142 temperatures were 30 and 22°C, respectively, with an average relative humidity of 80%
143 (Supplementary Table S1). The soil is classified as clay-loam (35% clay, 27% silt, 38% sand), and the
144 experiment was organized in randomly distributed blocks of 20 m^2 blocks with three replicates, each
145 block containing 80 plants. The seeds were sown in nursery trays and transplanted to the field 15 days
146 after the sowing. The irrigation was calculated according to the FAO (Rao et al., 2016), taking into
147 account the evapotranspiration rate (ETo) provided by a nearby meteorological station, property of the
148 island council, Cabildo de Tenerife (Supplementary Table S1). Soil humidity was monitored within
149 the wet-bulb with the TEROS 12 FDR sensor (METER Group, Pullman, WA). Treatments consisted
150 of 20 mL of water for the controls or of 0.1 mM Put (CAS number: 333-93-7) or 0.5 mM Spd (CAS
151 number: 124-20-9) for the treated plants, both compounds purchased from Aldrich Chemical Co. (St.
152 Louis, MO, USA). The treatment was applied twice directly to the wet bulb as drenching for direct
153 availability for the root system, two and four weeks after transplanting. After the second application,
154 the water restriction for the drought variant started, and these conditions were maintained until the
155 harvest of the maize cobs.

156 4.2.1 Biomass, Yield, and Water Status measurement

157 　　　Fifteen and thirty days after the onset of the water restriction, the relative water content (RWC,
158 %) was calculated (Barrs and Weatherley, 1962). 20 disks of 1 cm diameter per variant were excised
159 from the last fully expanded leaf and immediately weighted to register the fresh weight (FW, mg).
160 After that, the disks were kept submerged in distilled water for 24 h to determine the turgid weight
161 (TW, mg). Finally, the disks were oven-dried at 85°C for 48 h to register the dry weight (DW, mg).
162 RWC for each disk was calculated as

163    $RWC = (FW - DW)/(TW - DW)$ (Eq. 3)

164   Yield-related parameters were evaluated at the harvest, forty-five days after the onset of the stress. The
165   number of adequately developed maize cobs per plant and variant was counted. Ten random plants per
166   variant were selected to determine morphometric parameters such as plant length (from insertion to the
167   soil until the base of the flower) and width (at the middle of the stem length), and the length and width
168   of the last fully developed leaf. The cobs to evaluate production were also obtained from the selected
169   plants. The cobs were weighed to record the fresh weight and then oven-dried at 65°C for one week to
170   reduce the possible bias due to different moisture levels. The dry cob weight, the number and total
171   weight of all kernels per cob, and the weight of 100 kernels (in triplicate from each cob) were
172   registered. The total yield considering 40,000 plants/ha for the used plantation frame was also
173   calculated using the following equation with the following formula:

174    $Yield/ha = Avg\ Kernel\ Weight/cob * Avg\ Cob\ nº/plant * Estimated\ Plants/ha$ (Eq. 4)

175   The Harvest Index (HI) was also calculated from the total kernel weight (Total KW) per cob and the
176   average cob fresh weight (Avg cob FW) as follows:

177    $HI = Total\ KW/Avg\ Cob\ FW$ (Eq. 5)

178   The production water use efficiency (WUEp) in maize was also estimated based on Kiziloglu et al.,
179   (2009). The accumulated effective crop evapotranspiration (ETc, mm) was calculated through the
180   experiment (Supplementary Table S1). This term corrects the deficiencies of the ETo values by a Kc
181   factor that depends on the moisture soil level, crop characteristics, and the stage of the crop vegetative
182   cycle (Eq. 6). For maize, Kc values are estimated at 1.2 in the initial phases to 0.6 at the final stages
183   (FAO).

184    $ETc = ETo\ x\ Kc$ (Eq. 6)

185   The water use efficiency (WUEp, kg mm^{-1}) was then calculated as the ratio between the accumulated
186   ETc and the final yield (kg/ha).

187   4.2.2 Protein, Carbohydrates, and Mineral composition of the maize flour

188   All kernels from the selected cobs were ground to a fine powder to evaluate quality parameters.
189   50 mg of the powder per sample was used for the total protein content calculated using the Kjeldahl
190   Method (Kirk, 1950), multiplying the total nitrogen content by 6.25. An additional 100 mg of the
191   powder was used to determine total carbohydrates using the Phenol Sulphuric Acid method modified
192   in multi-well plates (Jiménez-Arias et al., 2019). Finally, one gram of the flour powder per sample was
193   used to analyze the mineral content (Ca, Mg, K, P, Na, Cu, Zn, and Fe). Each sample was converted to
194   ash in a muffle stove at 480°C and mineralized by the dry method with 6 N HCl. The mineral levels
195   were determined by ICP OES Avio 500 (Perkin Elmer, Waltham, MA, USA).

196   **4.3 Data analysis**

197   All plant growth and production parameters were used to calculate the Plant Biostimulant
198   Characterization Index (PBCI), a very visual and helpful tool to reduce all considered variants into a
199   single number for better biostimulant characterization (Ugena et al., 2018; Sorrentino et al., 2021). The

200   PBCI was used to select the best-performed concentrations in the first experiment (dose optimization)
201   and evaluate the PA application impact on maize production.

202   Data were evaluated using different statistical approaches; two-way ANOVA ($p \leq 0.05$)
203   followed by multiple comparisons with LSD posthoc test was used for parametric data and Kruskal
204   Wallis' test ($\alpha = 0.05$) for nonparametric data. For better visualization, multivariate statistical analyses
205   with the yield-related parameters were also carried out. One principal component (PC- Dim) analysis
206   and matrix correlation were constructed in RStudio V. 2021.09.1+372 using the packages *factoextra*,
207   *ggplot2,* and *corrplot*.

3   Results

3.1   Nursery Tray Dose Optimization

210   The dose optimization was performed in nursery trays following the protocol established by (Jiménez-
211   Arias et al., 2022). The parameters considered to evaluate the efficiency of the dose were the plant
212   weight (shoot and root), the RGR (%), and WUE (Supplementary Table S2). Table 1 displays the
213   heatmap obtained from the computed parameters, transformed with the Log2 and relativized to the
214   untreated maize seedlings. The drenching with Put at 0.5 and especially 0.1 mM enhanced the plant
215   biomass (weight), RGR (%), and WUE compared to untreated seedlings when grown under a water
216   limitation of 50% field capacity, ending with a positive PBCI value (Table 1). Contrarily, the
217   application of Spd did not improve the seedlings performance under the same growth conditions (Table
218   1, Supplementary Table S2). The plants treated with 0.5 mM Spm presented less negative PBCI (Table
219   1). Thus, we selected the 0.1 mM Put and 0.5 mM Spd to evaluate their impact on maize production
220   under optimal and water limitation conditions.

3.2   The application of polyamines enhanced the RWC in maize plants

222   The changes in soil water content were recorded through the experiment and represented in Figure 1A.
223   The plants under well-watered conditions were irrigated according to their water requirements
224   estimated by FAO's reference evapotranspiration (ETo) (Supplementary Table S1). The water supply
225   was reduced by 20% of the crop's water requirements for the water stress variants. As a result, we
226   observed that the volumetric water content in the well-watered plants was maintained between 1.7 and
227   1.6 m^3 m^{-3} (Figure 1A). However, in the stress variant, the water was drastically reduced to a volumetric
228   water content of 1.4 m^3 m^{-3} for the first four days and maintained for additional 15 days. After that, it
229   fluctuated between 1.3 and 1.1 m^3 m^{-3}.

230   The water status of the plants was estimated twice by the RWC, at 15 (t_1) and 30 (t_2) days after the
231   water restriction onset (Figures 1B and C). According to ANOVA, there was a significant interaction
232   between growth conditions and treatment for $RWCt_2$ but not for $RWCt_1$. The water restriction reduced
233   the $RWCt_1$ in all plants compared to well-watered ones. However, the application of Put and Spd
234   increased the $RWCt_1$ values compared to their respective controls. Put and Spd kept the same trend,
235   with an $RWCt_1$ of 4 and 2.6% for WW and 9 and 4% for WD conditions, respectively, compared to
236   the corresponding untreated plants (Supplementary Table S3). Interestingly, no differences in $RWCt_2$
237   were observed among variants; only the untreated plants significantly reduced the $RWCt_2$ to 80%
238   (Figure 1C, Supplementary Table S3). Thus, Put and Spd application increased significantly $RWCt_2$ to
239   14.9 and 15.8% in the maize plants, respectively, compared to the untreated plants under WD (Table
240   A1, Supplementary Table S3).

241 **3.3   The application of Spd by drenching reduced the biomass-related parameters under water**
242 **limitation**

243 The biomass production was evaluated by monitoring several parameters such as stem diameter, the
244 plant length (measured from the transition to the roots, up to the emission of the anthers), length and
245 width of the flag leaf, and the ratio length/width (L/W) for the flag leaf (Figure 2, Supplementary Table
246 S4). The parameters were then represented in a parallel coordinate plot, and the values were used for
247 the PBCI calculation. None of the treatments enhanced plant biomass production in any water regime,
248 so all presented negative PBCI values (Figure 2). The parallel coordinate plot illustrated that only Put
249 slightly enhanced the biomass production under WW conditions; the rest of the treatments and growth
250 conditions presented a negative effect. The thicker stem was reported for the WW control plants, with
251 Put plants having the thinnest stem for both irrigation conditions. Interestingly, the Spd-treated plants
252 were the only ones keeping the stem thickness irrespectively of the growth conditions (Figure 2,
253 Supplementary Table S4). The water limitation slightly reduced the plant height, flag leaf length, and
254 width without significant differences (Supplementary Table S4). Overall, the highest plant length and
255 leaves were found in the Put treated maizes under WW conditions, while the shortest plants were
256 reported for the WD with Spd application. Regarding the leaf L/W ratio, the highest values were
257 reported for Put and Spd in WW conditions, followed closely by the Control treatment and Put under
258 WD (Figure 2 and Supplementary Table S4). The lowest L/W ratio was observed in the maize plants
259 treated with Spd under WD, confirming that this treatment modified the plant stress response and
260 hence, the growth profile.

262 **3.4   Put enhanced yield and WUEp under optimal conditions, and Spd under water**
263 **limitations**

264 As a first step in evaluating maize's yield, the total number of cobs produced per variant was counted
265 and represented in Figure 3A. Put was the most effective treatment under WW, so its plants increased
266 the total production by 22.78%, with 97 cobs compared to 79 obtained in the controls. On the other
267 hand, Spd reduced the production to only 59 cobs. Water limitation reduced the final yield in all
268 treatments, except in Spd treated plants that maintained similar production as observed under WW
269 conditions (Figure 3A) and enhanced the production by 62.78% compared to control plants under WD.
270 However, the Put application reduced the production by 11.43% (Figure 3A)

271 Crop physiologists initially considered the Water Use Efficiency (WUE) as the amount of carbon
272 assimilated and crop yield per unit of transpiration (Viets, 1962), although the definition evolved to
273 biomass or marketable yield produced per unit of transpiration. In this sense, this parameter is essential
274 to better characterize the productivity of crops, especially in a water scarcity scenario. In this work, the
275 production WUE was calculated to understand better the relationship between plant production and the
276 water used by the plants (Figure 3B). A significant interaction between treatment and growth condition
277 was obtained according to ANOVA ($p = 0.002$). In control plants, no differences in WUEp were
278 observed among growth conditions. However, the PA treatments enhanced the WUEp of the treated
279 plants under WW conditions, but only Spd improved this parameter under WD (Figure 3B). These
280 results pointed to WUEp increase induced by PAs as one of the primary factors conditioning maize
281 yield under optimal and water limitation conditions.

282 Other parameters related to the cobs and kernel production were also determined (Supplementary Table
283 S5). Among them, the fresh weight and length of the cobs and the final yield per hectare considering

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284 the kernel were the production-related parameters that showed a significant interactive effect on the
285 treatment and growth conditions according to ANOVA ($p \leq 0.05$). The treatment with Put and Spd
286 improved the fresh cob weight but reduced the length compared to the controls under WW, but not
287 under WD (Figure 4, Supplementary Table S5). Contrarily, the PA treatments reduced the cob length
288 in both growth conditions except for Spd under WD. PAs significantly increased the yield per ha under
289 ($1.5*10^6$ g/ha for Put and $1.6*10^6$ g/ha for Spd) compared to untreated plants Control ($1*10^6$ g/ha)
290 under WD condition, reaching the level of the WW plants (Figure 4, Supplementary Table S5).
291 Regarding the harvest index (HI), Put-treated plants under WD overcame the levels of controls under
292 WW conditions.

293 To integrate the production data, two additional PBCIs were estimated (right panel, Figure 4). As a
294 final result, we observed that Put improved maize yield (positive values for PBCI) under WW and
295 W.D. conditions. However, Spd only enhanced maize production under WD conditions and negatively
296 affected the plants under WW (right panel, Figure 4). Altogether, the PA application by drenching
297 enhances the quality of maize production, especially under water restrictions.

3.5 PA application modifies the quality of the maize flour

299 As the final step, we evaluated the percentage of carbohydrates (CH) content and protein in the kernel
300 powder obtained from each treatment and growth conditions (Figure 5, Supplementary Table S6).
301 According to ANOVA, both parameters were affected by the interaction between the treatment and
302 growth conditions. Under WW conditions, the PA application did not change the carbohydrate content
303 (Figure 5A). The reduced water availability significantly decreased the CH content for the control
304 treatment; curiously, both PA treatments resulted in significantly higher CH content than the WD
305 control, leveling it up to the control treatment under WW conditions (Supplementary Table S6). The
306 water availability significantly affected the protein content (%), with higher values for the kernels from
307 the controls under WD than under WW conditions (Figure 5B). The PA application presented a
308 different pattern (Supplementary Table S6). Put significantly increased the protein content under WW
309 conditions but reduced them under WD compared to the respective controls. Contrarily, Spd did not
310 affect the protein content under WD but significantly reduced it under the protein content compared to
311 the controls under WW conditions (Figure 5B).

312 The mineralogical profile of the flour obtained from the dry grains was also analyzed (Table 2 and
313 Supplementary Table S7). Significant changes were observed in the flour mineral compositions due to
314 the treatment, growth conditions, and their interaction. Na, P, and Cu were the most sensitive
315 parameters because their changes were due to the interaction between treatment and growth conditions
316 ($p\leq0.006$, $p\leq0.001$, $p\leq0.047$, respectively). The treated plants presented the biggest changes in Cu,
317 mainly due to a significant increment in those treated with Spd (Table 2 and Supplementary Table S7).
318 The WD conditions significantly reduced the Na levels of the non-treated plant, but the application
319 with Put and Spd kept them at the same levels as the WW-variants. The water restriction significantly
320 induced the P, K, and Mg accumulation in the plants under WD, except the Spd treated ones that kept
321 the lower values observed in the flour from the WW plants. The opposite situation was observed for
322 Ca, where only the Spd treated plants from W.D. kept the levels of the WW ones (Table 2 and
323 Supplementary Table S7). Altogether, the PA application modifies maize flour chemical and mineral
324 composition. However, the changes are also highly influenced by the growth conditions.

3.6 The multivariate statistical analysis uncovers the different effects of Put and Spd in maize

326 To better visualize and integrate the biomass, productivity, hydric status of the plants, and the kernel
327 nutritional profile, we performed a principal component analysis (PCA) and correlation matrix (Figure

328   6). The two first PCs explained 67.3% (Dim1 = 42%; Dim2 = 25.3%) of the total model variation. As
329   the first result, Dim1 separated the non-treated plants due to the irrigation regime (Figure 6A).
330   However, whereas stressed Put treated plants were located close to the irrigated plants, Spd was located
331   opposite to these plants, pointing to a very different mechanism of action between these two PAs. Put
332   treated plants under WD strongly correlated with the leaf biomass expressed as flag leaf length/ width
333   ratio and the Zn and Fe levels. This result was also evident in the negative correlation between Fe, p,
334   K, and Mg with many production-related parameters, the plant RWC and Ca in the flour, observed in
335   the correlation matrix (Figure 6A). Contrarily, Spd treated plants under WD presented longer (Cob_L)
336   and heavier (Cobs_FW) cobs, with higher content of Cu in the flour (Figure 6A). It is worth mentioning
337   that the growth condition affected CH and protein content, presenting a higher correlation with the
338   RWC, Kernel-related parameters, and final yield. It was clear that the PA activated strategy conditioned
339   the biomass production (vegetative or reproductive biomass) and the composition of the final product,
340   in this case, the quality of the flour.

341   **4   Discussion**

342   Nowadays, many human deaths have been related to the low consumption of fruits and vegetables
343   (FAO/WHO, 2004). Therefore, it becomes essential for research topics to focus on understanding how
344   the principal abiotic stresses such as drought affect crop yield. Besides, due to the promising
345   biostimulant efficiency to mitigate the plants' stress effect, new evidence is needed to know if they
346   provide any quantitative or qualitative benefit in the final yield. This study aimed to evaluate the use
347   of small molecules-based biostimulants (e.i. polyamines and amino acids) to improve maize production
348   under optimal and water restriction conditions. Their impact on maize biofortification is also
349   investigated. When used as a foliar application, the concentration range 0.1- 1mM has elicited positive
350   responses in stressed plants (De Diego and Spíchal, 2020). In this work, we investigated their
351   application via fertirrigation because it is considered an efficient agricultural method to enable plants
352   to cope with the consequences of the water limitation during the growth and the fruit production
353   (Agliassa et al., 2021). The main reason is that the fertirrigation provides the plant with a longer nutrient
354   uptake window than foliar application (Parkunan et al., 2011). However, it must be considered that
355   different application methods can trigger different responses in the plants (Paul et al., 2019), so
356   optimizing the dose for the root treatment was an essential step, ending with 0.1 mM Put and 0.5 mM
357   Spd the most effective concentrations (Table 1).

358   The application of PAs reduced the RWC losses when plants are subjected to water limitations (Figure
359   2) compared to untreated plants. Many studies have also obtained similar results and reported that the
360   PA-induced better water balance is due to decreasing the stomatal conductance and increasing proline,
361   anthocyanins, and soluble phenolics levels, improving membrane properties and enhancing the activity
362   of catalase and superoxide dismutase (Farooq et al., 2009; Hassan et al., 2018). Their application has
363   also been described as improving plant osmotic adjustment mechanisms (Choudhary et al., 2022).
364   However, the improvement in the water balance did not influence the plant biomass but instead reduced
365   it in the PA-treated plants (Figure 3). Similar results were also observed in other maize species (Li et
366   al., 2018) and crops (Ullah et al., 2012; Liu et al., 2018). One possible explanation is that this type of
367   treatment simulates moderate stress in the plants, so-called hardening, to be ready for fighting future
368   adverse conditions (Duarte-Sierra et al., 2020).

369   The "no improvement" of the plant growth might also be an energy-saving mechanism to redirect the
370   PA-induced/accumulated resources to the enhancement of the production of the crop. This concept has
371   already been proposed for fruit tree management, where the application of plant growth retardants has

been explored to reduce the vegetative growth and obtain higher production (Köhne, 1989). According to this assumption, we expected that both PA treatments improved maize production under WW and WD. However, different responses were observed; Put enhanced the total maize yield under WW but reduced it under WD (Figure 3). Despite the reduced total maize yield, drenching with 0.1 mM Put ended with a positive PBCI under WD due to a higher number of cobs per plant, higher kernel weight, or yield per ha (Figure 4, Supplementary Table S5). The exogenous application of PAs, including Put, has improved flowering and yield in many plant species (Reviewed by González-Hernández et al., 2022) and also the production of eggplant (El-Tohamy et al., 2008) and wheat (Mostafa et al., 2010). These results could partially explain the higher number of cobs per plant. Besides, the exogenous application of 0.1 mM Put enhanced yield in winter wheat under WD and the plant biomass (Gupta and Gupta, 2011). These results agree with ours, so the Put-treated plants presented the largest leaves under WD (Figures 4 and 6A).

Regarding plant production, a much higher yield was obtained with Spd than with Put under WD (Figures 3 and 4, Supplementary Table S5). This could be because vital biological processes such as embryogenesis, and seed settings, have been related more to the levels of Spd than Put (Feng et al., 2011; Chen et al., 2019). The benefit of Put could be only due to its condition as a precursor of Spd synthesis (Feng et al., 2011). Furthermore, a recent study showed that high endogenous levels of Put but not of Spd could condition grain filling of wheat, and hence, yield, under drought because it induced the accumulation of endogenous ethylene and ABA in the grains, which worsened the adverse stress effects (Liu et al., 2016). However, this response must be concentration-dependent and conditioned by the endogenous levels of the different PA forms and their crosstalk with other phytohormones. For example, the crosstalk between ethylene and PAs has also been reported to condition the seed setting in maize (Feng et al., 2011). These authors also demonstrated that high levels of PAs are needed to avoid aborted kernels. It is well known that ethylene and PAs compete for the same precursor S-adenosyl-methionine (SAM), the activated form of methionine (Podlešáková et al., 2019). This could explain that high PAs and low ethylene levels promote plant flowering and embryogenesis [reviewed by (Chen et al., 2019)], where Spd is the leading PA form regulating these processes.

The application of PAs as small molecule-based biostimulants also modified the yield quantity and quality. The first two parameters analyzed were the CH and protein content (%) in the flour powder obtained from the seeds (Figure 5, Supplementary Table S6). Regarding CH, it was shown that the crosstalk between PAs and the ethylene pathway could condition plant yield and determine the grain filling and carbohydrate translocation in cereals (Yang et al., 2017). Therefore, we expected PA supplementation to induce a good CH transport, enhancing the kernel set and the final yield. As a result, WD reduced the CH content compared to the WW conditions in untreated plants, as previously observed by (Hussain et al., 2020; Abbas et al., 2021). However, opposite results were also published. For example, it has been reported that drought during the vegetative stage of maize plants induced an increment in glucose and amino acids on the grains (Harrigan et al., 2007) or did not affect the CH content (Barutcular et al., 2016). Interestingly, the PA application increased the flour CH content in the plants under WD conditions over the levels of the WW plants (Figure 5A, Supplementary Table S6). Besides, clear evidence was found about a positive correlation between CH, the leaf RWC, and the Na content, which also positively correlates with the weight of 100 kernels and the total kernels weight (Figure 6B). It can be because the application of PAs under stress has been reported to protect the photosynthetic apparatus (ensuring the synthesis of photosynthates) and increase the osmotic adjustment of the plants under stress conditions (Chen et al., 2019; Jing et al., 2019). Therefore, it is not surprising that those treatments reported the same yield as the proper irrigated plants and were higher than the WD control plants.

418   Low water availability increased the protein content in the flour, as described by (Lu et al., 2015;
419   Barutcular et al., 2016; Abbas et al., 2021). However, other studies showed no alterations in the protein
420   content by the water restriction (Hussain et al., 2020). Our work showed that the PA levels could be a
421   relevant factor in determining the protein content in the flour. However, opposite responses were
422   obtained by the Put or Spd application under both WW and WD conditions (Figure 5B). There is a
423   direct link between the source-sink ratio during the filling stage and the final protein content in the
424   kernels (Borrás et al., 2002). As mentioned above, Spd is considered essential for a good grain filling.
425   Its exogenous application reduced the production and the flour protein content under WW conditions,
426   whereas it improved the yield but not the protein level under WD (Figures 4 and 5B). Contrarily, the
427   Put application enhanced the production and protein content under WW but reduced both under WD.
428   In wheat, Spd application may affect grain filling by regulating protein synthesis and posttranslational
429   modification, together with a better antioxidative response under drought conditions (Li et al., 2020).
430   This way, the maize-Spd treated plants could deal better with the water limitations, ensuring a better
431   production under this adverse condition.

432   On the other hand, Put exogenous application induced the increment of N content in cotton plants
433   (*Gossypium barbadense* L.) under control conditions and salt stress (Darwish et al., 2013). Our results
434   partially agree since we only reported a protein increment under control conditions. The different
435   behavior observed for Put and Spd application in the plant growth and yield quantity and quality could
436   be because they regulated PA synthesis, conversion, and terminal catabolism differently. In this regard,
437   it has been proved that at least the synthesis of Spd and Spm via the activity of the enzymes spermine
438   synthase (SPMS) and spermidine synthase could condition the flowering (reviewed by Chen et al.,
439   2019). Additionally, their oxidation via polyamine oxidases has also been reported to regulate the plant
440   reproductive phases (reviewed by Yu et al., 2019). In this last case, the back conversion from
441   thermospermine or spermine to Spd was the most often identified step conditioning fertility and floral
442   development. Altogether, it is clear that the effect of the PA application in maize production is due to
443   the endogenous levels and the interconversion between the different PA forms. Further studies are
444   needed focusing on the enzymatic changes to clarify the mode of action of these compounds.

445   As the last step, we analyzed the mineral content of the flour. Only the limitation of water availability
446   has induced very controversial results regarding the flour mineral composition. For example, some
447   studies did not see any effect (Feil et al., 2005), whereas others observed a reduction in K, P, and Fe
448   (Abbas et al., 2021). Contrarily, Avila et al., 2017 reported the increment of P and Mg on grains of
449   maize plants subjected to water limitations. These contrasting results point to a very complex response
450   of the plants that affect the metabolism in the grains and condition their mineral composition, which is
451   regulated by the interaction of variety, genotype, and stress intensity. Our study clearly showed that
452   the application with PAs modified the composition under WW and WD (Table 2, Supplementary Table
453   S7). Only the Spd treatment increased the Zn content in WW plants, whereas all applications enhanced
454   the Cu under WW and WD. These two minerals have been reported to accumulate under drought stress
455   (da Ge et al., 2010; Avila et al., 2017). Zn deficiency reduces plant growth and nutritional quality (Liu
456   et al., 2019). Cu is also needed for growth and development and is an essential cofactor for many
457   metalloproteins and various enzymes involved in different physiological and cellular processes such
458   as oxidation and reduction reactions (Rajput et al., 2018). Besides, both minerals are considered
459   essential for human health (White and Broadley, 2009; Garg et al., 2018). It is worth mentioning that
460   the deficiencies in Cu also play a vital role in COVID-19, altering the disease outcomes and prognosis
461   (Altooq et al., 2022). In this context, crop biofortification by applying compounds or fertilizers
462   increases attention to assure plant and human health. Our results showed that a simple PA application

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463　could improve the content of these two minerals in the flour, especially when the plants are treated
464　with Spd (five times more Cu under WD or three times more Zn under WW). Additionally, the Spd
465　application also induced a significant accumulation of Ca. From the human nutritional point of view,
466　higher Ca content is an advantage because a recent study associates a dietary low calcium intake with
467　a higher risk of all-cause mortality (Yoo et al., 2022). Altogether, we demonstrate that the exogenous
468　application of PAs improves plant performance under stress conditions and can also be an efficient
469　biofortification approach, especially in the case of Spd.

470　**5　Conclusion**

471　The PA application by drenching can improve maize production under optimal and stress conditions.
472　However, different PAs induced different responses, conditioned by the growth conditions. Put was
473　the most effective treatment under WW because it enhanced fruit production, WUE, and the content
474　of Ca and Cu (Figure 9). The Spd application showed better results under WD, with better water
475　balance, WUE, higher production, and better nutritional composition of the flour by increasing CH
476　content, Cu and Ca (Figure 9). These results point to the PA supplementation as an exciting approach
477　for crop biofortification. However, it is evident that product and crop specificities exist, so it is expected
478　to respond differently to treatments according to the genotype (Shahrajabian et al., 2021). For that, a
479　higher effort should be put into elucidating and characterizing the use of these substances for crop and
480　site-specific locations.

481　**6　Conflict of Interest**

484　**7　Author Contributions**

485　A.E.H, D.J-A., A.A.B., and N.D.D designed the idea of the project. A.E.H, D.J-A., and S.M-S
486　performed the experiments. A.E.H and N.D.D analyzed the data and wrote the manuscript. All authors
487　agreed with the last version of the manuscript.

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490　**9　Funding**

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Table 1. Heatmap summarizes the parameters estimated in maize seedlings treated with Put or Spd at five concentrations (0.01, 0.1, 0.5, 1 or 2 mM) grown under water limitation (50% field capacity). The studied parameters were plant dried weight (Plant W, mg), relative growth ratio (RGR, %), and water use efficiency (WUE, mg mL^{-1}). The table represents the ratio (log2) between the original values of each variant and the untreated plants (Control). The right panel shows the obtained PBCI values. Blue indicates a stress alleviator under optimal conditions; orange indicates a stress inductor.

Compound	Concentration	Biomass	RGR	WUE	PCBI
Put	0.01	-0.003	-0.001	-0.003	**-0.008**
	0.1	0.050	0.026	0.059	**0.135**
	0.5	0.014	0.008	0.017	**0.039**
	1	-0.033	-0.018	-0.039	**-0.091**
	2	-0.021	-0.011	-0.025	**-0.057**
Spd	0.01	-0.089	-0.049	-0.106	**-0.244**
	0.1	-0.085	-0.046	-0.101	**-0.232**
	0.5	-0.044	-0.024	-0.052	**-0.120**
	2	-0.078	-0.042	-0.092	**-0.212**
	1	-0.119	-0.066	-0.143	**-0.328**

Table 2. Heatmap containing the log2 values of the different mineral elements content (ppm) in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under optimal conditions (WW) or water deficit (WD). The values are relative to the control treatment under WW. Different letters indicate significant differences according to the LSD test after two-way ANOVA, p=0.05.

Growth conditions	Treatment	Ca	Fe	K	Mg	Na	P	Zn	Cu
WW	Control	0 ab	0 a	0 ab	0 ab	0 b	0 ab	0 a	0 a
	Put	0.58 ab	-2.00 a	-0.42 a	-0.24 a	0.00 b	-0.15 a	-1.77 a	0.77 a
	Spd	0.20 ab	0.38 a	-0.27 ab	-0.11 a	-0.07 b	0.14 bc	1.45 b	0.31 a
WD	Control	-0.42 a	-0.70 a	0.33 c	0.25 c	-0.22 a	0.24 c	-0.41 a	1.23 a
	Put	-0.14 ab	0.17 a	0.14 bc	0.22 bc	0.03 b	0.23bc	-0.48 a	0.19 a
	Spd	0.72 b	-1.86 a	-0.17 ab	-0.15 a	-0.03 b	-0.08 a	-1.53 a	2.46 b

Figure 1. Water-related parameters in maize plants under optimal conditions and water limitation. A) Volumetric water content of the soil (m3 m-3) measured with the FDR soil moisture sensor from the onset of the water restriction. Red arrows indicate the two dates (t1, t2), when the Relative Water Content (RWC, %) was measured in the maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd under optimal conditions (WW) or water deficit (WD). B) RWCt1 of the plants, t1 corresponds to15 days after the onset of the stress). C) RWCt2 (%) of the plants, t2 corresponds to 30 days after the onset of the stress. Mean ± s.e . N= 24. Different letters for the RWC values indicate significant differences according to the LSD test after two-way ANOVA (p≤0.05).

Figure 2. Parallel coordinate plot and PBCI regarding the biomass–related parameters. Stem diameter (mm), plant length (cm), flag leaf length and width (cm), and length/width ratio (L/W) of maize plants treated with 0.1 mM Put or 0.5 mM Spd under optimal conditions (WW) or water deficit (WD). The plot represents the ratio (log2) between the original values of each variant and the untreated plants under WW conditions. The right panel shows the obtained PBCI values.

Figure 3. Total production and WUEp in PA treated maize plants. A) Total production expressed as the sum of all cobs produced per variant in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under well-water (WW) or water restriction (WR). Enclosed in the left upper table, the percentage of increment (blue, upside arrow) or reduction (orange, downside arrow) of Put and Spd treatment relative to the respective control in WW and WD conditions. B) Water Use Efficiency calculated as the ratio between the fresh biomass production and the ETo adapted to Maize crop with the FAO Kc. Different letters indicate significant differences between the treatments and growth conditions according to the LSD test after two-way ANOVA , p<0.05.

Figure 4. Parallel coordinates plot representing log2 of the production parameters relativized with the control of each growth condition; cobs per plant (Cobs/Pl), cobs fresh (Cob FW) and dry weight (Cob DW) (g), length (L(K)) (kernels in a line), circumference (Circ. (K)) (kernel circumference) and diameter (Diam.) (mm), the weight of 100 kernels (100 K DW) (g) and the total kernel weight (Total KW) (g), the total number of kernels (Total K), the yield per hectare, considering the dry kernel production (Yield(K)/ ha), harvest index (HI) (Total Dry kernels weight/ Avg Cob fresh weight), and the WUE in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under well-water (WW, A) or water restriction (WR, B). The right panel represents the PBCI, which summarizes the plot in a single number positive (Blue color) for growth promotor or negative (orange) for stressor.

Figure 5. Content of carbohydrates (A) and protein (B) expressed as % of the weight of the sample WUE in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under well-water (WW) or water restriction (WR). Different letters indicate significant differences between the treatments and growth conditions according to the LSD test after two-way ANOVA, p<0.05.

Figure 6. Principal component analysis A) and correlation matrix B) of the studied biomass parameters [flag leaf length (Leaf_L), flag leaf width (Leaf_W), flag leaf length/width ratio (leaf_LW)], relative water content at 15 and 30 days after the stress onset (RWC 15d, RWC30d), productivity parameters [cob length (Cob_L), cob circumference (Cob_circ.), cob diameter (Cob_diam.), cob fresh weight (Cob_FW), cob dry weight (Cob_DW), weight of 100 kernels (KW_100), total number of kernels per cob (Total_Kernel); total weight of the kernels per cob (Kernel_TW), harvest index (HI), yield, water

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use efficiency (WUE), protein content (Protein), carbohydrates content (CH), and mineralogical profile of the kernels (P, K, Mg, Fe, Zn, Ca, Na and Cu) in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under well-water (WW) or water restriction (WR).

Figure 7. Summary of the variation of the observed parameters in maize plants untreated (Control) and treated with 0.1 mM Put or 0.5 mM Spd grown under well-water (WW) or water restriction (WR). Bold formatting of the font means the statistical effect on the increment or reduction; the Total Production is expressed as the sum of every cob produced per treatment and condition. The parameters displayed are the water use efficiency (WUE); leaf relative water content (RWC); flag leave length/ width ration (L/W); cob fresh weight (g) (Cob FW); cob length, as kernels in the longitudinal line (Cob L); cob circumference, as the kernels in the perimeter at the center of the cob (Circumf.); average cobs produced in a plant (Cobs/ pl); the weight of 100 kernels (g) (100 K); harvest index (HI); yield (10^6g/ha) (Yield); % of carbohydrates; % of protein; and minerals (ppm) such as Ca, Zn, K, Mg, Na, P, and Cu.

Figure 1.JPEG
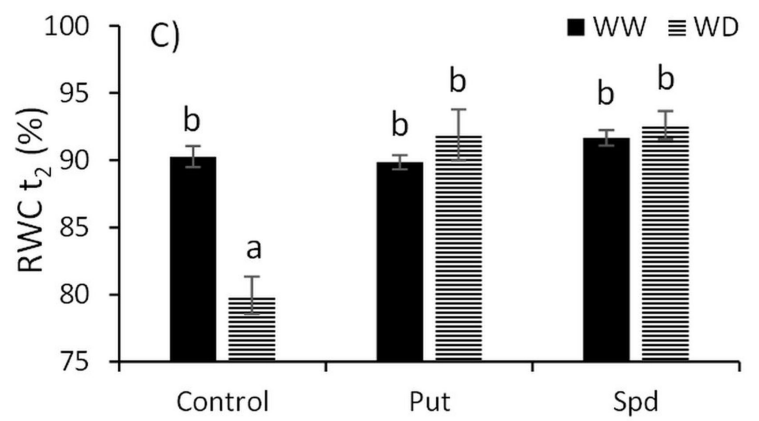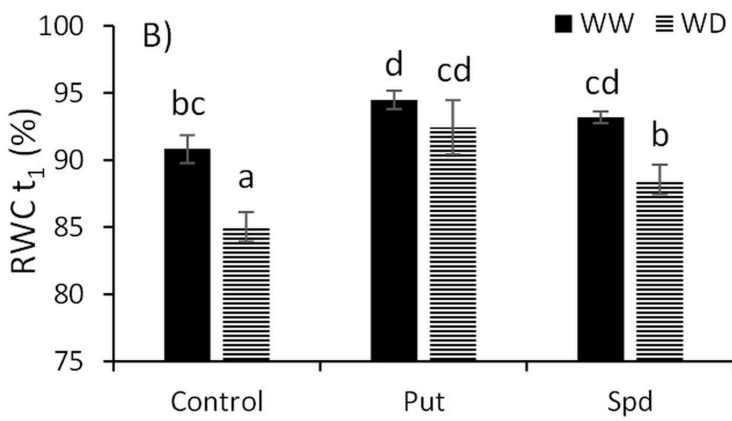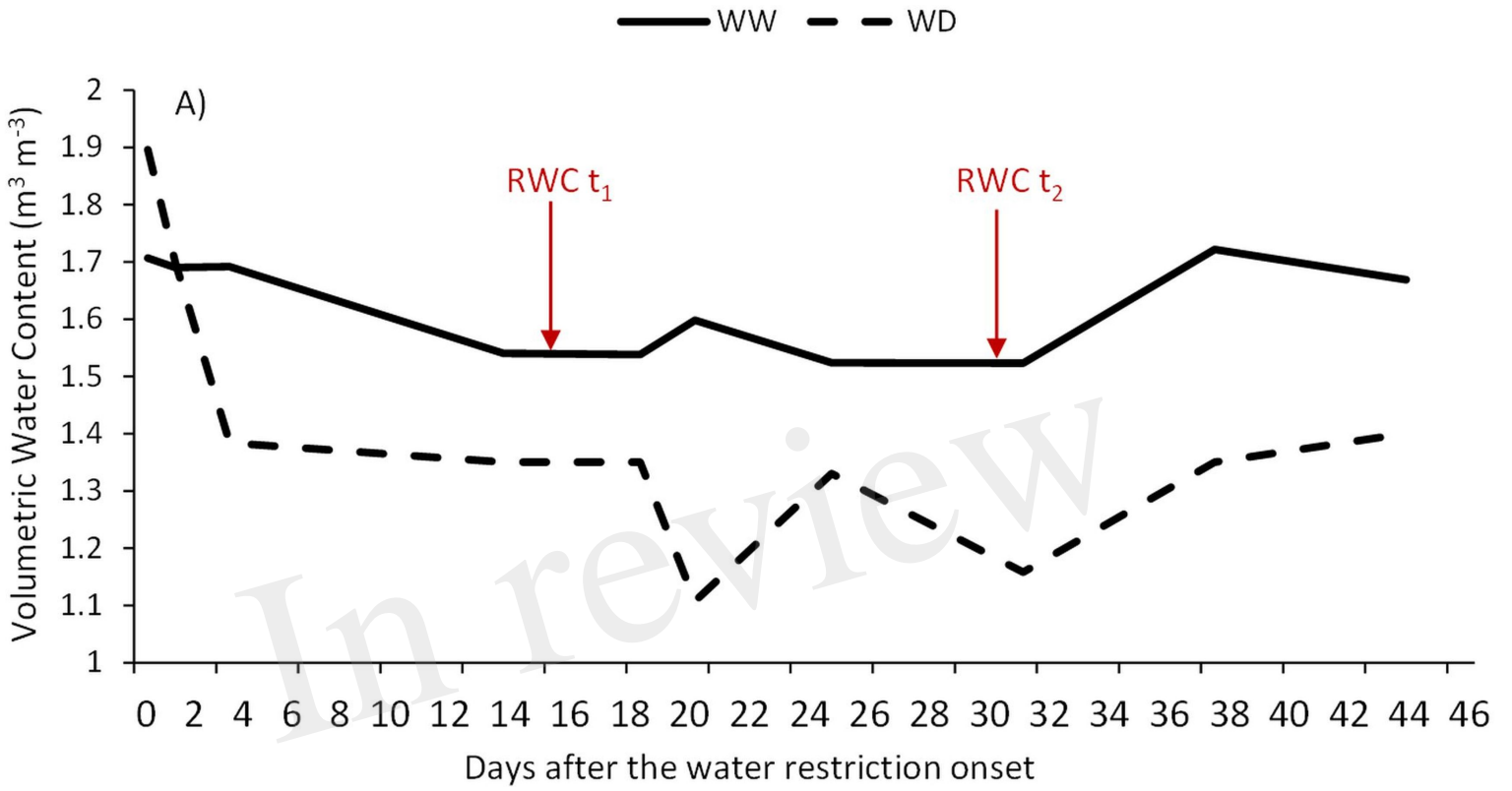
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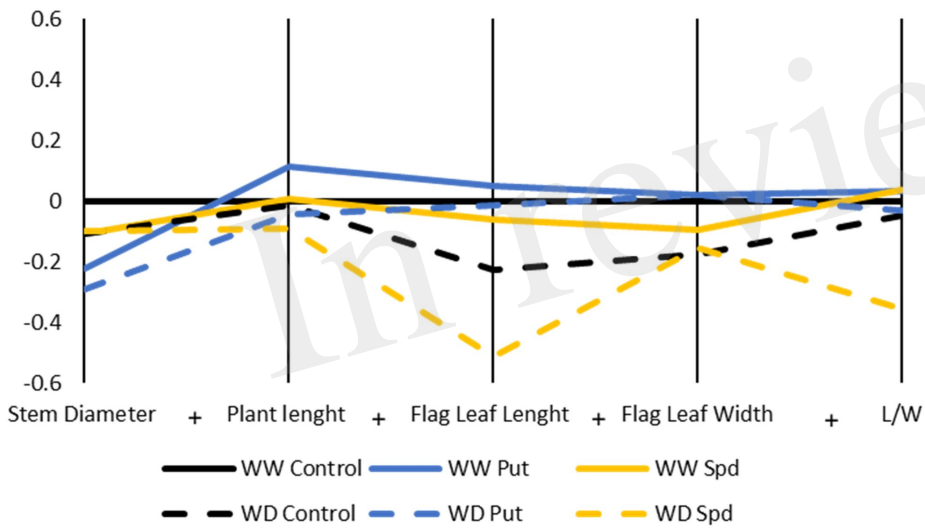
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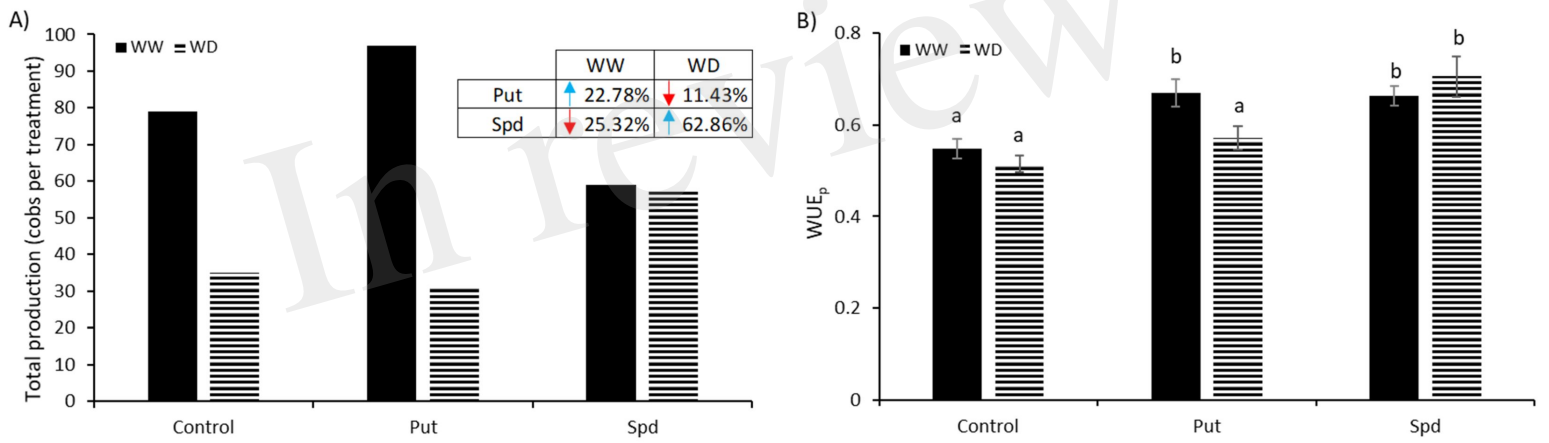
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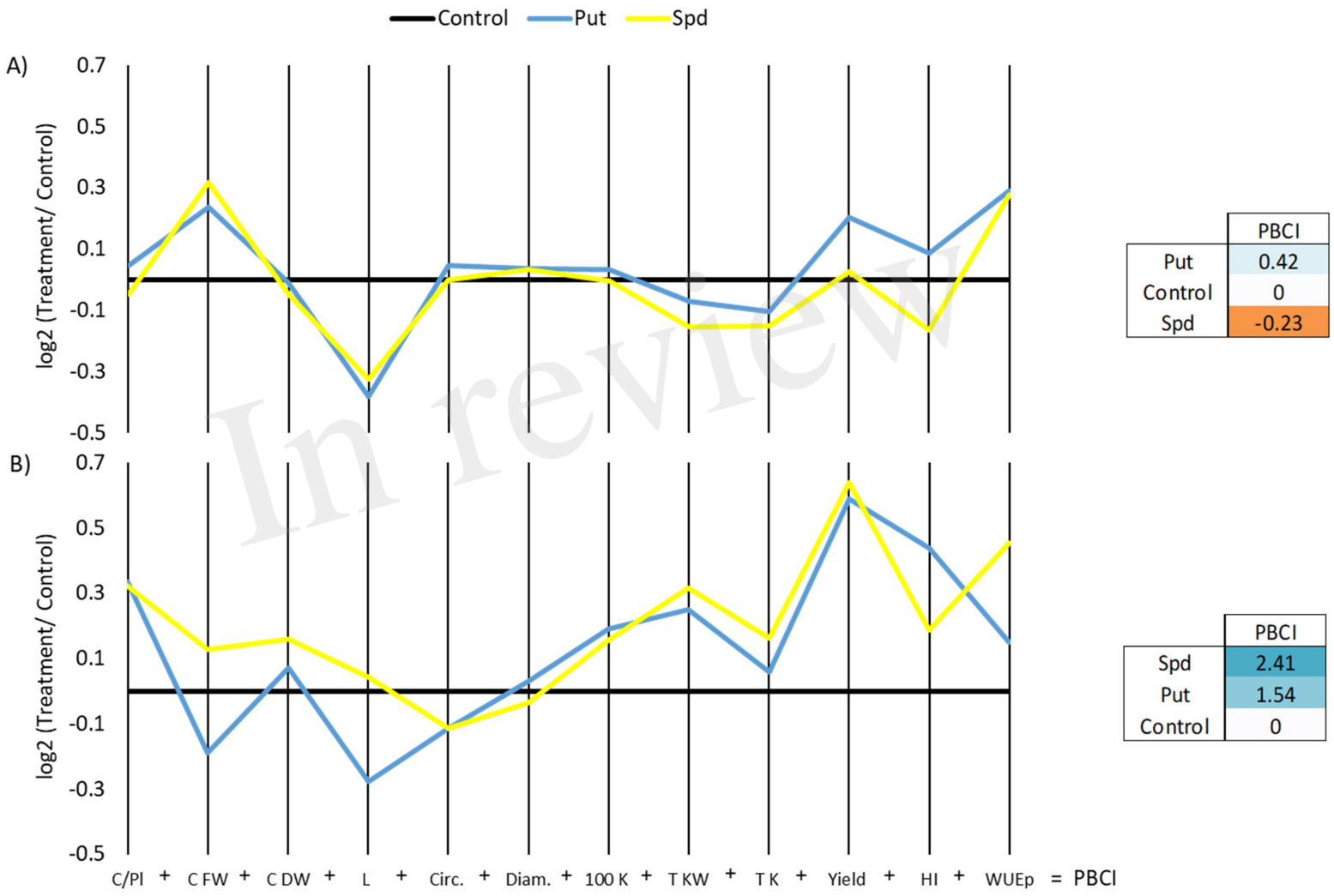
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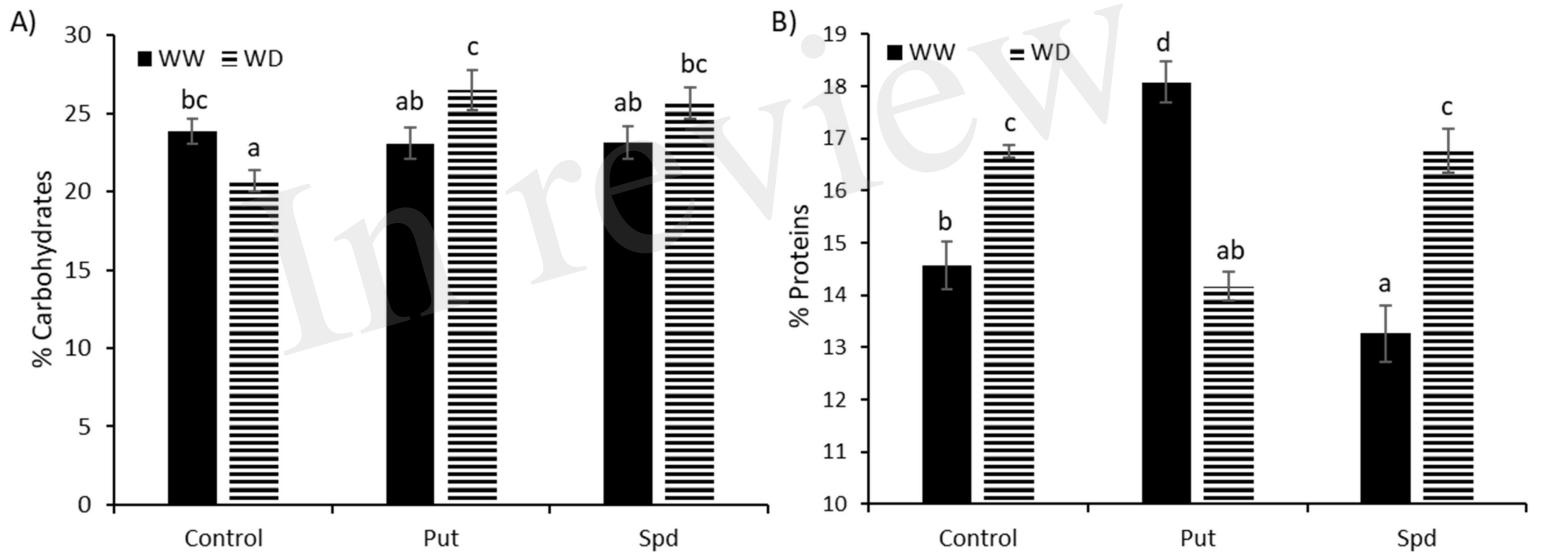
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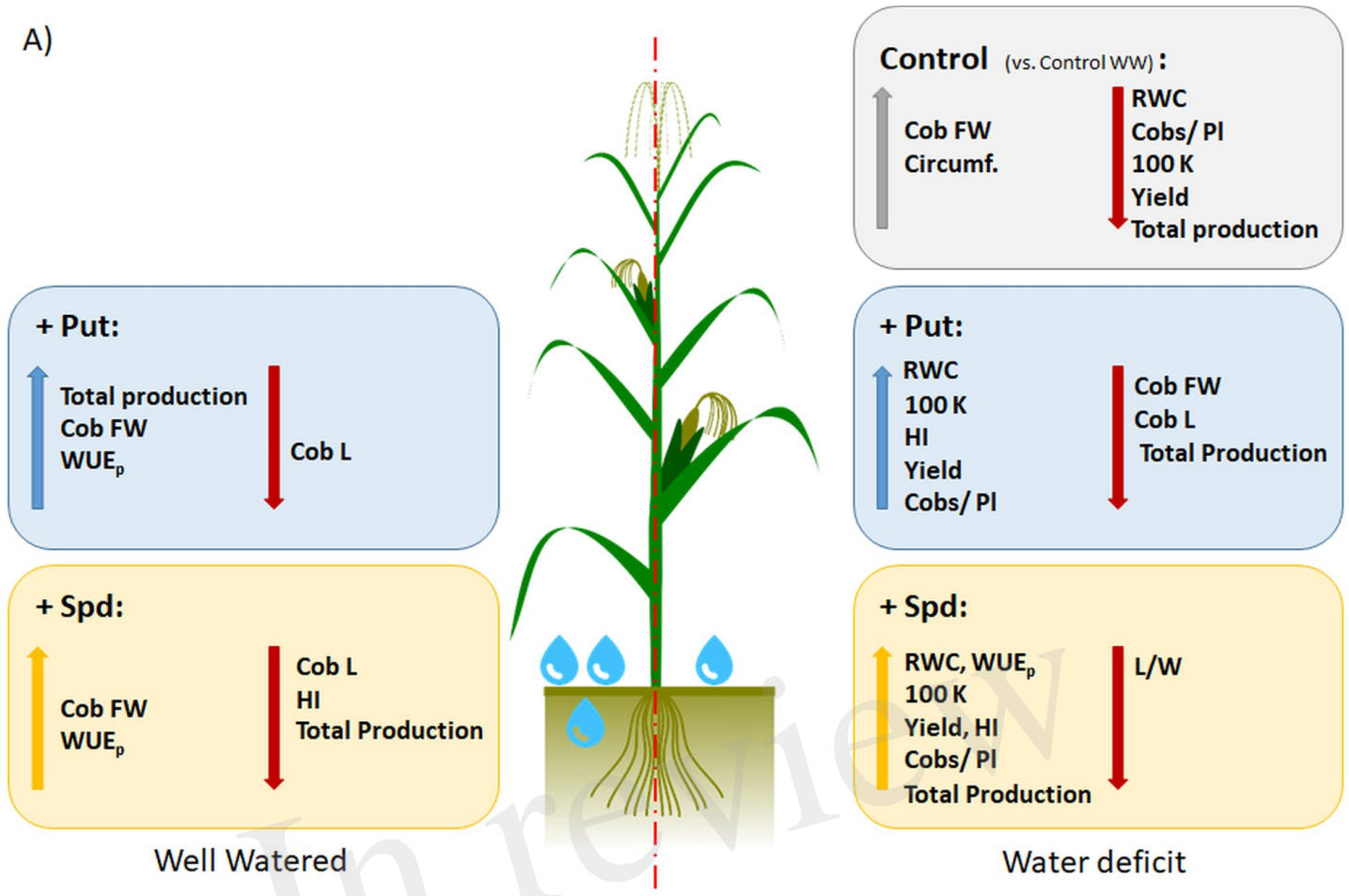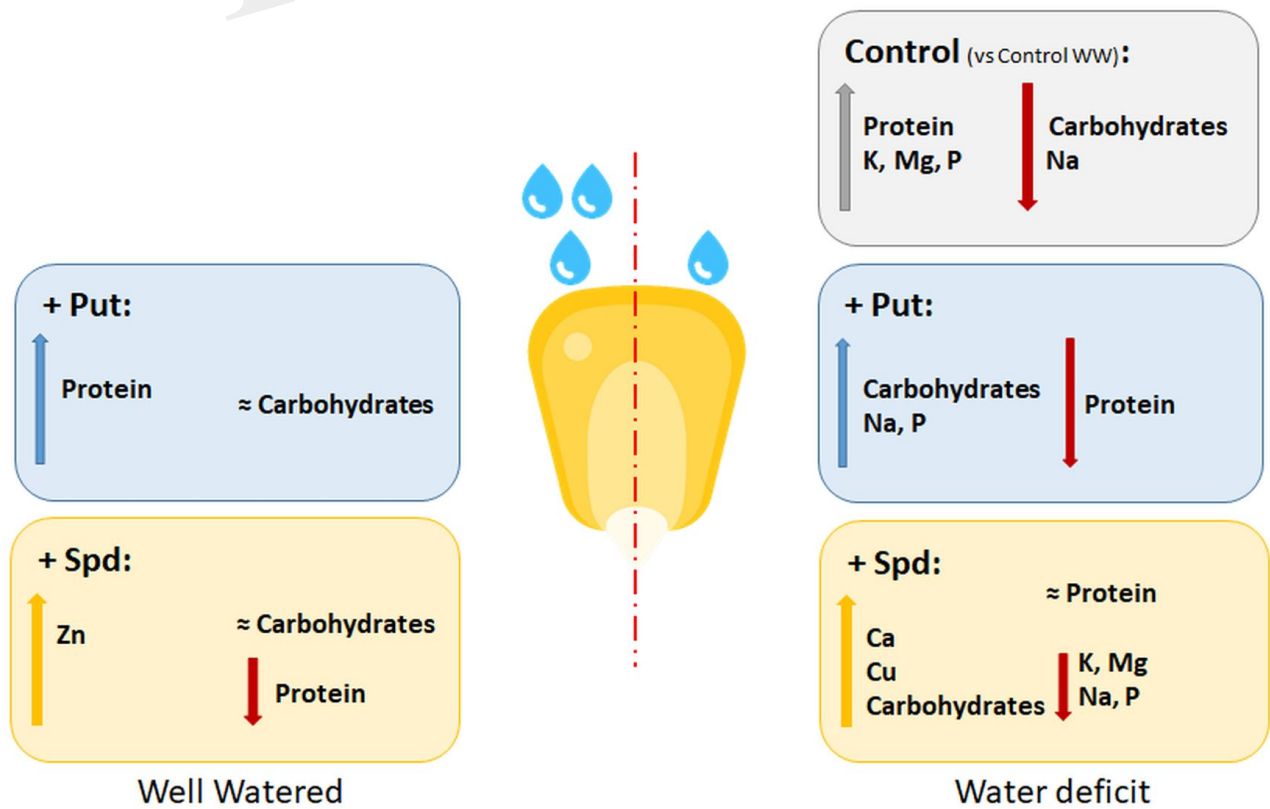
SUPPLEMENT V:

Priming with Small Molecule-Based Biostimulants to Improve Abiotic Stress Tolerance in Arabidopsis thaliana

Hernándiz, Alba E., Carlos E. Aucique-Perez, Sanja Ćavar Zeljković, Nikola Štefelová, Sara Salcedo Sarmiento, Lukáš Spíchal, and Nuria De Diego. 2022
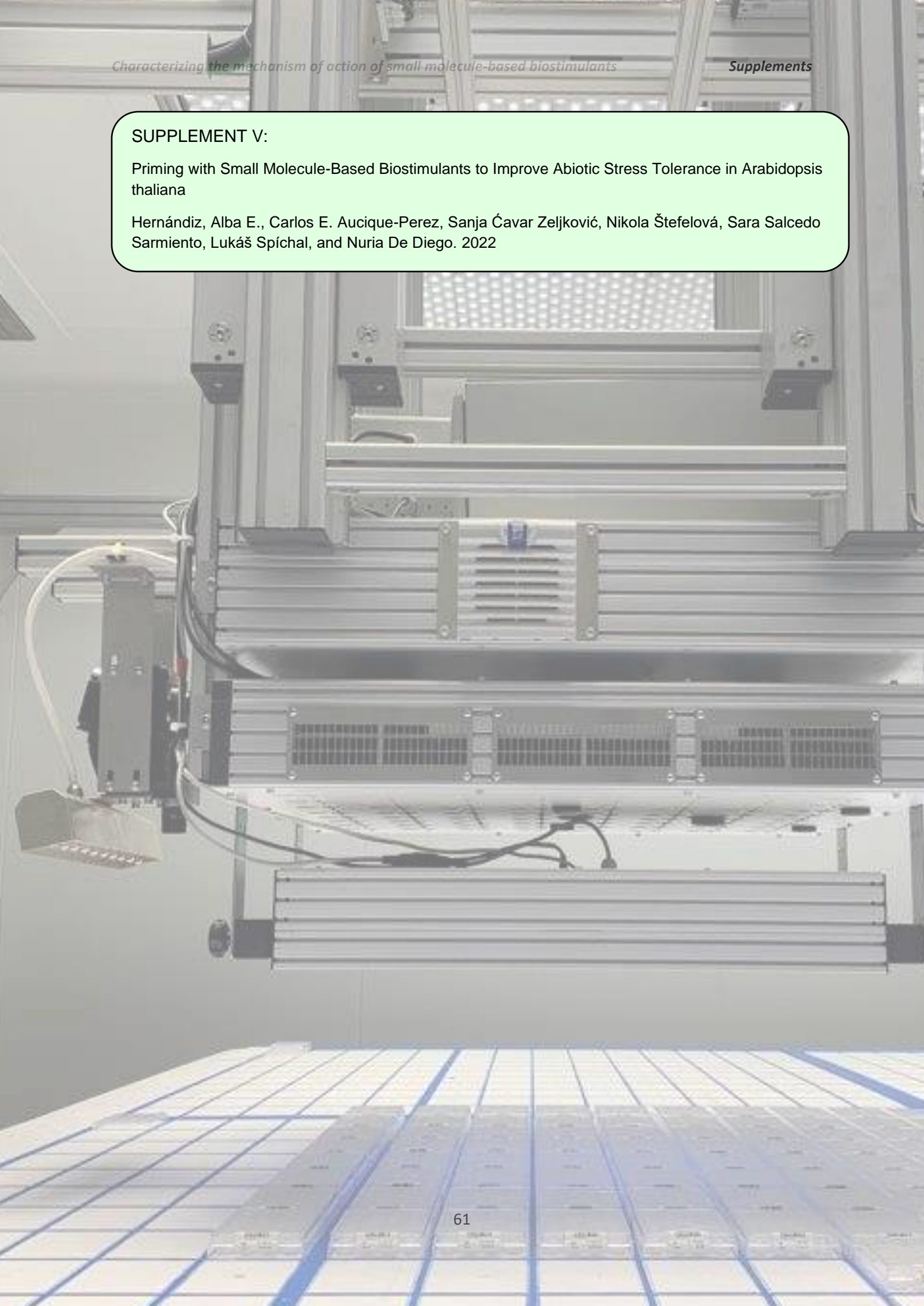
Priming with Small Molecule-Based Biostimulants to Improve Abiotic Stress Tolerance in *Arabidopsis thaliana*

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Abstract: Biostimulants became a hotspot in the fight to alleviate the consequences of abiotic stresses in crops. Due to their complex nature, it is challenging to obtain stable and reproducible final products and more challenging to define their mechanism of action. As an alternative, small molecule-based biostimulants, such as polyamines have promoted plant growth and improved stress tolerance. However, profound research about their mechanisms of action is still missing. To go further, we tested the effect of putrescine (Put) and its precursor ornithine (Orn) and degradation product 1,3-diaminopropane (DAP) at two different concentrations (0.1 and 1 mM) as a seed priming on in vitro Arabidopsis seedlings grown under optimal growth conditions, osmotic or salt stress. None of the primings affected the growth of the seedlings in optimal conditions but altered the metabolism of the plants. Under stress conditions, almost all primed plants grew better and improved their greenness. Only Orn-primed plants showed different plant responses. Interestingly, the metabolic analysis revealed the implication of the N-acetylornithine and Orn and polyamine conjugation as the leading player regulating growth and development under control and stress conditions. We corroborated polyamines as very powerful small molecule-based biostimulants to alleviate the adverse abiotic stress effects.

Keywords: abiotic stress; growth; plant phenotyping; biostimulant

1. Introduction

Climate change globally affects the earth's temperature and rainfall pattern, causing extreme meteorological phenomena, amplifying drought events, their frequency, duration, and intensity [1,2]. One of the impacts of extended drought conditions is the significant increase in salinity in the root-zone soil, most likely occurring in irrigated areas but not exclusively [3]. These two phenomena, drought and salinity, already affect 60 and 10.5 million km^2, respectively [4,5]. The entailed reduction of arable land becomes another obstacle to meeting the food demand. For that, food production is under threat due to exposure of the crops to numerous abiotic and biotic stresses that negatively affect plant growth and productivity, leading to reduced yields and quality [6]. This situation jeopardizes agricultural production and food security and entails long-term socioeconomic impacts. Consequently, the arable land availability will decline, so it is expected that the land for tropical and temperate crops will be reduced by 2.3 and 10.9%, respectively, in the year 2100. In addition, the prediction suggests that this reduction is expected to escalate up to 14.9% and 18.3% by 2500 [7].

Moreover, international organizations have alerted that the high food demand for a world population with a growth expectation of 9.1 billion by 2050 should increase by 50 to 70% of the current production [8]. Therefore, the climatic crisis and, consequently, the loss in food security are the target center of the world discussion, both in science and economic scenarios. In this context, drought has caught the attention of many researchers since water is a major component of the fresh biomass of the plants and plays a vital role in various physiological processes such as plant growth, development, and metabolism [9]. To counteract the adverse effects of drought on food production, scientific initiatives and research aim to improve the plant stress response through classic breeding strategies associated with genetic engineering tools to accelerate the obtaining of tolerant plants [10–12]. Concurrently, better agricultural practices have been developed to optimize the production systems and the study of the potential use of biostimulants as products that might contribute to easing the enormous pressure that climatic factors are putting on agriculture. According to the Regulation (EU) 2019/1009 of the European Parliament, a biostimulant is "a product stimulating plant nutrition processes independently of the product's nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, and availability of confined nutrients in soil or rhizosphere". Under this definition, there are comprised a wide range of substances that are currently classified according to their source of origin [13], providing the following categories: humic and fulvic acids, seaweed and botanical extracts, protein hydrolysates, and N-containing compounds, chitosan and other biopolymers, inorganic compounds and beneficial fungi, and bacteria. Biostimulants are commonly a mixture of several substances since the circular economy has seen in this sector a niche to give a second life to waste and by-products [14,15]. Nevertheless, the use of mixtures presents some disadvantages, such as the possible lack of homogeneity of the batches and the difficulty in identifying the active substances [16]. Therefore, there are limitations to studying and accurately describing the mode of action of those substances to prove their effectiveness, but it is a challenge that can be overcome considering the new technologies that exist.

Another possibility is the use of small molecule-based biostimulants. They are natural compounds such as amino acids, carbohydrates, polyamines, plant growth regulators, or other nanoparticles, which can be easily found in plants and help them deal with stress [17,18]. They are also involved in plant–microorganism communication [19], giving the plant higher tolerance to abiotic stress or resistance to biotic stress [18,20]. Our previous works demonstrate that priming Arabidopsis seeds with small molecules such as polyamines, especially Put and Spd, improves plant growth under optimal conditions and increases salt stress tolerance. However, the mechanism of action that the seed priming with these compounds activates in the plant is still unclear. To go further in this issue, we decided to study a precursor [ornithine (Orn)], a product [putrescine (Put)], and a final product [1,3-diaminopropane (DAP)] of the polyamine (PA) metabolism.

Orn is an amino acid produced from glutamate (Glu), essential in nitrogen assimilation metabolism. Orn participates in the arginine (Arg) synthesis, the urea cycle, and as a precursor of many stress-related compounds, including PAs synthesis and the amino acids proline (Pro) and γ-aminobutyric acid (GABA), which, in turn, is linked to the tricarboxylic acids cycle (TCA) [21]. Only one publication reported the beneficial effect of the exogenous Orn application on beetroot plants subjected to drought stress [22]. Put is the first to be PA synthesized, and it has been extensively reported for its positive contributions to the plant response to several stresses, improving the photosynthetic capacity, contributing to the enhancement of amino-oxidases, maintaining the membrane stability, contributing to reactive oxygen species (ROS) scavenging mechanisms, or enhancing plants growth and germination [23–25]. The Put catabolism can end with the DAP formation. This diamine has been reported to repress ethylene biosynthesis [26]. In addition, its acetylated product diacetyl-DAP, originated by the action of the *N*-acetylaminotransferase 1 (NATA1, E.C 2.6.1.11.) enzyme, has been described as an antagonist of the abscisic acid (ABA)-regulated

stomatal closing [27]. However, no studies exist about its effects as a priming agent to mitigate the adverse stress effects.

Both Orn and DAP are directly linked with metabolic pathways that control plant stress tolerance. We hypothesize that the exogenous application with DAP and Orn as small molecule-based biostimulants could be a simple and efficient approach for improving plant stress tolerance. To validate our hypothesis, deep characterization of DAP, Orn, and Put as seed priming agents was performed using *Arabidopsis thaliana* plants growing in vitro under optimal conditions or subjected to osmotic and salt stresses based on [28]. In addition, the combination of phenomic and metabolomics data will uncover the critical metabolic steps in which these metabolites affect and condition the Arabidopsis phenotype under the different growth conditions studied.

2. Results

2.1. The Priming with DAP, Orn, and Put Improved Plant Performance under Abiotic Stress but Not under Optimal Growth Conditions

As the first step for studying DAP, Orn, and Put as priming agents, we analyzed the Arabidopsis rosette growth under optimal conditions and osmotic and salt stress. Osmotic and salt stress reduced 70% and 78%, respectively, the rosette size of Arabidopsis compared to the seedlings grown under control conditions (Figure 1). The seed priming with DAP, Orn, and Put modified the growth compared to the seedling from non-priming seeds, but the effect depended on the interaction between the compound concentration and growth condition. For example, the priming with DAP and Put did not alter Arabidopsis rosette growth under optimal conditions or osmotic stress, but they improved it under salinity (Figure 1). The low Orn concentration also improved plant growth under salt stress. Contrarily, 1 mM Orn reduced the rosette growth under optimal and stress conditions (Figure 1B,E,H).
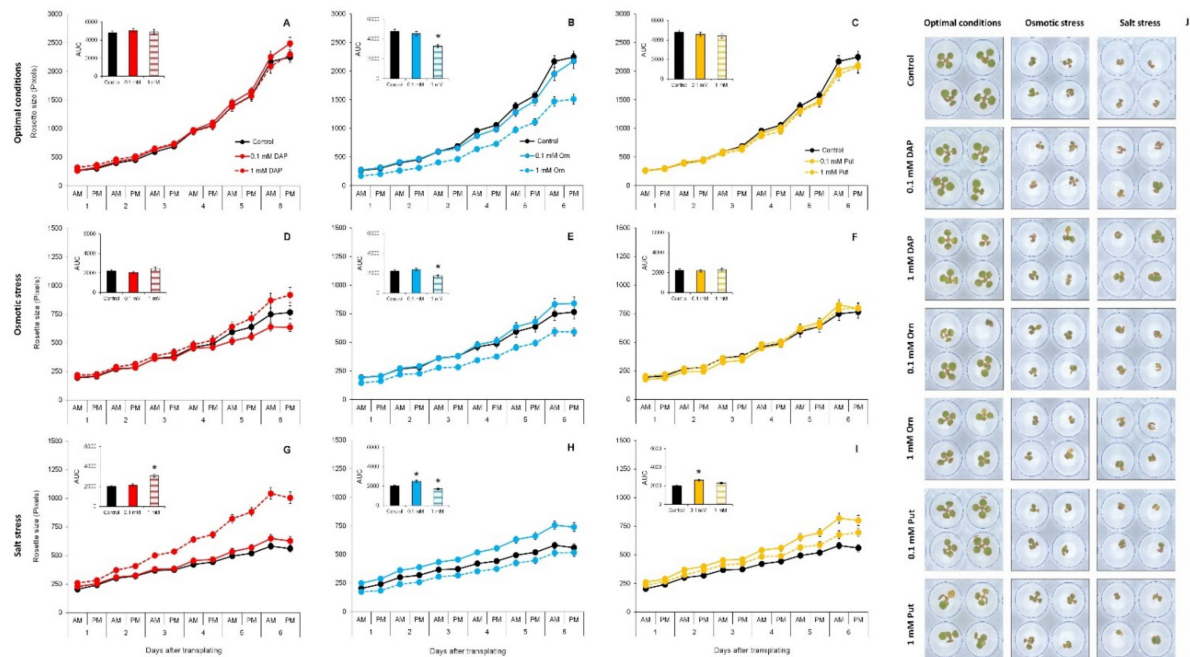

Figure 1. Rosette growth (green pixels, left panel) of *Arabidopsis thaliana* (Col-0 ecotype) primed with 1,3-diaminopropane (DAP), Ornithine (Orn), or Putrescine (Put) at two concentrations (0.1 or 1 mM) under optimal conditions (**A**–**C**), osmotic stress (**D**–**F**), and salt stress (**G**–**I**). RGB images (**J**) of the Arabidopsis seedlings 7 days after the transfer to the well-plates with different growth conditions. The corresponding area under the curve (AUC) for each growth curve was calculated and displayed in the bar chart. According to Kruskal–Wallis' tests, asterisks show significant differences compared to the respective control treatment ($p < 0.05$). Bars correspond to standard error. $n = 48$. Well diameter = 10.4 mm.

The phenotyping traits extracted from the RGB images were represented in a parallel coordinate plot (Figure 2, left panel) [29]. The obtained value per trait is recalculated using log2 and represented in a parallel coordinate plot (Figure 2, left panel) [29]. Finally, the values for each trait are summed individually per treatment and growth condition to obtain the PBCI, which is included in an independent table in the right panel of Figure 2. The treatments presenting positive values will be considered plant growth promotors under optimal conditions or stress alleviators under stress conditions. Contrarily, the negative values will describe the stress inductors.
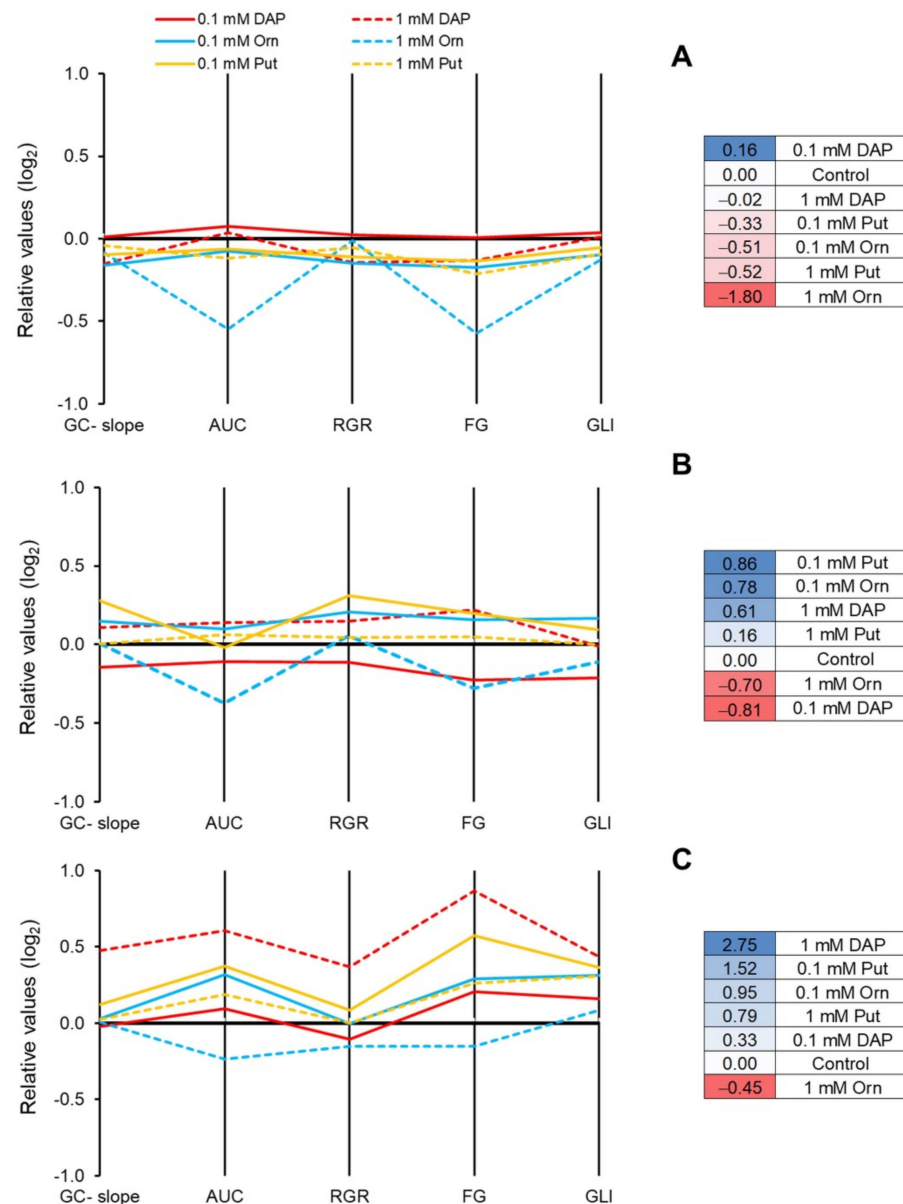
Figure 2. Parallel coordinates plot (left panel) representing all the phenotyping traits; the slope of the growth curve (GC-slope), the AUC of the growth curve, the relative growth ratio (RGR), the final size of the seedlings at the end of the experiment (FG), and the green leaf index (GLI) in Arabidopsis seedlings from primed seeds with DAP, Orn, or Put at two concentrations (0.1 or 1 mM) grown under optimal growth conditions (**A**), osmotic (**B**), and salt stress (**C**). Plant Biostimulant Characterization Index (PBCI) (right panel) is calculated as the sum of the values represented for each phenotyping trait in the parallel coordinates plot. The blue color indicates plant growth promotor under optimal conditions or stress alleviator under stress. The red color indicates a stress inductor.

For the optimal growth conditions, none of the treatments enhanced the plant performance for the studied parameters but instead had a negative effect, ending with negative values of the PBC index for all the treatments (Figure 1A). The only plant growth promotor was 0.1 mM DAP, presenting a positive PBCI due to a better relative growth ratio (RGR) and the green leaf index (GLI) (Figure 2A and Supplementary Table S1).

Although no apparent effect was observed in the growth curves of the seedlings from primed seed compared to their controls under osmotic stress, a deeper analysis in which parameters such as RGR, final size, or the color index were extracted showed a positive effect on the plants (Figure 2B). The seed priming with 1 mM DAP and 0.1 mM Orn provided the highest values of PBCI and the best seedling performance. The Put application slightly improved the plant performance (Figure 2B). However, 1 mM Orn and 0.1 mM DAP showed negative PBCI values due to decreased traits such as the slope of the curve, RGR, final rosette size, or GLI (Figure 2B and Supplementary Table S1).

All treatments alleviated the salt stress adverse effects (Figure 2C). Only 1 mM Orn showed a negative PBCI, but it improved GLI compared to the non-primed plants. The best salt stress alleviator was 1 mM DAP, which greatly enhanced all phenotype parameter. Similar trends were observed in the rest of the treatments, with 0.1 mM DAP as the less effective stress alleviator (Figure 2C).

2.2. Seed Priming Induced Changes in the N-Related Metabolites, and the Changes Were Due to the Interaction between the Priming Agent, Its Concentration, and Growth Conditions

We analyzed the metabolite changes on the rosettes collected after the last phenotyping measurement in the following step. Due to the involvement of Orn, Put, and DAP in the amino acid and PA metabolism, we performed a targeted metabolomic analysis for their quantification (Figure 3 and Supplementary Materials Table S2). The most abundant free amino acids were Pro, aspartic acid (Asp), and glutamine (Gln) in Arabidopsis plants from all the treatments and growth conditions (Figure 3 and Supplementary Table S2). Contrarily, cystine (Cis) was the amino acid that appeared in the lowest concentration (Figure 3). Although there were not many significant changes in the content of free amino acids, some of them changed by the effect of the treatment and the growth conditions (Figure 3). For example, all primed plants accumulated N-acetylornithine (AcOrn) under optimal and salt stress conditions, except for the 1 mM or 0.1 mM Put-treated plants under optimal or salt stress conditions, respectively. Met was significantly accumulated with 1 mM DAP and 0.1 mM Orn under optimal conditions. Only the primed seedlings with 1 mM DAP or 0.1 mM Put significantly accumulated Pro under optimal and osmotic stress conditions, respectively. However, 1 mM Orn reduced the Pro content. Glu was only significantly accumulated in 1 mM Put-primed plants and reduced in Put-treated ones under osmotic stress (Figure 3). The Put-treated seedlings also reduced the content of Cis under optimal conditions and in the high concentration under osmotic stress. Finally, the 0.1 mM DAP or 1 mM Orn reduced the levels of Asp under osmotic stress (Figure 3).

Regarding PAs, both free and total (sum of conjugated and free PAs) Put, Spd, and agmatine (Agm) were the most abundant in all plants, whereas tyramine (Tyra) appeared in low concentrations (Supplementary Table S3). Only 1 mM DAP, 0.1 mM Orn, and both Put concentrations induced significant increments for the total forms under optimal conditions (Figure 3, left panel). All primed plants presented higher total thermospermine (ThSpm) and spermine (Spm) levels than controls under optimal conditions. Total ThSpm was also accumulated in 1 mM DAP and Put-primed seedlings under osmotic stress. They also accumulated higher total Cadaverine (Cad) levels but reduced the free form under optimal conditions (Figure 3, left panel). Finally, the uncommon PA homospermidine (HomoSpd) was more abundant in the Arabidopsis seedlings from 1 mM Orn and Put-primed seeds than in the untreated plants under optimal conditions. However, the 0.1 mM Put-treated plants accumulated norspermidine (NorSpd) under optimal conditions, osmotic stress, and salt stress when plants were primed with 0.1 mM DAP and 1 mM Orn and Put (Figure 3).

It is worth mentioning that the acetylated form AcPut significantly increased in 0.1 mM Orn treated plants under optimal conditions (Figure 3).
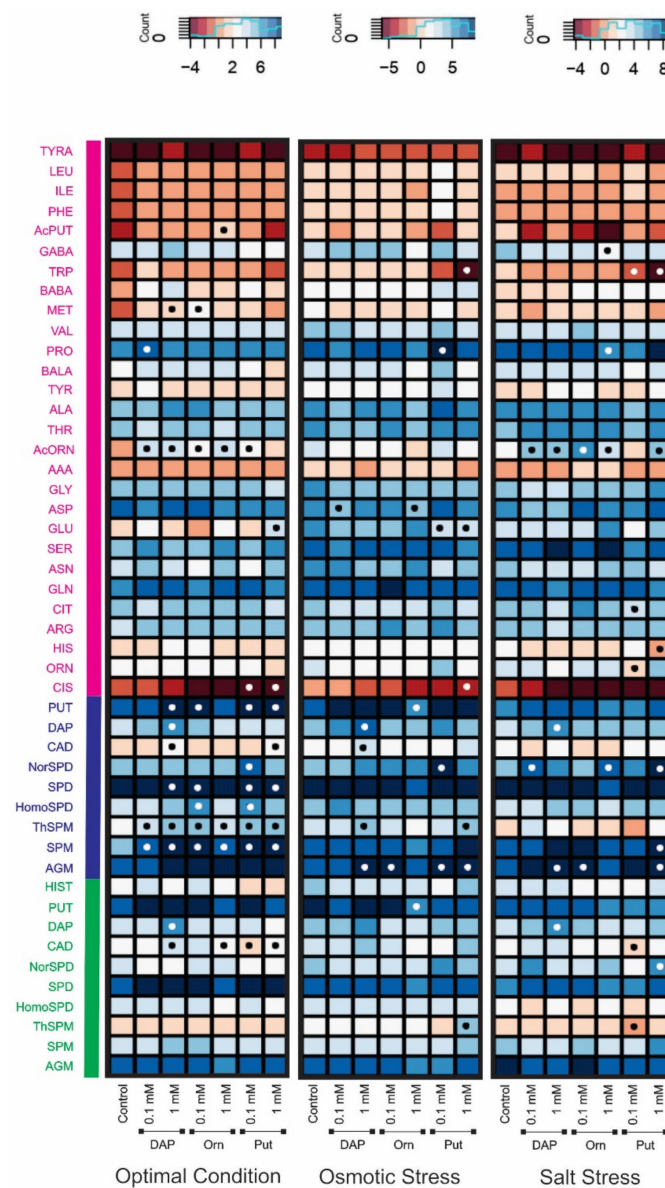

Figure 3. Metabolic profile of free amino acids (pink) [tyramine (TYRA), leucine (LEU), ileucine (ILE), phenylalanine (PHE), N-acetylputrescine (AcPUT), γ-aminobutyric acid (GABA), tryptophan (TRP), β-aminobutyric acid (BABA), methionine (MET), valine (VAL), proline (PRO), β-alanine (BALA), tyrosine (TYR), alanine (ALA), threonine (TRH), N-acetylornithine (AcORN), 2-aminoadipic acid (AAA), glycine (GLY), aspartic acid (ASP), glutamate (GLU), serine (SER), asparagine (ASN), glutamine (GLN), citruline (CIT), arginine (ARG), histidine (HIS), ornithine (ORN), cystine (CIS)], total polyamines (blue) [putrescine (PUT), 1,3-diaminopropane (DAP), cadaverine (CAD), norspermidine (NorSPD), spermidine (SPD), homospermidine (HomoSPD), thermospermine (ThSPM), spermine (SPM), agmatine (AGM)], and free polyamines (green) [histamine (HIST), PUT, DAP, CAD, NorSPD, SPD, HomoSPD, ThSPM, SPM, AGM] in *Arabidopsis thaliana* (Col-0 ecotype) seedlings from primed seeds with 1,3-diaminopropane (DAP), Ornithine (Orn), or Putrescine (Put) at two concentrations (0.1 or 1 mM) grown under optimal conditions, osmotic stress, and salt stress. Data were normalized through a natural logarithm. Each cell represents the values of four biological replicates (*n* = 4). Red and blue colors indicate a decrease and increase, respectively, for the level of each metabolite. Mean values containing black or white points show significant differences among treatments evaluated by Tukey's test (*p* < 0.05).

2.3. Seed Priming Induced Changes in the N-Related Metabolites, and the Changes Were Due to the Interaction between the Priming Agent, Its Concentration, and Growth Conditions

To better visualize and integrate the metabolomic with the morphologic results, we performed three principal component analyses (PCA) and correlation matrices individually for each growth condition (Figures 4–6). The two first PCs explained 60.68% (PC 1 = 37.47%; PC 2 = 23.21%, for optimal conditions), 63.93% (PC 1 = 37.84%; PC 2 = 26.09%, osmotic stress), and 57.07% (PC 1 = 35.79%; PC 2 = 21.28%, salt stress) of the total model variation. Under optimal conditions, the control treatment was related to Cis, NorSpd, and dry biomass, whereas Put-primed plants positively correlated with final growth, Glu, and total PAs, especially NorSpd and Agm (Figure 4A). Control plants were also located opposite the acetylated compounds N-acetylputrescine (AcPut) and AcOrn. Contrarily, DAP-treated plants correlated with almost all free amino acids and PAs, including Pro (Figure 4A). The accumulation of Pro also presented a negative correlation with the dry biomass. However, this morphological parameter and the final growth were positively correlated with the free ThSpm, Spm, and spermidine (Spd). Finally, the accumulation of free amino acids such as Glu, serine (Ser), asparagine (Asn), Arg, and Gln was negatively correlated to the final growth (Figure 4B). Altogether, the priming agents that induced the accumulation of free amino acids, especially Pro, and those related to Glu metabolism reduced free PAs levels like Spd and limited plant growth under optimal conditions.
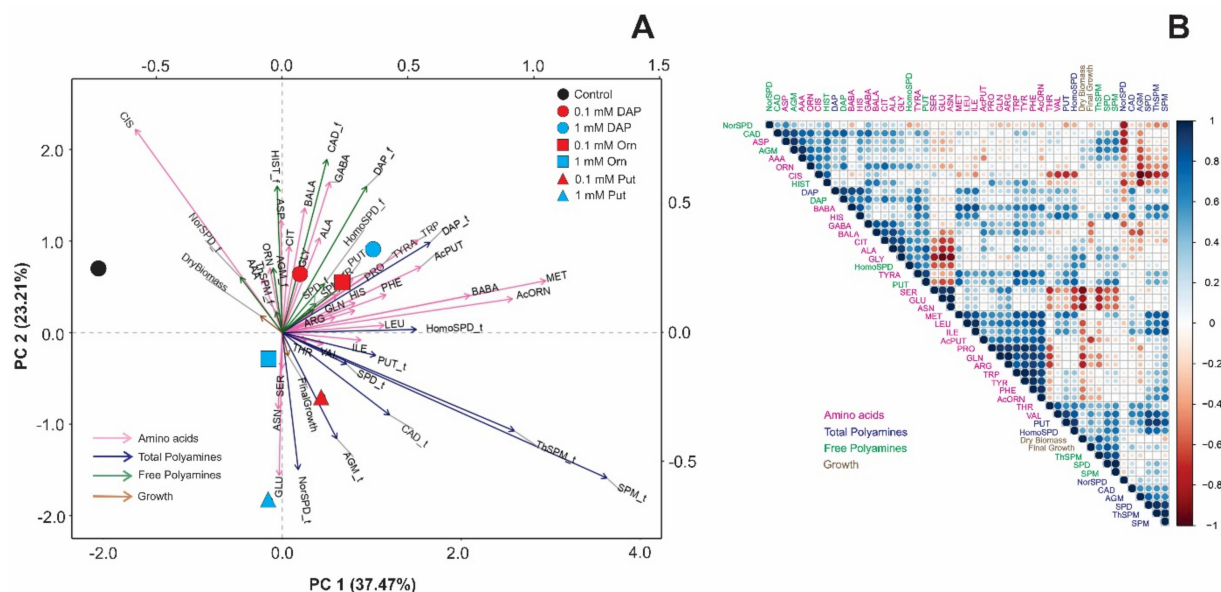

Figure 4. Principal components analysis (PCAs) (**A**) and correlation matrix (**B**) of the N-containing metabolites [free amino acids (pink arrows), total polyamines (blue arrows), free polyamines (green arrows)] and growth (dry biomass and final growth) in *Arabidopsis thaliana* (Col-0 ecotype) seedling from unprimed (control, black circle) or primed seeds with DAP (circles), Orn (square), or Put, (triangle) at two concentrations [0.1 mM (red symbols) or 1 mM (blue symbols)] grown under optimal conditions. Data were normalized using the natural logarithm. Each geometric figure (circle, square, and triangle) represents the mean values of four biological replicates (*n* = 4). Blue and red colors in the correlation matrix indicate positive and negative values, respectively.
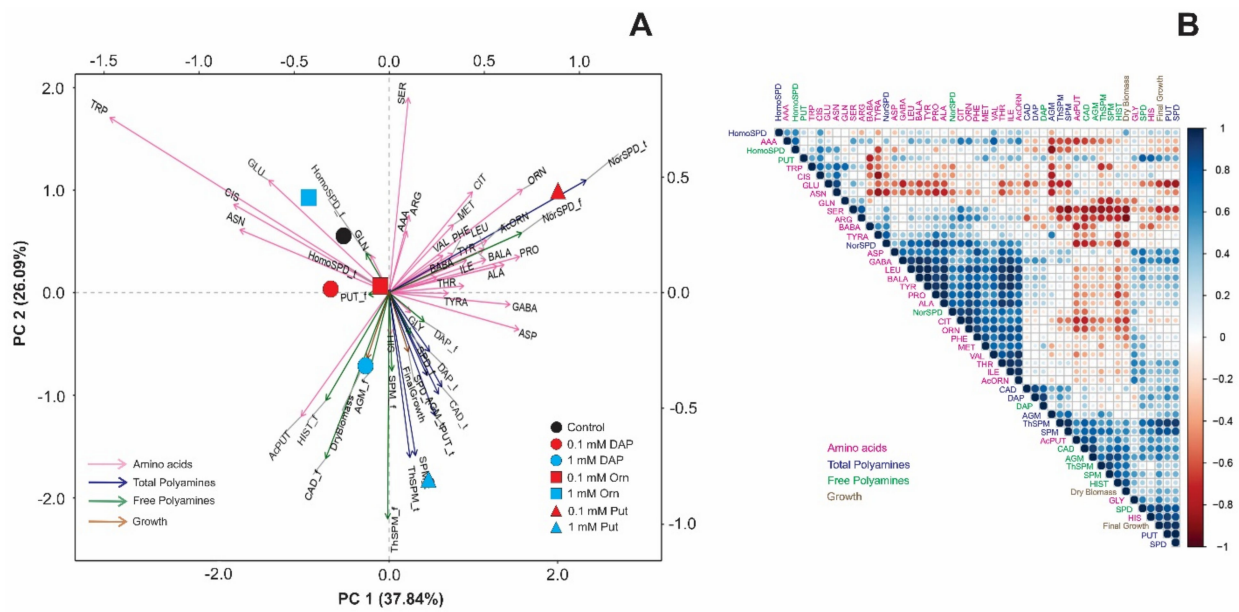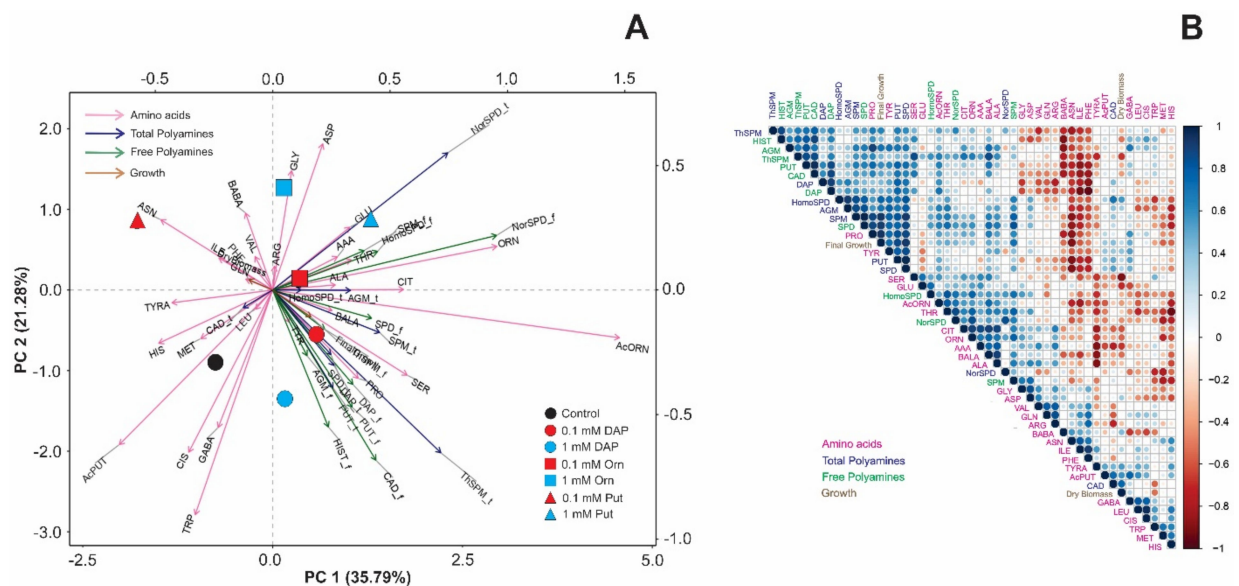
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Figure 6. Principal components analysis (PCAs) (**A**) and correlation matrix (**B**) of the N-containing metabolites [free amino acids (pink arrows), total polyamines (blue arrows), free polyamines (green arrows)] and growth (dry biomass and final growth) in *Arabidopsis thaliana* (Col-0 ecotype) seedling from unprimed (control, black circle) or primed seeds with DAP (circles), Orn (square), or Put, (triangle) at two concentrations [0.1 mM (red symbols) or 1 mM (blue symbols)] grown under salt stress. Data were normalized using the natural logarithm. Each geometric figure (circle, square, and triangle) represents the mean values of four biological replicates (*n* = 4). Blue and red colors in the correlation matrix indicate positive and negative values, respectively.

Under osmotic stress, the metabolic profile in unprimed or primed Arabidopsis seedlings showed substantial alterations compared to optimal conditions (Figure 5). Whereas the seedlings from 0.1 mM Put-primed seeds accumulated the highest number of free amino acids, the free and total NorSpd, 1 mM Put, and DAP-treated plants showed higher total and free PAs and bigger final growth and dry biomass (Figure 5A). The rest of the treatments were located close to the controls, positively correlated to Cis, tryptophan (Trp), Glu, and the total and free HomoSpd. Similar results were obtained in the correlation matrix, whereas a strong correlation was observed between the final growth and the total Put, Spd, ThSpm and Spm, free Put, and the free amino acids GABA and Asp but less Glu and Ser. Dry biomass positively correlated with free PAs but negatively with Cit, Orn, Arg, Ser, and NorSpd (Figure 5B).

Different spacial distribution was observed in the PCA performed for the Arabidopsis seedlings grown under salt stress (Figure 6). For example, the controls correlated with GABA, methionine (Met), Cis, and AcPut. Their response was opposite to 1 mM Put-primed seedlings that accumulated higher levels Glu and Orn, free and total HomoSpd and NorSpm, the free Spm. Meanwhile, the DAP treatment was related to Pro, almost all the PA forms (free and total), including Spd and Cad, and the plant biomass (Figure 6). The correlation between high levels of Pro, Cad, and Spd with the final biomass was then corroborated by the correlation matrix with a highly significant linear correlation (Figure 6B). Contrarily, the accumulation of β-aminobutyric acid (BABA), Asn, isoleucine (Ile), phenylalanine (Phe), Met, and histidine (His) was opposite to the final growth (Figure 6B). Altogether, it is clear that the effectiveness of the seed priming with Orn, Put, and DAP for improving plant growth and stress tolerance depends on the type of growth conditions.

3. Discussion

Seed priming has earned recognition as an innovative and affordable technology to counteract the harmful effects of abiotic stress since it enhances the plant defense responses [30]. The use of natural compounds or biostimulants as priming agents has shown promising results in improving plant performance under suboptimal conditions [29,31]. The use of these substances is more sustainable and environmentally friendly compared with the use of other materials. In addition, some of them regulate plant growth, development, and health by controlling plant–microorganism interaction [18,19]. In this context, studying the mechanism and mode of action of pure substances or complex formulations with biostimulant potential is essential to describe better the plant defense process, standardize formulations, and assist the development of new products by the agrochemical industry [32]. Moreover, priming causes changes in the growth pattern and alterations in the plant metabolism and, hence, in the plant stress tolerance and resistance [33,34]. Plant phenotyping platforms are valuable tools for characterizing plant biostimulants or other priming agents, so they can quickly scan a multitude of variables and/or parameters, allowing a high-throughput pipeline [35,36]. Previous studies performed in the XYZ system (Olophen phenotyping platform) have already been proven as an accurate method to evaluate the efficiency of the seed priming for our in vitro assays based on *A. thaliana* rosette growth [29,31]. Using our previously described approach, we evaluated the use of small molecules-based biostimulants by applying DAP, Orn, or Put as priming agents to improve plant growth and tolerance. As a result, no concrete profile was observed in the primed plants because the response changed due to the interaction effect between the compound, the concentration used, and the growth conditions.

Under optimal conditions, the seed priming did not affect the plants (Figure 3). We speculated that the seed treatment induced a hardening process to prepare the plant for future stress situations or activate a plant eustress response [37] to become a more efficient organism (less energy expended and more growth). To solve these two possible hypotheses, we analyzed the content of free amino acids due to their involvement in plant stress response [38,39]. However, only the AcOrn was significantly increased in all primed plants, except those with 1 mM Put, compared to the controls. There is not much information about

the biological relevance of this non-protein amino acid. Only a few studies have linked this metabolite as nitrogen storage or a precursor of the Orn biosynthesis [40,41]. Recently, it has also been related to the plant stress response. For example, [42] observed the AcOrn accumulation in a heat stress-tolerant and sensitive wheat lines under heat stress alone or combined with drought. It could be that plants accumulate AcOrn under stress as part of their plant defense, so it has been reported to promote the synthesis of methyl jasmonate in Arabidopsis [40]. Similar results were observed in our work because the unprimed Arabidopsis seedlings showed higher levels of this metabolite when grown under stress conditions (Figure 3, Supplementary Table S2). In addition, the seed priming enhanced this accumulation in all treated plants under salt stress, except for those with 0.1 mM Put, but not under osmotic stress. One possible explanation is that under salt stress, the plants suffer double stress induced by the osmotic pressure of the solute concentration and the toxic effect of the ion accumulation [31]. This was mainly visible in the plant growth, with the most miniature plants obtained under salt stress conditions. It is worth mentioning that the 1 mM Put-primed plants did not accumulate significant levels of AcOrn under optimal conditions but increased the Glu content (Figure 3 and Supplementary Table S2). Moreover, the Arabidopsis seedlings primed with 0.1 mM Put did not show significant changes of AcOrn compared to the unprimed ones under salt stress and reduced their levels of Orn and Cit. Considering that AcOrn is part of the Glu metabolism to produce Orn [43] and that both Cit and Orn are precursors of the PA biosynthesis, we could conclude that their changes are relevant players regulating plant growth and stress response.

Regarding Pro, it is the most stress-related amino acid in plants and is highly accumulated under stress conditions [24]. This metabolite is considered an osmoprotectant and regulator of the plants' cellular homeostasis, redox balance, and energy status. In our study, the most significant accumulation of Pro occurred mainly in the best performing variants; the plants primed with 0.1 mM DAP under optimal conditions and 0.1 mM Put under osmotic stress. Contrarily, 1 mM Orn reduced it and GABA content. As [24] described, the metabolism of GABA, Pro, and PAs is closely connected to regulating plant growth and development. Their interconversion activates many biological processes in the plants, with the antioxidative response as the main one under stress conditions. Altogether, we could conclude that the seed priming with the polyamines Put and DAP activate the accumulation of stress-related amino acids under optimal conditions, most likely to activate the antioxidative response of the plant to deal with the following stressful situation. In addition, this accumulation permits the plant to perform better under abiotic stress, ending with better plant growth. However, the most exciting phenotype was observed in 1mM Orn-treated plants that showed a negative PBCI due to the inhibition of growth under salt stress. However, they improved the greenness GLI, the levels of AcOrn, and total NorSpd but reduced GABA and Pro. Further studies will be needed to explain the contrasting response of these plants.

The seed priming also affected the PA content of PAs (Figure 3). The most significant changes were observed for the total content of ThSpm and Spm, followed by the total Put content under optimal conditions. However, the primed plants grown under stress conditions mainly accumulated total Agm. An increase in the total content of specific PAs but not in their free forms pointed to a relevant increase in conjugation as a primary consequence of the seed priming. Although very little is known about the biological relevance of the conjugation PAs, [44] recently reviewed the current state of the art. They described that PAs could be conjugated with phenolic compounds forming a class of secondary metabolites called phenolamides or hydroxycinnamic acid amides (HCAAs). These metabolites play a critical role in plant growth and developmental processes, including cell division, cytomorphogenesis, flowering, cell wall cross-linking, tuberization, and stress responses [44–46]. As an example of their implication in the plant stress response, it has been shown that p-coumaroylagmatine, the major HCAA accumulated in Arabidopsis, inhibits *Phytopthora infestans* spore germination in vitro [47]. However, the increase of p-coumaroylagmatine and feruloylagmatine have also been identified in Arabidopsis leaves

after infection with the pathogen *Alternaria brassicicola* [48]. One possible explanation of these controverted results is that the plant could perceive the presence of the pathogen and activate the jasmonic acid (JA)/ethylene (ET) signaling pathways that, at the same time, activate the transcription factor ORA59, which can bind the promoter of an Arabidopsis agmatine coumaroyl transferase (AtACT) and enabled its expression and HCAAs biosynthesis [49]. Thus, enhancing the pool of conjugated PAs in Arabidopsis seedlings from primed seeds could be clear evidence of stress response and, hence, plant hardening for better performance under future stress conditions.

To further this idea, we investigated the possible role of the conjugated forms with Spm or ThSpm. However, no HCAAs connected to Spm or ThSpm have been detected in Arabidopsis. This means that the high increase of their conjugated forms could have a different role. It has been reported that PAs can also bind particular proteins as post-translational regulators, and the induced modifications may have a specific functional role under concrete growth conditions [50]. Recently, [51] reviewed the interaction of the main PAs, Spm, Spm, and ThSpm, with the translation machinery of the plants.

4. Conclusions

In summary, this study corroborates that seed priming with PAs, not only the usual ones (Put) but also the uncommon such as DAP, can be an efficient technological approach for plant hardening and improving plant stress tolerance. The efficiency of the priming depends on the changes that the priming agent induces in the part of the Glu metabolism related to the PA synthesis, with AcOrn, Orn, and Cit as crucial players regulating plant performance under optimal and stress conditions (Figure 7). However, the seed priming with Orn induced contrasting responses, mainly 1 mM Orn inhibited plant growth. Two possible scenarios can explain this plant reduction: the activation of a conservative strategy to deal better with the stress or the induction of negative stress (distress) in the plant. In this regard, foliar application with Orn to sugar beet (*Beta vulgaris* var. *saccharifera* L.) alleviated the adverse effect of the water deficit by increasing the biomass production and accumulation of photosynthetic pigments, proteins, and total soluble sugars, as well as the antioxidant defense [22]. Further studies will be needed to understand better the effect of the exogenous Orn application. Finally, our results also pointed to the conjugation of PAs as the most relevant change induced by the seed priming. The conjugation could be by binding phenolic compounds or other macromolecules RNA or proteins as post-translation regulation, most likely to determine the resilience of the plants. However, these responses are defined by the interaction between the priming agent concentration and the plant growth conditions.
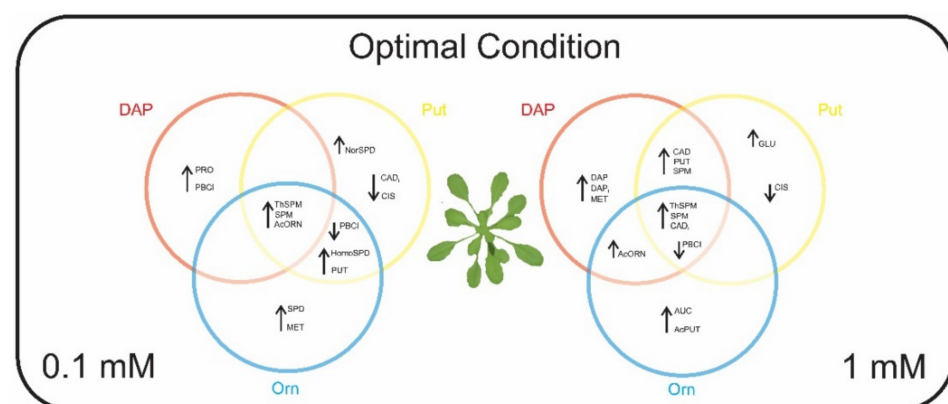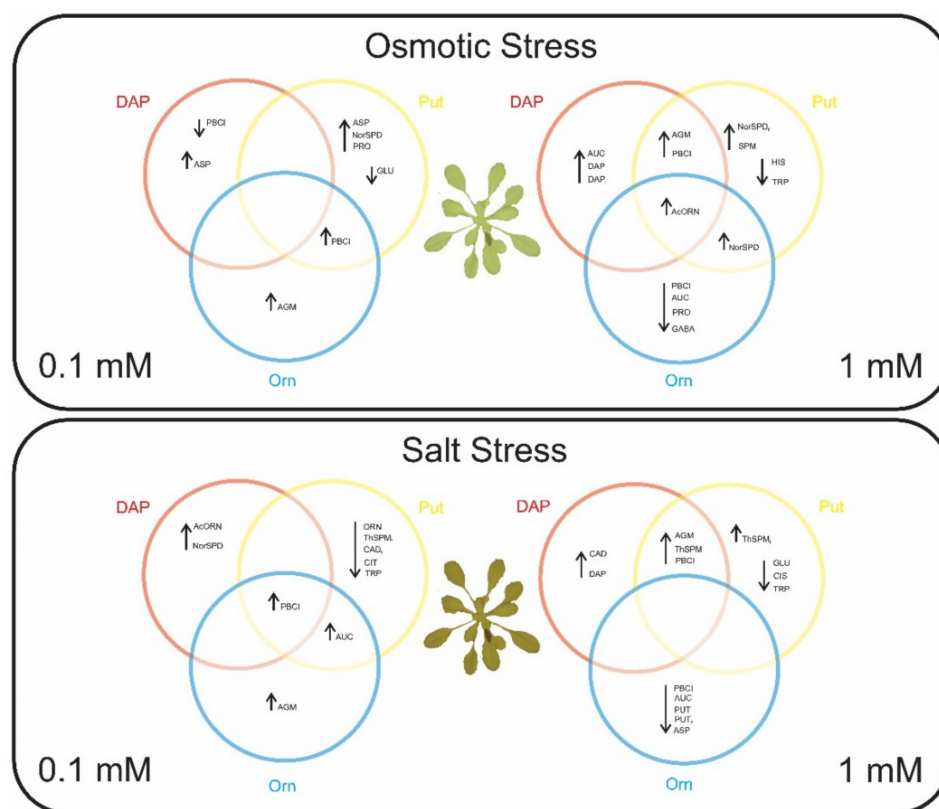

Figure 7. *Cont.*

Figure 7. Summary of the reported effects of the seed primings with DAP, Orn, and Put at 0.1 and 1 mM on the *Arabidopsis thaliana* seedlings exposed to optimal growing conditions, osmotic, and salt stress. Red circles are for DAP, blue circles are for Orn, and yellow circles are for Put; on the left side of the plant is represented the lower concentration priming, while on the right is represented the higher priming concentration. Inside the circles, significant changes regarding growth (AUC), the potential biostimulant effect (PBCI), free and total polyamines, and free amino acids are represented. An upside arrow indicates significant increment, while a downside arrow means significant reduction compared to the unprimed treatment within the same growing condition. Significant factors situated in the intersection of two or all primings mean that that parameter's change was common for the involved primings.

5. Materials and Methods

5.1. Plant Material, Priming, and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. (Col-0 ecotype) were surface sterilized with 70% Ethanol plus 0.01% Triton X-100 following the protocol described in [31]. The sterilized seeds were distributed homogeneously with the help of toothpicks autoclaved on a sheet of autoclaved filter paper moistened with sterile water under laminar flux chamber conditions. After 3–4 min, the filter paper with the seeds was transferred to a square plate (120 × 120 mm, P-Lab, Ref. 212358.2) containing sucrose-free half-strength solid Murashige and Skoog (Phytotechlab M519) medium. For the priming treatments, 1,3-diaminopropane (DAP), Ornithine (Orn), and Putrescine (Put) at two final concentrations (0.1 and 1 mM) were added to the medium. Control treatment consisted of the seeds sown in the square plates with the plain medium. After the sowing, the square plates were led with micro-pore tape and kept in the dark at 4 °C for 4 days. The plates were then positioned vertically in the growth chamber under controlled conditions: temperature of 22 °C, 60% relative humidity, 16/8 h (light/dark), and 120 µmol photons s^{-1} m^{-2} [28] for five days.

5.2. Plant Growth under Optimal Conditions and Osmotic and Salt Stresses

Three days after germination, seedlings of similar size were manually transferred, under laminar flux chamber conditions, into 48 and 24-well plates (Jetbiofil, Guangzhou,

China), 1 plant per well. Each of the 48 and 24-well plates was previously filled with $1\times$ MS growth medium (pH 5.7; supplemented with 0.6% Phytagel) for the optimal growing condition. The growth medium was supplemented with Mannitol (osmotic stress) and NaCl (salt stress), both at 100 mM for the stress conditions. After the transfer, the well plates were sealed with transparent film, manually perforated to allow the water and gas exchange and avoid water condensation that might have made the image analysis difficult. Finally, the sealed well plates with plants were transferred to the OloPhen platform "Olophen. Available online: http://www.plant-phenotyping.org/db_infrastructure#/tool/57 (Accessed on 10 March 2021)", consisting of the PlantScreenTM XYZ system where the growth conditions were set to simulate a long day (16/8 h light/dark cycle) with a temperature regime of 20/22 °C, an irradiance of 120 µmol photons s^{-1} m^{-2}, and relative humidity of 60%.

5.3. Plant Phenotyping Determinations

The phenotyping platform is placed within a controlled conditions growth chamber (cold-white and far-red LED lighting system, temperature, and humidity) (Photon Systems Instruments, Brno, Czech Republic). The system consists of a robotically driven arm lodging an RGB camera with a customized lighting panel and a table where the plates are located. The robotic arm moves above the plates, taking top view pictures [28]. The robotic arm was programmed to acquire RGB images twice per day (10:00 and 16:00) for seven consecutive days, as described in [31]. The RGB camera (top-view; resolution 2560 × 1920) was located 20 cm high on the well-plate. The outcome is the individual picture of the 48 Arabidopsis seedlings per treatment (genotype vs. priming vs. growth condition) as biological replicates for the analyzed phenotypical traits.

5.3.1. Rosette Growth Analysis

The images were stored and analyzed to extract the underlying information using the software described in a previous report [28] "GITHUB, Available online, https://github.com/UPOL-Plant-phenotyping-research-group/In-vitro-plant-growth-analyzer (accessed on 15 March 2021). After the image processing, the leaf rosettes of the seedlings were segmented, and the number of pixels corresponding to each plant was taken as the indicator of the plant area. From this data, different traits were calculated: the slope of *A. thaliana* rosette growth curves (GC-slope) and the area under the curve (AUC), the relative growth ratio (RGR), and the final size of the rosettes at the end of the experiment (FG):

$$AUC = \sum((s_i - s_{(i-1)}))/2$$

where s_i the rosette size at the moment *i*, and $s_{(i-1)}$ the rosette size in the immediate previous measurement:

$$RGR = [(\ln(s_2) - \ln(s_1))/(t_2 - t_1)]$$

where s_1 and s_2 are the initial and final size, respectively; and t_1 and t_2 are the initial and final time, respectively.

All those traits were used to characterize the behavior of the different genotypes under stress and the influence of the priming, and its combination allowed the calculation of the Plant Biostimulant Characterization Index (PBCI) [31]. The PBCI is the sum of the number obtained by the $\log_2$ of the ratio between the treatment (compound and concentration) and the controls (umprimed seeds) at each growth condition for each phenotyping trait.

5.3.2. Rosette Color Indices

The software used for the segmentation also provides valuable information about the particular color channels red (R), green (G), and blue (B) extracted from each pixel within the plant mask. the greenness of the *A. thaliana* seedlings and possible change in leaf

color index (GLI) due to stressors and/or treatments, which has been correlated with plant biomass, nutrient status, and abiotic stress [52–54], was calculated using the equation:

$$GLI = [(2G - R - B)/(2G + R + B)]$$

5.4. Targeted Metabolomic Analysis

At day 7 of growth, the Arabidopsis rosettes of all variants were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C. The samples were lyophilized, and the obtained DW was used for the targeted metabolomic analysis. The analysis of the free amino acids and total and free polyamines was performed as described by [42]. Briefly, for the analysis of free amino acids, pulverized plant material (3–5 mg) was mixed with 1 mL of 50% EtOH and sonicated for 10 min (Bandelin, Germany). After centrifugation (Prism, Labnet, St. Louis, MO, USA) at $14,500 \times g$, the supernatant was transferred into the new vial and kept at 20 °C until analysis. A 200 μL of supernatant was evaporated to dryness and redissolved in the mobile phase. For the quantification, UHPLC-MS/MS analysis was performed on Nexera X2 UHPLC (Shimadzu Handels GmbH, Kyoto, Japan), coupled with MS-8050 (ShimadzuHandels GmbH, Kyoto, Japan). Chromatographic separation was performed on an Acquity UPLC BEH AMIDE (50 × 2.1 mm; 1.7-μm particle size) with an appropriate pre-column. All target amino acids were separated using a binary gradient consisting of 15 mM formic acid, pH 3 (component A), and 0.1% formic acid in ACN (component B).

For the polyamines, 200 μL of 2 M NaOH was added to 200 μL of supernatant from the amino acid extraction, followed by 2.5 μL of benzoyl chloride (in MeOH, 50:50, *v:v*), and after vortexing for 5 s, the reaction mixture was stirred for 40 min at 25 °C. About 500 μL of saturated NaCl was added, and benzoylated polyamines were extracted with 2 μL x 500 μL of diethyl ether. The solvent was evaporated under the vacuum at 40 °C, and dry samples were dissolved in 200 μL of the mobile phase and analyzed according to the method described before by [39]. To quantify total polyamines, 200 μL of supernatant was acidified with 50 μL of conc. HCl and shaken for 16 h at room temperature. The 200 μL of 6 M NaOH was added, and derivatization was performed as described above.

5.5. Data Analysis

Kruskal–Wallis was used to determine the differences in the AUC of the plants' growth, with the Infostat software. Multi-ANOVA was performed to determine the differences in the plant metabolites. Data were log-transformed to normalize them before analysis. Duncan's test was used as a post hoc test for the multiple comparisons between the variants. Multivariate statistical analysis was also carried out. Principal component analysis (PCA) was conducted using singular value decomposition, and PCA biplots were constructed. Heatmaps with dendrograms were produced. Pearson correlations were computed and displayed. All analyses were performed in RStudio (R Software version 4.1.0).

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Characterizing the mechanism of action of small molecule-based biostimulants.

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CONTENTS

AIMS OF THE PH.D.THESIS

This thesis aims to use phenotyping approaches to determine, characterize, and describe the tolerance mechanisms of different plant species grown under abiotic stress to identify the main metabolite pathways and, consequently, specific molecules, such as small molecule-based biostimulants, that enhance their performance under adverse growth conditions. To achieve this goal, we proposed the following partial objectives:

Objective n°1: To evaluate the potential of the Olophen platform to be used for studying different plant species grown under different abiotic stresses.

Objective n°2: To evaluate the effect of two polyamines (PAs), putrescine (Put) and spermidine on the production, yield, and quality yield parameters of maize subjected to water deficit. This work was performed as the following study of the positive results obtained by Ugena, (2019), in which the seed priming with small molecule-based biostimulants improved maize emergence under optimal and stress conditions.

Objective n°3: To characterize the mode of action of the selected metabolites, the non-proteinogenic amino acid ornithine (Orn) as a precursor of the PA biosynthesis, Put as the most effective PA improving plant growth and stress tolerance, and 1,3- diaminopropane (DAP) as a product of the final PA catabolism of the polyamines, using Arabidopsis (*Arabidopsis thaliana* L.) as a model plant.

Objective n°4: To evaluate the use of Ornithine as a possible small molecule-based biostimulant in crops of agricultural interest, such as barley (*Hordeum vulgare* L.).

INTRODUCTION

Agriculture is a key activity to accomplish the Sustainable Development Goals (SDGs) set by the United Nations (UN), specifically the SDG-2: Zero Hunger (Viana et al., 2022). This activity produces the majority of the calories, proteins, and fats consumed by the world's population (Steinfeld et al., 2006; Cassidy et al., 2013). The food demand will rise by 50% by 2050 due to the increment in the world's population (FAO, 2017). To meet this demand, it is necessary to increase agricultural efficiency. However, the pressure to transition to greener and environmentally friendly practices and climate change are two extra-added challenging conditions. Arable land and groundwater resources are "impoverished" (Fróna et al., 2019), and the use of agrochemicals entails the threat of soil and groundwater contamination (Srivastav, 2020). Contrarily, climate change-induced changes in the rainfall pattern cause extreme meteorological phenomena such as the amplification of drought events and their frequency, duration, and intensity (Bouras et al., 2019; Tramblay et al., 2020). Combined with high temperatures, all these can cause the agricultural drought phenomena (Maracchi, 2000), which negatively affect plants' development and performance. Moreover, the drought might increase the salinity in the root zone (Corwin, 2021), causing salinity stress, which is considered a major environmental constraint to crop productivity throughout the arid and semi-arid regions (Carpici et al., 2009).

Plant response to stress is a highly dynamic and complex process dependent on the severity and duration of the stress and the fitness and preparedness of the plant itself and its developmental stage (Claeys and Inzé, 2013). The unfavorable environmental factors such as drought reduce plant growth and yield, but when it is prolonged or very intensive can cause damage to the plant structures and even death (Sofia et al., 2013; Mosa et al., 2016). Due to the sessile nature of the plants, they developed various mechanisms to cope with the stress, made possible by physiological, morphological, phenological, biochemical, and molecular responses.

Water is the major component of the plant's fresh biomass and plays a vital role in many physiological processes related to plant growth, development, and metabolism (Brodersen et al., 2019). One of the earliest effects of the drought on plants is the reduction of the relative water content (RWC) (Farooq et al., 2009); the reduction of the turgor affects the cell expansion and division processes (Shamsi, 2010), and therefore the plant growth and establishment (Bhargava and Sawant, 2013). Water deficit also affects the number of leaves and their anatomy, which negatively impacts the photosynthesis rate (Ding et al., 2013). The reduced stomata number and its closure might lead to an imbalance between the light and dark phases of the photosynthesis and increase the excessive accumulation of reactive oxygen species (ROS) in the chloroplasts (Bhargava and Sawant, 2013; Nezhadahmadi et al., 2013). The root system is also affected by the water limitations, being more developed in some species to increase the capacity for water uptake (Franco, 2011; Bhargava and Sawant, 2013). In general, water deficit reduces the ion uptake, and therefore, it alters the mineral nutrition (Rana et al., 2013; Sapeta et al., 2013).

Salinity stress happens when the salt content in the root zone is high enough to disturb plant growth and development, although the damage depends on the plant species, variety, growth stage, environmental factors, and nature of the salts (Yadav et al., 2011). The response of the plants to salinity is generally explained in two phases. First, the ion-independent shoot response is related to the Na+ sensing and signaling (Gilroy et al., 2014; Roy et al., 2014), which induces stomatal closure and leaf rolling (Munns and Termaat, 1986). And then, the ion-dependent response accumulates ions in toxic concentrations, especially in old leaves, causing premature senescence (Munns and Tester, 2008). Salinity induces water stress, nutritional imbalance, specific ion toxicity, and/or the combination of these three factors (Ashraf, 1994) and the accumulation of excessive ROS (Ahmad et al., 2019). The root system is negatively affected by salinity by decreasing its length and surface, the number of lateral roots, root hairs, and dry matter content, and disrupting the cell membrane ion homeostasis (Shannon and Grieve, 1998; Otsuka et al., 2021). It also affects the absorption of essential nutrients (e.i. K^+, Ca^{2+}, and NO_3^-) negatively (Shaterian et al., 2005) and enhances the accumulation of Na^+ and Cl^- ions happens, which are toxic for plants and disturb physiological and biochemical processes such as photosynthesis, protein synthesis and damage cell organelles (Zörb et al., 2019). The salt concentration also affects shoot growth by reducing cell expansion and lateral bud development (Munns and Tester, 2008). Moreover, the shoot length, total leaf area, leaf area index, and the number of branches are negatively affected by salinity (Läuchli and Grattan, 2007). The photosynthesis can be reduced (Kitayama et al., 2020), as well as the mineral uptake (Kaya et al., 2009), from which limitations in N and Mg affect the biosynthesis of the chlorophylls, thus leading to a chlorophyll reduction (Zhu et al., 2020).

One of the most promising strategies to deal with plant stress is the application of biostimulants (Bulgari et al., 2019), as they can enhance the growth and productivity of the plants while causing no harm to the environment. These substances are classified according to the source of the compound, commonly accepted in seven categories: humic and fulvic acids, seaweed and botanical extracts, protein hydrolysates and N-containing compounds, chitosan, and other biopolymers, inorganic compounds, and beneficial fungi and bacteria (du Jardin, 2015). While it is widely extended to work with a mixture of these substances, the research focused on a pure product, or small molecule-based biostimulants (smbBS), presents the advantage of the identification and description of its mode of action, thus facilitating the certification and registration processes (Yakhin et al., 2017). Some of the most well-known smbBS compounds that enhance plant growth under stress are menadione sodium bisulfite (MSB) (Borges et al., 2014) and γ-aminobutyric acid GABA (Li et al., 2017), and melatonin (Arnao and Hernández-Ruiz, 2019). Our research team has also proved that polyamines are interesting compounds to be considered smbBS. Concretely, priming of Arabidopsis seeds with putrescine (Put) and spermidine (Spd) improved plant growth under salt stress conditions (Ugena et al., 2018); since they are related directly to the antioxidative response of the plant (Hussain et al., 2019; Taie et al., 2019).

The technological advances made possible the development of high throughput phenotyping platforms (HTTP), which had successfully been integrated for breeding programs, alleviating traditional phenotyping practices' work and time cost (Araus and Cairns, 2014; Tardieu et al., 2017). Nevertheless, these platforms have more potential to assist other activities besides breeding programs since they can increment the accuracy and precision of the phenotype assessment at the whole level of the plant and enhance the data integration and experimental design (Omari et al., 2020). Combining the multi-source data can better explain biological phenomena such as biotic and abiotic stress resistance (Song et al., 2021). It is possible to screen model plants as barley to provide key drought stress traits, as well as to identify sensitive mutants (Marchetti et al., 2019); but also to analyze *in vitro* massive experiments, as was done by De Diego et al., 2017, with *Arabidopsis thaliana* grown under stress conditions to collect growth information and colorimetric data. Considering the proven sensitivity and suitability of the HTP platforms to monitor large amounts of plants and provide many physiological traits, these devices are very convenient for biostimulant screenings. In this regard, (2018) reported the utility of the XYZ PlantScreen robot to categorize diverse substances into plant growth promotors/inhibitors or stress alleviators through an in vitro protocol and describe their mechanism of action. The possibility of evaluating the biostimulants through *in vivo* experiments is also possible, Ugena, 2019, in her Ph.D. thesis revealed that the polyamine spermidine (Spd) used as seed priming enhanced the synchronicity and other emergence-related parameters for maize germinated under salt stress in an emergence assay under controlled conditions. In summary, the HTP technologies are key factors in developing faster and more efficient methods to characterize the biostimulants.

MATERIAL AND METHODS

1. Biological materials and growing protocol

Seeds of Arabidopsis thaliana (L.) Heynh. (Col-0 ecotype) were used for Supplement I and V. The germination protocol was based on (Ugena et al., 2018); seeds were surface sterilized and germinated in square plates containing sucrose-free half-strength MS with the chemical agent as priming. The seedlings were transferred to 48 and 24 well plates (one seedling per well) three days after the germination. Then the plates were brought to the PlantScreen^{TM} XYZ system under controlled conditions (16/ 8 h of light/dark; 22°C/20°C night/day, a light intensity of 120 µmol photons s^{-1} m^{-2}, and 60% RH). The plants were monitored twice per day for 7 consecutive days.

Green pea (*Pisum sativum* L.) cv. "Walor" (PlantiCo Zielonki Sp. zoo., https://plantico.pl), was used for the physiological characterization of the plants under drought stress included in Supplement II. Seeds were germinated in humid vermiculite and transferred to pots once germinated. Plants were grown in controlled conditions (16/8 h of light/dark at 25°C/ 23°C day/night, a light intensity of 300 µmol m^{-2} s^{-1}, and 40% RH), and were kept fully irrigated for five days. Then they were let to dry until reach pf 4.2 which was the stress threshold. This level was maintained for 7 days and after re-watered for 3 days. For the physiological

characterization of pea responses under drought stress, the plants were monitored with the PlantScreen™ Compact system of the OloPhen platform for 10 days.

A local maize variety from Gran Canaria Island (*Zea mays* L. c.v. Lechucilla) provided by a local nursery was the material employed for Supplement III and IV. Seedlings from a nursery were kept in controlled conditions [22°C, 16 h light /8 h dark, 300–400 µmol m^{-2} s^{-1}), 60–70% RH]. According to the protocol described by Jiménez-Arias et al., (2022), an initial dose optimization was performed in the nursery trays. Additionally, for Supplement III another dose optimization in hydroponic conditions was carried out, slightly modified from the Jiménez-Arias et al., (2019b) protocol. The plants were transplanted to a greenhouse fifteen days after the sowing. Two root treatments were performed fifteen and thirty days after the transplantation to the soil. The last application was the onset of the water restriction.

Seeds of barley (*Hordeum vulgare* L.) cultivar Steptoe and an induced mutant (AZ34) partially deficient in the abscisic acid (ABA) synthesis were used as plant material for the Preliminary Results. Two assays were performed; on the first, transpiration curves were recorded for excised leaves in optimal conditions (water) or simulated osmotic stress (PEG) with or without 1 mM Orn, slightly modified from (Ceciliato et al., 2019). According to (Marchetti et al., 2019), the second assay was performed with 1 mM Orn as a foliar treatment.

2. Chemicals

The four compounds (N^9-Substituted CK derivatives with a fluorinated carbohydrate moiety) tested in Supplement I, were synthesized by a slightly modified one-step synthesis (Wan et al., 2005) of 9-(2´-deoxy-2´- fluoro-β-D-arabinofuranosyl) hypoxanthine with benzylamine or isopentenylamine hydrochloride as appropriate in the presence of BOP and DIPEA in DMF.

For Supplement III, L-pyroglutamic acid (PG) (CAs number: 98-79-3) and glycine betaine (GB) (CAs number: 590-46-5) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA).

The polyamines (PAs) putrescine (Put) (CAS number 333-93-7) and spermidine (Spd) (CAS number 124-20-9) used for Supplement IV were purchased from Aldrich Chemical Co. (St. Louis, MO, USA).

For Supplement V, the PAs Put and 1,3-diaminopropane (DAP) (CAS number 333-93-7, and 109-76-02, respectively) were both purchased from Aldrich Chemical Co. (St. Louis, MO, USA). The amino acid (AA) ornithine (Orn) was purchased from Acros Organics (CAS number 3184-13-12).

3. Instrumentation

For Supplement II, the measurement of soil pF was performed with a ProCheck dielectric water potential sensor (MPS-6; Decagon Devices, Pullman, USA).

For Supplements III and IV, the absorbance was measured at 490 nm in the multi-detection plate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) for the carbohydrates determination. The mineral content of the samples was determined with the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Avio® 500 (Perkin Elmer, Waltham, USA).

For Supplements I and V, the OloPhen platform used was the PlantScreenTM XYZ system (Photon Systems Instruments, Brno, Czech Republic).

For Supplement II and Preliminary Results, the OloPhen platform used was the PlantScreen™ Compact System (Photon Systems Instruments, Brno, Czech Republic).

For Supplement V, the sample preparation prior to free AAs and PA analysis was carried out in a sonic bath (Bandelin, Germany), centrifuge (Prism, Labnet, USA), and vacuum (Centrivapm Labconco, USA) were used. Then, UHPLC-MS/MS analysis was performed on Nexera X2 UHPLC (Shimadzu Handels GmbH, Kyoto, Japan) coupled with an MS-8050 (Shimadzu Handels GmbH, Kyoto, Japan). Chromatographic separation was performed on an Acquity UPLC BEH AMIDE (Waters, Milford, USA) (50 × 2.1 mm; 1.7 μm particle size) with the appropriate pre-column.

Gas exchange analysis performed for the Preliminary Results was obtained using the Ciras3 (PPSystems, Amesbury, USA). For the chlorophyll quantification, an incubator ThermoMixer C (Eppendorf, Hamburg, Germany), a Centrifuge 5415R (Eppendorf, Hamburg, Germany), and a spectrophotometer Synergy H4 Hybrid Reader (Biotek, Winooski, USA) were used.

4. Biometric determinations

4.1 Growth related parameters
From the images obtained by the phenotyping platforms, the following parameters were obtained: area under the growth curve (AUC), relative growth ratio (RGR), and average growth ratio (AGR).

The biomass production was evaluated as fresh and dry weight (FW and DW, respectively). Also, manual measurements were performed to determine the plant length (from insertion to the soil until the base of the flower) and width (at the middle of the stem length), and the length and width of the last fully developed leaf.

The water use efficiency (WUE) was determined for seedlings' biomass.

The relative water content (RWC) was estimated to assess the hydric status of the plants.

4.2 Colorimetric index
The colorimetric parameters green leave index (GLI), normalized green red difference index (NGRDI), and visible atmospherically resistant index (VARI) were computed from the information obtained from the RGB images.

4.3 Production parameters

The WUE for the production was evaluated using the reference evapotranspiration (ETo) and the crop coefficient (Kc) to obtain the crop evapotranspiration (ETc). The yield per hectare and the harvest index (HI) were also calculated.

In addition, the number of adequately developed maize cobs per plant and variant was counted, and from those, a representative sample was taken to record the fresh and dry weight (FW, DW), the number and total weight of all kernels per cob, the weight of 100 kernels (in triplicate from each cob), the length and circumference of the cob (number of kernels) and the cob diameter (cm).

5. HPLC

The targeted metabolic analysis for the free amino acids, free and total polyamines was performed as described by (Abdelhakim et al., 2022), using 3-5 mg of homogenized lyophilized plant material.

6. Nutritional composition of the flour

The total protein content was estimated through the Kjeldahl method (Kjeldahl, 1883; Kirk, 1950), using 50 mg of each flour sample.

For the total carbohydrates (CH) determination, 100 mg of each sample flour was used for the slightly modified Phenol Sulphuric Acid method in multi-well plates (Jiménez-Arias et al., 2019a).

One gram of flour powder per sample was used to analyze the mineral content. Each sample was converted to ash in a muffle stove at 480°C and mineralized by the dry method with 6 N HCl. The mineral levels were determined by ICP OES Avio 500 (Perkin Elmer, Waltham, USA).

7. Chlorophyll quantification

The chlorophylls were extracted following the Cross et al., (2006) protocol, through two consecutive extractions with 80% ethanol, followed by the last extraction with 50% ethanol. The extract was then placed in the spectrophotometer and read at 645 nm and 665 nm. The chlorophyll content was then calculated with the formulas described in Porra et al., (1989).

8. Data Analysis

To assess differences between the parameters related to growth, production and physiological parameters, different approaches were used: ANOVA with post-hoc Fisher, Tukey's HSD, and LSD HSD ($p<0.05$) for parametric data; and Kruskal Wallis ($\alpha= 0.05$) for non-parametric data. These analyses were performed with the software Infostat and STATISTICA.

The data were log-transformed to assess the differences between the metabolite content and analyzed with Multi-ANOVA.

The principal component analysis (PCA) and correlation matrices were performed in RStudio (V. 1.1.463 – 2009-2018; V. 2021.09.1+; V. 4.1.0)

RESULTS

1. Plant Phenotyping Systems to study abiotic stress tolerance in plants (Supplement I and II).

The PlantScreen™ XYZ System was suitable for testing the efficiency of four new N^9-Substituted cytokinin derivatives with a fluorinated carbohydrate moiety (Supplement I). Three out of four CK derivatives were efficient as seed priming; moreover, differences in their effect due to different concentrations were also detected. The other calculated parameters (RGR, AGR, slope, and final rosette area) were improved by the hormopriming, more notably for the osmotic and salt stress conditions. The combination of these parameters allowed the calculation of the PBCI, which revealed that all the compounds worked as plant growth regulators and stress alleviators at some concentrations. The greenness of the seedlings represented by the colorimetric indices GLI, NGRDI, and VARI showed that the priming contributed to enhancing the greenness index and, therefore, has antisenescent properties. (Bryksová et al., 2020)

The second platform PlantScreen™ Compact System was used to perform an integrative phenotyping study of pea (*Pisum sativum* L.) plants grown under water-limited conditions (Supplement II). This device provided the data to corroborate that the drought-induced biomass reduction was linked to changes in chlorophyll fluorescence parameters. This was obvious due to the drought-induced reduction of the ΦPSII and ΦP, whereas ΦNPQ increased. In addition, the foliar temperature reported significant differences since the beginning of the water restriction, showing itself as a potent stress biomarker. (Blicharz et al., 2021)

2. Characterization of small molecule-based biostimulants to the production and nutritional quality in maize (Supplement III and IV)

Two dosage optimization for the application of glycine betaine (GB) and L-pyroglutamic acid (PG) as smbBS on maize plants subjected to water restrictions were tested (Supplement III). The first approach was based on a hydroponic approach (Jiménez-Arias et al., 2019b), and the best performing doses were 0.1 mM GB and 1 mM PG. The optimization on the nursery trays (Jiménez-Arias et al., 2022b) confirmed the results obtained in the previous step. Therefore, those concentrations were used in the greenhouse experiment. The application of both BS ameliorates the hydric status of the plants, represented by the RWC, which dropped after the water restriction onset for the non-treated plants.

The leaf width and length were severely affected by the water condition. However, the treated plants reduced the differences compared to non-stressed plants. This trend was also reproduced for the reproductive development, represented by the cobs number, weight of 100 kernels, and total grain weight. The water use efficiency (WUE) was also improved by the GB and PG applications. The analysis of the nutritional properties of the kernels revealed that both carbohydrates and protein content decreased with the water restriction, and both BS prevented that. The mineral profile of the flour shows that water availability has a significant influence on the Ca, P, and Mg content. Ca content decreases with the water availability, whereas P and Mg were accumulated. PG under water restricted conditions significantly accumulates Mg. Other minerals such as Fe, Cu, and Zn did not significantly differ from the control plants. However, their levels were increased in the treated plants. Overall, the application of GB and PG as drench treatment proved to improve the WUE, preventing evapotranspiration losses and maintaining the nutritional benefits of the maize. (Jiménez-Arias et al., 2022a)

As the previous step in the field experiment, the dose optimization on nursery trays provided that Put at 0.1 mM was the more beneficial treatment, while Spd did not substantially benefit the water-restricted seedlings. Therefore, a less prejudicial concentration (0.5 mM) was selected. The drench application of both PAs improved the hydric status of the plants (RWC) compared to non-treated controls (Supplement IV). The treatment with Put seemed to improve the plant growth, whereas Spd harmed the biomass production. The contribution of the treatments to the production was affected by the irrigation regime. While Put enhanced the production for the WW conditions, Spd had a negative effect; and the opposite was observed under WD conditions. As a result, the WUE related to production was enhanced by Put under WW conditions and by Spd under WD.

The PBCI collected the information regarding all considered productivity parameters to reveal that Put had a positive effect in general, but Spd was only beneficial under WD conditions. Regarding nutritional quality, the PA application induced the accumulation of carbohydrates in the kernels up to the levels of the WW controls. The protein content behaved differently, with Put enhancing the levels in the plants under WW conditions and Spd accumulating more CH under WD. Regarding the mineralogical composition of the flour, Na, P, and Cu were the elements more responsive to the interaction between water regime and treatment. ["*Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario"* Alba E. Hernandiz, David Jiménez-Arias, Sarai Morales-Sierra, Andres A. Borges, Nuria De Diego. WORK SUBMITTED TO: Frontiers of Plant Science (ID nº 944066)].

3. The use of Small Molecule – Based Biostimulants (smbBS) to improve Arabidopsis stress tolerance (Supplement V)

For the optimal growing conditions, none of the priming at any of their concentrations affected the growth of the rosettes, except for 1 mM Orn which negatively affected the plants' growth. This negative

effect was observed in all growing conditions. The priming with 0.1 mM DAP improved the plant growth under stress conditions. However, it was significant only for salt stress. Treatment with 0.1 mM Orn significantly improved the rosette growth when subjected to salt stress; nevertheless, this enhancement was not significant under osmotic stress. Priming with 1 mM Put significantly enhanced the rosette growth under salt conditions. All the parameters extracted from the images were used to compute the PBCI, which revealed that all priming agents improved plant growth under osmotic stress, except 1 mM Orn.

A targeted metabolomics assay was also performed to quantify the plants' free AAs and PAs. The most abundant free AAs for all treatments and growth conditions were Pro, aspartic acid (Asp), and glutamine (Gln), while cystine (Cis) appeared in the lowest concentration. All primed plants accumulated N-acetylornithine (AcOrn) under optimal and salt stress conditions, except for the 1 and 0.1 mM Put-treated plants. Under optimal and stress conditions, 1 mM DAP and 0.1 mM Put accumulated Pro, while 1 mM Orn had the opposite effect. Under optimal conditions, the priming 1 mM DAP, 0.1 mM Orn, and both Put doses induced increments in the PA free forms. Moreover, all primed plants presented higher total thermospermine (ThSpm) and spermine (Spm) than the unprimed plants. This trend was also observed in 1 mM DAP primed plants under osmotic stress and optimal conditions. Homospermidine (HomoSpd) accumulated greatly in 1 mM Orn and Put primed plants under optimal conditions. Norspermidine (NorSpd) accumulated after the 0.1 mM Put priming in optimal and osmotic conditions and under 0.1 mM DAP, and 1 mM Orn and Put under salt stress.

The multivariate statistical analysis also revealed that the control treatment was related to Cis, NorSpd, and dry biomass under optimal conditions, whereas Put-primed plants positively correlated with final growth, Glu, and total PAs, especially NorSpd and Agm. DAP-treated plants correlated with almost all free amino acids and PAs, including Pro. The accumulation of Pro presented a negative correlation with the dry biomass, which, together with the final growth, correlated positively with ThSpm, Spm, and Spd. Under osmotic conditions, the controls were positively correlated to Cis, Trp, Glu, and total and free HomoSpd. Both Orn priming and Put at 1mM showed a similar trend. On the other hand, 0.1 mM Put-primed seeds accumulated the highest number of free amino acids, and free and total NorSpd, whereas 1 mM Put and DAP-treated plants showed higher total and free PAs and bigger final growth and dry biomass. The final growth was strongly correlated with total Put, Spd, ThSpm and Spm, free Put, GABA, and Asp; and the dry biomass positively correlated with free PAs but negatively with Cit, Orn, Arg, Ser, and NorSpd. Under salt stress, the controls correlated with GABA, methionine (Met), Cis, and AcPut. Oppositely, 1 mM Put primed seedlings that accumulated higher levels of Glu and Orn, free and total HomoSpd and NorSpm, the free Spm. Meanwhile, the DAP treatment was related to Pro, almost all the PA forms (free and total), including Spd and Cad, and the plant biomass. The final growth was significantly correlated with Pro, Cad, and Spd; but negatively correlated to β-aminobutyric acid (BABA), Asn, isoleucine (Ile), phenylalanine (Phe), Met, and histidine (His). The effectiveness of the seed priming with Orn, Put, and DAP for improving plant growth and stress tolerance depends on the type of growth conditions. (Hernándiz et al., 2022)

4. The use of Ornithine as a foliar spray to enhance the tolerance to water deficit in sensitive barley (Preliminary results)

To perform a more integrative study of the Orn as stress alleviator, we studied how the foliar application of this aa affects the performance of two genotypes of barley (*Hordeum vulgare* L.), cultivar Steptoe as the wild type (WT) and the mutant AZ34 (AZ) which is partially deficient in the ABA synthesis. The transpiration curves revealed that AZ and WT had a similar A from the first assay. However, AZ line presented higher C_i, E, and g_s under optimal conditions. The addition of Orn incremented A, E, and g_s in the AZ plants. The Orn application only incremented E slightly in WT plants. The addition of PEG caused a sharp decrease in all parameters, especially in E and g_s.

Under stress conditions, the Orn treated variants reduced the photosynthesis-related parameters, keeping only higher values of C_i. Interestingly, the transpiration of the AZ treated with Orn dropped to similar values as the non-treated AZ plants, whereas the WT with Orn revealed lower E values than the non-treated WT plants. The Orn treatment tended to decrease the WUE both intrinsic and instantaneous compared to the non-treated plants. PEG increased both WUE parameters, but the Orn treated plants kept lower values, especially in WT line.

From the second assay, the different performance between WT and AZ plants was remarkable, being the mutant line 25% smaller than WT in optimal conditions. The water restriction limited the growth of both genotypes, reducing the plant height by 48% and 33.4% for the WT and AZ, respectively. Under WW conditions, the application of Orn significantly increased the size of the mutants, while under WD an enhancing trend can be observed. These results are supported by the fresh and dry weight measurements. The foliar treatment improved the relative water content (RWC) for the AZ plants in WW conditions, but no effect was observed for the WD. The chlorophyll content was also affected by the water regime. The levels of chlorophyll in AZ sprayed plants were higher than the non-treated ones in WW conditions, but no effect was observed in WD. As a result, the Orn application enhanced the total chlorophyll content for the AZ plants under WW conditions.

CONCLUSIONS AND FUTURE PERSPECTIVES

The present thesis pursues to confirm the adaptability and suitability of the protocols developed in our team based on high throughput phenotyping platforms (HTPPs). Moreover, it was intended to provide a deeper understanding of the mode of action of pure substances, which might be considered small molecule-based biostimulants (smbBS). The main conclusions of this work are:

- The suitability and accuracy of *in vitro* and *in vivo* protocols for PlantScreen™ XYZ System and PlantScreen™ Compact System have been proved. The protocols can be adapted to different plant

species and are robust and versatile enough to find stress biomarkers as the minimum temperature, in the case of Supplement II, or to evaluate the potential stress alleviator effect of newly developed compounds, as in the case of the Supplement I.

- The beneficial effect of the smbBS was proved by applying different compounds to water-limited maize plants and studying their performance. The reported benefits of using polyamines (PAs) used as seed priming are not lost when the PAs are applied via drenching to the roots. We described the different effects of putrescine (Put) and spermidine (Spd) on the development of the vegetative and reproductive stages of the maize. Moreover, PAs, and in general smbBS treatments affect the composition of the kernels. Therefore, smbBS as a biofortification tool is an exciting area to investigate further.

- The use of smbBS with well-known PAs and the uncommon ones DAP can be an efficient technological approach for plant hardening and improving plant stress tolerance. The results obtained with Orn were contradictory since the higher doses induced the growth inhibition of the plants, which, on the other hand, could be part of conservative response.

- As part of the preliminary results, we observed that the foliar application with Orn alters the physiology and metabolism of barley plants differently in WT (as control) and AZ34 (as sensitive), pointing to this metabolite as a vital regulator of PA metabolism and endogenous ABA, and hence, plants' water stress response.

Because of these results, the HTPPs are valuable tools for bioassays for any research that intends to provide a deeper understanding of the plant processes, either evaluating screening for stress markers or assessing the BS efficacy. The smbBS as a biofortification strategy could be considered to further studies involving more challenging growing conditions and testing multiple plant species. Finally, the results also revealed a vital role of Orn regulating PA metabolism and plant stress response, but further studies will be needed to understand its involvement deeper**.**

LIST OF PUBLICATIONS

1. Published articles and contributions

Mandarin irrigation scheduling by means of frequency domain reflectometry soil moisture monitoring

M.A. Martínez-Gimeno, M.A. Jiménez-Bello, A. Lidón, J. Manzano, E. Badal, J.G. Pérez-Pérez, L. Bonet, D.S. Intrigliolo, A. Esteban,

Agricultural Water Management, Volume 235, 2020, 106151, ISSN 0378-3774, https://doi.org/10.1016/j.agwat.2020.106151.

Hormopriming to Mitigate Abiotic Stress Effects: A Case Study of N9-Substituted Cytokinin Derivatives With a Fluorinated Carbohydrate Moiety

Bryksová Magdaléna, Hybenová Andrea, Hernándiz Alba E., Novák Ondřej, Pěnčík Aleš, Spíchal Lukáš, De Diego Nuria, Doležal Karel

Frontiers in Plant Science, 11, 2020, ISSN=1664-462X
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Phloem exudate metabolic content reflects the response to water-deficit stress in pea plants (*Pisum sativum* L.)

Blicharz, S., Beemster, G.T., Ragni, L., De Diego, N., Spíchal, L., Hernándiz, A.E., Marczak, Ł., Olszak, M., Perlikowski, D., Kosmala, A. and Malinowski, R. (2021),

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Innovative UAV LiDAR Generated Point-Cloud Processing Algorithm in Python for Unsupervised Detection and Analysis of Agricultural Field-Plots.

Polák, M.; Miřijovský, J.; Hernándiz, A.E.; Špíšek, Z.; Koprna, R.; Humplík, J.F.

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Applying Biostimulants to Combat Water Deficit in Crop Plants: Research and Debate.

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Priming with Small Molecule-Based Biostimulants to Improve Abiotic Stress Tolerance in Arabidopsis thaliana

Hernándiz, Alba E., Carlos E. Aucique-Perez, Sanja Ćavar Zeljković, Nikola Štefelová, Sara Salcedo Sarmiento, Lukáš Spíchal, and Nuria De Diego. 2022.

Plants 11, no. 10: 1287. https://doi.org/10.3390/plants11101287

Addressing the contribution of small molecule-based biostimulants to the biofortification of maize in a water restriction scenario

Alba E. Hernandiz, David Jiménez-Arias, Sarai Morales-Sierra, Andres A. Borges, Nuria De Diego.

WORK SUBMITTED TO: Frontiers of Plant Science (ID nº 944066)

2. Contribution to conferences

Alba E. Hernándiz. Affordable field phenotyping, basic concepts for beginners. Luhačovice, 2019

Carlos Eduardo Aucique-Pérez, Alba E. Hernándiz, Ondřej Vrobel, Nuria De Diego, and Lukáš Spíchal. Phenotyping of pea (Pisum sativum) lines under drought and rewatering conditions. EFB virtual conference, 2021

Karolina Komzaková, Carlos Eduardo Aucique-Pérez, Alba E. Hernándiz, Nuria De Diego Sanja Ćavar Zeljković. Response of selected Basil genotypes to different nitrate doses. EFB virtual conference, 2021

Alba E. Hernándiz, Carlos Eduardo Aucique-Pérez, Sara Salcedo-Sarmiento, Jonathan Cárdenas, Lukáš Spíchal, Nuria De Diego. Foliar application of L-Ornithine improves the resilience to water deficit in Barley sensitive lines. Agriculture and Food Sustainability, Madeira 2021

ABSTRACT

Biostimulants (BS) are an emerging trend that can alleviate the adverse effects of climate change on crops and help in the transition to greener agriculture. Among the different types of BS, the small molecule-based BS (smbBS) is an interesting option. They allow the study of the pure substances' mode and/or mechanism of action, simplifying the formulation and registration processes. In this scope, our research group already had positive results on using polyamines (PAs) as seed priming agents on *Arabidopsis thaliana* (L.) Heyhn., and maize (*Zea mays* L.) exposed to abiotic stress. These results inspired this work and aimed to unveil the mechanism of action of well-known PAs and other related metabolites naturally present in plants, used as smbBS using different –omics, phenomics performed into the OloPhen platform, and metabolomics mainly using targeted methods.

Two initial experiments were performed to assess the suitability and reproducibility of the protocols developed for these devices. The morphological and physiological characterization of pea plants (*Pisum sativum* L.) under drought stress revealed the link between the reduced biomass production and the increment of the non-photochemical quenching. Newly synthesized N^9-substituted cytokinin derivatives with a fluorinated carbohydrate moiety were evaluated as priming agents to improve plant growth and stress tolerance using an *in vitro* Arabidopsis rosette growth assay. The obtained information allowed to determine its role as growth regulators and/or stress alleviators. The next step was to study further the effect of the already proven beneficial PAs, putrescine (Put) and spermidine (Spd), on the vegetative and reproductive development of maize plants grown in a greenhouse under water restricted conditions. We reported a different mechanism of action of the PAs where Put enhanced the biomass production and the productivity of the plants in an optimal irrigation scenario. Contrarily, Spd negatively reduced biomass production but enhanced the yield under a water deficit scenario. Moreover, both treatments affect the nutritional parameters of the kernels, inducing the accumulation of some minerals which are interesting for human nutrition.

To understand further the PA mechanism/mode of action, an additional study using Put as a positive control, ornithine (Orn) as PAs precursor, and 1,3- diaminopropane (DAP) as a compound of the PA terminal catabolism was performed using the previously mentioned *in vitro* Arabidopsis rosette growth assay under optimal, osmotic and salt stress conditions. None of the primings affected the growth of the seedlings in optimal conditions but altered the metabolism of the plants. Under stress conditions, almost all primed plants grew better and improved their greenness. Only primed plants with high concentrations of Orn showed different plant responses.

Interestingly, the metabolic analysis revealed the implication of the N-acetylornithine and Orn and polyamine conjugation as the leading player regulating growth and development under control and stress conditions. To further understand the Orn mechanism of action and translate the findings into a crop with economic value, an experiment on barley (*Hordeum vulgare* L. cv. Wildtype; WT) and a sensitive mutant

(AZ34; AZ) was performed, using Orn as a foliar treatment. As preliminary results, we observed that Orn alters the physiology and metabolism of barley plants differently according to the genotype, pointing to this metabolite as a relevant regulator of polyamine metabolism and endogenous abscisic acid and, hence, the plants' water stress response.

SOUHRN (in Czech)

Použití biostimulantů (BS) je rozvíjejícím se trendem, který může zmírnit nepříznivé dopady změny klimatu na plodiny a pomoci při přechodu k ekologičtějšímu zemědělství. Zajímavou možností mezi různými typy BS je použití BS na bázi malých molekul (smbBS). Umožňují studovat působení anebo mechanismy účinku čistých látek a zjednodušují procesy formulace a registrace. V tomto rozsahu již naše výzkumná skupina měla pozitivní výsledky při použití polyaminů (PA) jako aktivátorů semen *Arabidopsis thaliana* (L.) Heyhn. a kukuřice (*Zea mays* L.) vystavených abiotickému stresu. Tyto výsledky inspirovaly k napsání této práce, která měla za cíl odhalit mechanismus působení dobře známých PA a dalších příbuzných metabolitů přirozeně vyskytujících se v rostlinách, používaných jako smbBS pomocí různých metod, fenomiky provedené na platformě OloPhen a metabolomiky.

Na počátku byly provedeny dva experimenty pro posouzení vhodnosti a reprodukovatelnosti protokolů vyvinutých pro toto zařízení. Morfologická a fyziologická charakterizace rostlin hrachu (*Pisum sativum* L.) ve stresu ze sucha odhalila souvislost mezi sníženou produkcí biomasy a nárůstem nefotochemického zhášení. Nově syntetizované *N9*-substituované deriváty cytokininu s fluorovanou sacharidovou skupinou byly hodnoceny jako aktivační činidla pro zlepšení růstu rostlin a odolnosti vůči stresu pomocí *in vitro* testu růstu rozety Arabidopsis. Získané informace umožnily určit jejich roli v regulaci růstu anebo ve zmírnění stresu. Následujícím krokem bylo další studium účinku již prokázaných prospěšných PA, putrescinu (Put) a spermidinu (Spd), na vegetativní a reprodukční vývoj rostlin kukuřice pěstovaných ve skleníku v podmínkách při nedostatku vody. Zaznamenali jsme odlišný mechanismus působení PA, kde Put zvýšil produkci biomasy a produktivitu rostlin při optimálním zavlažování. Naopak Spd snížil produkci biomasy, ale zvýšil výnos při vodním deficitu. Kromě toho aplikace obou látek ovlivnily nutriční parametry semen a vyvolaly akumulaci některých minerálů, které jsou zajímavé pro lidskou výživu.

Pro další pochopení mechanismu a způsobu účinku PA byla provedena další studie využívající Put jako pozitivní kontrolu, ornitin (Orn) jako prekurzor PA a 1,3-diaminopropan (DAP) jako sloučeninu terminálního katabolismu PA s použitím dříve zmíněného *in vitro* testu růstu rozety Arabidopsis v optimálních podmínkách a při osmotickém a solném stresu. Žádný z primingů neovlivnil růst semenáčků v optimálních podmínkách, ale změnil metabolismus rostlin. Vlivem sterových podmínek téměř všechny ošetřené rostliny lépe rostly a byly zelenější. Pouze rostliny ošetřené vysokými koncentracemi Orn ukazovaly různou reakci.

Je zajímavé, že metabolická analýza odhalila, že konjugace N-acetylornithinu a Orn a polyaminů hraje hlavní roli v růstu a vývoji při kontrolních i stresových podmínkách. Abychom lépe porozuměli mechanismu účinků Orn a výsledky aplikovali na ekonomicky významné plodiny, byl proveden experiment s Orn aplikovaným na listy ječmene (*Hordeum vulgare* L. cv. Wildtype; WT) a citlivého mutantu (AZ34; AZ). Jako předběžné výsledky jsme pozorovali, že Orn mění fyziologii a metabolismus rostlin ječmene odlišně v závislosti na genotypu, což ukazuje, že tento metabolit je relevantní regulátor metabolismu polyaminů a endogenní kyseliny abscisové, a tedy reakce rostlin na vodní stres.

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