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Laboratory of Growth Regulators & Chemical Biology and Genetics Department

Lidia Ugena García – Consuegra

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"It matters not how strait the gate,

How charged with punishments the scroll,

I am the master of my fate:

I am the captain of my soul"

-W. E. Henley: Invictus-

Dedicated to my parents,

A mis padres,

José Mª y Piedad

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❖ Declaration I

I hereby, declare that the presented Ph.D. dissertation is my original work. The literature used is listed in the Bibliography section.

In Olomouc on
Lidia Ugena

❖ Declaration II

I declare that my role in preparation of the papers listed below was as follows:

- **Supplement I**: First author - Bibliographical research, manuscript preparation.
- **Supplement II:** Co-author - High-Throughput Screening method (HTS) development, samples preparation, validation and optimization of the method, HTS measurements.
- **Supplement III**: First author - Design of experiments, multi-trait high-throughput screening method development and measurements, data analysis and manuscript preparation.
- **Supplement IV:** First author - Design of experiments, high-throughput bioassay development, seedling emergence measurements and manuscript preparation.

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List of papers

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I. Kateřina Podlešáková*, Lydia Ugena*, Lukáš Spíchal, Karel Doležal, Nuria De Diego. "Phytohormones and polyamines regulate plant stress responses by altering GABA pathway". *New Biotechnology;* vol.48, pp.53-65, 2019.

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IV. Lydia Ugena, Jan F. Humplík, Tomáš Fürst, Nuria De Diego, Lukas Spichal. "CroSeEm: a high-throughput emergence assay for screening maize seedlings under salinity" (*Under revision*).

Terms and abbreviations

AA	amino acid
AACP	amino acid containing products
ANOVA	analysis of variance
B	blue
C/N	carbon /nitrogen
Ca	calcium
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
Cl	chlorine
CO_2	carbon dioxide
Col-0	columbia-0
CroSeEm	crop seedling emergence
DAS	days after germination
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
EBIC	European Biostimulant Industry Council
EtOH	ethanol
FW	fresh weight
G	green
GABA	gamma- aminobutyric acid
GLI	green leaf index
GMO	genetic modified organisms
H_2O_2	hidrogen peroxide
HCP	hormone containing product
HS	humic substances
HTP	high-throughput phenotyping
HTS	high-throughput screening
INCYDE	2-chloro-6-(3-methoxyphenyl) aminopurine

ISR	induced systematic resistance
LED	light-emitting diode
K	potassium
MB	megabytes
MS	murashige-skoog
MTHTS	multi-trait high-throughput screening
N	nitrogen
Na	sodium
NaCl	sodium chloride
NGRDI	normalized green red difference index
P	phosphorus
PAR	photosynthetically active radiation
PA	polyamine
PBC	plant biostimulant characterization
PCR	polymerase chain reaction
PGPF	plant growth-promoting fungi
PGPR	plant growth-promoting rhizobacteria
PHs	protein hydrolysates
PNG	portable network graphics
Pro	proline
Put	putrescine
R	red
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
RGB	red-green-blue imaging
RGR	relative growth rate
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
Spd	spermidine
Spm	spermine

SSR	simple sequence repeats
TDZ	N-phenyl- N -1, 2, 3-thiadiazol-5-yl urea
T-Spm	thermo-spermine
VARI	visible atmospherically resistant index
VCs	volatile compounds
3D	three dimensions

I. Abstract

Plant response to stress is a highly dynamic and complex process dependent on the severity and duration of the stress, the fitness and preparedness of the plant itself and its developmental stage. Breeders worldwide have therefore focused on quantitative analyses of plant traits in order to accelerate the development of appropriate strategies for improving crops which are adaptable to resource-limited environments. Soil salinity is an important environmental factor that reduces plant germination and early seedling establishment and results in decreased crop productivity on a global scale.

The application of biostimulants represents one of the most innovative and promising strategies for minimizing stress impact, including salinity. The origin of biostimulants is diverse, and ranges from single compounds to complex matrices with different groups of bioactive components that have only been partly characterized. Irrespective of their complexity, biostimulants encompass different groups of plant signaling compounds such as plant hormones, amino acids, and polyamines among others. The exogenous application of these signaling molecules has been reported to ameliorate the adverse effect of stress through sophisticated crosstalk leading to the activation of conserved pathways. Their use also contributes to more sustainable and environmentally friendly agricultural practice, and offers an alternative to synthetic protectants.

Plant phenotyping platforms have become an important tool in plant biology and agriculture. They provide new possibilities for automated, fast scoring of several plant growth and development traits, followed over time using non-invasive sensors. These approaches allow simultaneous testing of a large number of potentially bioactive compounds in a wide range of concentrations and / or genotypes, under various growth conditions as well providing information about the developmental and physiological status of the treated plants and, analyzing traits like the scoring of seedling emergence. Altogether, we consider that the new protocols based on high-throughput screening (HTS) could accelerate identification of the mode of action of known biostimulants and the characterization of new ones.

II. Objectives

Plant response to stress is a highly dynamic and complex process dependent on the severity and duration of the stress. The application of biostimulants represents one of the most innovative and promising strategies for minimizing stress impact. However, there are obstacles to determining their mode of action. For this reason, the development of efficient, affordable and high-throughput agronomic techniques for identifying and validating the legitimacy of a product on the market of biostimulants is a priority.

The main objectives elaborated and discussed in this doctoral thesis are the following:

- Compilation of a literature review related to the topic of the doctoral thesis, specifically plant stress, biostimulants and high-throughput screening approaches.
- In depth study bringing together information on plants exposed to stress conditions, and discussion of the possible crosstalks among different groups of signaling molecules.
- Development of a highly reproducible *in vitro* HTS bioassay using *Arabidopsis thaliana* as a model plant to be used for selecting phenotypes, growth conditions and/or compounds that can confer stress tolerance.
- Development of a novel multi-trait high-throughput screening (MTHTS) of *Arabidopsis* for the identification of new biostimulants and their modes of action under different salt stress concentrations.
- Characterization of the seedling emergence using high-throughput screening assays in real crops such as maize (*Zea Mays* L.) under salt stress using the indoor phenotyping method and validation of the assay and characterization of the specific mode of action of the biostimulants.

CHAPTER 1

INTRODUCTION

1.1 Influence of global warming on crop production.

Climate is defined by the World Meteorological Organization in statistical terms as the means and variability of relevant parameters such as temperature, precipitation and wind over a period of around 30 years ranging from months to thousands or millions of years [1].

Climate change is a problem of the highest priority today with, influence on agricultural production worldwide. It implies atmospheric increase in the concentration of greenhouse gases, mainly carbon dioxide (CO_2) and methane [2]. Although these gases are naturally found in the atmosphere, their density has drastically increased recently due to human impacts on the environment, causing an increase in the average temperature of the

earth - global warming - and directly influencing rainfall patterns by causing longer periods of water deficit or, in contrast, heavy floods [3,4].

The human population is expected to increase to over 8 billion by the year 2025 [5] and the demand for food is expected to rise up to 50% [4]. Moreover, there is constantly more information on how climate variability and extreme climate conditions are affecting agricultural production. This is one of the main causes of severe food crises, increasing difficulty in resolving the challenge of ending hunger, achieving food security, improving nutrition and promoting sustainable agriculture [4]. For these reasons, improved crop production has become a research priority in the past decades [6].

1.2 Stress in plants.

Plants have to endure periods under unfavorable situations throughout their life cycle. Since environmental factors play a crucial role in crop yield, growth and other physiological, biochemical and morphological processes, unfavorable changes in the environment can reduce plant growth and, yield, cause permanent damage and even death [7,8]. There are a multitude of plants stressors with different modes of actions. In order to survive, plants have developed sophisticated defense mechanisms that, depending on their ability for signal perception and transduction, act as diverse responses to all these stimuli [9].

Plant stress was defined in 1987 by Walter Larcher as “a state in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance. If the limits of tolerance are exceeded and the adaptive capacity is overtaxed, permanent damage or even death may result” [10]. According to this definition, plant stress is a state where the plant experiences a change that demands a response and the stress strength and duration is directly related to the level of damage. Subsequently, Lichtenthaler in 1996 extended the definition with the concept of the regeneration phase, where the plant can undergo a recovery process when the stressors are eliminated [11]. Several other definitions of stress were expressed later [12–14], but regardless of the differences, all of them describe an alteration in the conditions that affect the plant, as well as the plant response to this change and the level of generated damage.

Taking into account the proposals of different authors, stress in plants involves a dynamic process that is divided into four phases, based on the duration and intensity of the stressor [8] **(Figure 1.1)**.

a. Response phase: alarm reaction.

The alarm phase is a change in optimal growth conditions, such as photosynthesis or the transport of metabolites, which takes place at the beginning of the stress. As a consequence, a deviation of the plant's normal physiological conditions, a vitality decrease, and a process where there is a higher rate of catabolism is produced [15]. However, the plant activates stress coping mechanisms and only those with low or no stress tolerance experience acute damage [15].

b. Restitution phase: stage of resistance.

This phase consists in an adaptation process where the stressors still affect the normal plant growth conditions, and repair and hardening processes. As a result, new physiological standards are established against a prolonged stress or a stress-dose overloading, reaching a maximum resistance level [15].

c. End phase: stage of exhaustion.

When the level of resistance is over and the stressor continues, the plant enters a period where the physiology and vitality are continuously reduced. If the stress conditions endure, the results is severe damage, chronic disease or death, depending on species, time and dosage [15].

d. Regeneration Phase.

However, if the stressor is removed, a partial or full regeneration of physiological functions may be achieved and the plant will be able to survive. On the other hand, if the senescence process predominates, the plant cannot regenerate and restore itself [13,15]. Depending on the time and stage of exhaustion when the stressor is removed, the new physiological status will range from minimum to maximum resistance. If the intensity and duration of the stress are not excessive, the plant will orient itself within the range set by the resistance level, and in some situations damage symptoms are not detectable [15].

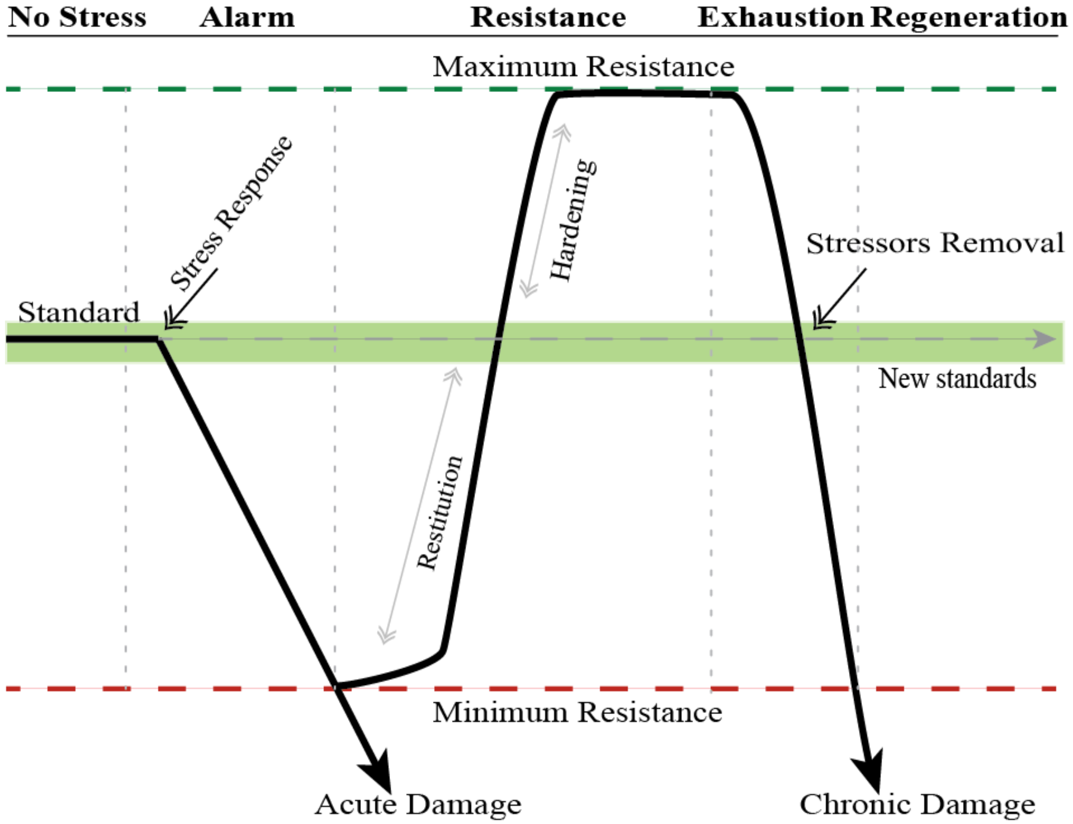

Figure 1.1: Sequence of phases and induced responses in a plant by exposure to a stress factor at the physiological level [8].

According to different factors, plant stress can be classified into various groups [8]:

- Plant stresses can be divided into those with positive effects for plant development, stimulating stress or "Eustress"; and severe stresses with negative effects and causing damage, or "Distress" [13,15].
- Moreover, they can be classified depending on the period of exposure on into "short-term stresses", where the plant can overcome the stress, or persistent or "long-term stresses" that result in significant and irreversible injuries [11,13].
- According to the factors that produce the stress, these may be "biotic stress", defined as a result of interactions among the plant and other living organisms that share the same environment and results in partial or significant damage. The organisms included in this type of stress are pathogenic and non-pathogenic bacteria, fungi or viruses, which produce stress

mostly in the form of diseases or parasitism, as well as nematodes and insects. Animals and plants (wild or cultivated) are also causes of biotic stresses by physical damage, competition and phyto-parasitism [13]. The other stress is "abiotic stress". It is generally well-known that abiotic stresses are the main restrictive factor in agricultural productivity [16,17]. They are caused by non-living factors (environmental or nutritional) such as drought, changes in temperature and, salinity, among others, that affect the plant's growth, reproduction and life [18,19].

1.2.1 Effects of salt stress on crop production.

The objective of this work is study of the plant response to salinity effects. Salinity is one of the major abiotic factors that affect plant growth. By the year 2050, more than 50% of all arable lands may suffer salinization, with an annual growth rate of 10% [20–22]. The prediction of yield losses are estimated at 20% and over 6% of world's is affected [23].

The concept of soil salinization includes diverse salt-affected soils defined by the nature and characteristics of the composition: saline soils contains a high content of neutral soluble salts, which crystallize on the surface, forming a white crust and they are in sufficient amount to have negative effects on plant growth. In the case that the soil contains a high sodium cation concentration of salts, it is defined as sodic soil. The nature of salts present in sodic soils, such as a high carbonates concentration, makes them alkaline. Cultivation of plants is difficult due to high pH, poor drainage and the toxic effects of sodium [16,24,25].

Natural processes such as weathering of parent materials or groundwater are some of the main drivers in the long-term accumulation of salts in soils. This is primary salinization. In contrast, when human activities introduce a change in the hydrologic balance, it is called secondary salinity. It is caused by poor drainage conditions, the use of salt-rich irrigation water schemes or the use of fertilizers which produce soil pollution [16,24].

High concentration of salt in soil affects plant growth response in two-phases: the first and rapid osmotic phase inhibits the growth of young leaves due to reduction in the ability to absorb water, and the second and slower ionic phase that accelerates senescence of mature leaves. In the osmotic phase, which starts immediately after the salt concentration

around the roots increases to a threshold level (around 40 mM NaCl for most plants or less for sensitive plants like, rice and *Arabidopsis*), the rate of shoot growth decreases significantly [16,26,27]. The second, ion-specific, phase of plant response starts when salt accumulates to toxic levels in the source leaves, which rapidly die. The accumulation of Na^+ and Cl^- ions are the main drivers of these detrimental effects. This last phase dominates in high salinity conditions or in sensitive species [16].

It is known that salinity affects nutrient uptake in plants and induces nutrient deficiencies, such as a reduction of Ca^{2+}, N, and K levels in different plant species [28–30]. Moreover, there is evidence that it can induce conditions of oxidative stress, such as generation and/or accumulation of reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals [31].

For these reasons, breeders worldwide use different strategies in order to accelerate the development of appropriate methodologies for improving crop production and alleviate the stress conditions.

1.3 Strategies of plant crop improvement.

To ensure the food security for the rising world population, total crop production will have to be considerably increased, with less arable land available, and under much more severe environmental conditions. The most important strategies of crop improvements against stresses include several agronomical, physiological and molecular approaches such as classical breeding programs, molecular breeding, genetic engineering, and/or environmental friendly practices, such as the use of biostimulants, among others [32] (**Table 1.1**). The option to improve crop growth and yield by irrigation techniques due to dry land is very expensive, provides a short-term solution and is decidedly not productive [22,31]. Different strategies for improving crop efficiency specifically against salt stress have been used, because the biochemical and physiological traits for salt tolerance are different from other types of stress tolerance.

1.3.1 Conventional breeding.

Conventional, classical or traditional breeding consists in the development of genotypes using conservative tools and natural processes. Early farmers selected seeds to develop new lines from the best looking plants, and saved them for planting the next season. Other operational technologies for plant breeding are now used [25,33].

In general, conventional breeding is time-consuming and labor-intensive, with a limit to the transference of genes [34]. Consequently, it is necessary to develop new approaches to confront the setbacks. Genetic engineering, it is an alternative, which is employed worldwide not only for improving stress tolerance but also for improving the quality and yield potential of most crops [34].

1.3.2 Molecular breeding.

Successful screening and selection of salt tolerant cultivars in conventional breeding program is limited by the significant influence of environmental factors [5]. The development of modern molecular breeding techniques using molecular marker technologies allow more effective and efficient discrimination to identify the most suitable individuals [35].

The use of molecular markers in salt stress tolerance has improved the efficiency of selection for those traits that are controlled by several genes and are highly influenced by environmental factors [20]. Moreover, it is widely accepted that the degree of salt tolerance varies during the plant life, and it has been observed that the regions of genes associated with salt tolerance in the germination stage in different crops are different from those associated at the early stage growth [36].

As a solution, the use of molecular breeding techniques enables the combination of genes, but it takes multiple generations and consequently many years. These problems could be avoided if it is possible to introduce the gene directly into the crop by genetic modification.

Table 1.1. Operational classification of technologies of plant breeding [25].

Classical/traditional tools	**Common use of the technology/tool**
Emasculation	making a completer flower female; preparation for crossing
Hybridization	crossing un-identical plants to transfer genes
Wide crossing	crossing of distantly related plants
Selection	the primary tool for discriminating among variability
Chromosome counting	determination of ploidy characteristics
Chromosome doubling	manipulation of ploidy for fertility
Male sterility	elimination of the need for emasculation in hybridization
Triploidy	achievement of seedlessness
Linkage analysis	for determining association between genes
Statistical tools	for evaluation of germplasm
Relatively advanced tools	
Mutagenesis	induction of mutations to create new variability
Tissue culture	for manipulating plants at the cellular or tissue level
Haploidy	creation of extremely homozygous diploid
Isozyme markers	facilitation of the selection process
In situ hybridization	detection of successful interspecific crossing
More sophisticated tools	
DNA markers	
– RFLP	more effective than protein markers (isozymes)
– RAPD	PCR-based molecular marker
Advanced technology	
Molecular markers	SSR, SNPs, etc.
Marker assisted selection	facilitation of the selection process
DNA sequencing	ultimate physical map of an organism
Plant genomic analysis	studying the totality of the genes of an organism
Bioinformatics	computer-based technology for predicting DNA sequence data
Microarray analysis	understanding of gene expression and for sequence identification
Primer design	for molecular analysis of plant genome
Plant transformation	for recombinant DNA work

1.3.3 Genetic Engineering.

The direct introduction of one or more genes by genetic engineering may be a powerful tool for understanding and manipulating the responses of plants to stress. Crops containing transgenes are described as genetically modified organisms (GMO).

Although transgenic approaches have considerably improved the qualitative and quantitative traits related to tolerance, plant response to salt stress is complex and involves changes in the expression of many genes. Under these circumstances, the possibility of improving crop salt tolerance by genetic modification is rather difficult and slow [5].

1.3.4 Application of stress alleviator compounds.

Over the last decades, the market demands and the need to satisfy the crop requirements has increased. The application of diverse compounds to plants to improve qualitative and quantitative traits, including tolerance to biotic and abiotic stresses could be an option.

1.3.4.1 Agrochemicals.

Agrochemicals or agrichemicals are products of chemical origin used in agriculture. In most cases, the term agrochemical includes a broad range of pesticides, as insecticides, herbicides, fungicides and nematicide chemicals. The term also encompasses synthetic fertilizers, hormones, and other chemical agents that promote plant growth [37].

Even though these compounds increase plant crop production, overuse deteriorates environment and has harmful impacts. Excessive use of fertilizers; substances composed of high levels of N, phosphorus (P) and/or K, or because of the insufficient uptake by plants, results in leaching into water bodies through rainwater, causing eutrophication and affect living beings, including growth-inhibiting microorganisms. Furthermore, excess of these compounds use causes depletion of the water holding capacity, soil fertility and disparity in

soil nutrients [38,39]. Another problem is persistence. Some pesticides, such as the insecticide DDT (dichlorodiphenyltrichloroethane), have remained active in the environment for many years. Nowadays, these materials are carefully regulated, and the safety requirements for each product is spelled out in detail [37]. The health and environmental effects have prompted us to consider the use of such compounds as undesirable. Other nonchemical methods of enhancing crop nutrient uptake and dealing with crop pests are required.

Commercially available plant growth regulators are used in agricultural practice for the management of plant growth and development [40]. These comprise several types of natural plant hormones, their synthetic analogues and compounds regulating their biosynthesis, metabolism and transport [40]. As example, strategies leading to the stabilization of cytokinin (CK) levels by exogenous application look highly promising as a tool for yield management in agriculture. However, despite the large number of studies describing positive effects of exogenous application of CKs in various crops, the practical use of CKs still remains unresolved [40]. They can improve biological yield in cereals by increasing the number of productive tillers and reducing the number of nonproductive tillers among others, but these responses appears to be dependent on the type of CK applied, crop, application time and growth conditions [40].

Likewise, other examples of synthetic compounds are used in agriculture, such as analogues of CKs and synthetic auxins [41,42]; 2-chloro-6-(3-methoxyphenyl) aminopurine (INCYDE) or Thidiazuron (TDZ) (N-phenyl- N -1,2,3-thiadiazol-5-yl urea), both inhibitors of cytokinin oxidase/dehydrogenase (CKX), an enzyme that degrades CKs [43–47]. The potential of INCYDE as a valuable compound in agriculture by playing an important role as enhancing the antioxidant defense system by overproduction of antioxidant enzymes and photosynthesis efficiency in NaCl-stressed plants, is supported in diverse studies. This could be linked to increased levels of endogenous CK induced by the application of INCYDE [45]. In the case of TDZ, this has been reported to increase the lifetime of CKs and their effects in plants [43]. TDZ can also promote the growth of various fruits, delay senescence of cut and potted flowers, and increase the stress tolerance and yield of several crops. Its effect could be mediated through the activation of all the CKX receptors in plants and their downstream associated signaling pathways, or indirectly, through the inhibition of CKX enzymes [46].

To recapitulate, a number of studies support available alternatives to harmful agrochemicals but it remains true that there is still a challenge and they are not yet in widespread use.

1.3.4.2 Plant biostimulants.

Plant biostimulants could assist in making agriculture more sustainable and environmentally friendly and companies are introducing innovative products composed of different ingredients [48]. These compounds, offer an alternative to synthetic protectants. They have been gaining interest because their application activates several physiological processes in plants to stimulate growth, improve plant tolerance to environmental disturbances, alleviate stress-induced limitations and to increase yield [49]. The issue with them is the lack of harmonized legal framework from a regulatory point of view due to, lack of formal definition and acceptance of the concept.

Throughout the years, there have been several attempts to define plant biostimulants. However, despite the efforts, the term “biostimulant” is still not well-defined due to a diversity of contributions with a broad conceptual range. Various proposals have suggested that plant biostimulants should be defined by the mode of action, origin, or based on the beneficial impact on plant productivity.

One of the definitions formally established was by the European Biostimulant Industry Council (EBIC) where “Plant biostimulants contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality” [50].

The majority of biostimulants have an undefined composition made by complex mixtures of compounds derived from a biological process or extracted from biological materials. The interaction of these complex formulations is essential for the performance of the biostimulants as their properties cannot be elucidated a priori by knowing the activity of the individual components.

Considering that they will play an important role in agriculture in the future, it is important to establish a legal framework for marketing and regulation of these products to solve the problematic just described.

In the same manner as the definition, the classification of plant biostimulants has had an evolution throughout time. It is worth noting the controversy surrounding different classifications based on multiple factors. Understanding of the biological characteristics of a biostimulant is necessary with the aim of identifying and targeting specific physiological responses in the plants that breeders are interested in. To illustrate this, an example is that biostimulants can contain traces of natural plant hormones and hence they should be classified as plant growth regulators. However, their mode of action is not attributed to this and hence they would have a different classification [51]. Origin, composition, use and mode of action have been proposed as the basis for division into different groups.

One of the first classifications was conducted by Filatov who classified 4 main groups of "biogenic stimulants", including carboxylic acids and hydroxy acids, unsaturated acids and phenolic aromatic acids [52]. In 2007, Kauffman *et al.* [53] summarized the definition of biostimulants as substances with a variety of formulations classified into three major groups depending on their original source and content: humic substances (HS), hormone containing products (HCP), and amino acid containing products (AACP). The classification of plant biostimulants by origin does not contribute information a priori about the mechanism of action but it could be useful to use it for a comparison between similar products.

It was not until 2015, when du Jardin [54,55] provided a broad classification of seven groups of compounds reported as biostimulants. In contrast, Bulgari *et al.*[51] proposed a classification based on their physiological effect on plants rather than the composition, indicating the physiological targets and metabolic network involved.

Many categories of biostimulants have been widely recognized by scientists, covering both substances and microorganisms, and they have been extensively reviewed.

- Humic substances are natural organic compounds produced by the biodegradation of organic materials such as dead cell materials in soils. HS are categorized humic acids, fulvic acids and humins and can be found in soil, peat and lignites [51,55,56]. There is a high variability in these compounds due to the source, environmental conditions, plant species and dose of

HS application. They have been recognized as contributors to soil fertility, indicating the potential of these substances by improving uptake of macro- and micronutrients in roots, carbon and oxygen exchange between soil and atmosphere, ROS scavenging and stimulating shoot elongation and leaf nutrient accumulation among others [51,55–57]. Moreover, the stimulatory effect of humic acids have been reported in enhanced tolerance to salinity [58,59]. Therefore, they have gained more attention not only for their use in agriculture, but also for solving many other environmental problems.

- Seaweed extracts are a complex mixture containing a wide range of organic and mineral compounds including complex polysaccharides such as laminarin, alginates, eckol or plant hormones [55,58,60]. They may vary according to the source, season of collection and extraction process. Generally they belong to the brown algae (*Ascophyllyum nodosum, focus, luminaria*) but they can include red and green macroalgae that represent 10 % of marine productivity [56,58].They are applied on soils, in hydroponic solutions or as foliar treatments [55], showing mainly positive effects in seed germination, plant growth, yield, flowering and increasing tolerance to biotic and abiotic stresses [55,58,61].

- There are five main inorganic beneficial elements as inorganic salts and insoluble forms including aluminum, selenium, silicon, cobalt and sodium. All these elements present in the soil positively affect plant growth, increase the quality of plant products and enhance plant stress responses [55]. Inorganic salts from essential chemical elements, such as phosphates and phosphites, silicates, chlorides or sulphates act as a protection against soil microorganisms. They either directly suppress fungicidal effects or indirectly sustain plant defense reactions among others [54].

- Chitosan is a deacetylated form of the biopolymer chitin. Both have an important role in signaling stress responses and in development regulation because they can bind with a broad range of cellular components and specific receptors involved in defense gene activation [55]. In the field of the agriculture, they are used against fungal pathogens and salt/ drought stress as well as in the enhancement of traits related to primary and secondary metabolism [54].

- Microorganisms. Over 80% of plants worldwide are colonized with diverse microflora with which they create a symbiotic relationship and which subsequently may reduce the adverse effects of biotic and abiotic stresses [62]. Root-microbe interactions or the

interactions between microorganisms are the main types of rhizosphere interactions that can be either neutral, beneficial or harmful. The beneficial microbes positively affecting plants were firstly defined as plant growth-promoting rhizobacteria (PGPR) [63]. This is a dynamic complex of plant roots with soil microorganisms, particularly bacteria or fungi, for which the rhizosphere form a very beneficial habitat [58,64]. Taking into consideration that at least a million microbial species is estimated to exist on earth, to date only around 10,000 have been identified [65]. PGPR are multifunctional and influence all aspects of plant life such as plant growth by increasing plant biomass, yield, enhancing water retention, tolerance to osmotic and ionic stress, root proliferation and by production or even degradation of plant hormones [55,56,66]. Beneficial rhizosphere fungi promoting plant growth are called plant growth-promoting fungi (PGPF) [67]. In addition, PGPF also have the ability to protect the plant against deleterious microorganisms. They have also demonstrated positive effects on seed germination, plant growth and stress tolerance by hormone synthesis, activation of induced systematic resistance (ISR), among others [68,69]. Recent studies have reported that certain PGPF strains promote plant growth through the production of plant growth-promoting compounds such as phytohormones and volatile compounds (VCs) [70]. Plant growth promotion of VCs is not limited to beneficial microorganisms. It includes many different phytopathogens [71,72]. These substances are essential for many plant-microbe interactions and can promote the growth and flowering of plants by facilitating nutrient uptake, photosynthesis and defense responses [66,71].

- <u>Protein hydrolysates (PHs)</u> are “mixtures of polypeptides, oligopeptides and amino acids (AAs) that are manufactured from protein sources using partial hydrolysis” [73] that are obtain by chemical, thermal and enzymatic hydrolysis of animal wastes and plant biomass [74]. They can be applied close to the root or as foliar spray and they are available as liquid extracts, soluble powder and granular form [75]. PHs play an important role in the modulation of molecular and physiological processes in plants increasing the yield and alleviating the effects of diverse types of the main abiotic stress described above [49,58,74].

The accumulation of other nitrogenous molecules included in this group are beatines, polyamines and non-protein AAs [55].

Polyamines (PAs) are low molecular weight ubiquitous polycations with an important role in many regulatory and cellular processes such as plant growth and

development, senescence and stress mitigation. For this reason, they are considered multifunctional regulators of plant physiological processes [76–78]. Putrescine (Put), spermidine (Spd), spermine (Spm) and the spermine isomer thermo-spermine (T-Spm) are the most studied. Under stress conditions, oxidation of Put and Spd is a mechanism in the antioxidative response of plants and contributes directly to the synthesis of the non-protein AA γ- Aminobutyric acid (GABA) [79].

Recently, several studies have reported the importance of PAs to the tolerance to abiotic stresses and many approaches have been developed to manipulate PA metabolism: specific inhibitors, mutants, and in transgenic plants [80–83]. In many cases, stress results in the accumulation of PAs, suggesting that their biosynthesis could be a component of plant response to stress [83]. However, it is not clear how their synthesis, the catabolism or a combination of both are involved in stress tolerance processes of plants. Plant species, plant developmental stages, duration of stress treatment and its intensity are other factors modifying the response in terms of PA fluctuation [80]. Some might produce the accumulation of PAs in response to stress, while others do not or even decrease their endogenous content when exposed to harsh environments. Specifically in salinity, the mechanisms involved are generally less understood than the ones caused by osmotic-stress [81,83–86].

The accumulation of AAs is another strategy that plants use as stress response. Among AAs, proline (Pro) is considered to be the most stress-related compound in plants, particularly under salt and drought stress [87]. Proline plays an important role in the osmotic regulation in plants and it is also involved in regulation of cellular homeostasis, including redox balance and energy status. It can act as a signaling molecule to modulate mitochondrial function, influence cell proliferation or cell death and trigger specific gene expression [88].

GABA is a four-carbon non-protein amino acid whose its main roles is to control stress responses, as well as herbivore deterrence, pH regulation, redox regulation, energy production and maintenance of the carbon/nitrogen (C/N) balance [89]. Besides the synthesis of GABA from glutamate, there are several studies that also report its production via the degradation of PAs [90,91]. High levels of GABA accumulate rapidly in plant tissues in response to a variety of biotic and abiotic stresses and it appears to have a protective role in stressed plants by elevating the endogenous levels of osmolytes and by decreasing the oxidative injury to cells through upregulation of antioxidants [92].

There is relevant crosstalk between PAs and Pro regulating stress. As example, the exogenous application of Put induced stress tolerance and Pro accumulation in some species during drought, but not in others under salt stress [93,94]. These opposite results could be species dependent or due to the type and intensity of the stressor, or a combination of both factors.

In summary, all the studies reviewed point to the existence of a highly conserved pathways that regulate plants under stress condition. Thus, plants perceive stress signals that induce modifications in the AA and PA pathway and their interconnection regulating the stress response and hence the tolerance capacity. However, the specific compounds that take part in the crosstalk, at what moment and at what levels depend on the type and intensity of stress and the plant tissue and species. In the event, the known aspects of the mode of action of these compounds, strongly suggest that their application has potential use in mitigating the adverse effects of multiple stresses.

1.3.4.2.1 Composition of biostimulants and mode of action.

The complex composition of many biostimulants beleaguers identification of their mode/mechanism of action. For a large number, no specified mode of action or a mechanism of action has been identified. Mode of action is defined as a “specific effect on a discrete biochemical or regulatory process” [49]. Some of the principal reported modes of action that biostimulants produce in agriculture are shown in **Figure 1.2**. On the other hand, the mechanism of action could be defined as the impact generated at biochemical, molecular or physiological levels [49].

Companies in the agricultural sector play a key role in the registration of products to ensure their practical, safe and legitimate application. Given that legal regulatory practices in diverse regions require identification of each compound in a product, this makes the regulation of biostimulants virtually impossible. It remains however, that there is a real need to ensure that all products on the market have clear benefits to crop productivity.

With the difficulty in determining the mode/mechanisms of action of a complex multicomponent product in mind, the main aim of this thesis was to show a possible method for determining the biological activity of a biostimulant. The approach is predicted to be

suitable for the characterization of mode of action, as a bioassay for quality studies in batches, and /or identification of new biostimulants, as a powerful tool in the research and development in this field.

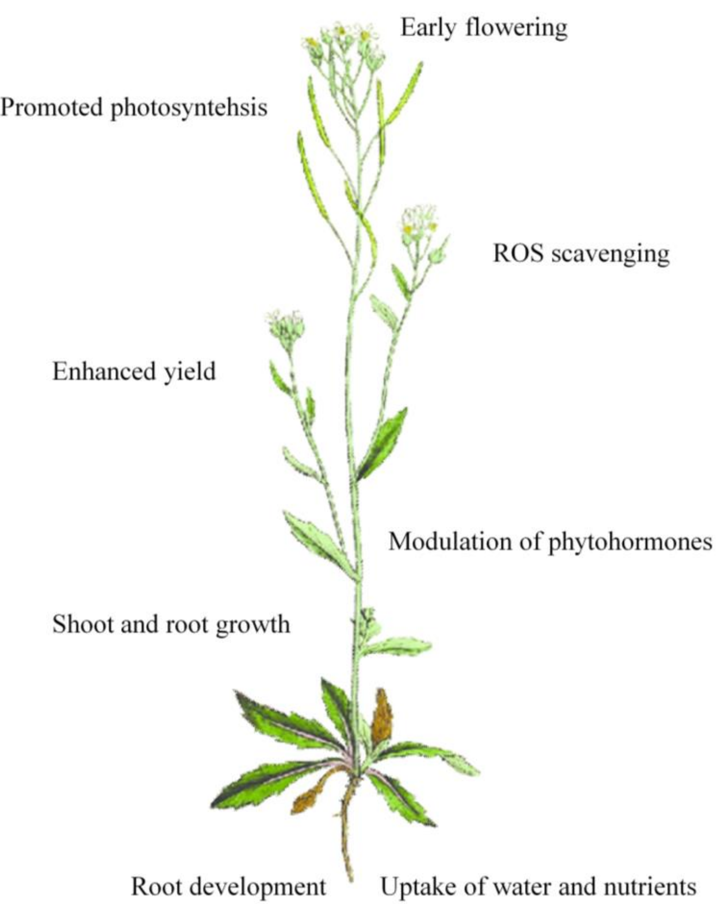

Figure 1.2: Main reported modes of action that biostimulants produce in agriculture (iconography from English Botany: 115, 1863, under the basionym of *Arabis thaliana* L.)

1.4 Plant phenotyping platforms for high-throughput screening bioassays.

In order to ensure higher plant productivity and better adaptation to various climatic modifications, it is necessary to understand the interaction between genotype and ambient defined as the phenotype of a plant [95,96]. Plant phenotyping is defined as a set of various methodologies and protocols to assess parameters and characteristics that can be expressed quantitatively or qualitatively [97,98]. On the other hand, throughout the evolution of plant breeding, hundreds even thousands of measurements have been necessary to select specific individuals or identify particular regions in the genome and this, is expensive, time consuming and tedious.

High-throughput phenotyping (HTP) has become an important tool in agriculture and contributes significantly to plant breeding and management approaches [99]. HTP facilities are divided based on the scale of the experiment performed and they offer the opportunity to combine various automated, simultaneous and non-destructive online methods (or sensors) monitoring multiple morpho-physiological plant traits. Small-scale screening platform bioassays based on RGB image allows for miniaturization of the assays to assess the biological response to different abiotic stress treatments [100]. They provide a complex picture of the plant growth and vigour in one run, and time-course measurements during the plant's life-span, showing the progression of growth. In addition, it reduces cost, labor and time for the analyses by improved data integration and remote sensing [100,101]. The broad spectrum of plant traits can be described by integrative phenotyping in multi-sensoric phenotyping platforms including imaging sensors for visible imaging (RGB imaging) and/or 3D imaging, imaging spectroscopy (hyperspectral imaging), thermal infrared imaging, and chlorophyll fluorescence [101–103]. The integrative phenotyping approach allows us to obtain more complex images on the possible mode of action of a biostimulant under specific environmental conditions [100].

Biostimulant functional characterization can be monitored in high-precision and high-resolution in a specific stage of plant development and can characterize the response to environmental conditions [104]. For this purpose, the use of high- throughput phenotyping platforms were recently proposed in order to characterize the biostimulant mode of action

and as an efficient tool for finding new bioactive substances [100,105]. Using HTS methods, these compounds can be applied in a broad concentration range under different growth conditions and in combination, followed by other species-based bioassays confirming their applicability for crops [104]. Although the creation of massive libraries of compounds and identification of their mode of action is a major challenge in agriculture, there are several examples that point to the HTS approaches as adequate tools for solving this issue. As an example, *Arabidopsis* root and leaf growth grown in 24 multi-well *in vitro* plates were used for the screening and evaluation of several important plant growth traits from a library of 10,000 diverse compounds [106]. Moreover, the success transferability of the results obtained previously to other crops of commercial interest, such as tomato, lettuce, carrots, among others has been demonstrated [106]. Focusing on *Arabidopsis* root development, 800 natural compounds for qualitative effects were screened using the Microphenotron platform [107].

Methods for the evaluation and validation of the mode(s) of action of complex biomolecules in higher plants like tomatoes using advanced phenotyping platforms [108–110], combined with metabolomic analysis have been also reported [111]. The effect of borage extracts on lettuce has also been analyzed using image analysis approaches [112]. In summary, non-invasive image analysis-based methods have allowed us to classify the effect of a compound application on plants under control or different stress conditions, pointing to this technology as a key for a faster and more efficient characterization of biostimulants.

CHAPTER 2.

MATERIALS AND METHODS

This section briefly summarizes the experimental equipment and methods required to carry out the analyses in the attached publications.

2.1 Plant material and growth conditions.

In ***Supplement II*** the optimization and validation of a phenotyping protocol was established in plants under control and salt stress conditions (**Figure 2.1**). *Arabidopsis thaliana* (accession Col-0) was used as plant material in all experiments. Seeds were surface-sterilized with a solution of 70% of EtOH and 0.01% Triton-X, sown on square plates (12 cm x 12 cm) containing 0.5x Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) (pH 5.7) supplemented with a gelling agent 0.6% Phytagel (Sigma–Aldrich, Germany) at a density of about one seed per 1.5 cm^2 to facilitate the manipulation in the subsequent transfer and maintained for 3 days at 4 ºC in the dark. After cold stratification, the plates were

transferred into a growth-chamber (CMP6010, Conviron Adaptis) with controlled conditions (22ºC, 16/8 h light/dark cycle, a photon irradiance of 120 µmol photons of PAR m^{-2} s^{-1}) and placed in a vertical position, thus preventing growth of the root into the solid media. Three days after germination, seedlings of similar size (one seedling per well) were transferred under sterile conditions into the multi-well plates [12- and 24-well plates (Jetbiofil, Guangzhou, China)]. Each well contained 2.7 mL (12-well plate) or 1.3 mL (24-well plate) of full MS medium (pH 5.7; supplemented with 0.6% Phytagel). Plates were sealed with perforated transparent foil allowing gas and water exchange.

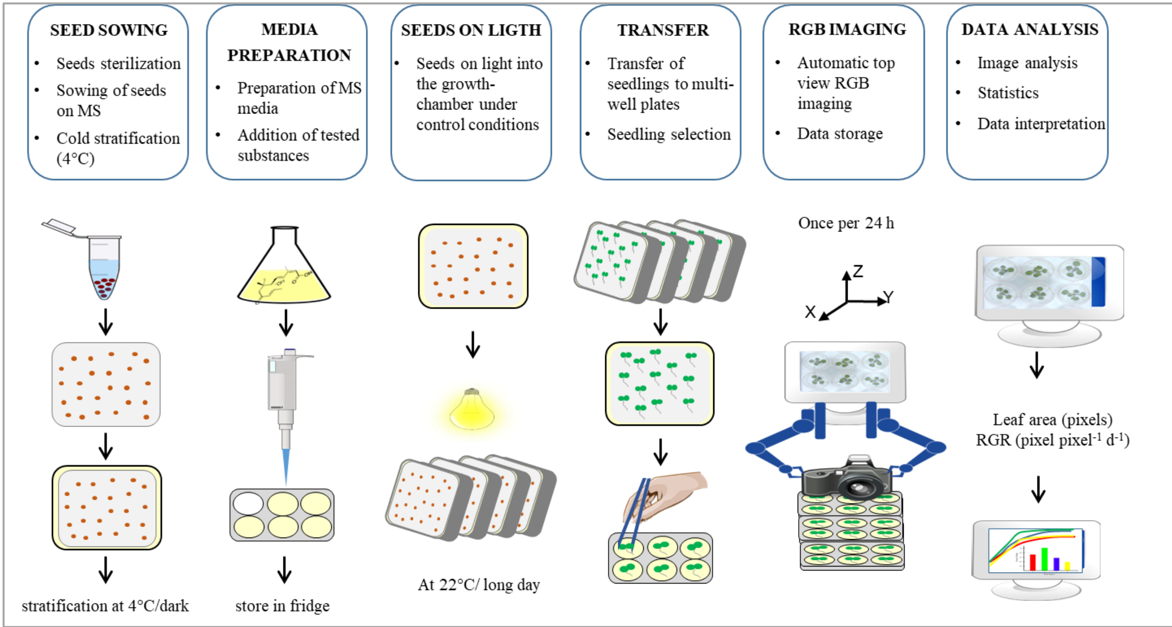

Figure 2.1: Protocol for high-throughput phenotyping for screening of *Arabidopsis* rosette growth in multi-well plates (more details in ***Supplement II, figure 2***).

To standardize the normal conditions of the protocol that could affect *Arabidopsis* rosette growth, different concentrations of MS (1x, 0.5x, or 0.25x) supplemented with sucrose (0, 0.1, or 1%) (pH 5.7; containing 0.6% Phytagel) were used. In the salt-stress experiment, 12- and 24-well plates were filled with 1x MS medium (pH 5.7; containing 0.6% Phytagel) supplemented with different concentrations of NaCl to achieve specific salinities (50, 75, 100, or 150 mM NaCl). To deal with interacting growth conditions and treatments,

12-well plates containing different MS concentrations (1x, 0.5x, or 0.25x) with or without salt stress (75 mM NaCl identified as representing medium salt stress) were used in a different experiment. The consecutive steps to perform the whole experiments included in the protocol were the same than previously described **(*Supplement II*)**.

A preliminary experiment using *Arabidopsis* seedlings supplemented with different concentrations of GABA (Sigma-Aldrich, Inc.) was performed to analyze the effect of some simple compounds as biostimulant. *Arabidopsis* seeds were sown and after 4 days in the dark and 3 days of germination, seedlings were transferred for continued growth into 24 multi-well plates filled with 2.71 mL 1x MS medium with/without three concentrations (0.1, 1 or 10 mM) of the compound and four different growth conditions: control and three concentrations of NaCl solution (50, 75 or 100 mM).

To improve the high-throughput of the protocol described in ***Supplement II***, for testing the application of biostimulants in *Arabidopsis* under control and salt stress condition, the use of 48-well plates was tested and validated. Put (1, 4-butanediamine dihydrochloride), Spd (N-(3-aminopropyl)-1, 4-butanediamine trihydrochloride), Spm (N-(3-Aminopropyl)-1,4-butanediamine trihydrochloride) and Pro (all purchased from Sigma-Aldrich, Inc.) were used for seed priming. Seeds were placed on square plates with MS individually supplemented with those compounds at four concentrations (0.001, 0.01, 0.1 or 1 mM) **(*Supplement III*)**. After 4 days in the dark and 3 days of germination, seedlings were transferred into 48 multi-well plates filled with 850 mL 1x MS medium with/without salt (75 or 150 mM NaCl solution) addition. Due to the high mortality of seedlings under severe salt stress conditions, three plates for the seedling in 150 mM NaCl were used to obtain sufficient reproducible data and an adequate number of measurable individuals.

An additional protocol was established for the performance of high-throughput emergence assays in maize (*Zea mays* L.) hybrid Koblens (KWS Osiva s.r.o., Czech Republic) (**Figure 2.2**). Seeds were imbibed with distilled water for 16 hours at 4ºC in the dark. Meanwhile, nursery trays TEKU JP 3050/160 T were filled with soil substrate (Substrat 2, Klassmann Deilmann, Geeste, Germany) and cut to fit into hydroponic inserts for standard PlantScreenTM measuring trays (Photon Systems Instruments, Brno, Czech Republic). The cut trays had 110 cells (volume of one cell equals to 21.5 mL), and they were also cut in the corners to allow for watering using a funnel. One seed per cell was then sown 1 cm deep into

the substrate. Each tray was watered to its full capacity with tap water or with a solution of NaCl at two concentrations: 75 or 150 mM NaCl. Afterwards, all trays were watered using 0.5 L of tap water (average conductivity around 56 mS/m) every third day until the end of the experiment. The trays were assigned to the control, moderate salt stress, and severe salt stress groups randomly at the beginning of the experiment and the experiment was repeated twice over different days to evaluate the reproducibility of the bioassay ***(Supplement IV)***.

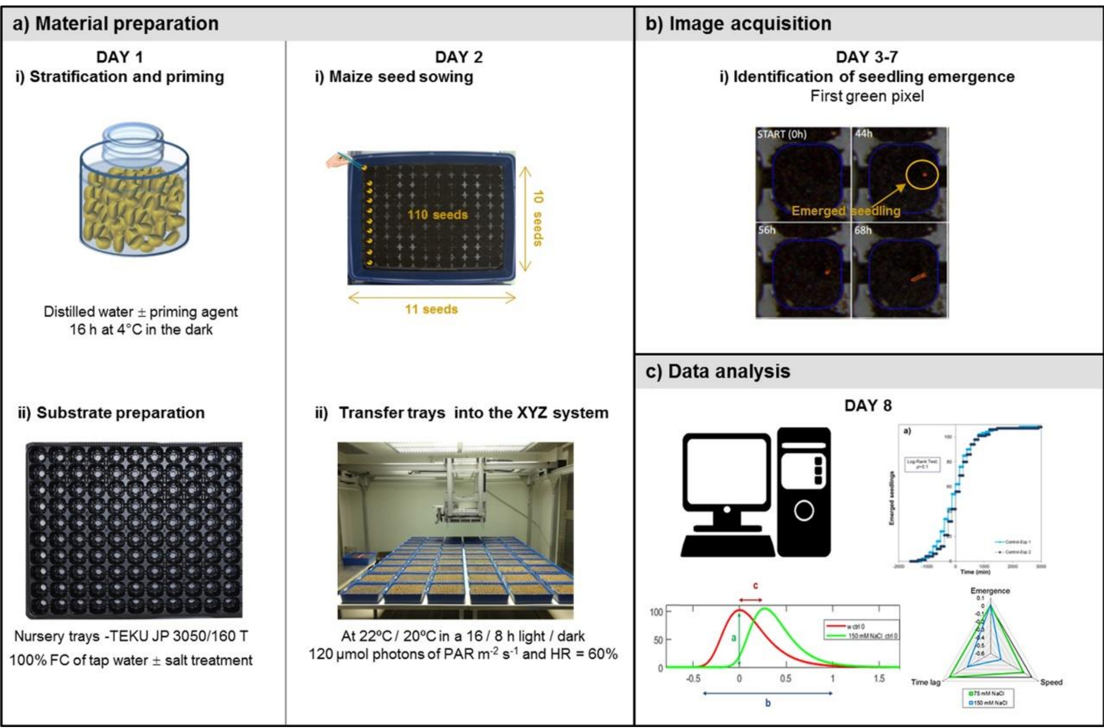

Figure 2.2: Protocol used for high-throughput screening of maize seedlings emergence under salt stress conditions. (a) Stratification of 110 maize seeds and sown into soil (b) Determination of seedling emergence as first "green pixel". (c) Analysis of the data by empiric emergence curve fitted to the Gompertz curve.

The power of the bioassay for the characterization of plant biostimulants was also evaluated. Maize seeds were primed with the same three PAs; Put, Spd and Spm in three concentrations (0.01, 0.1 or 1 mM). The treatment was applied during the imbibition phase described above. The solutions were removed before seed sowing and each treatment was evaluated for the control and in moderate and severe salt stress conditions (0, 75 or 150 mM,

respectively). In each of two independent experiments, one tray per variant (treatment and/or concentration) was analyzed, with a total of 21 trays including controls.

2.2 Phenotyping platform, experimental setup and assay conditions.

The multi-well plates with the transferred *Arabidopsis* seedlings were placed onto the OloPhen platform that uses the PlantScreen™ XYZ system installed in a growth chamber with a controlled environment and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czech Republic). The conditions were set to simulate a long day with a regime of at 22ºC/20ºC in a 16/8 h light/dark cycle, an irradiance of 120 µmol photons of PAR m^{-2} s^{-1} and a relative humidity of 60%. The PlantScreen™ XYZ system consists of a robotically driven arm holding an RGB camera with customized lighting panel and growing tables with a total area of approximately 7 m^2 with a capacity of 572 multi-well plates fixed in customized trays for accurate positioning of every plate. The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. RGB images (resolution 2500 x 2000 pixels) of a single plate with a file size of approximately 10 MB in the PNG compression format were 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 12 and 24 multi-well plate was performed once per day ***(Supplement II)*** and each 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days ***(Supplement III)***.

The trays with the sown maize seeds were placed onto the OloPhen platform described above. In an emergence experiment, the capacity of the system was 60 customized trays. Images were taken once every two hours over 5 days and the time of emergence was set as the first imaging time when the seedling was already visible. Some of the seedlings may not have emerged at all until the end of the experiment. For these, the total duration of the experiment was recorded and they were denoted as “censored” ***(Supplement IV)***.

The data of the whole experiments included in this section were automatically stored in PlantScreen XYZ database, exported by PlantScreen Data Analyzer software and analyzed using an in-house software routine implemented in MatLab R2015 ***(Supplement II,III, IV)***.

2.3 Biometric parameters.

In ***Supplement III***, the changes in the green area (pixels) were measured twice per day in each *Arabidopsi*s seedling using the aforementioned automatic system. From the obtained data, the relative growth rate (RGR) per hour or day was estimated for each replicate and variant as follows:

$$RGR = \frac{\ln\left(A_{G,t_i}\right) - \ln\left(A_{G,t_{i-1}}\right)}{t_i - t_{i-1}} \quad (2.1)$$

where t*i* is the *i* time (h or days) and A_G is the green area.

2.4 Determination of leaf color in *Arabidopsis* rosette under control and salt stress conditions.

For non-invasive estimation of the changes in leaf color, three vegetative indices (NGRDI, GLI, and VARI) were calculated which correlate with the plant biomass, nutrient status and tolerance to abiotic stress [113–115] (***Supplement III***). The values corresponding to particular color channels (red = R, green = G, and blue = B) were extracted for each pixel within the plant mask, and the vegetative indices were calculated as follows:

Normalized green red difference index (NGRDI):

$$NGRDI = (G-R) / (G + R) \quad (2.2)$$

Green leaf index (GLI):

$$GLI = (2G-R-B) / (2G + R + B) \quad (2.3)$$

Visible atmospherically resistant index (VARI):

$$VARI = (G-R) / (G + R - B) \quad (2.4)$$

Subsequently, indices representing particular seedlings were determined by calculating the mean values for each plant mask. The mean value for each 48-well plate was then calculated.

2.5 Statistical analysis and data presentation.

To assess the differences between the projected areas (pixels) of two or more groups of plants at a particular time-point, the non-parametric Kruskal-Wallis one-way analysis of variance by ranks was used in ***Supplement II*** and the parametric one-way analysis of variance (ANOVA) in ***Supplement III***. The Kruskal-Wallis test compares the medians of the samples in the respective groups, and calculates a *p*-value for the null hypothesis that all samples are drawn from the same population. ANOVA compares the variance between sample means with to the within group variances. When ANOVA was significant, the differences were determined using the Dunn-Sidák's correction. For analysis of multidimensional data, visual representations in the form of box plots, histograms, and animations, created using the MatLab R2015 software, were used ***(Supplement II)***. The relationship among traits was analyzed via Pearson's correlation. The significance of the regression was determined by applying a Student's t-test to the linear curves and after linearization of non-linear curves ***(Supplement III)***.

For each maize seed, the time of emergence was recorded ***(Supplement IV)***. The resulting data set consisted of several predictor variables (severity of the salt stress, presence and concentration of the biostimulant).

Firstly, for testing the difference in seedling emergence among various subgroups, the nonparametric log-rank test was used. This test is used to compare two or more emergence curves and determine whether the difference among them is significant. However, this test it is not suitable for capturing differences in various aspects of the emergence process. Parametric methods are more suitable for this purpose. The emergence of maize seedlings was secondarily analyzed by fitting the Gompertz curve to the empirical cumulative distribution function. The Gompertz curve is given by:

$$y(t) = A \exp(-x(t)) \tag{2.5}$$

where

$$x(t) = \exp(-k(t - t_m)) \tag{2.6}$$

where the factors A, k and t_m are described in the Results section.

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CHAPTER 3

RESULTS

3.1 Interactions involved in plant responses to stress conditions.

The first purpose in this doctoral thesis was to bring together information from plants exposed to salt stress conditions and discuss the possible links among the different groups of signaling molecules. In depth study was carried out on up to date information on the GABA pathway, synthesis and catabolism, and further analyses of the interactions involved in plant responses to stress conditions, suggesting highly conserved pathways connecting primary and secondary metabolism, with an overlap of regulatory functions related to stress responses and tolerance among phytohormones, AAs and PAs (***Supplement I***).

Once the knowledge about the complex mechanisms for perceiving the signals related to different stresses was reviewed, the development and optimization of a growing

protocol suitable for HTS of *Arabidopsis* rosette growth *in vitro* in multi-well plates under salinity was performed (***Supplement II***).

3.2 Standardization of the bioassay for HTS of *Arabidopsis* rosette growth in normal and stressed conditions.

The design of an assay for automated large-scale analysis of *Arabidopsis* rosette growth *in vitro* using multi-well plates was performed. To ensure an appropriate experimental procedure, we established and discussed the protocol schematized in ***Supplement II*** used for optimization and validation of the method, using plants grown under control and salt stress conditions. The protocol takes a total of 18 days and consists of several steps including seed sowing, cold stratification, transferring the seedlings into multi-well plates, time-course RGB-imaging, data processing and analysis (**Figure 2.1**).

After seedling transfer, the multi-well plates are placed into the PlantScreen^{TM} XYZ system and after about 24 h of acclimation, automated RGB-imaging is performed every day for the next 9 days.

3.2.1 Bioassay optimization and validation.

The experimental design for the assay requires a standardized protocol resulting in maximum homogeneity of plant growth on a single plate and among replicates. To define the most suitable screening conditions 12- and 24-well plates were prepared following the experimental scheme described in Section 2.1 with nine and six replicates per variant and analyzed for 9 days. The outcome of the analysis is represented as a single growth curve describing the increase in green area over time. In both cases there is a similar profile, showing highly significant exponential growth (***Supplement II, figure 3A***). Further, a curve describing the relative growth rate shows the same tendency but with higher values for those grown in 12-well plates (***Supplement II, figure 3B***). This indicates that the volume and space of the well are the main factors determining the observed difference. To optimize the assay, we used the Kruskal–Wallis test for evaluating statistically significant differences in rosette area between the plate replicates, which were randomly distributed within the growth

chamber. Unexpectedly, significant differences in the average green areas in both types of multi-well plate, in some cases reaching almost 50%, were observed (***Supplement II, figure 4)***. Taking into account the experimental set-up of the assay, the possible reasons for the differences could be either different micro-climatic conditions in the growth chamber or the non-randomized selection of the seedlings at the time of transfer. Experimentally discarded as the first rationale, more careful selection of seedlings resulted in standardization of the population heterogeneity between plate replicates (***Supplement II, figure 5***). Finally, to test the reliability of the method, we compared the green area estimated by automated RGB imaging with the weight of the rosettes determined manually. The rosettes of individual plants were harvested on the last day of measurements and their fresh weight (FW) was determined. Subsequently, correlations between the green area and FW were calculated using Pearson's coefficient and the significance determined using ANOVA to obtain highly significant correlations (R= 0.94 and 0.85, respectively) (***Supplement II, figure 6***). The relationship between green area and biomass of *Arabidopsis* rosettes showed more homogeneous size of the plants grown in 0.5x MS compared to 1x MS, suggesting that the nutritional conditions contribute to the population phenotype.

3.2.2 Standardization of control conditions for the bioassay.

To select our standardized normal conditions, we tested whether MS concentration and the addition or not with sucrose influenced *Arabidopsis* rosette growth. First, MS medium without sucrose was used in different concentrations; 0.25x, 0.5x or 1x. A clear concentration-dependent increase in rosette area was found, indicating that 1xMS is the best growing medium for *Arabidopsis* seedlings *in vitro* (***Supplement II, figure 8A***).

The presence of sucrose in the growing medium led to substantial changes conditioning seed germination and modifying plant metabolism. Hence, we tested how the exogenous addition of sucrose alters the growth of *Arabidopsis* seedlings and no significant differences were found (***Supplement II, supplementary figure S2***). Taking into account these results, we decided to use 1xMS without sucrose as the standard growing medium for our assay.

3.2.3 Use of the bioassay in salt-stress studies.

To illustrate the potential of our assay, we further optimized the methodology for evaluating plant response to stress. An experiment in which the effect of salt on *Arabidopsis* rosette growth was tested using 1x MS medium supplemented with different concentrations of NaCl (50, 75, 100 or 150 mM) was performed. Three replicates of 24-well plate were used for each tested variant, with no significant differences among them throughout the experiment (***Supplement II, figure 9***). Both time-dependent increase in shoot area and RGR were found to be negatively affected by NaCl treatment in a dose-dependent manner (***Supplement II***, ***figures 10A, B***). The severe salt-stress conditions (100 or 150 mM NaCl) showed very dramatic growth inhibition and fast senescence (yellow tissues) leading to death. Overall, these results proved the potential of the assay to be used as a tool for salt-stress studies.

In our experiments, it was also observed that salinity induced growth inhibition and *Arabidopsis* tolerance capacity are defined by an interaction between salt and the nutrient concentrations in the growing medium. The effect of 75 mM NaCl on *Arabidopsis* rosette growth was tested in MS medium of three different strengths. Each variant comprised three replicates on a 12-well plate and no statistical differences among them were found (Kruskal-Wallis test, ***Supplement II, supplementary figure S3***). Higher tolerance to salinity was found in plants grown in the 0.5x MS medium than those grown in 0.25x or 1x MS (***Supplement II, figure 11 and table 3***), and a higher number of dead and smaller plants were observed for the combination 1x MS and salinity (***Supplement II, supplementary figure S4***). These results suggest that specific nutrients may be responsible for plant sensitivity to stress and that a reduction in their concentration could delay the senescence effect characteristic of the second phase of the stress response.

Finally, to evaluate the use of our high-throughput method for testing libraries of compounds, we performed a preliminary study with *Arabidopsis* plants treated with GABA, as an important molecule regulating plant stress response, (***Supplement I***) under control and salt stress conditions. During the experiment, we used a 1x MS medium supplemented with three concentrations of NaCl (50, 75 or 100 mM) and for GABA treatment, the medium was supplemented with three different concentrations (0.1, 1 or 10 mM). The 4-day old seedlings

were transferred for continued growth under four different conditions: control, slight salt (50 mM NaCl), moderate salt (75 mM NaCl) and severe salt (100 mM NaCl). Using this approach, each compound could be simultaneously tested at different concentrations and plant growth conditions.

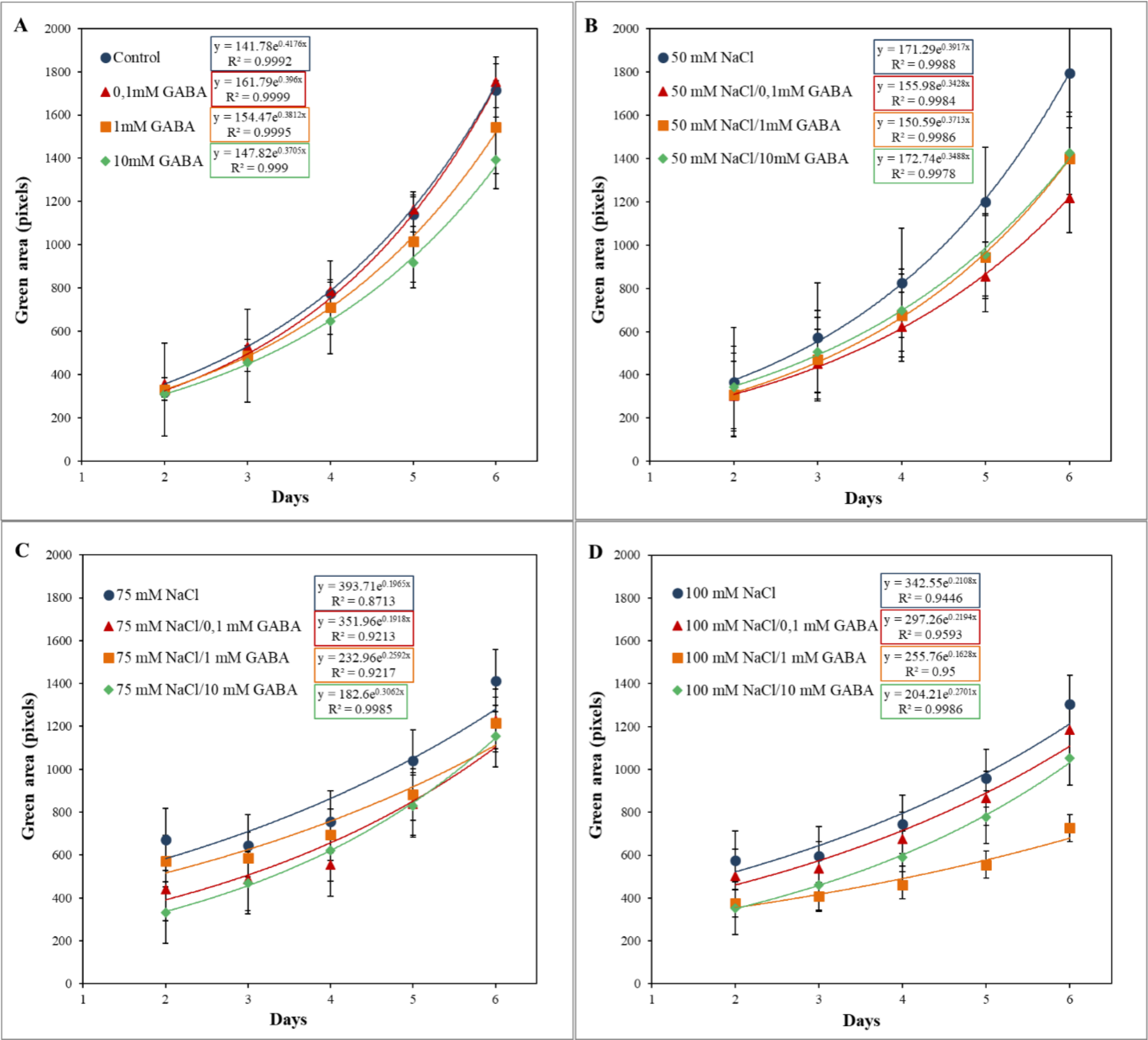

Figure 3.1: Variation in *Arabidopsis* rosette growth from primed seeds with slight, moderate and severe salt stress. Green area (pixels) of four DAG *Arabidopsis* seedlings primed with GABA at three concentrations (0.1, 1 or 10 mM) and grown for 6 days in 48-well plates under **A)** control, **B)** slight (50 mM NaCl), **C)** moderate (75 mM NaCl), and **D)** severe (100 mM NaCl) salt stress conditions. *Mean* ± *SE*. The equation of the curve and the Pearson's correlation coefficient were calculated.

As shown in **Figures 3.1** and **3.2**, seedlings grown on media supplemented with GABA did not result in significant differences in *Arabidopsis* rosette growth. In moderate and severe conditions higher growth with lower concentrations of GABA up to the fourth day are possible. There was no growth after that. On the other hand, no stress-alleviating effect was observed with any treatment or growth condition. Consequently, GABA was discarded as a potential compound to be used as plant stress mitigator.

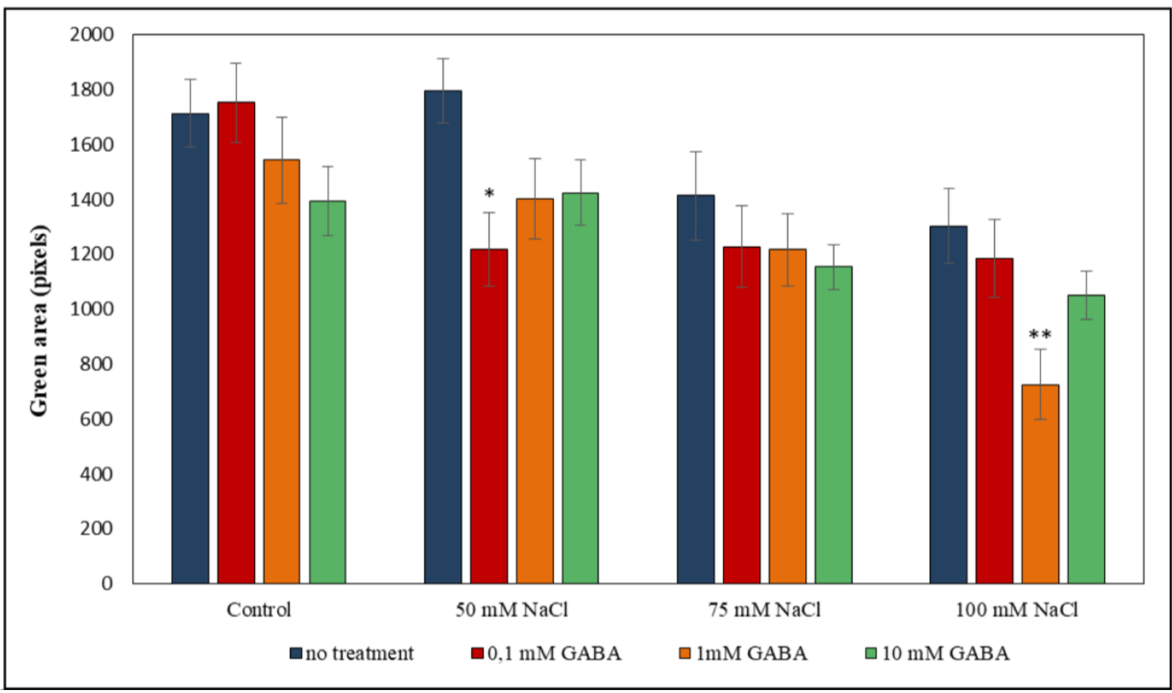

Figure 3.2: Comparison of *Arabidopsis* rosette growth grown for 6 days in 48 well-plates under four different growth conditions and primed with GABA at three concentrations (0.1, 1 or 10 mM). Statistical analysis was performed with the Kruskal-Wallis test. Asterisks indicate differences relative to the non-treated variant ** $p < 0.01$; * $p<0.05$.

3.3 HTS of *Arabidopsis* rosette growth as a suitable assay for the characterization of biostimulants under control and salt stress conditions.

To characterize the mode of action in different biostimulants, we optimized our protocol described in ***Supplement II*** based on the analysis of early seedling development in *Arabidopsis* plants under salt stress conditions. The sensitivity of the analysis allows scoring differences in the rosette area over only 2-h-long intervals. This allows for the possibility for further assay miniaturization to increase the number of plants per variant and measurements per day. Therefore, 48 well plates were used, with four biological replicates randomly distributed in the platform. The seedlings were measured twice per day (at 10:00 h and at 16:00 h) for 7 days, ending with 14 data points per plant (***Supplement III, supplementary figure S1***). As in previous experiments, we found a negligible difference in the green area among replicates (***Supplement III, figure 4A***), which was also exhibited by similar RGR. The effect of seed size on the variability of early seedling development was another factor to take into account in the optimization of the previous protocol, with the objective of increasing the technical precision of the assay. The seed batch was separated into three different size categories: 250–280, 280–300, and >300 µm, and given their abundance and good growth performance, 280–300 µm seeds were selected as the standard for subsequent experiments. In total, we developed a very fast *in vitro* bioassay that enables a simultaneous study of different growth conditions without compromising the number of variants, replicates or plants per treatment.

Seed priming with certain compounds (e.i., biostimulants) is an innovative alternative for coping with negative stress effects. For this reason, they can improve the efficiency of crop production and yield under sub-optimal conditions. Many biostimulants contain complex mixtures of biologically active compounds and, hence, the testing should be performed over a broad concentration range allowing evaluation of concentration dependent effects. This fact renders necessary comparison of a wide number of variants per run only possible using high-throughput approaches as we have described above.

To go further in the optimization of our bioassay for the characterization of biostimulants, we decided to treat *Arabidopsis* plants with simple compounds related to plant

stress response. In this case, based on the negative results obtained using GABA as a treatment, we decided to apply three PAs (Put, Spd, Spm) and the AAs Pro over the concentration range (0.001, 0.01, 0.1 or 1 mM) as priming agents. The results revealed differences in the mode of action for the four compounds applied to *Arabidopsis* rosette growth (***Supplement III, figures 3–9***) and, resulted in significant differences in rosette growth and RGR, in both control and salt stress conditions. Put and Spd were identified as plant growth promotors and stress alleviators, whereas Spm was less efficient and its positive effect was concentration dependent (***Supplement III, figures 5, 10, and 11***). Spm can therefore be classified as a plant growth promotor rather than a stress alleviator. The priming with Pro was less effective than with PAs, and the most positive effect was in the germination rate under a severe salt stress. This may have resulted from the fact that enhanced Pro levels in plants occur in the first phases of seed germination and the seed-to-seedling transition. Thus, integrating a wide range of concentrations in the same bioassay combined with different stress levels for the testing of biostimulants constitutes a viable strategy for biostimulant mode of action characterization.

3.3.1 Effect of biostimulants on *Arabidopsis* seedling establishment.

Analysis of the results obtained from the above bioassay revealed the effect of seed priming on early seedling establishment. The green area of the *Arabidopsis* seedlings immediately after the transfer to 48 well plates was analyzed, corresponding to time zero of the HTS. Without salt stress, the size of seedlings established from primed seeds differed significantly from the size of seedlings resulting from non-primed seeds (***Supplement III, figure 8***). These results showed that our method can record traits in a complex manner that describes the effect of priming on all important stages of early development.

3.3.2 Effect of biostimulants on leaf color of *Arabidopsis* rosettes under control and salt stress conditions.

The degradation of chlorophyll, manifested as a change in leaf color, represents an important marker in stress-related plant studies. As described in the Methods section, the leaf color of the *Arabidopsis* rosettes and the three vegetation indices as indicators of leaf color change was determined. The values obtained depended on the seed priming treatment and salt intensity (***Supplement III, figures 9A, B***). Of the three indices, GLI exhibited the highest sensitivity to salt stress. The seed priming with Put and Spd generated *Arabidopsis* rosettes with the highest greenness under control and salt stress conditions (***Supplement III, figure 9E***).

3.3.3 PBC Index for estimating biostimulant mode of action.

Plant Biostimulant Characterization (PBC) index is a tool created to yield straight-forward information allowing simple selection of the best treatment under each condition. The index can represent up to four traits: seed germination rate (%), seedling establishment (green pixels after transfer to 48 well plates), growth capacity (pixels) and the leaf color index (GLI) for primed and non-primed seeds. The calculation is performed by the $\log_2$ of the differences among the controls of the different growth conditions and variants (compound and concentration). A parallel coordinate plot is the representation of the values that allows better visualization of the variant-induced changes in each trait (***Supplement III, figures 10, 11***).

The concentration effect of the tested compound under different growth conditions was determined by summing the relative changes ($\log_2$) obtained for the previous representation. This sum yielded a total that can reach a positive (biostimulant- blue) or negative (inhibitor-red) value. The new results were plotted in a multidimensional graphic “radar chart” using concentrations as a quantitative variable (***Supplement III, figure 12***).

In conclusion, with this approach we confirm that Put was the most efficient plant growth promotor and stress alleviator with higher values in each concentration and growth condition, compared with the controls. The remaining compounds exhibited a concentration and growth-condition-dependent response. These results confirm that the presented MTHTS approach is an adequate tool for fast and simultaneous analysis of various concentrations and growth conditions for identification and, especially, characterization of the mode of action associated with new biostimulants.

3.4 CroSeEm as HTS of maize emergence for characterizing priming agents in control and salt stress conditions.

The following aim of this Ph.D. dissertation is the transference of the interesting results observed in the characterization of biostimulants mode of action to a crop (biological translation) and the evaluation under different growth conditions to cope with the decrease in agricultural production worldwide. For this reason, it is necessary to develop more sophisticated high-throughput bioassays for testing different priming agents and to provide information about the developmental and physiological status of the treated plants. Such understanding may contribute to advancing selection of the most adequate compound, concentration per crop, stress condition and intensity. Crop Seedling Emergence (CroSeEm) is a high-throughput bioassay that automatically monitors the first appearance of the coleoptile in maize under control and saline conditions.

3.4.1 Setup for *CroSeEm* analysis.

Nonparametric log-rank test was used for testing the difference in seedling emergence in two different experiments. The test is used to compare two or more emergence curves and find whether the difference among them is significant. However, the results obtained conclude that the log-rank test compares two or more emergence curves but it is not suitable for capturing differences in various aspects of the emergence process. A more detailed description of this deduction is given in ***Supplement IV***.

Therefore, the emergence of maize seedlings was analyzed by the parametric method Gompertz curve (described in Materials and Methods section) to the empirical cumulative distribution function. From the fitted emergence curves three traits were extracted:

1. **Final emergence rate** (i.e. the total number of seedlings that had emerged at the end of the experiment, divided by the total number of seedlings per tray = 110) described as coefficient A in Eq. (2.5).

2. **Time lag** (i.e. difference between the positions of the peak for the control and the treatment) measured by t_m in Eq. (2.6).

3. **Emergence synchronicity** (i.e. a measure of the width of the peak of the derivative of the fitted Gompertz curve) also denoted as emergence "speed" which corresponds to k in Eq. (2.6).

As a result, we observed a delay in the speed of maize seedling emergence compared to the control in two independent experiments when they were sown with 75 mM NaCl (***Supplement IV, figure 6***). This trait, together with the time lag, was particularly affected when 150 mM NaCl was used. However, the final germination rate was almost unchanged, suggesting that this trait was less of a stress indicator. These results showed that the three extracted traits should be independently analyzed because the growth conditions altered them to different extents. Thus, analyzing them separately is a more sensitive and reproducible approach for the characterization of maize emergence under salinity.

3.4.2 Characterization of priming agents.

To evaluate the use of HTS of maize emergence for the characterization of priming agents, maize emergence was analyzed using seeds primed with the same PAs and concentrations used in ***Supplement III*** in control and salt stress conditions (**3rd experiment-** ***Supplement IV, table 1***).

Several aspects of the crosstalk between the compounds and the growth conditions can be observed. None of the PAs affected the final germination rate under any growth

conditions. Under control conditions, all the traits remain largely unaffected with the possible exception of time lag –primed seeds tend to emerge slightly sooner than the control (***Supplement IV, figure 7***). However, under salt stress, the effect of the PAs becomes visible. Under moderate salt stress, Spd tends to increase the time lag and decrease synchronicity in all three tested concentrations, whereas priming with Put at high concentrations increased the emergence speed without modifications in the time lag. Under severe salt stress, the effect becomes even more pronounced. Put in all the three tested concentrations increased the time lag and in 0.01 mM concentration improved the emergence synchronicity (***Supplement IV, figure 7***). Overall, priming of maize seeds with Put was the most efficient treatment. Interestingly, similar positive effects of priming with Put were found in ***Supplement III***. Altogether, the results showed that CroSeEm is good approach for characterizing priming agents in maize populations.

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

The present thesis address the development of reproducible HTS bioassays to be used for selecting and characterizing biostimulants and their modes of action under different salt stress concentrations.

- For this purpose, in depth review about the plant response to stress was performed. It pointed to the existence of a highly conserved pathway expressed in plants under stress, in which the crosstal of phytohormones, PAs and/or GABA define plant stress tolerance. The mode of action of these compounds strongly suggest them as potential candidates to mitigate the adverse effects of multiple stresses.

- The development and optimization of HTS method based on *Arabidopsis* rosette growth in multi-well plates for the characterization of biostimulants mode of action was achieved. Based on their contribution to the plant development and stress tolerance, their

mode of action could be define such as plant growth promotor/inhibitor and/or stress alleviator.

- In order to create strategies for improving crops, a HTS method of seedling emergence "CroSeEm" was developed. It is suitable for characterizing different maize lines and/or seed priming agents against salinity.

- Overall, it was demonstrated that the use of PAs as seed priming agents can be a useful biotechnological practice to improve salt stress response of plants.

In summary, we consider that the new protocols based on HTS methods could make easier and faster the identification of the mode of action for known biostimulants and help in the identification of new ones.

Further research lines raised while working for this Ph.D. dissertation are based in the extension of the application of these methods to several crops. Moreover, the developed technology is also suitable for the selection and studies of different genotypes and transgenic lines under different stress conditions. To conclude, the transference of the knowledge acquired on the bioassays of this work for the performance of field high-throughput screening phenotyping experiments is another plan to accomplish.

CHAPTER 5.

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Review Article

Phytohormones and polyamines regulate plant stress responses by altering GABA pathway

Kateřina Podlešáková[1], Lydia Ugena[1], Lukáš Spíchal, Karel Doležal, Nuria De Diego*

Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, CZ-78371, Czech Republic

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ABSTRACT

In plants, γ-aminobutyric acid (GABA) accumulates rapidly in response to environmental stress and variations in its endogenous concentration have been shown to affect plant growth. Exogenous application of GABA has also conferred higher stress tolerance by modulating the expression of genes involved in plant signalling, transcriptional regulation, hormone biosynthesis, reactive oxygen species production and polyamine metabolism. Plant hormones play critical roles in adaptation of plants to adverse environmental conditions through a sophisticated crosstalk among them. Several studies have provided evidence for the relationships between GABA, polyamines and hormones such as abscisic acid, cytokinins, auxins, gibberellins and ethylene, among others, focussing on the effect that one specific group of compounds exerts over the metabolic and signalling pathways of others. In this review, we bring together information obtained from plants exposed to several stress conditions and discuss the possible links among these different groups of molecules. The analysis supports the view that highly conserved pathways connect primary and secondary metabolism, with an overlap of regulatory functions related to stress responses and tolerance among phytohormones, amino acids and polyamines.

Introduction

Plants are exposed to many unfavourable environmental alterations defined as abiotic or biotic stresses and have developed complex mechanisms for perceiving the signals related to different stresses. These mechanisms permit them to retard their growth and metabolism, thus maximizing their ability to survive under stress [1]. However, plant response to stress is a highly dynamic process dependent on the severity and duration of the stress, as well as on the fitness and preparedness of the plant itself and its developmental stage [2].

γ-Aminobutyric acid (GABA) is a ubiquitous four-carbon non-protein amino acid which is conserved from bacteria to plants and vertebrates. In plants, one of its main roles is to control the stress responses [3]. Postulates to explain alterations in GABA metabolism include roles in herbivore deterrence, pH regulation, redox regulation, energy production and maintenance of the carbon/nitrogen (C/N) balance [4]. However, only a limited number of studies have sought to integrate its complex mode of action.

Many other plant molecules play important roles in integrating stress signals and controlling downstream stress responses by modulating gene expression and regulating a range of transporters/pumps and biochemical reactions [5]. They include calcium (Ca^{2+}), the hormones abscisic acid (ABA), jasmonates (JA) and salicylic acid (SA), amino acids such as proline (Pro) and polyamines (PA). In recent years many studies have evaluated the effect of their exogenous application as stress alleviators or as a means of understanding stress responses and tolerance. Moreover, being natural compounds, their use offers an alternative to synthetic protectants, contributing to more sustainable and environmentally compatible agricultural practices. In addition, elevated endogenous levels of these compounds in food have shown several health benefits, reducing the risk of many diseases, preventing obesity and supporting longevity [6,7].

In plants, exogenous application of GABA increases leaf turgor and Pro accumulation under stress condition [8–10]. The application of plant hormones or PAs can also ameliorate the adverse effect of stress by activating an anti-oxidative [11–13]. Here, we summarize the recent

Abbreviations: ABA, abscisic acid; CK, cytokinin; GABA, γ-aminobutyric acid; IAA, indole-3-acetic acid; JA, jasmonic acid; SA, salicylic acid; PA, polyamines; Pro, proline; Put, putrescine; Spd, spermidine; Spm, spermine

* Corresponding author.

E-mail addresses: katerina.podlesakova@upol.cz (K. Podlešáková), lydia.ugena@upol.cz (L. Ugena), lukas.spichal@upol.cz (L. Spíchal), karel.dolezal@upol.cz (K. Doležal), nuria.de@upol.cz (N. De Diego).

[1] These authors contributed equally to this work.

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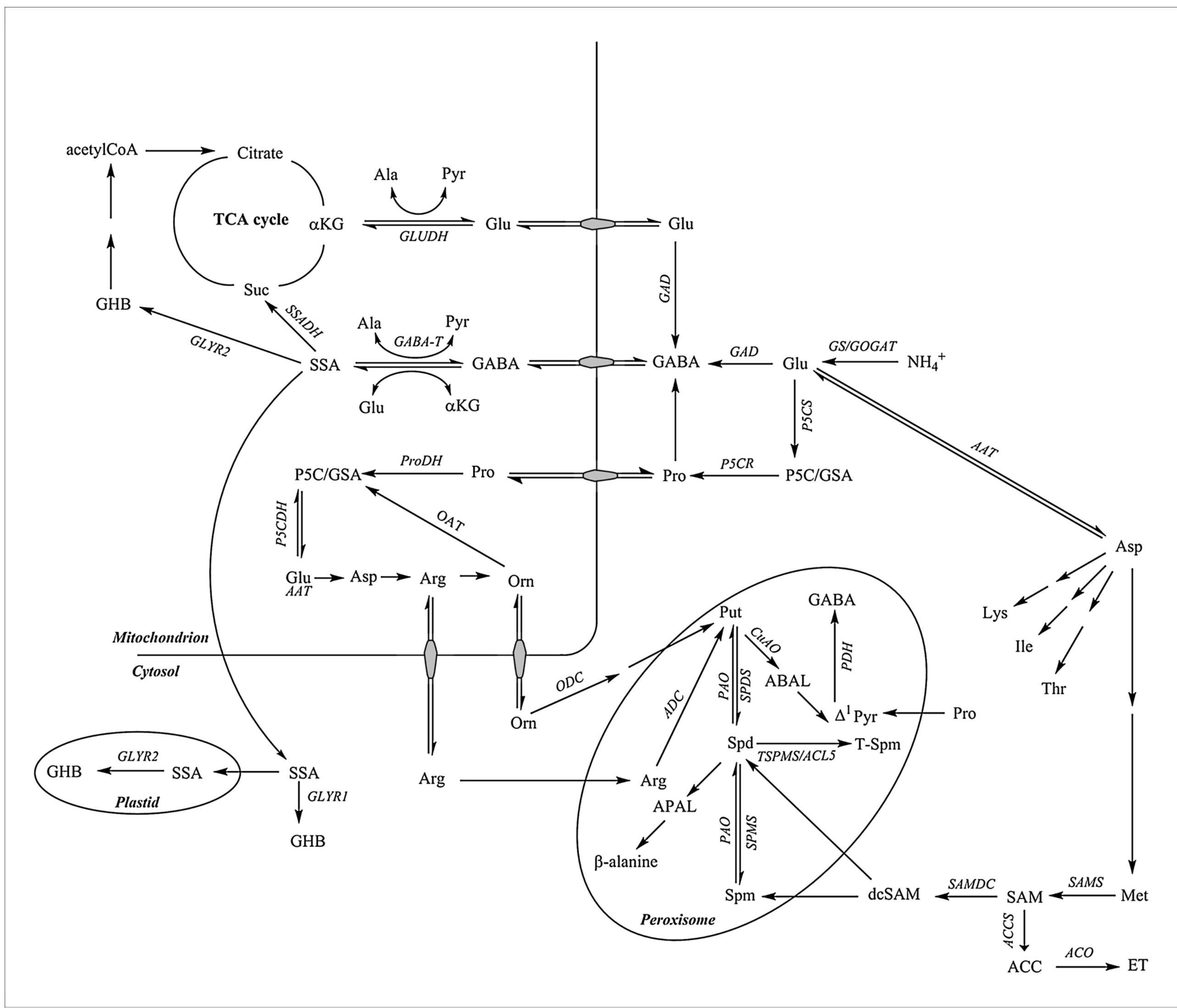

Fig. 1. GABA metabolism. AAT (Asp aminotransferase), ABAL (4-aminobutanal), ACC (1-aminocyclopropane-1-carboxylic acid), ACO (ACC oxidase), ACCS (ACC synthase), ADC (arginine decarboxylase), Ala (alanine), AMADH (NAD^+-dependent aminoaldehyde dehydrogenases), AO (amine oxidase), APAL (3-aminopropanal), Arg (arginine), Asp (aspartate), CuAO (copper-containing AO), dcSAM (decarboxylated SAM), ET (ethylene), GABA (γ-Aminobutyric acid), GAD (glutamate decarboxylase), GABA-T (GABA transaminase), GHB (γ-hydroxybutyrate), Glu (glutamate), GLUDH (glutamate dehydrogenase), GLYR1 (glyoxylate reductase 1), GLYR2 (glyoxylate reductase 2), GSA (Glu-semialdehyde), GS/GOGAT (glutamine synthetase/glutamate synthase), αKG (α-ketoglutarate), Ile (isoleucine), Lys (lysine), Met (methionine), OAT (Orn-d-aminotransferase), ODC (Orn decarboxylase), Orn (ornithine), PAO (polyamine oxidase), PDH (Δ1-pyrroline dehydrogenase), P5C (pyrroline-5-carboxylate), P5CDH (P5Cdehydrogenase), P5CR (P5C reductase), P5CS (P5C synthase), Pro (proline), ProDH (proline dehydrogenase), Put (putrescine), Δ^1 Pyr (pyrrolidin-1-yl), Pyr (pyruvate), SSA (succinic semialdehyde), SSADH (SSA dehydrogenase), SAM (S-adenosyl-methionine), SAMDC (SAM decarboxylase), SAMS (SAM synthetase), Spd (spermidine) Spds (Spd synthase), Spm (spermine), SPMS (Spm synthase), Suc (succinate), T-Spm (Thermo-spermine), TSPMS/ACL5 (T-Spm synthase), Thr (threonine).

knowledge about the GABA pathway, synthesis and catabolism, and further analyse interactions involved in plant responses to stress conditions, suggesting that important crosstalk between GABA, PA pathway and phytohormones exist, regulating stress tolerance.

The GABA pathway

GABA synthesis

GABA is synthesized mainly from L-glutamate (Glu), catalysed by glutamate decarboxylase (GAD) (Fig. 1). GAD has an acidic pH optimum and is activated by the increasing cytosolic H^+ and Ca^{+2} ions which usually accompany stress. Ca^{+2} ions, in turn, complex with calmodulin which binds and activates some GAD isoforms [14,15]. The *Arabidopsis* genome contains five genes encoding *GAD*. *GAD1/2/4* possess a C-terminal calmodulin (CaM)-binding domain, which allows *in vitro* activity to be activated at neutral pH by Ca^{2+}/CaM. However, *in silico* analysis suggests that *GAD3* and *GAD5* proteins are CaM independent [16]. This has been also described for rice and apples [17,18]. In *Arabidopsis*, expression of *GAD1* is predominantly in roots [19]. The *GAD2* isoform is constitutively expressed in all organs [20,21], though expression is lower in siliques [22], while expression of the other *GAD* genes is generally weak in all organs with the exception of *GAD4* and *GAD5* in flowers. *GAD3* and *GAD4* are present at moderate levels in young leaves, sepals, carpels and immature siliques [23].

The expression of different *GAD* genes can change during different plant processes and according to growth conditions (Table 1). The ubiquitous expression of *GAD2* in *Arabidopsis* was enhanced during senescence [24] and under high salt stress [25], but downregulated in hypoxia [22], whereas, *GAD1* expression did not respond to hypoxia

Table 1
Expression (↑- upregulation and ↓- downregulation) of genes related to GABA and polyamines pathway during different plant processes and growth condition.

		Species	Tissue	GABA pathway	Polyamines pathway	Reference
Plant processes	Senescence	*Arabidopsis*	Leaves	*GAD1* ↑	–	[154]
	Senescence + N-Remobilization	*Arabidopsis*	Leaves	*GAD2* ↑	–	[24]
	Fruit ripening	*Tomato*	Fruits	*SlGAD2; SlGAD3* ↓ *SlGAD1* ↑ *SlGABA-T2* ↓	–	[155]
	Pollen germination	*Nicotiana tabacum*	Pollen tube	*GAD1; GAD3* ↑	–	[156]
	Germination	*Rice*	Seedlings	*OsGAD; OsGABA-T* ↓	*OsADC; OsODC; OsDAO; OsPAO* ↑	[157]
			Plantlets	*OsGAD3* ↑; *OsGABA-T* ↓	*OsODC 1-3; OsDAO* ↑	
Plant stress	Salinity	*Arabidoposis*	Seedlings	–	*ADC1; ADC2* ↑ *SAMDC2;SPMS* ↑ *ACL5* ↓	[158]
		Arabidopsis	Plantlets	*GAD1*↓; *GAD2*↑; *GAD4*↑; *GAD3 nd; GAD5 nd; GABA-T*↑; *SSADH*↑	–	[25]
		Arabidopsis	Seedlings	*GAD1* ↓ *GABA-T ; SSADH* ↑	–	[159]
		Arabidopsis	Shoots and Roots	*GAD 3/4* ↑	–	[160]
		Arabidopsis	shoots	*GAD 4* ↑	–	[26]
		Rice	Seedlings	–	*SAMDC* ↑	[161]
		Maize	Leaves & Roots	*ZmGAD1* ↑	–	[162]
		Tomato	Fruits	*SlGAD2; SlGAD3* ↓	–	[163]
	Drought	*Arabidopsis*	Seedlings	–	*SPMS* ↑ *ACL5* ↓	[158]
		Arabidopsis	Leaves	–	*ADC2* ↑ *SPDS1 ; SPMS* ↑	[86]
		Arabidopsis	Shoots	*GAD1* ↑ *GAD 3/4* ↑	–	[160]
		Arabidopsis	Seedlings	*GAD1*↓ *GAD4 ; GABA-T ; SSADH* ↑	–	[159]
		Rice	Seedlings	–	*SAMDC* ↑	[161]
		Maize	Leaves & Roots	*ZmGAD1* ↑	–	[162]
		Lotus tenuis	Shoots	–	*ADC* ↑	[164]
	Salinity (S)- Drought (D)	*Rice*	leaves	–	*ADC2, SPD/SPM2, SPD/SPM3*↑ *S-SAMDC4*↑ and *D-SAMDC2*↑	[165]
	Osmotic	*Arabidopsis*	Shoots	*GAD1* ↑ *GAD3/4* ↑	–	[160]
		Arabidopsis	Leaves	–	*ADC2* ↑	[161]
	H_2O_2	*Panax ginseng*	Seedlings	*PgGAD* ↓	–	[27]
	Hypoxia	*Arabidopsis*	Roots	*GAD4* ↑ *GAD2* ↓	–	[22]
		Arabidopsis	Leaves	*GLYR1; GLYR2* ↑	–	[166]
		Melon	Roots	–	*DAO; PAO* ↑ *ADC; ODC; SAMDC* ↑	[71]
	Anoxia	*Camellia sinensis*	Leaves	*CsGAD1 ; CsGAD2* ↑	–	[70]
	Anoxia + Mechanical damage	*Camellia sinensis*	Leaves	*CsGAD2* ↑	–	[28]
	Cold	*Arabidopsis*	Plantlets	–	*SAMDC* ↑	[167]
		Arabidopsis	Plantlets	–	*ADC1* ↑	[168]
		Arabidopsis	Leaves	*SSADH*↑	–	[159]
		Rice	Roots	–	*SPDS* ↑	[161]
		Maize	Leaves	*ZmGAD1* ↑	–	[162]
		Panax ginseng	Seedlings	*PgGAD* ↑	–	[27]
	Cold + Spm exogenous	*Camellia sinensis*	Seedlings	–	*CsADC; CsSPMS* ↑	[169]
	Wounding	*Arabidopsis*	Shoots	*GAD1* ↑ *GAD 3/4* ↑	–	[160]
		Arabidopsis	Leaves	–	*AOS* ↑ *ADC2* ↑	[170]
		Panax ginseng	Seedlings	*GAD* ↑		[27]
	UV-B	*Arabidopsis*	Shoots	*GAD1* ↑ *GAD 3/4* ↑	–	[160]
	Zinc	*Nicotiana tabacum*	Shoots	*GAD* ↑	–	[34]
	Selenium	*Arabidopsis*	Roots	*GAD4* ↑	–	[171]
	Cadmium	*Arabidopsis*	Leaves	*GAD1* ↑	–	[32]
		Arabidopsis	Leaves & Roots Roots	*GAD2* ↓ *GAD3 ; GLYR1* ↑	–	[33]
	Pathogenes	*Arabidopsis*	Rosette	*GAD1; GAD4; SSADH* ↑ *GLYR2* ↓	–	[130]
	Microorganism volatiles	*Arabidopsis*	Leaves	*GAD 2* ↑	–	[134,135]
		Arabidopsis	Leaves	*GABA-T* ↓	–	[132]

[22]. Under conditions of low O_2 and salinity, mainly the expression of *GAD4* was increased (Table 1) [22,26]. In this last report, however, the expression of *GAD1* and *GAD2* to salinity was unaltered [16,26]. This suggests that different growth conditions and the intensity and type of stress could modulate *GAD* expression differentially. Thus, it was reported that, in *Panax ginseng,* the expression of the only *GAD* isolated changes with stress [27]. In addition, the transcription levels were highly up-regulated by cold and wounding but declined severely with oxidative stress (Table 1). Interestingly, anoxia and wounding also increased the accumulation of high levels of *CsGAD2* mRNA but not *CsGAD1* in tea (Table 1) [28]. However, it was also shown that *CsGAD1* but not *CsGAD2* is activated by binding CaM, pointing to other Ca^{2+}-independent mechanisms for activation of *GAD* expression in some species under stress conditions.

Other studies have also established concentration- and species-dependent connections between heavy metals and GABA accumulation. For example, cadmium stress induced GABA accumulation in different tomato organs [29,30] but not in white lupin [31]. In *Arabidopsis*, some genes related to the GABA pathway have been also reported to be influenced by cadmium, but with contradictory results [32,33]. In tobacco, GABA accumulation and high *GAD* expression also conferred stress tolerance under moderate zinc stress but not with high concentrations (Table 1) [34]. It is clear that GABA metabolism is altered in plants under metal stress, but the intensity depends on the species, the type of metal and its concentration.

GABA catabolism

In plants, GABA initially accumulates mainly in the cytosol and is then transported into mitochondria [35]. Catabolism of GABA provides a substrate for both the TCA cycle and the electron transport chain, so it can serve as a useful metabolic substrate, providing energy and carbon skeletons under stress [36]. Thus, GABA is converted to succinic semialdehyde (SSA) by GABA transaminase (GABA-T) that uses either α-ketoglutarate (αKG) (GABA-TK), or pyruvate (GABA-TP) as amino acceptors, producing Glu or alanine (Ala) respectively (Fig. 1) [19]. Recent studies have shown that some plants also use glyoxylate as an amino acceptor to produce glycine, linking GABA metabolism and photorespiration [37].

In *Arabidopsis,* there is only one *GABA-T* encoding gene, *POP2* (*At3 g22200; Pollen-Pistil Incompatibility 2*), which is essential for growth and other processes [38]. Alteration in growth conditions can modify the *GABA-T* gene expression (Table 1). During salinity, deficiency of *GABA-T* expression causes root and hypocotyl developmental defects and alterations in cell wall composition [39].

In subsequent steps of GABA catabolism, SSA is oxidized to succinate by SSA dehydrogenase (SSADH) (Fig. 1) [40,41], or reduced to γ-hydroxybutyrate (GHB) by SSA reductase (SSR), also called GHB dehydrogenase (GHBDH), in the cytosol and chloroplast (Fig. 1) [42,43]. The reduction usually takes place under anaerobic conditions when the cellular $NADH:NAD^+$ ratio increases and as a consequence SSADH activity is inhibited (Table 1). *Ssadh* mutants are dwarfs with reduced leaf area that show necrosis, bleached spots, lower chlorophyll content and fewer flowers compared to wild type [44]. Under exposure to UV-B light or heat stress, *ssadh* mutants accumulate SSA, GHB and reactive oxygen intermediates which induce cell death [45]. GHB is also accumulated in response to cold stress, salinity and drought [46]. Finally, although there is still insufficient evidence from plants regarding GHB receptors, signalling and extracellular sensing, there is clear evidence that the quorum-sensing signal in some bacteria is modulated by GABA and GHB [47–49].

Recently, it was reported that enzymes catalysing reduction of glyoxylate to glycolate, namely glyoxylate reductase (GR1 and GR2), can also effectively reduce SSA, and in this case they are designated GLYR (Fig. 1) [42,50]. Two GLYR isoforms exist¸ NADPH-dependent cytosolic (GLYR1) and plastidial/mitochondrial (GLYR2) succinic semialdehyde/glyoxylate reductase, respectively [42,51,52]. *Arabidopsis GLYR1* and *GLYR2* are moderately expressed throughout the plant, including roots and imbibed seed, but *GLYR2* expression is more highly associated than *GLYR1* with leaves. Biochemical studies in *Arabidopsis* have revealed that these two *GLYR* isoforms appear to have physiological implications for the stress response, thus representing an alternative pathway that contributes to redox balance through the detoxification of both SSA and glyoxylate [46]. However, recent studies showed that GLYRs from *Arabidopsis,* apple and rice display higher affinity and catalytic efficiency for glyoxylate than for SSA. It is also known that approximately 85% of the cellular GLYR activity is attributed to cytosolic GLYR1 and 15% to the plastidial/mitochondrial GLYR2 [52]. The higher affinity of GLYRs for glyoxylate has clear physiological relevance in coping with toxic levels of glyoxylate that increases due to the increased rubisco oxygenase activity after stress induced stomatal closure [52].

GABA and other stress related amino acids

The next section is an overview of the interconnections of GABA with other stress related amino acids such as proline, alanine and those derived from aspartate pathway.

Proline

Traditionally, Pro has been considered to be the most stress-related amino acid in plants. The accumulation varies among species, but it can be 100-times greater under stress than under control conditions [53]. Apart from being osmoprotectant, Pro is involved in regulation of cellular homeostasis, including redox balance and energy status. It can act as a signalling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for recovery from stress [54]. However, although some studies consider its mode of action to be long-term and related to plant recovery and hardening [55,56], others take into account all the processes involved in the Pro pathway, including the regulation of synthesis, catabolism and transport, and these point to Pro homeostasis as being key to the plant's response [57]. The relationship between GABA and Pro in regulating stress tolerance has been also well discussed. For example, the exogenous application of GABA improved heat and drought tolerance in *Agrostis stolonifera* by enhancing osmotic adjustment and Pro synthesis, which maintained cell membrane stability and delayed leaf senescence [8]. On the other hand, in plants exposed to biotic stress caused by the bacterial pathogen *Agrobacterium tumefaciens*, Pro antagonizes the plant GABA-defence mechanism and a lower level of Pro accumulation induces less severe tumour symptoms [49,58].

Like GABA, Pro is derived from Glu *via* its reduction to Glu-semialdehyde by Glu-5-semialdehyde dehydrogenase (GSADH). Glu-semialdehyde can spontaneously convert to pyrroline-5-carboxylate (P5C), which is further reduced by P5C reductase (P5CR) to form Pro (Fig. 1). Pro is degraded in mitochondria by Pro dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to Glu (Fig. 1). The alternative synthetic pathway uses ornithine as precursor, which can be transaminated to P5C by the mitochondrially-located enzyme ornithine-d-aminotransferase (OAT) [54]. In addition, recently Pro has been shown to be a precursor in the synthesis of GABA through a non-enzymatic reaction [59]. Spontaneous decarboxylation of Pro induces the formation of pyrrolidin-1-yl, which can easily be converted to Δ^1-pyrroline, the substrate of Δ^1-pyrroline dehydrogenase (PDH), which produces GABA in peroxisomes (Fig. 1). The interconnections between Glu, GABA and Pro could thus be key in understanding the roles of these amino acids in plant development and stress responses [55,60,61].

Alanine

GABA can also be produced through the action of the widely distributed alanine aminotransferase (AlaAT), which regulates the

reversible conversion of alanine (Ala) and α-ketoglutarate (αKG) into pyruvate and Glu (Fig. 1). AlaAT has been described as being responsible for the synthesis of Ala during hypoxia, which is necessary to avoid the accumulation of pyruvate that provides carbon skeletons for ethanolic fermentation [62], and *vice versa*, to assist during the period of recovery after low-oxygen stress by facilitating a rapid switch to pyruvate as C source [63]. However, it has also been shown that the *Arabidopsis AlaAT1* mutant (*alaat1-1*) accumulates Ala *via* the activity of GABA-T using pyruvate as co-substrate, thus bypassing AlaAT. Similarly, *GABA-T* null mutants accumulated only slightly less Ala upon hypoxia compared with wild-types [22]. These results suggest that there is at least partial redundancy of *AlaAT* and *GABA-T* under hypoxic conditions.

Amino acids derived from aspartate pathway

Synthesis of some amino acids as a consequence of the connection between the GABA shunt and C and N metabolism has been described under both normal and stress conditions [64]. Glu-to-αKG conversion is used to donate an amino group in multiple transamination reactions that allow conversion of Glu to methionine (Met), threonine, isoleucine and lysine *via* the plastidial aspartate-family pathway [4]. Under stress, the expression of lysine–ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH), which form the bifunctional polypeptide LKR/SDH involved in lysine catabolism, is induced [65], allowing the reverse conversion of lysine to Glu, which can be subsequently reincorporated into GABA. Altering Met levels by overexpressing genes involved in Met synthesis has also been reported to affect the GABA pathway, intensifying the flux of Glu to succinate through the GABA shunt, most probably to produce other metabolites such as PAs that are essential for plant development and stress responses [66]. Furthermore, the generation of ROS under stress conditions has been shown to oxidize Met residues by the addition of two electrons to form the *R* or *S* configurated sulphoxide. This in turn can be reduced back to Met by methionine sulphoxide reductases, which form part of the ROS response mechanism and expression of which is necessary to control oxidative damage and photoinhibition.

GABA and polyamines

Plant stress responses have been related to the synthesis and catabolism of polyamines (PAs) (Fig. 1), of which putrescine (Put), spermidine (Spd), spermine (Spm), and the spermine isomer thermospermine (T-Spm) are the most studied. Under stress conditions, oxidation of Put and Spd represents a mechanism that contributes directly to GABA production [67]. The enzymes involved in the oxidative deamination of PAs are amine oxidases (AOs): diamine oxidases (DAO), copper-containing AOs (CuAOs, also known as primary-amine oxidases), and FAD-dependent AOs (FAD-AOs), also known as monoamine oxidases (MAOs). CuAOs convert 1,3-diaminopropane to 3-aminopropanal (APAL) or Put to 4-aminobutanal (ABAL), H_2O_2 and NH_3. Plant PAOs catabolize primarily Spd and Spm through oxidation of the carbon on the endo-side of their N^4-nitrogens to produce, respectively, ABAL and *N*-(3-aminopropyl)-4-aminobutanal, in addition to 1,3-diaminopropane (DAP) and H_2O_2. ABAL is then spontaneously cyclized to form Δ^1-pyrroline. Finally, Δ^1-pyrroline can be converted to GABA by pyrroline dehydrogenase (PDH) (Fig. 1). There are eight putative *DAO*s in *Arabidopsis* which are differentially distributed in tissues throughout plant development. Their expression is highly stress-inducible and reported to show the opposite behaviour to that of *GADs* (Table 1) [67]. This confirms the relevance of crosstalk between PAs and GABA under stress. For example, 39% of the GABA accumulation in soybean roots under salt stress was attributed to PA oxidation due to increased DAO activity [68]. In fava beans under hypoxic conditions the contribution was almost 30% [69], whereas in *Camellia sinensis* L. under anoxia, only around 25% of GABA accumulation derived from the PA degradation pathway [70]. Changes in O_2 availability and cellular redox balance due to stress may directly influence the activities of DAO and GAD, thereby restricting GABA formation [67]. During hypoxia, the roles of PAs and GABA are probably reversed and GABA reduces the effects of short-term hypoxia by increasing the biosynthesis of free Put, Spd and Spm, as demonstrated in melon roots and corroborated by exogenous application of GABA [71].

The crosstalk between PAs and Pro in regulating stress tolerance has also been widely described, but with many conflicting results. The exogenous application of Put induced stress tolerance and Pro accumulation in *Trifolium repens* L. during drought [72,73], but not in *Melissa officinalis* L. supplied with Spd under salinity [74]. These opposite results could be species dependent or due to the effect of each PA regulating stress response. To resolve this issue, Cvikrová et al. [75] analysed *P5CSF129A* transgenic tobacco, which accumulated higher Pro, Put and Spd levels, drought tolerance and recovery capacity than wild type. Many new studies combining genomic and metabolomic approaches will be needed to clarify whether all these metabolites are interconnected, regulating plant tolerance under different stress conditions.

GABA and plant hormones

Abscisic acid

Abscisic acid (ABA) is considered to be a key endogenous messenger in plant responses to abiotic stresses and is also required to fine tune growth and development under non-stress conditions [76]. One of the most important plant processes is stomatal opening. The transport of ions and water across the plasma and vacuolar membranes through channel proteins alters turgor and guard cell volume, thereby regulating stomatal aperture and controlling transpiration and CO_2 assimilation [77]. ABA-induced stomatal closure causes the activation of an inward-rectifying Ca^{2+}- influx channel and a transient rise in cytosolic Ca^{2+} (Fig. 2). Under stress conditions, plants produce ROS that increase cytosolic Ca^{2+} either directly by activating Ca^{2+} in channels or indirectly by inducing nitric oxide (NO) synthesis [78,79]. An intracellular increase in Ca^{2+} levels leads to Ca^{2+-}CaM-induced dimerization of C-terminal domains, resulting in the activation of GADs [80], which convert Glu to GABA (Figs. 1 and 2). Subsequently, the increased GABA regulates aluminium-activated malate transporter membrane channels (Fig. 2). In *Arabidopsis*, some members of the aluminium-activated malate transporter (*ALMT)* family genes (*ALMT6, ALMT9* and *ALMT12)* have been reported to regulate guard cell movement (Table 2) [81–83]. In barley (*Hordeum vulgare*), when expression of the gene *HvALMT1* was reduced, alteration in stomatal closure as well as in grain formation and seed germination were also observed [84]. Recently, it was observed that *ALMT2* but not *ALMT1* was transcriptionally induced by salinity [26] (Table 2). Therefore, it was speculated that they have different physiological functions, with *ALMT2* involved in a GABA-mediated salt response and unrelated to aluminium toxicity [26]. Other *ALMT* members in other species have been also related to stresses independent of Al^{3+} (Table 2). Although the possible role of ALMTs as "GABA receptors" has not been thoroughly confirmed, all those tested from wheat, barley, grapevine, *Arabidopsis,* and rice were sensitive and downregulated to low concentrations of GABA [85]. However, the high expression of *ALMT2* observed in salt stressed *Arabidopsis*, which also accumulated GABA levels compared to control [26], suggested that ALMT proteins appear to be clear candidates not only for transducing GABA signals but also for others signals.

In *Arabidopsis*, the PA-related genes arginine decarboxylase 2 (ADC2), spermidine synthase 1 (SPDS1) and spermine synthase (SPMS) are strongly induced by drought stress, and these responses are mostly impaired in ABA-deficient (*aba2-3*) and ABA-insensitive (*abi1-1*) mutants [86]. In rice, one of strategies deployed to resist chilling stress is to raise ABA levels and, in turn, to enhance the ADC-mediated synthesis of

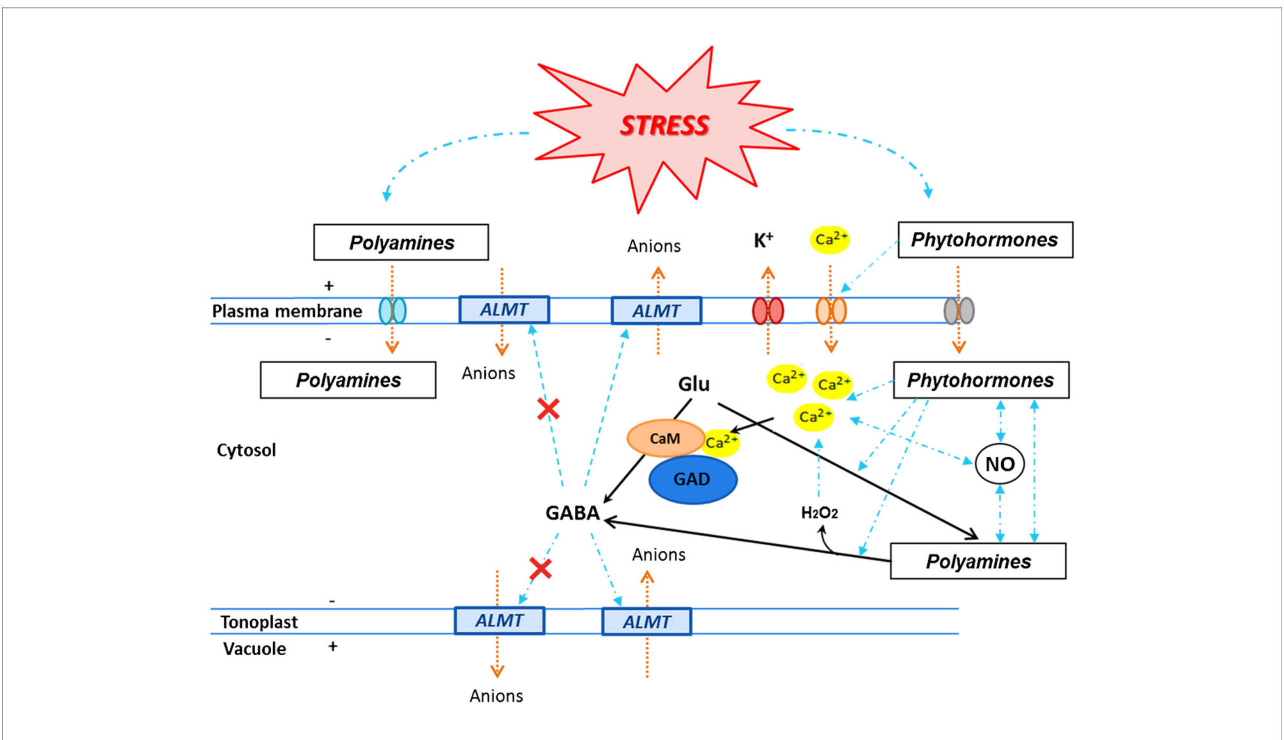

Fig. 2. Diagrammatic model of plant stress responses mediated by hormones, polyamines and GABA in different plant cell types, including guard cells and root cells based on Bown and Shelp [152]. Continuous black arrows indicate metabolic pathways, discontinuous blue and orange arrows indicate regulation and transport, respectively.

Put (Table 1). It was reported [87] that in the guard cells of *Vicia faba* the H_2O_2 generated from Put oxidation catalysed by copper amine oxidase (CuAO) may contribute to the increase in Ca^{2+} which occurs in response to ABA and induce stomatal closure (Fig. 2). In contrast, Spd and Spm did not contribute to ABA-promoted H_2O_2 generation in *V. faba* guard cells but induced stomatal closure. Thus, some PAs may regulate stomatal closure through different signalling routes, possibly through the activation of OST1. In guard cells, it is postulated that ABA is sensed by the pyrabacin resistance 1(PYL)/ PYR1-like (PYL)/regulatory components of ABA receptors (RCAR) [79]. PYLs change their conformation and then interact and inhibit group A type 2C protein phosphatases (PP2Cs). Then, PP2Cs interact with subclass III plant-specific sucrose non-fermenting 1-related subfamily 2 (SnRK2s) protein kinase open stomata 1 (OST1), leading to dephosphorylation of Ser/Thr

Table 2
Summary of known functions and localization of *ALMTs* from several species (reviewed by Sharma et al. [153] and Palmer et al. [83]).

Species	ALTM	Tissue	Function	Transport	Reference
Arabidopsis	*AtALMT1*	Root	Biotic and abiotic tolerance	organic acids	[103]
	AtALMT2	Shoot	Salinity tolerance		[26]
	AtALMT6	Stomata	Opening	organic acids	[172]
	AtALMT9	Stomata	Opening	organic acids	[81]
	AtALMT12	Stomata	Opening	organic acids ions Cl^-, NO_3^-, SO_4^{2-}	[82]
Maize	*ZmALMT1*	Root	Al^{3+} tolerance	organic acids and other anions	[173]
	ZmALMT2	Root	mineral nutrient acquisition and transport	organic acids and other anions	[174]
Barley	*HvALMT1*	Stomata Root	Al^{3+} and acid soil tolerance	organic acids	[175]
	HvALMT1	Stomata Grain development Germination		organic acids	[84]
Wheat	*TaALMT1*	Root	Al^{3+} and acid soil tolerance	organic acids ions Cl^-, NO_3^-, SO_4^{2-}	[176]
Rye	*ScALMT2*	Root tips	Al^{3+} and acid soil tolerance	organic acids	[177]
Brachypodium distachyon	*BdALMT1*	Root	Al^{3+} tolerance	organic acids	[178]
Holcus lanatus	*HlALMT1*	Root	Al^{3+} tolerance	organic acids	[179]
Glycine max	*GmALMT1*	Root	Al^{3+} and acid soil tolerance	organic acids	[180]
Lotus japonicus	*LjALMT2* *LjALMT3* *LjALMT4* *LjALMT5* *LjALMT6*	Stem and leaves All tissues nodules All tissues Stem and leaves		organic acids and other anions	[181]
Brassica napus L	*BnALMT1*	Root	Al^{3+} tolerance	organic acids	[182]
	BnALMT2	Root	Al^{3+} tolerance		
Apple tree	*Ma1* *Ma2*	Fruit	affect the taste and flavour	organic acids	[183]
Grape berry	*VvALMT9*	Fruit	Acidity	organic acids	[184]

residues present at the activation loop of the SnRK2s, resulting in its inactivation. However, recent work has reported that OST1 can be active in response to some stimuli independent of ABA and PYR/RCAR receptors [88]. Another possibility could be GABA produced *via* PA degradation by generating ROS. Recently, it was demonstrated that *Arabidopsis* mutants deficient in GABA synthesis exhibited deformed stomata and had lost the capacity to close them [89]. In addition, plants with single loss-of-function mutations in NAD^+-dependent aminoaldehyde dehydrogenase (AMADH) homologs, which are involved in the oxidation of ABAL and APAL to GABA and β-Ala, were more sensitive to salinity than wild-type plants, and this phenotype was accompanied by a reduction in GABA accumulation [90]. Another possible means by which PAs regulate stomata closure could be the production of NO, which functions as an intra- and intercellular messenger (reviewed [91]). NO also modulates cytoplasmic Ca^{2+} levels, which again activate plant stress responses. In addition, studies performed on isolated epidermal peels showed that *Atnos1* mutant stomata produced less NO and showed a reduction in closure when treated with ABA [92]. However, it was also reported that NO negatively regulates ABA signalling in guard cells by inhibiting OST1/SnRK2.6 through *S*-nitrosylation [93], an action also associated with PAs [91]. Thus, a dual role for NO exists in guard cells, promoting and attenuating the ABA leading to the stomatal closure. Overall, these findings have established that there is clear synergy among ABA, PAs and GABA in the regulation stomatal closure.

Auxins

Indole-3-acetic acid (IAA) is the most active auxin and its endogenous levels are altered in plants exposed to stress. For example, salt stress reduced IAA levels in the leaves of tomato plants but induced IAA accumulation in the roots [94]. In sweet peppers, however, salinity did not affect the IAA level in the aerial part, but it reduced the content in the roots, and the difference from the control level increased when plants were exposed to a high concentration of CO_2 [95]. IAA has been also reported to play a major role in the regulation of drought tolerance in pines by inducing leaf epinasty as a stress defence mechanism, and by stimulating secondary root formation [96,97]. Many members of gene families involved in IAA metabolism are also associated with stress/defence responses [98]. *TLD1/OsGH3.13*, encoding IAA-amido synthetase, was shown to enhance the expression of *LEA* (late embryogenesis abundant) genes [99], which mediate responses to abiotic stresses by conferring desiccation tolerance [100,101]. In the halophyte *Thellungiella salsuginea* the dehydration tolerance was mediated by, among other mechanisms, *LEA* overexpression and GABA accumulation [102]. Lastly, IAA/ABA-induced transcription of the aluminium-activated malate transporter family gene *AtALMT1*, which is expressed at the plasma membrane of the epidermal cells of the root tip, has been described as being critical for aluminium resistance and for the recruitment of beneficial rhizobacteria that induces immunity in *Arabidopsis* [103]. Thus, GABA modulation of ALMT activity results in changes in root growth and altered root tolerance to alkaline pH, acid pH and aluminium ions (Fig. 2). Furthermore, some ALMT proteins have been identified as Rapid or Quick activating Anion Channels (R/QUAC-type) [104]. Processes that depend on the function of R-type anion channels include inhibition of hypocotyl growth by auxin, but not by other phytohormones such as ethylene or cytokinins [105]. Root growth and dehydration tolerance have also been related to GABA accumulation and the crosstalk between ABA and IAA [56,97]. In barley, IAA-mediated ROS generation induced root inhibition under cadmium treatment and the effect was reversed by salicylic acid (SA) [106]. Similar antagonistic effects between phytohormones inhibiting root growth under stress conditions was described in the roots of transgenic *Arabidopsis* seedlings expressing a *DR5* (Synthetic auxin-responsive promoter)*::GUS* fusion, which activity was suppressed by ABA, SA, cold and salt treatment [107]. Significant interactions among ABA, IAA and some CK derivatives have also been shown to play roles in nitrogen signalling, one factor which determines plant development and also root growth (reviewed [108]). Thus, it is clear that the crosstalk between auxin and other phytohormones interferes with GABA signalling in different plant processes involved in plant stress tolerance, root growth, nutrient uptake, stress related-ROS response and metabolism.

It is also worth mentioning here that the crosstalk between phytohormones and GABA present both overlap and differences in regulation of stress related responses. The exogenous application of GABA, PAs and hormones activate the antioxidative response in many species [109–112], but there are many other biological processes that condition plant stress response and tolerance. Thus, the exogenous application of ABA, SA and GABA induced drought stress tolerance in *Agrostis stolonifera*, through different metabolic pathways [113]. Exogenous GABA and SA application elevated threonine, serine and Pro, whereas ABA and GABA significantly accumulated several organic acids like malic acid. Malate release has also been reported to be induced by *AtALMT1* as consequence of stress responses, closing the loop between IAA, ABA and GABA, and the regulation of *ALMT* members in different plant species under varied growth conditions. However, it did not clarify the upregulation of some *ALMT* under stress condition when plants accumulate GABA.

Cytokinins (CKs)

CKs are hormones involved in many processes, including stress response. Modulation of CK levels through either upregulation of synthesis or deregulation of their degradation has been reported to increase plant stress tolerance. Plants transformed with an isopentenyl transferase (*ipt*) gene, which encodes a CK biosynthetic enzyme, under the control of a stress- or senescence-activated promoter (*SAG12-ipt*) showed improved drought tolerance in rice and cassava [114,115]. A functional connection between GABA and CKs can be illustrated using the example of barley expressing the cytokinin dehydrogenase 1 gene from *Arabidopsis* (*AtCKX1)* under the control of a weak root-specific β-glucosidase promoter from maize [116]. These transgenic lines overexpressed the GABA related gene *GAD* and *ALMT* in roots (Table 1) [116]. Interestingly, a follow-up study showed that these plants had upregulated expression of two GABA related genes, other transcription factors implicated in stress responses, some genes involved in the methionine pathway and in ABA sensitivity during drought stress and recovery [117]. However, in contrast to the auxin-related stress response, the CK deficient plants showed downregulation of *LEA* genes, and also of glyoxylate reductase (GLYR), an enzyme involved in GABA catabolism. These results conflict with those of recent biochemical studies conducted in plants under stress, in which the expression of the two *Arabidopsis* GLYR isoforms contributed to an alternative pathway to control redox balance by detoxifying both succinic semialdehyde and glyoxylate [46]. In addition, *Arabidopsis* treated with GABA or with the CK N^6-benzyladenine showed a significant reduction in root growth and a high degree of overlap between downregulated and upregulated genes, including those related to sucrose addition and nitrate starvation [118].The crosstalk between GABA and CK regulating stress tolerance has been also corroborated in tobacco under metal stress, where the zinc tolerance of transformed plants (SAG12 promoter with *ipt* gene) was associated with accumulation of Pro, methionine and GABA [119].

Stress modifies xylem differentiation and this effect is related to ABA accumulation [120]. However, it was shown [121] that xylem differentiation is mediated by T-Spm (Fig. 1), the level of which also modulates auxin signalling. Recently, it has been reported that PA oxidase (PAO5), which controls the homeostasis of T-Spm, participates in a tightly controlled interplay between auxin and CKs that is necessary for proper xylem differentiation [122]. Taken together, an interaction between GABA and PA metabolism and hormone production is likely to modulate several physiological processes in plants and requires further research to determine the extent to which the changes of each compound condition the others.

Other plant hormones

In plants, ethylene (ET) is regulated by internal signals in response to environmental stimuli including biotic and abiotic stresses, in which GABA is also accumulated. ET is produced from *S*-adenosyl-methionine (SAM), the activated form of methionine, and the rate-limiting step is the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (Fig. 1). SAM, which is also a precursor in the synthesis of PAs, represents the connection between ET and GABA. Moreover, the oxidation of PAs or hydroxylamines and ROS could induce NO production [123], which also controls ET production and plant stress tolerance [124]. To study this connection, exogenous GABA was applied to sunflower and up to a 14-fold increase was observed in the rate of production of ET by enhancing ACC synthase transcript abundance [125]. However, others showed that the action of GABA in downregulating *14-3-3* genes in *Arabidopsis* seedlings is dependent on ET and ABA signalling, raising the question of which of these compounds acts first [126]. During wounding or mechanical damage to tissues, GABA accumulates very rapidly, taking only 30 s, whereas the rate of ET production increases only after about 20 min [125]. This could mean that other plant hormones respond faster and that ET production is only a consequence of their action. As an example, in tomato plants exposed to salinity, ET production was related to ABA concentration and to the modulation of resource partitioning between shoot and roots [94]. ET-induced growth inhibition in roots also requires auxin biosynthesis, transport, signalling and response [127].

Plant defence against pathogens and insects has been shown to be regulated mainly by cross-communication between SA, jasmonates (JA) and ET [128,129]. In addition, a study investigating the roles of these hormones using *Arabidopsis* infected with various pathogens with different modes of attack revealed that, as with JA, SA and ET levels, *GAD4* was strongly upregulated after the first 12 and 24 h of infection with *Pseudomonas syringae* and *Alternaria brassicicola* (Table 1) [130]. Simultaneously, expression of *GAD1* was upregulated 270-fold in the first 12 h after commencing *Pseudomonas syringae* treatment. *GAD4* was significantly upregulated within 1 h after treatment with exogenous methyl jasmonate (MeJA) (Table 1). Furthermore, MeJA induced protection in barley against powdery mildew infection, also altering PA metabolism and upregulating CuAO [131], the first step in PA catabolism that ends with the synthesis of GABA (Fig. 1) [67].

To explore plant tolerance to biotic stress in greater depth, we considered other studies that analysed the phenotypes of *gaba-t* mutants [132,133]. Mirabella et al. [132] showed that E-2-hexenal-response (*her*) mutants encoding a *GABA-T* gene accumulate high levels of GABA, which also reduced the toxic effect induced by the accumulation of the green leaf volatile E-2-hexenal, normally produced by plants upon herbivory or during pathogen infection. The authors also reported that the plant response to E-2-hexenal was independent of the biotic stress related hormones JA, SA and ET [132]. These results suggested that the volatile-inducing plant response is probably regulated by the action of other signals. Interestingly, recent work evaluating the positive effect of volatiles compounds emitted by phytopathogens without physical contact with the plant, revealed that CKs play an essential role in the regulation of plant responses, as reflected in enhanced plant growth, and in increased photosynthesis and starch accumulation [134]. Furthermore, transcriptome analysis showed that a *CuAO* gene was upregulated in plants exposed to volatiles (Table 1). Follow-up studies using *pgi* mutants, with altered C metabolism, growth, photosynthesis, starch accumulation and CK content, showed that volatile compounds stimulate these biological processes, in the mutants just as in wild type plant, through upregulating the expression of *GAD2* and genes involved in C metabolism (Table 1), and the accumulation of soluble carbohydrates and amino acids including Pro, Asp, Glu, Gln, Ala and GABA [135]. If this result is compared with the expression of other *GAD* forms in *Arabidopsis* grown under salinity, drought, and other abiotic stresses, we could suggest that in general, *GAD* is upregulated under all stress condition but the isoform depends on the type of stress (Table 1). Furthermore, during salinity stress, a deficiency in *GABA-T* expression causes developmental defects in the root and hypocotyl and alterations in cell wall composition [39]. However, plants exposed to volatile compounds showed downregulated expression of *GABA-TP* and altered expression of genes related to cell wall composition, suggesting that changes in plant growth during exposure to salinity in plants with a deficiency in *GABA-T* expression were due primarily to an alteration in the GABA shunt which controls redox balance under growth conditions in which the photosynthetic capacity is reduced [55]. Thus, the GABA accumulation seems to represent a preventive response, when plants perceive the volatiles, in order to increase disease resistance. We suggest that the plant may activate GABA synthesis as a biotic stress response to volatile compounds irrespective of the type of emitting organism. In addition, CKs are sufficient to alter developmental patterns of defence metabolites in many plant species, confirming the importance of these hormones in regulating plant responses to both abiotic and biotic stress [136].

Gibberellins (GAs) have been also related to plant stress response [137,138]. However, their involvement is controversial. *Arabidopsis* grown under salinity showed reduced levels of GAs and increased accumulation of DELLA proteins, named after the conserved Asp-Glu-Leu-Leu-Ala N-terminal motif, which constrain the processes of cell proliferation and expansion that drive plant growth [139]. In *Populus* seedlings, however, an overexpression of two DELLA subfamily members, GA insensitive gene (*GAI*) and repressor of GA1-like (*RGL1*), increased GA level by 12- and 64-fold, respectively, and presented a dwarf phenotype with bigger root growth [140]. The mutation also induced metabolic changes with 3-fold higher GABA levels compared to wild type. Conversely, increased GA biosynthesis and signalling promote growth in plant and escape responses to shading and submergence [137]. Exogenous application of GA_3 in combination with N ameliorated the adverse effects of salt stress, enhancing the growth of *Brassica juncea* L., which also accumulated Pro and GB. Exogenous GA application has been also reported to increase the endogenous content of GABA in rice seeds and grapes fruits [6,141]. High endogenous levels of active forms of GA, together with the auxins IAA and IBA, were also related to salt tolerance in GABA treated *Cassia italica* [142]. However, these plants had reduced levels of ABA and JA. Downregulation of the ABA pathway and signalling by GAs were observed in *Capsicum annuum* under stress conditions, but SA related genes were upregulated [143]. These results are in agreement with those from *Arabidopsis* mutants lacking four of the five DELLAs, which exhibited high levels of SA-dependent resistance when challenged with the hemibiotrophic pathogen *Pseudomonas syringae* [144]. However, these mutants showed attenuated induction of the expression of the JA-reporter gene *PDF1.2*, and this was correlated with enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola*. Thus, DELLAs could modulate the balance of SA/JA signalling during plant immunity, promoting JA perception and/or signalling, and repressing SA biosynthesis and signalling, although this response is species-dependent [138]. In addition, DELLAs have been reported to modulate early defence by mediating GA-ABA crosstalk in seeds, influencing the expression of ABA related genes by recruiting some of the *LEA* genes, whose products confer osmotolerance upon the embryo under harsh environmental conditions [145]. GAs also regulate Ca^{2+} cell content, thus activating plant stress responses (for review, see [146]). Finally, it should be noted that not only GAs, but also ABA, ET, SA and NO, have been reported as regulating the levels of Pro in plants under stress conditions, although further studies must be performed to clarify whether they do so *via* PAs and/or through direct synthesis *via* Glu [146].

There is also a connection between PAs and hormones regulating plant defence response [147]. Microarray analysis of plants overexpressing arginine decarboxylase (*ADC2*) revealed both up- and downregulation of hormone-related genes, encoding transcription factors belonging to the APETALA2/ethylene responsive factor domain family,

Fig. 3. Hypothetical timed hierarchy of consecutive events in plant response to stress based on Maffei et al. [129].

genes involved in the biosynthesis of auxin, ET, ABA, GAs and SA, genes for auxin transport, and genes for auxin-responsive proteins, ET- and ABA-responsive transcription factors, and JA-induced proteins. They also showed that these plants had alterations in Ca^{2+} signalling. In this regard, it was suggested [148] that long-distance signalling by these hormones is mediated by waves of ROS and Ca^{2+} rather than directly by the movement of the hormones themselves. These findings support the conclusion that the stress response is a complex interaction between all plant hormones, PAs and GABA, including ROS response, NO production and the levels of Ca^{2+} (Fig. 2).

Finally, the strigolactones (SLs) have been also related to plant stress responses [149]. In this context, a recent transcriptomic study performed in SL-related mutants showed that they exhibit hypersensitivity to drought and salinity. In these mutants, *GAD5* and *GAD2* were upregulated after 4 h of drought (Table 1) [150]. These results supported the hypothesis that the expression of the *GAD* isoforms is dependent on the type of stress and mediated by the action of different phytohormones.

Conclusion

All the studies cited above point to the existence of a highly conserved pathway that is expressed in plants under stress, one in which different combinations of phytohormones regulate plant processes and responses *via* changes in Glu metabolism (Fig. 2), and thence in PA and/or GABA pathways that regulate the plant ROS response and NO production, which in turn also regulates phytohormone signalling (reviewed [151]). Plants perceive stress signals that induce modifications in the endogenous levels of hormones and/or PAs (Fig. 2). This alters the expression of genes involved in primary metabolism and those related to the PA and/or amino acid pathways, ROS, NO and, as a consequence, Ca^{2+} signalling. An increase in the level of Ca^{2+} then regulates the expression of different *GAD* isoforms according to the type of stress and the plant tissue (Table 1). Glu is converted to GABA, which regulates the ALMT membrane channels (Fig. 2) [152], to control stress response processes such as stomata closure, turgor maintenance and ion homeostasis (Table 2) [83,153]. Secondary metabolites, amino acids, PAs and phytohormones then crosstalk in order to provide an appropriate response to each particular stress by, for example, altering growth pattern, photosynthetic capacity *etc*. However, which specific compounds take part in the crosstalk, at which moment and at what levels depend on the type and intensity of stress, the plant tissue and species (Fig. 3). In any case, the known aspects of the mode of action of these compounds strongly suggest that their application can be potentially used to mitigate the adverse effects of multiple stresses. This represents a possible alternative technology to exogenous application of synthetic compounds to improve the efficiency of crop production and stabilization of yield under suboptimal conditions. The detailed knowledge of the GABA crosstalk brings also new possibilities in utilization of simple technologies improving the quality of food products with added benefits such as nutraceuticals and functional components.

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❖ *Supplement II*

Nuria De Diego, Tomáš Fürst, Jan F. Humplík, Lydia Ugena, Kateřina Podlešáková, Lukáš Spíchal. "An automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates and its validation in stress conditions". *Frontiers in Plant Science*, vol.8, Art. 1702, 2017.

METHODS
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An Automated Method for High-Throughput Screening of *Arabidopsis* Rosette Growth in Multi-Well Plates and Its Validation in Stress Conditions

Nuria De Diego[1], Tomáš Fürst[1], Jan F. Humplík[1,2], Lydia Ugena[1], Kateřina Podlešáková[1] and Lukáš Spíchal[1]*

[1] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czechia, [2] Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czechia

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Correspondence:
Lukáš Spíchal
lukas.spichal@upol.cz

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High-throughput plant phenotyping platforms provide new possibilities for automated, fast scoring of several plant growth and development traits, followed over time using non-invasive sensors. Using *Arabidops*is as a model offers important advantages for high-throughput screening with the opportunity to extrapolate the results obtained to other crops of commercial interest. In this study we describe the development of a highly reproducible high-throughput *Arabidopsis in vitro* bioassay established using our OloPhen platform, suitable for analysis of rosette growth in multi-well plates. This method was successfully validated on example of multivariate analysis of *Arabidopsis* rosette growth in different salt concentrations and the interaction with varying nutritional composition of the growth medium. Several traits such as changes in the rosette area, relative growth rate, survival rate and homogeneity of the population are scored using fully automated RGB imaging and subsequent image analysis. The assay can be used for fast screening of the biological activity of chemical libraries, phenotypes of transgenic or recombinant inbred lines, or to search for potential quantitative trait loci. It is especially valuable for selecting genotypes or growth conditions that improve plant stress tolerance.

Keywords: high-throughput screening assay, *Arabidopsis*, multi-well plates, rosette growth, stress conditions

INTRODUCTION

Large-scale plant phenotyping has become an important tool in plant biology and agriculture and contributes significantly to cutting-edge plant breeding and management approaches needed to meet future food and fuel demands. However, the application of high-throughput approaches is still severely limited by a lack of appropriate instrumentation and experimental standards, which would allow better communication of the experimental results and outcomes of any analyses. Identifying good practices associated with performing high-throughput phenotyping of large plant populations is a current challenge for achieving high genotyping capacity and expanding our knowledge of

plant development in different environments (Humplík et al., 2015a; Rousseau et al., 2015). In this context, the use of non-invasive imaging techniques has potential for revealing morphological and physiological traits related to plant responses, such as growth. Usually, this trait is described as biomass formation, determined as the weight of the whole plant or part of it (most often the shoots) at a given point in its lifespan. However, classical biomass determination involves the destruction of the plant thus allowing only end-point analysis; this means that the developmental course (kinetics) of the single organ cannot be monitored. To address this, many phenotyping platforms take advantage of relatively simple red–green-blue (RGB) imaging and subsequent software image analysis for non-destructive assessment of the growth of intact plants (Skirycz et al., 2011; Rahaman et al., 2015). Besides, new integrated analysis platform has been also designed combining imaging data analysis obtained from different spectra (Klukas et al., 2014; for review see Humplík et al., 2015b).

Non-invasive techniques for plant growth determination have demonstrated high correlations between the projected area and the biomass, expressed as fresh or dry weight of the shoot, in many plant species including *Arabidopsis*. Although without agronomic significance, *Arabidopsis* offers important advantages for high-throughput screening (HTS). It is a small plant, well-characterized in terms of growth-regulating molecular mechanisms, making this species highly suitable for phenotypic analysis. In addition, new studies have demonstrated the possibility of extrapolating results obtained for *Arabidopsis* using HTS methods to other crops that are of commercial interest, such as tomato, lettuce, carrots, etc. (Rodriguez-Furlán et al., 2016). However, there are still limitations to the actual automated phenotyping methodologies for *Arabidopsis*. In recent years, the development of new techniques has allowed an increase in the number of plants in an experiment: from 3–6 plants per treatment in manual phenotyping studies (Mishra et al., 2014) to 200–1000 plants per whole experiment, depending on the level of automation, platform capacity and the number of variants (Vasseur et al., 2014; Flood et al., 2016). Thus, the maximum number of experimental variants per experiment, e.g., the number of simultaneously studied growth conditions, is determined by the number of plants per variant and the number of technical replicates of each variant. Recently, new methods using semi-automated systems of image acquisition by microscope or scanner for scoring *Arabidopsis* growth *in vitro* in 15 cm Petri dishes and 24-well plates, respectively, were published, allowing an increase in the number of plants per treatment and number of replicates (Rodriguez-Furlán et al., 2016; Tomé et al., 2017).

Several potential complications and methodological difficulties have been identified in some phenotyping platforms; these included spatial and temporal variability of micrometeorological conditions within a growth chamber, differences in soil moisture maintenance, and plant growth capacity after sowing (Granier et al., 2006). Thus, the real HTS of a phenotyping platform is highly dependent on the experimental design selected, which needs to be precisely optimized and standardized to minimize the number of variables influencing the accuracy and reproducibility of the procedure. Methods to improve image acquisition and recommendations for data handling can be found in the literature (e.g., Li et al., 2014; Krajewski et al., 2015). Nevertheless, despite the fact that the correct experimental setup, including selection of the plant material, has significant influence on the success of automated high-throughput phenotyping, there is little published information explaining its relevance.

In the work presented here, we report on the development and optimization of growing protocol suitable for HTS of *Arabidopsis* rosette growth in multi-well plates under salinity as plant stress condition. This approach will allow simultaneous testing of a large number of potentially bioactive compounds in a wide range of concentrations and/or genotypes, under various growth conditions. The relevance of choosing the appropriate experimental design is emphasized and examples illustrating its importance are presented for each case studied and then discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (accession Col-0) was used in all experiments. Seeds were surface-sterilized, sown on square plates (12 cm × 12 cm) containing 0.5× Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) (pH 5.7) supplemented with a gelling agent 0.6% Phytagel (Sigma–Aldrich, Germany) and maintained for 3 days at 4°C in the dark. Thereafter, the plates were transferred into a growth-chamber with controlled conditions (22°C, 16/8 h light/dark cycle, a photon irradiance of 120 μmol photons of PAR m^{-2} s^{-1}) and placed in a vertical position. Three days after germination, seedlings of similar size were transferred under sterile conditions into the multi-well plates [12- and 24-well plates (Jetbiofil, Guangzhou, China)] one seedling per well and the plates were sealed with perforated transparent foil allowing gas and water exchange. Each well contained 2.7 mL (12-well plate) or 1.3 mL (24-well plate) of full MS medium (pH 5.7; supplemented with 0.6% Phytagel). For optimization, different concentrations of MS (1×, 0.5×, and 0.25×) and sucrose (0, 0.1, and 1%) (pH 5.7; containing 0.6% Phytagel) were also used. In the salt-stress experiment 12- and 24-well plates were used filled with 1× MS medium (pH 5.7; containing 0.6% Phytagel) with the addition of NaCl to achieve specific salinities (50, 75, 100, and 150 mM NaCl). In the experiment dealing with interacting growth conditions, 12-well plates containing different MS concentrations (1×, 0.5×, and 0.25×) with or without salt stress (75 mM NaCl) were used.

Phenotyping Platform, Experimental Setup and Assay Conditions

The multi-well plates with the transferred *Arabidopsis* seedlings were placed onto the OloPhen platform[1] that uses the PlantScreen™ XYZ system installed in a growth chamber

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

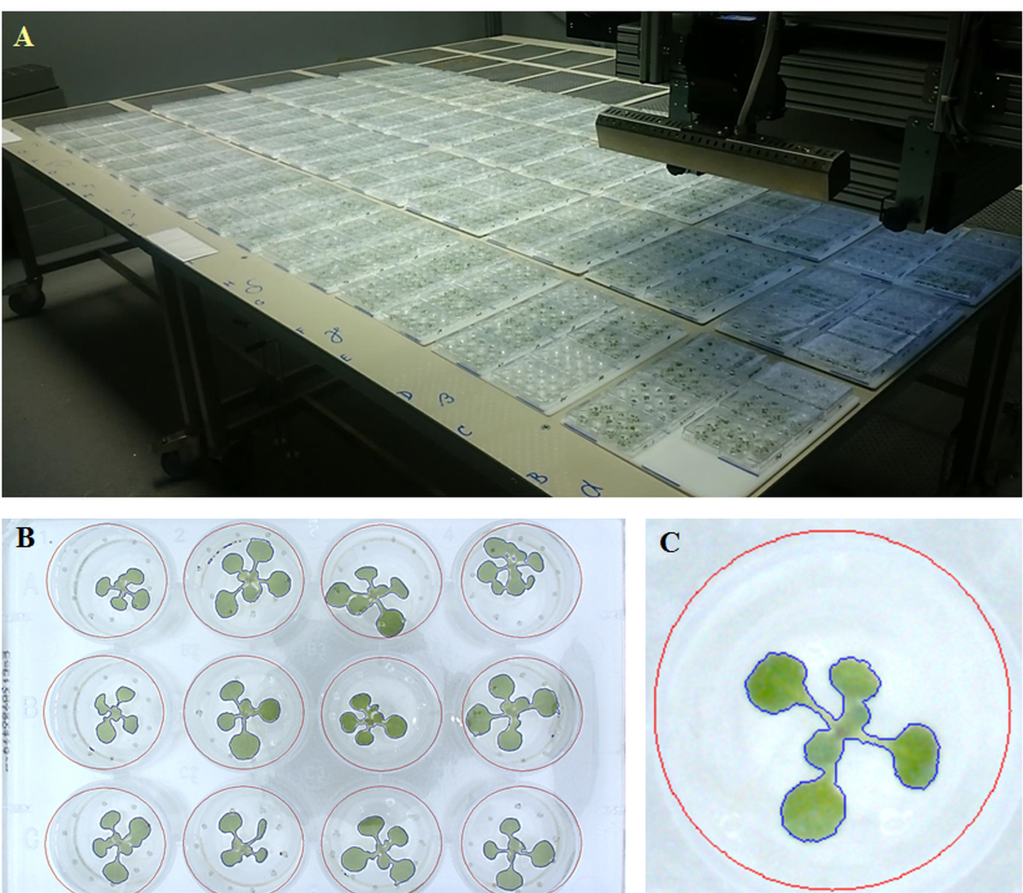

FIGURE 1 | High-throughput phenotyping platform for growing *Arabidopsis* in multi-well plates. **(A)** XYZ PlantScreen^{TM} growth-chamber with automatic top view RGB imaging. **(B)** RGB images of 9 DAG old *Arabidopsis* seedlings. **(C)** RGB image of an individual well-plate with the blue boundary mark created by the in-house software for analysis.

with a controlled environment and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czech Republic). The conditions were set to simulate a long day with a regime of at 22°C/20°C in a 16/8 h light/dark cycle, an irradiance of 120 μmol 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 and growing tables with a total area of approximately 7 m^2 with a capacity of 480 multi-well plates fixed in customized trays for accurate positioning of every plate (**Figure 1A**). The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. RGB images (resolution 2500 × 2000 pixels) of a single plate with a file size of approximately 10 MB in the PNG compression format (**Figure 1B**) were 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 data were automatically stored in PlantScreen XYZ database, exported by PlantScreen Data Analyzer software and analyzed using an in-house software routine implemented in MatLab R2015 (for details see Results). The application can be used without any charge upon obtaining a license from the author. The license can be obtained by e-mail to Palacky University upon agreeing not to use the application for commercial purpose. After obtaining the license, the end-user will be provided (free of charge) with the MCRInstaller.exe. MCRInstaller simulates the MatLab environment on computers where MatLab is not installed and enables to execute the applications. To obtain the application executable files, please contact the author Tomas Furst by email tomas.furst@upol.cz. The email must contain the following statement: "Neither the application nor the MCRInstaller will be used for any commercial purpose".

Statistical Analysis and Data Representation

To assess the differences between the projected areas of two or more groups of plants at a particular time-point, the non-parametric Kruskal and Wallis one-way analysis of variance by ranks was used. The test compares the medians of the samples in the respective groups, and returns a *p*-value for the null

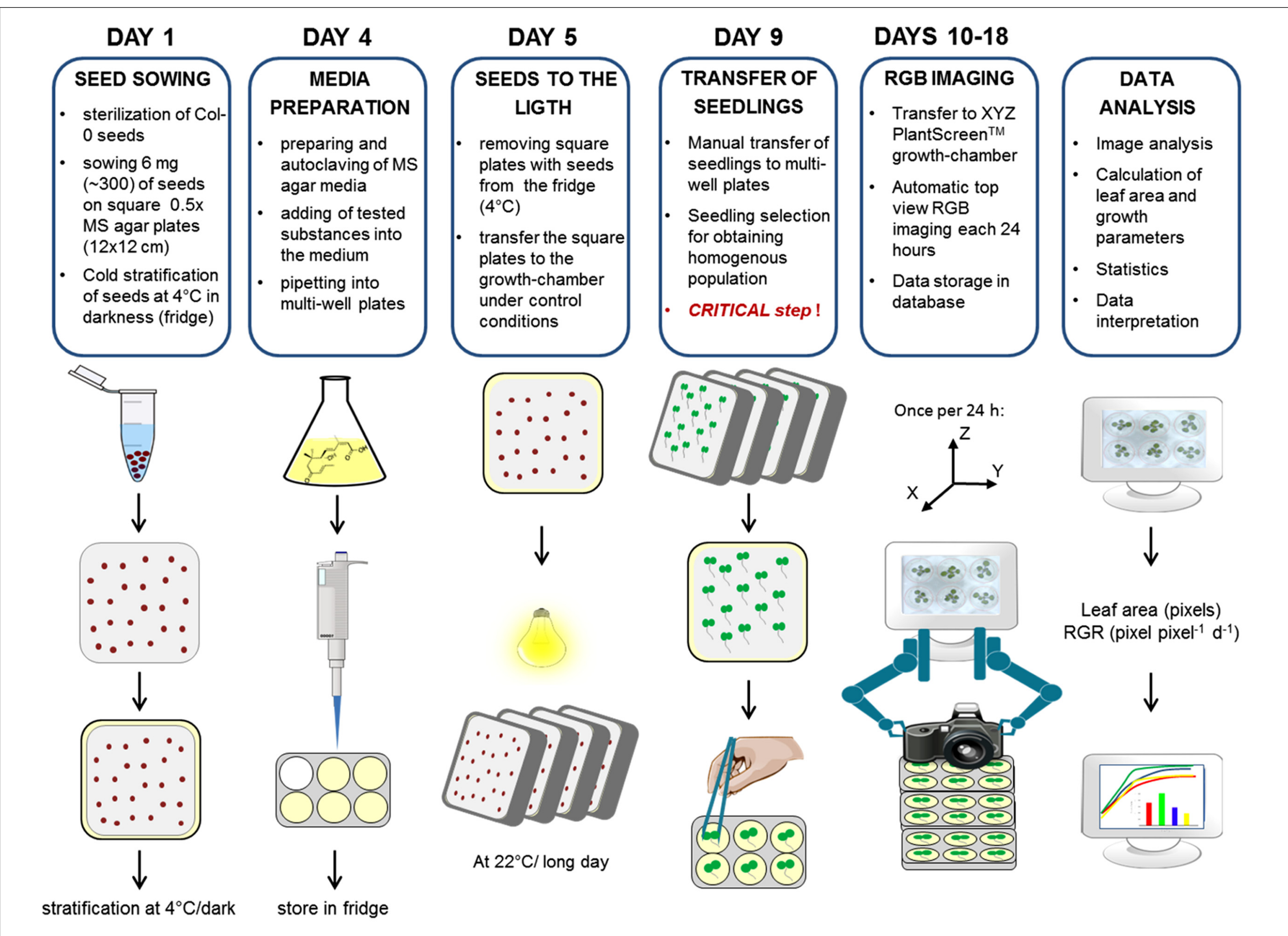

FIGURE 2 | Protocol for high-throughput phenotyping for screening of *Arabidopsis* rosette growth in multi-well plates. Col-0 were germinated in 0.5× MS under long day controlled conditions. Seedlings of similar sizes were transplanted into multi-well plates with fresh MS medium 4 days after cold stratification. Plates were transferred to a XYZ PlantScreen^{TM} growth-chamber for automatic RGB imaging and data analysis.

hypothesis that all samples are drawn from the same population. For the analysis of multidimensional data, visual representations in the form of box plots, histograms, and animations, created using the MatLab R2015 software, were used to capture the time dimension of the problem.

RESULTS

The Assay Workflow

The presented assay was designed for automated large-scale analysis of *Arabidopsis* rosette growth *in vitro* using multi-well plates. The *in vitro* cultivation of the plants confers the advantage of precise control of the growth media and easy supplementing and dosing of tested factors. Importantly, it allows easy introduction of generalized randomized block designs (GRBDs), the statistical theory of the design of experiments that is used to study the interaction between blocks and treatments. In our method, a block is represented by a multi-well plate containing an array of plants. The plates can then be randomized within the growth area and replicated in the case of optimization of the method when a blocking factor is tested as the potential source of variability. Typically, such a source is differences in the growth chamber microclimate, however, one usually underestimated factor that introduces nuisance variables is the operator preparing the treatment.

Thus, to ensure an appropriate experimental procedure, we established the protocol schematized in **Figure 2**; this was used for optimization of the method, its validation, salt stress response and growth interaction studies. The protocol takes a total of 18 days and consists of several steps including seed sowing, cold stratification, transferring the seedlings into the multi-well plates, time-course RGB-imaging, data processing and analysis (**Figure 2**). The seeds are sown onto the square plates (12 cm × 12 cm) containing compound/stressor-free medium at a density of about one seed per 1.5 cm^2, facilitating manipulation during subsequent seedling transfer. After cold stratification, the plates are placed vertically in the growth chamber, thus preventing growth of the root into the solid media. This minimizes any possible damage to the tiny 3 days after

germination (DAG) old seedlings during the transfer into the multi-well plates (one seedling per well). The transfer of the seedlings was introduced into the protocol as an important step to achieve a method in which the effect of the tested conditions (stressors, chemicals, etc.) is scored not earlier than during the stage when the cotyledons are expanded, thus avoiding the possible effect on the process of germination. As described later in the text, the selection of seedlings of similar size for the transfer into multi-well plates represents a critical point in this method. After seedling transfer, the multi-well plates are placed into the PlantScreen^{TM} XYZ system and after about 24 h of acclimation, automated RGB-imaging is performed every day for the next 9 days. When the platform is at capacity, the whole imaging run takes 70 min, thus in theory there can be 12–13 imaging runs within the 16-h-long light period, producing a dense-point growth curve. In the case of a well optimized assay, the sensitivity of the analysis allows scoring differences in the rosette area over only 2-h-long intervals. As shown in the example of two independent 12-well plates, the average increase in the green area in 2 h is 2-3% (**Supplementary Figure S1**). This offers the possibility for further optimization to increase the assay through reduction of the entire time of the assay and/or use of multi-well plates with a larger number of smaller wells. In the presented protocol, the growth of the green area was recorded every 24 h (typically at midday) during the 9 days. The imaging data were processed using in-house software described in the following section.

Software for Image Processing

The RGB imaging data were analyzed using an in-house software routine, examining all the files covering the entire experiment, i.e., images of up to 1000s of plates taken at many pre-defined time-points. In the first step, fish-eye correction of each image is performed. Next, the image is registered automatically so that the positions of the wells are correctly identified. For this step, blue boundary marks on the trays are used (**Figure 1C**), together with an edge detection routine. The registration step is somewhat sensitive to errors, therefore several suggestions for the correct registration are computed and returned in order of decreasing probability. The most probable registration is tried first and the image is registered and cropped to contain only the plate with no surroundings. The plants are automatically detected by thresholding the image in the HSV color space. Since we are looking for green pixels in a generally white background, the threshold need not be very intelligent, a fixed cut-off value is used separately in each of the three HSV channels and the results are combined by means of the logical operator "AND." If any green area is detected outside the wells, the software recognizes that it made a mistake in the registration step and returns to try the registration step with another set of plausible registration parameters. When the segmentation is successful, i.e., there is no green area detected outside the wells, the green areas of all the plants are computed and a pre-view of the registered and segmented image is saved to disk. The pre-views can be reviewed manually and any remaining errors corrected. After all the images have been analyzed, a single XLSX data file is produced which contains, in each row: the name of the file, date of acquisition, (x, y) position of the camera, and subsequently the list of 12, or 24 numbers, which represent the green areas (in pixels) of the plants in the wells. The wells are numbered column-wise. On a standard PC, the processing of a single 10 MB PNG file takes approximately 10 s with most of the time spent on the fish-eye correction routine. Since the experiment itself usually lasts for days, there was no need for any speed-optimization of the MatLab routine.

For correct data handling, we must take into account the fact that the data produced by these experiments are naturally multi-dimensional. Usually, several different treatments in various concentrations are tested together with one or more controls, with the option to include different numbers of wells per plate. Thus, there are at least three independent predictors of the green area: time, type of treatment (together with its concentration), and the type of plate (6-, 12-, or 24-well). It is also important to keep track of plants that come from the same plate because it is possible that there is more correlation between the green areas of plants from the same plate than among plants from different plates. These multidimensional data are not easy to handle using standard table-processing software (e.g., Excel) because of a richer data-structure, so a table with more than two dimensions is needed. Thus, the data processing was also performed using MatLab software, creating a data structure with the same length as the number of PNG files. Each item in the structure contains the following terms: time from the beginning of the experiment, type of treatment, numerical code of the treatment, type of plate, position on the table, and a list of the green areas of the plants. The same data structure was used for the statistical analysis and data representation.

The Assay Optimization and Validation

The right experimental design for the assay requires introduction of a standardized protocol resulting in maximum homogeneity of the recorded trait, in this case similar plant growth on a single plate and among plate replicates. This allows application of the statistical methods to describe significance of the differences between the tested variants. To define the most suitable screening conditions to achieve HTS using *in vitro* conditions, first the response of 4-day-old *Arabidopsis* seedlings grown in 1× MS [recommended for *Arabidopsis* growth in the protocol published by Cold Spring Harbor Protocols (Recipe, 2010) and by the Arabidopsis Biological Resource Center[2]] was evaluated using a different culture plate format, with a higher number of replicates randomly distributed across the growth area. The 12- and 24-well plates were prepared following the experimental scheme (**Figure 2**) with nine and six replicates *per* variant (represented by a single plate), respectively, and the rosette size (represented by the green area) was analyzed for 9 days. The outcome of the analysis can be either a single growth curve describing the increase in green area over time (**Figure 3A**), or a curve showing the relative growth rate calculated as described Hoffmann and Poorter (2002) (**Figure 3B**). The curves for seedling growth in the 12-well and 24-well plates had similar profiles, showing that during the 9 days when data were collected, the green

[2]https://abrc.osu.edu/seed-handling

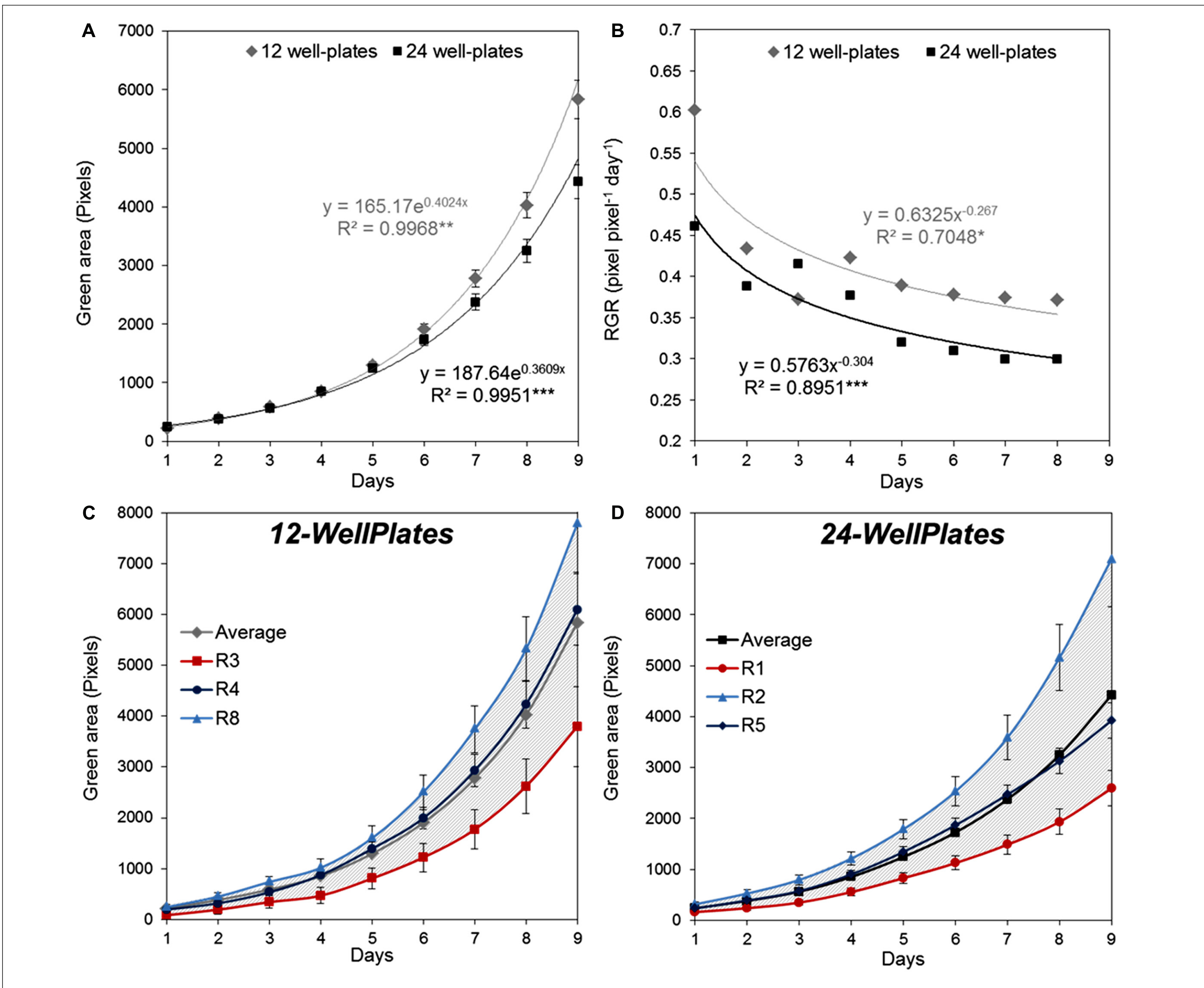

FIGURE 3 | Natural variation in *Arabidopsis* rosette growth in multi-well plates under control conditions. **(A)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates (n = 108) or 24-well plates (n = 144) with 1× MS medium for 9 days. *Mean* ± *SE* **(B)** Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of 4 DAG *Arabidopsis* seedlings. The equations of the curves and the Pearson's correlation coefficients with significance according to ANOVA after linearization were calculated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(C)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings' growth in independent 12-well plates (replicates). **(D)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings' growth in independent 24-well plates (replicates). Gray striped area represents the variation between replicates compared to the average treatment effect.

area of the seedling exhibits high significant exponential growth (**Figure 3A**). Although the rosette area of the seedlings grown in the 12- and 24-well plates starts to differ after 1 week of cultivation, the relative grow rate (RGR) of the seedlings shows the same tendency, with parallel curves but with higher values for the those grown in 12-well plates (**Figure 3B**). This indicates that the volume and space of the well are the main factors determining the difference observed after the sixth day.

To optimize the assay we used a Kruskal–Wallis test to evaluate statistically the differences in rosette area between the plate replicates, which were randomly distributed within the growth area. Unexpectedly, significant differences in the average green areas, in some cases reaching almost 50%, were observed within the plate replicates in both types of multi-well plate at the analyzed time-points (**Figure 4**). Analysis of the rosette growth in the replicates with the smallest, intermediate and largest average growth areas (R3, R4 and R8 or R1, R2 and R5 for 12- and 24-well plates respectively) revealed similar profiles (**Figures 3C,D**), with significant differences between the two extremes (**Table 1**). Taking into account the experimental set-up of the assay, the possible reasons for the differences in the average rosette size in the randomly distributed replicates could be either different micro-climatic conditions in the growth chamber or the non-randomized selection of the seedlings at the time of transfer with respect to developmental stage, resulting from the natural heterogeneity of the population. Measurements of the

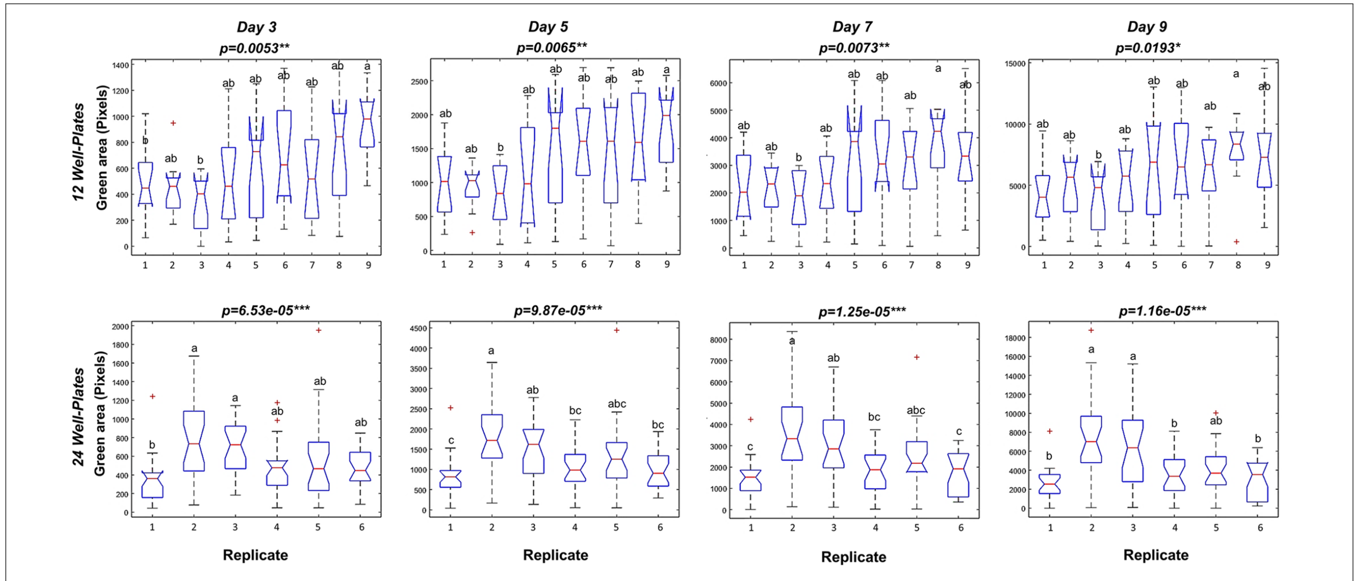

FIGURE 4 | Variation between repeated well plates in *Arabidopsis* rosette growth under control conditions. Box plots representing the green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates (9 biological replicates) or 24 well-plates (6 biological replicates) with 1× MS medium for 9 days. Different letters indicate significant differences according to Conover's test after Kruskal–Wallis' test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

micro-meteorological conditions in the phenotyping chamber did not reveal any differences. For this reason, in the next step we increased the number of germination plates to increase the population of the seedlings. This allowed us to improve our selection of the 4-day-old seedlings, ensuring that they were all a similar size at the time of transfer, discarding any particularly large or particularly small seedlings. The more careful selection of seedlings did, indeed, result in standardization of population heterogeneity between plate replicates, and almost no statistically significant differences between the average rosette areas of the nine replicates were found at the different time-points (**Figure 5**).

Finally, to test the reliability of the method, we compared the green area estimated by automated RGB imaging with the weight of the rosettes determined manually. The rosette of individual plants grown *in vitro* in 24-well plates containing 1× MS and 0.5× MS medium, respectively, were harvested on the last day of measurements and the fresh-weight (FW) of individual plant rosettes was determined. Subsequently, correlations between the green area and FW were calculated using Pearson's coefficient and the significance determined after ANOVA. In both growing conditions, a highly significant correlation was obtained with correlation coefficients of 0.94 and 0.85, respectively (**Figure 6**). The relationship between green area and FW of *Arabidopsis* rosettes showed more homogeneous size of the plants grown in 0.5× MS compared to 1× MS. In 1× MS conditions the distribution of the population was broader, with higher number of smaller (<10 mg of FW) and bigger plants (>40 mg of FW), suggesting that the nutritional conditions contribute to the population phenotype.

Standardization of the Assay for HTS of *Arabidopsis* Rosette Growth in Normal and Stressed Conditions

State-of-the-Art of Conditions Used for *In Vitro* Growth of *Arabidopsis*

To standardize our assay so that it can become a universal HTS tool suitable for analysis of *Arabidopsis* rosette growth, we first performed an in-depth literature review of the typical conditions used for *in vitro* growth of *Arabidopsis*. The main goal for the

TABLE 1 | Statistical differences among replicates of *Arabidopsis* rosettes grown under control conditions.

Well-Plate	Replicate	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
12WP	R3	a	a	b	b	b	b	b	b	b
	R4	a	a	ab	ab	ab	ab	ab	ab	ab
	R8	a	a	a	a	a	a	a	a	a
24WP	R1	b	b	a	a	a	a	a	a	a
	R2	a	a	b	b	b	b	b	b	b
	R5	ab	ab	a	a	a	a	a	a	a

Different letters indicate significant differences in green area (pixels) of 4 DAG old Arabidopsis grown in 1× MS medium over time for selected replicates of 12-well plates and 24-well plates according to Conover's test after a Kruskal–Wallis test ($p < 0.05$).

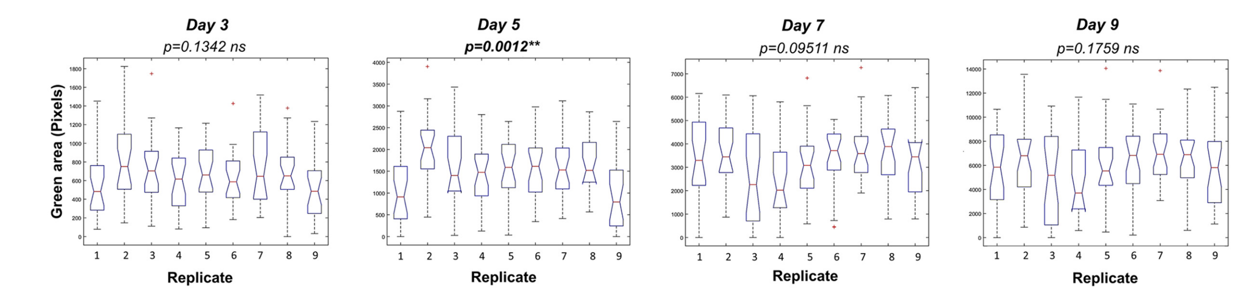

FIGURE 5 | Variation among replicates in *Arabidopsis* rosette growth under control conditions after seedling selection. Box plots representing the green area of 4 DAG *Arabidopsis* seedlings grown in 24-well plates (9 biological replicates) with 1× MS medium for 9 days. Statistical analysis was performed using Kruskal–Wallis' test $^{**}p < 0.01$; ns – non-significant.

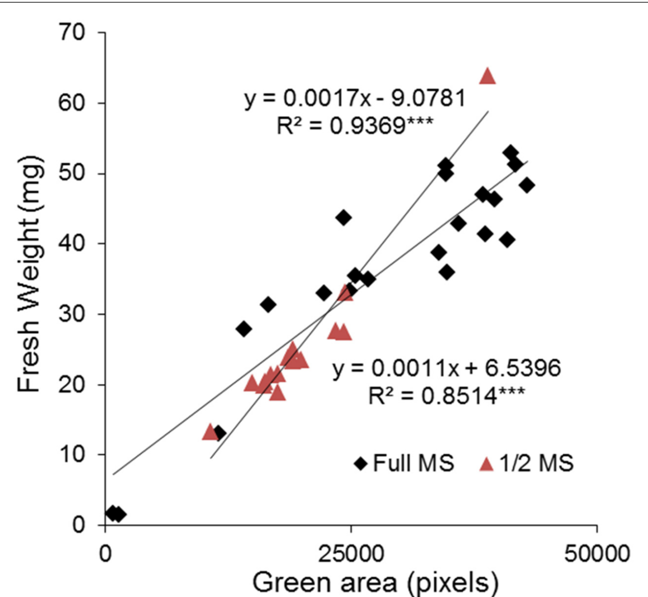

FIGURE 6 | Validation between high-through phenotyping and traditional biometric methods for the analysis of *Arabidopsis* rosette growth in multi-well plates. Correlation between projected green area (pixels) and fresh weight (mg) of 13 DAG *Arabidopsis* seedlings grown in 24-well plates with 1× and 0.5× MS for 9 days. The equation of the curve and the Pearson's correlation coefficient with significance according to ANOVA were calculated. $^{***}p < 0.001$.

presented assay is evaluation of rosette growth (green area) under normal and stressed conditions. Thus, we tried to investigate which growth conditions are typically used by plant biologists to represent "normal" for *Arabidopsis* growth *in vitro* and which conditions are chosen to study stress responses, with the focus on salinity. To achieve this, we analyzed "materials and methods" sections of research articles published in the five research journals with the highest impact factor in the category "Plant Science" (based on the ranking of Web of Science). We attempted: (1) to find the growing medium composition used most often for *in vitro* growth of *Arabidopsis* for publications from 2016, and (2) to determine the conditions used to study its response to salinity in publications from the last 5 years (2012–2016). To determine what is meant by "normal *in vitro* conditions," we analyzed 242 articles published during 2016. As presented in **Figures 7A,B**, a high diversity with respect to plant growth conditions was found. Approximately 70% of the studies used MS medium as a source of nutrients, with many variations in the concentration of sucrose and gelling agent. Half strength MS (0.5× MS) was used in about 60% of studies, followed by full strength MS (1× MS) in about 8% of studies and even quarter strength MS (0.25× MS) in 1% of studies. Surprisingly, in about 30% of the articles examined, information about the type of growing medium was missing (**Figure 7A**). The use of sucrose as a source of energy for *in vitro* grown *Arabidopsis* was also highly variable. About 55% of studies reported using sucrose in different concentrations, only 5% of the studies did not use sucrose in the growing medium, and surprisingly, about 40% of published articles did not specify whether sucrose was used (**Figure 7B**). Among the work that did mention the use of sucrose, the most common concentration was 1% and higher, only in 10% of the cases was a concentration less than 1% used (**Figure 7B**). Next, we analyzed the growth conditions used in the salt-response studies (**Figures 7C,D**). Of 64 articles, about 70% reported using MS medium (with a clear preference for 0.5× MS over 1× MS), while in the rest of the studies salinity was applied through hydroponics, soil, or an unspecified medium (**Figure 7C**). With respect to the concentration of salt applied, we found that 62% of the articles on the subject used only one concentration of NaCl, whilst the remaining 38% reported using a range of salt concentrations (**Figure 7D**). More than half of the studies used 100–200 mM NaCl; of the remaining studies, there were similar numbers that used concentrations either lower than 100 mM, or higher than 200 mM; and, surprisingly, in a few cases an extremely high concentration of NaCl (≥300 mM) was used (**Figure 7D**).

Standardization of Control Conditions for the Assay

To select our standardized normal conditions we tested experimentally whether MS concentration influenced *Arabidopsis* rosette growth and also evaluated the need for sucrose as a component of the growing medium. First, MS medium without sucrose was used in different concentrations;

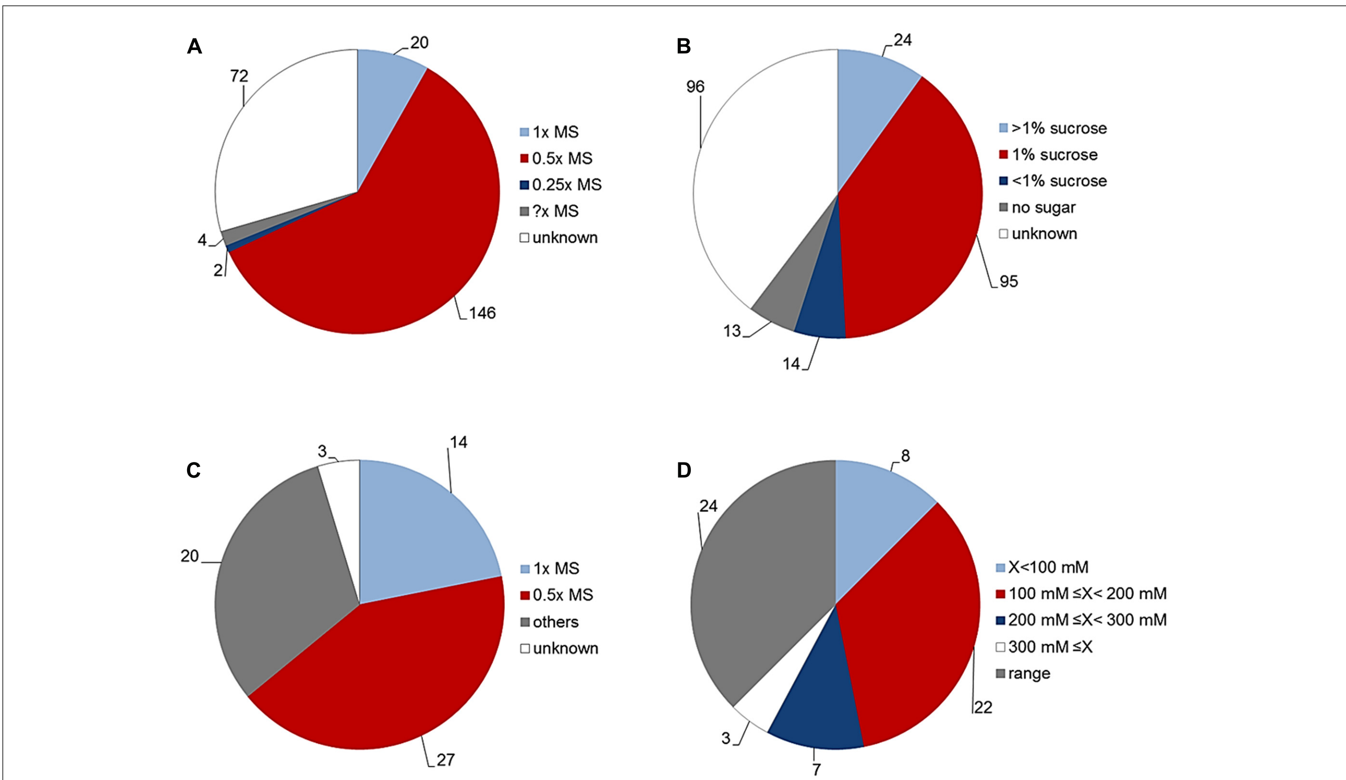

FIGURE 7 | "Normal" *in vitro* growth conditions for *Arabidopsis* plants. **(A)** Culture medium used for *Arabidopsis* grown *in vitro* according to articles (n = 242) published in the five highest impact Plant Science journals (Web of Science) during 2016. MS = Murashige and Skoog basal salt mixture. **(B)** Concentration of sucrose added to the culture medium for *Arabidopsis* grown *in vitro* in the same publications. **(C)** Culture conditions used for salt-stress studies of *Arabidopsis* according to articles (n = 64) published in the five highest impact Plant Science journals (Web of Science) from 2012 to 2016. **(D)** Concentration of salt published in the same articles for stress studies of *Arabidopsis*.

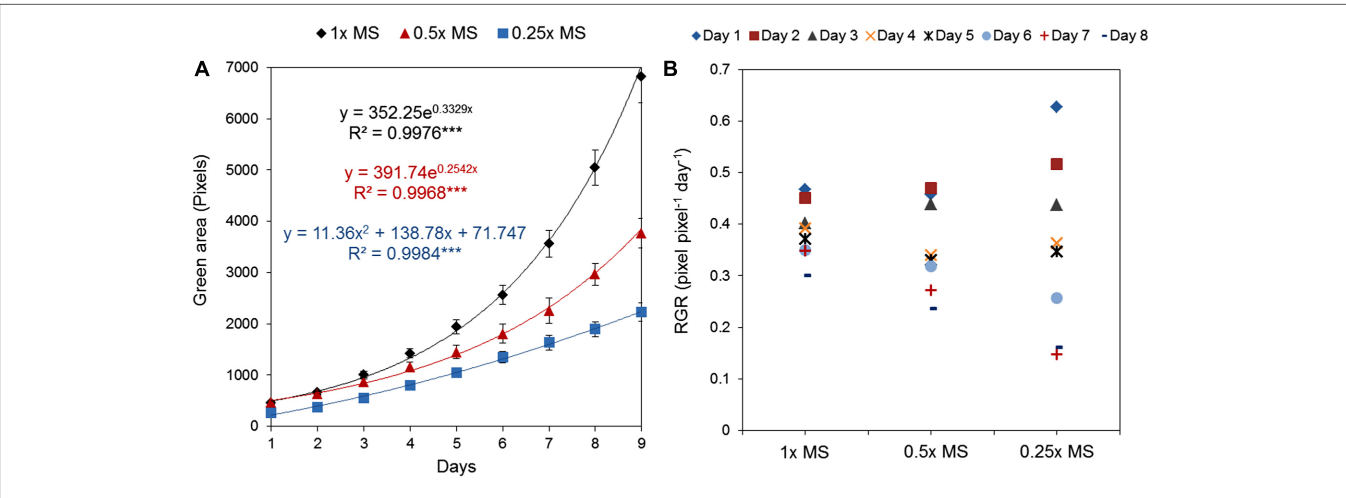

FIGURE 8 | Natural variation in *Arabidopsis* rosette growth in different MS media. **(A)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates (n = 36) with different MS media for 9 days. *Mean* ± *SE*. The equations of the curves and the Pearson's correlation coefficients with significance according to ANOVA after linearization were calculated. $^{***}p < 0.001$. **(B)** Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of 4 DAG *Arabidopsis* seedlings.

0.25×, 0.5×, and 1×. A clear concentration-dependent increase in rosette area was found, indicating that 1× MS is the best growing medium for *Arabidopsis* seedlings *in vitro* (**Figure 8A**). Although the RGR of the seedlings grown on 0.5× MS was comparable to those on 1× MS during the first 4 days, in the second half of the growth period the development of

these seedlings slowed and their RGR was 13% lower than for seedlings on 1× MS, decreasing to 25% during the last 2 days (**Figure 8B**). This suggests that use of lower MS concentration than 1× can result in a change from optimal to suboptimal growth conditions during the period that the experiment is running and the seedlings are inadvertently subjected to low nutrient stress during their, otherwise, exponential growth period.

As mentioned above, the use of sucrose in growth media is also generally very variable, ranging from a concentration of 0 to 3%. As discussed later, the presence of sucrose in the growing medium leads to substantial changes in the physiology of the developing seedling, conditioning seed germination and modifying plant metabolism (Ohto et al., 2001; Eckstein et al., 2012). Hence, we tested how the exogenous addition of sucrose alters the growth of *Arabidopsis* seedlings in optimal nutritional conditions and compared plant performance when grown on 1× MS medium containing 0, 0.1, and 1% sucrose. No significant differences were found in the increase of the rosette areas of the seedlings grown with and without sucrose over the duration of the experiment (**Supplementary Figure S2**). Taking into account these results, we decided to use 1× MS without sucrose as the standard growing medium for our assay.

Use of the Assay in the Salt-Stress Studies

Our platform has sufficient capacity to allow simultaneous testing of large numbers of variants. This can be employed for evaluation of chemical libraries and/or genetic populations in normal and stressed conditions and for cross-testing of a wide range of concentrations of stressors and/or tested compounds. To illustrate the potential of our assay to be used as a tool for large-scale stress-response studies, we performed an experiment in which the effect of salt on *Arabidopsis* rosette growth was tested using 1× MS medium supplemented with different concentrations of NaCl (50, 75, 100, and 150 mM). Three replicates of a 24-well plate were used for each tested variant, with no significant differences among them throughout the experiment (**Figure 9**). Both time-dependent increase in shoot area and RGR were found to be negatively affected by NaCl treatment in a dose-dependent manner (**Figures 10A,B**). Even after 2 days, significant differences in the rosette area were recorded between the controls and the plants grown in the presence of 100 mM and 150 mM NaCl (**Figure 10A** and **Table 2**), due to a very fast decrease in RGR: 56 and 84%, respectively (**Figure 10B**). After 5 days, significant differences in the rosette area were also apparent between the controls and the lowest salt treatments (**Table 2**). Interestingly, salt treatment modified the population distribution causing changes in the quartiles. The moderate salinity (50 mM) increased the rosette areas of the plants of the first and the third quartiles (Q1, Q3) until the fifth day, after which the salinity started to have the expected negative effect on rosette growth (**Table 2**). The severe salt-stress conditions (100 mM and 150 mM NaCl) had clear negative effects on the rosette growth and, moreover, reduced the plant size in both quartiles. The Q1 for the plants treated with 150 mM NaCl was reduced to zero on the fifth day of the salt treatment and the survival of plants in this variant reduced from 67% at day 7 to 50% at day 9 (**Figure 10C**). Overall these results proved the potential of the assay to be used as a tool for salt-stress studies.

Large Scale Testing Can Reveal Unexpected Interactions between Conditions/Treatments

In the previous text we described the effect of different salt concentrations on the *Arabidopsis* rosette grown under optimal nutrient conditions (1× MS). As mentioned above, analysis of the typical conditions used in salt-stress studies, 0.5× MS was mostly chosen as the source of nutrients (**Figure 7C**). This fact led us to perform an experiment in which the effect of 75 mM NaCl (identified in this study as representing medium salt stress) on *Arabidopsis* rosette growth was tested in MS medium of three different strengths, i.e., 0.25×, 0.5×, and 1×. Each variant comprised three replicates on a 12-well plate and no statistical differences among them were found according to a Kruskal–Wallis test (**Supplementary Figure S3**). When the green area of the different treatments was analyzed we obtained an unexpected result: a significant interaction between MS concentration and salt treatment (**Table 3** and **Figures 11A,B**). Both time-dependent increase in the green area of the rosette and decrease in RGR were higher in salt-stressed plants grown in 0.5× MS than those in 1× MS (**Figures 11A,B**). When they were compared with the plants grown in different MS without salt (**Figure 8A**), we observed that whereas plants grown in 1× MS without salt had at least 2-4 times bigger rosettes compared to the salt stressed ones after 7 and 9 days of the treatment, respectively, no significant differences were observed between those *Arabidopsis* grown with and without 75 mM NaCl in 0.25× and 0.5× MS (**Figures 11C,D** and **Table 3**). Interestingly, whereas the salt treatment reduced the quartiles, median and average rosette area of the plants grown in 1× MS by a factor of four, whilst keeping similar minimum and maximum values, it improved the Q1 and Q3, and the minimum size of the plants grown in the low nutrient media (**Table 3**). These results were further confirmed by the population distribution of each treatment over time (**Supplementary Figure S4**), where the salt treated 0.25× and 0.5× MS variants presented a narrower distribution and more homogeneous populations with plants of similar rosette size compared to their respective controls. The analysis also revealed the same average green areas, size heterogeneities and similar distribution of the populations of the plants grown in 0.25× MS and plants grown in 1× MS with 75 mM NaCl (**Table 3**, **Figures 8**, **11**, and **Supplementary Figure S4**). These results revealed the existence of a crucial interaction between the concentration of nutrients and the salt treatment that conditions the stress response and growth capacity of the plants through the heterogeneity of the plant population.

DISCUSSION

Recent advances in high-throughput phenotyping allow simultaneous screening of multiple quantitative traits of plant

Day 3 | Day 5 | Day 7 | Day 9

50 mM NaCl — Green area (Pixels): p=0.2092 ns | p=0.1111 ns | p=0.2938 ns | p=0.1999 ns

75 mM NaCl — Green area (Pixels): p=0.2503 ns | p=0.1376 ns | p=0.7066 ns | p=0.9513 ns

100 mM NaCl — Green area (Pixels): p=0.821 ns | p=0.6158 ns | p=0.2393 ns | p=0.3486 ns

150 mM NaCl — Green area (Pixels): p=0.0560 ns | p=0.9003 ns | p=0.9035 ns | p=0.0999 ns

Replicate | Replicate | Replicate | Replicate

FIGURE 9 | Variation among replicates in *Arabidopsis* rosette growth under salt-stress. Box plots representing the green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 24-well plates with 1× MS medium and different NaCl concentrations for 9 days. Statistical analysis was performed using Kruskal–Wallis' test. ns, non-significant.

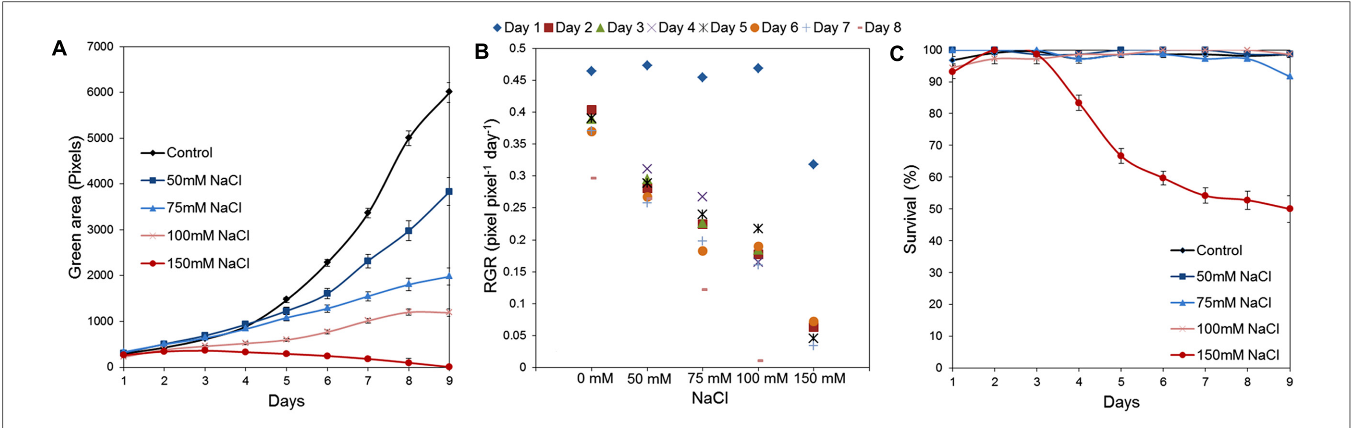

FIGURE 10 | Natural variation in *Arabidopsis* rosette growth under salt-stress. **(A)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 24-well plates (n = 48) in 1× MS with different NaCl concentrations for 9 days. *Mean* ± *SE* **(B)** Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of 4 DAG *Arabidopsis* seedlings. **(C)** Survival (%) of the seedlings.

growth under different environmental conditions (Humplík et al., 2015a; Rahaman et al., 2015). However, many of the existing phenotyping systems still have a limited capacity to measure a large number of plants in a short time. For example, the GROWSCREEN FLUORO system can measure 30 plants per run, and after each run the plates must be manually exchanged (Jansen et al., 2009). Other published protocols have improved the total throughput to 200 plants (Awlia et al., 2016), or even 800 plants per hour (Arvidsson et al., 2011), however, actual throughput is in fact defined by the number of variants being tested, the number of replicates, and the number of plants per variant/replicate. For HTS approaches, transferring to *in vitro* conditions allows the miniaturization of the bioassay and an increase in the number of both variants and replicates. One example is the new work recently published for HTS of 1000s of compounds with growth regulator activity using *Arabidopsis* grown *in vitro* in 24-well plates (Rodriguez-Furlán et al., 2016). However, in this method, it takes 20 min per plate for image analysis using a scanner, which defines the number of variants and plates that can be used as replicates. It is important to mention here that 20 min per plate or series, especially in plant species like *Arabidopsis* with short life cycles and very fast growth, can be problematic. In our study we observed changes in the size of the green area of 8-day-old plants in periods as short as 2 h (**Supplementary Figure S1**). Thus, the long intervals associated with semi-automated systems that have slow image acquisition can introduce significant bias. Similar limitations can be detected in the recently published rosettR method, where the image acquisition is performed by microscope and the plates are changed manually (Tomé et al., 2017). Thus, our main goal was to develop a fast, robust and reproducible high-throughput *in vitro* bioassay for *Arabidopsis*. Our system delivers the advantage of fast fully automated measurements of the rosette growth of 11,000 *Arabidopsis* plants in less than 2 h, allowing a simultaneous study of different growth conditions without compromising the number of variants, replicates and plants per treatment, as summarized in **Table 4**. To achieve this, fully automated image-processing software and data analysis for evaluating the reproducibility of *in vitro* growth conditions using *Arabidopsis* as the plant material was developed and various growing conditions and experimental set-ups were tested. The optimal growing conditions for *Arabidopsis in vitro* growth in different well format plates (12-well and 24-well plates) were full MS + 0.6% agar without sucrose. In preliminary tests, we detected significant differences between replicates over time in both types of well plate used (**Figure 2**). After several testing runs we identified the preliminary selection of the plant seedlings for transplantation into the multi-well plates as the main influencing factor within the bioassay, significantly affecting reproducibility. Indeed, the way that germination timing influences phenotypic

TABLE 2 | The effect of salinity on *Arabidopsis* rosette growth.

Days	Control			50 mM NaCl			75 mM NaCl			100 mM NaCl			150 mM NaCl		
	Q1	Q3		Q1	Q3		Q1	Q3		Q1	Q3		Q1	Q3	
Day 1	157.5	400.0	a	226.3	442.5	a	228.3	403.5	a	106.5	334.3	b	132.8	409.3	a
Day 2	278.0	568.5	bc	390.8	664.0	a	409.5	628.3	ab	248.8	495.0	c	234.8	554.5	c
Day 3	403.5	846.3	a	508.3	935.3	a	447.3	812.3	a	312.8	601.3	b	253.8	549.0	b
Day 4	473.5	1291.3	a	676.8	1337.5	a	528.0	1139.8	a	388.0	708.3	b	96.3	461.5	c
Day 5	894.0	2059.5	a	892.0	1773.0	ab	724.8	1441.3	b	437.0	838.0	c	0	546.8	d
Day 6	1514.5	3040.0	a	1271.0	2289.3	b	914.0	1816.5	b	554.8	1004.8	c	0	594.5	d
Day 7	2142.3	4496.5	a	1413.3	2731.0	b	1084.3	2080.5	c	597.8	1233.8	d	0	641.3	e
Day 8	2937.5	6321.0	a	1614.8	3657.8	b	1317.8	2400.0	c	645.5	1421.5	d	0	680.0	e
Day 9	3689.0	7976.0	a	1963.5	5311.8	b	1249.8	2728.5	c	604.3	1554.0	d	0	659.5	e

The first quartile (Q1), the third quartile (Q3) and statistical differences among treatments in green area (pixels) of 4 DAG old Arabidopsis grown in 1× MS medium supplemented with different NaCl concentrations. Different letters indicate significant differences according to Conover's test after a Kruskal–Wallis test ($p < 0.05$).

TABLE 3 | The interaction between MS concentration and salinity for *Arabidopsis* rosette growth.

Treatment	Day 7						Day 9					
	Q1	Median	Q3	minimum	Maximum	*SE*	Q1	Median	Q3	Minimum	Maximum	*SE*
1x MS	4030	5530 a	6528	386	7919	342.5	5008	7007 a	9031.75	381	11156	499.4
1x MS + Salt	9334	1981 c	2948	193	7307	257.2	1004	2043 d	3802	194	9528	339.3
0.5x MS	1924	2911 b	3785	410	7375	210.1	2440	3719 bc	4700	454	10493	289.7
0.5x MS + Salt	2362	3359 b	3890	1320	6331	191.9	2916	4139 b	5000.25	1423	7699	267.0
0.25x MS	1154	2108 c	2553	346	3785	150.3	1424	2504 d	2889.25	371	4588	175.2
0.25x MS + Salt	2059	2528 bc	2816	556	3669	115.4	2216	2631 cd	3500.5	490	4625	153.8

The first quartile (Q1), the median, the third quartile (Q3), the minimum (min.), the maximum (Max.) green area (pixels) of 4 DAG old Arabidopsis after 7 and 9 days grown in different MS media supplemented with 75 mM NaCl. Different letters indicate significant differences according to Conover's test after a Kruskal–Wallis test ($p < 0.05$).

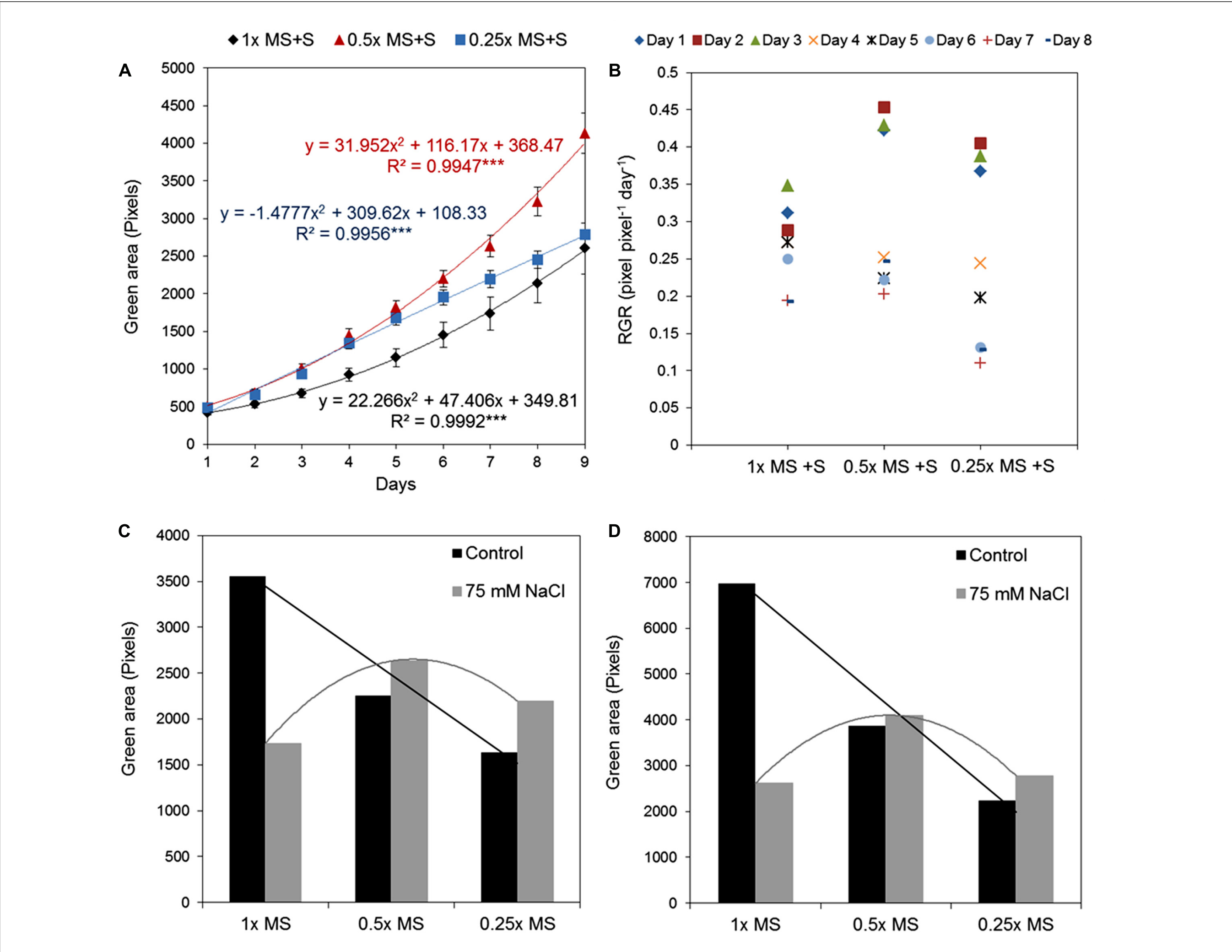

FIGURE 11 | Natural variation in *Arabidopsis* rosette growth in different MS media with salt. **(A)** Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates ($n = 36$) with different MS media and 75 mM NaCl for 9 days. *Mean* ± *SE*. The equations of the curves and the Pearson's correlation coefficients with significance according to ANOVA after linearization were calculated. ***$p < 0.001$. **(B)** Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of 4 DAG *Arabidopsis* seedlings grown under the same conditions. **(C)** Comparison among 11 DAG *Arabidopsis* seedlings grown in different MS media with or without 75 mM NaCl. **(D)** Comparison among 13 DAG *Arabidopsis* seedlings grown in different MS media with or without 75 mM NaCl.

TABLE 4 | The capacity of the high-throughput *in vitro Arabidopsis* bioassay using different well plates.

Type of well plate	No. plants	Replicates	Platform capacity	Total plants	No. variants	Assay duration
6-Well Plates	6	3		2880	160	14 days
12-Well Plates	12	2	480 Plates	5760	240	9 days
24-Well plates	24	1		11520	480	9 days

expression post-germination in *Arabidopsis* (Donohue, 2002), and affects plant survival (Joosen et al., 2012) have been described previously. Thus, careful selection of the plants to be used in the experiment has been highlighted and implemented in some research, ensuring synchronization of plant germination and then selecting seedlings that germinated at the same time and/or are at the same developmental stage (Humplík et al., 2015b; Awlia et al., 2016). In a recent study, specific software was presented for this purpose (Clauw et al., 2015). In our work, the selection of similar 4-day-old *Arabidopsis* all at the same developmental stage allowed us to obtain a reproducible methodology for growing, avoiding significant differences between replicates used in the different treatments (**Figures 4**, **6**). In addition, our method also conserved population heterogeneity over time, thus permitting rapid identification of the differences in rosette area among phenotypes (**Supplementary Figure S2**).

After optimization of the standard conditions for the *Arabidopsis* HTS, we further optimized the methodology for

evaluating plant response to stress. As an example of a form of stress condition, we examined the effect of salinity on plant growth. Salinity is the main environmental factor responsible for decreasing crop productivity, affecting more than 20% of the cultivated land worldwide (Gupta and Huang, 2014). Salt stress affects plant growth in two phases: the first and rapid osmotic phase that inhibits growth of young leaves, and the second and slower ionic phase that accelerates senescence of mature leaves. In the osmotic phase, which starts immediately after the salt concentration around the roots increases to a threshold level (around 40 mM NaCl for most plants or less for sensitive plants like rice and *Arabidopsis*), the rate of shoot growth decreases significantly (Munns and Tester, 2008). The second, ion-specific, phase of plant response starts when salt accumulates to toxic levels in the source leaves, which rapidly die. This last phase dominates in high salinity conditions or in sensitive species. In our work we have demonstrated that the growth of a sensitive species such as *Arabidopsis* is not so highly affected by salt stress of 40 mM and the growth inhibition is only apparent over the time. On the other hand, the plants grown in salt concentrations of 150 mM showed very dramatic growth inhibition and fast senescence (yellow tissues) leading to death (**Figure 10**), most probably because of reaching the second salinity phase. Similar plant survival was obtained in *Arabidopsis* ecotype Col-0 grown *in vitro* using MS medium and 150 mM NaCl after 4 days [≅ 15% (Zhao et al., 2013)] or 10 days [≅ 50% (Feng et al., 2015)] of exposure. Interestingly, similar curves for plant growth over time were also presented in a recently published HTS technique for studying salinity tolerance in *Arabidopsis* using soil, where 50 mM NaCl did not significantly affect the growth until 8 days of exposure, but very rapid growth inhibition and chlorosis in plants was indiced when 150 mM NaCl was applied (Awlia et al., 2016).

Surprisingly, in our experiments we also observed that salt-induced growth inhibition and *Arabidopsis* tolerance capacity are defined by an interaction between salt and the nutrient concentrations in the growing medium. Higher tolerance to salinity was found in plants grown in the 0.5× MS medium than those ones grown in 0.25× or 1× MS (**Figure 11** and **Table 3**), and a higher number of dead and smaller plants was observed for the combination 1× MS and salinity (see **Supplementary Figure S4**). These results suggest that some specific nutrients may be responsible for plant sensitivity to stress and that a reduction in their concentration could delay the senescence effect characteristic of the second phase of the stress response, even in a sensitive species as *Arabidopsis*. It is known that salinity affects nutrient uptake in plants and induces some nutrient deficiencies, such as a reduction of Ca^{2+}, N, and K levels in different plant species (Pérez-Alfocea et al., 1996; Gunes et al., 2007; Koksal et al., 2016). Tuna et al. (2007) studied the effect of nutrient supplementation with $CaSO_4$ for mitigating salt stress-induced losses in crop production. They showed that tomato plants exposed to 5 mM $CaSO_4$ exhibited improved salt tolerance, increasing the concentration of specific ions in the plant such as K^+, Ca^{2+}, and N and reducing the levels of Na^+. In accordance with this, it has been shown that Ca^{2+} can move very rapidly through the plant and activates a rapid plant response to stress (Choi et al., 2014). Ca^{2+} is one of the main nutrients present in MS medium together with N. Due to the fact that salinity greatly affects the activity of many enzymes involved in the N metabolism (Dubey, 1997), we propose that plants growing in 1× MS + salt are absorbing too many nutrients and exceeding their assimilation capacity under these conditions. However, more studies are needed to corroborate this suggestion. This might also explain the discrepancies in some published works using *Arabidopsis* plants grown in 0.5× MS medium with high salt concentrations (Pitzschke et al., 2014; Wang et al., 2015; Dolata et al., 2016). To clarify these results, more simultaneous studies of these two variables (salinity and nutrients) are needed. In addition, to avoid controversial results and to ensure that different studies are comparable, we think there is a need for a standard global protocol, specifying the *in vitro* growing conditions for *Arabidopsis*.

CONCLUSION

In this work we present a highly reproducible *in vitro* HTS assay using *Arabidopsis* that offers simplified scoring of phenotypes and permits large-scale miniaturized screening over a short time, allowing faster identification of phenotypes with different tolerances and the evaluation of possible candidate molecules that can offer a simple solution to the production problems caused by salinity.

AUTHOR CONTRIBUTIONS

NDD, TF, JH, and LS designed the experiments and performed the data analysis. LU and KP performed the experiments. NDD and LS supervised the study and the concept of the project. All authors discussed the results. NDD, TF, and LS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01702/full#supplementary-material

FIGURE S1 | Green area (pixels) of 9 DAG *Arabidopsis* seedlings grown in independent 12-well plates (replicates, R1 and R2) at 12:00 and at 14:00 H.

FIGURE S2 | Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates (n = 36) with 1× MS medium with different sucrose concentrations (0, 0.1 or 1%) for 8 days. *Mean ± SE.*

FIGURE S3 | Variation among replicates in *Arabidopsis* rosette growth in different MS media with salt. Box plots representing the green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates containing different MS media with or without 75 mM NaCl for 9 days. Statistical analysis was performed using Kruskal–Wallis' test. **p* < 0.05; ns, non-significant.

FIGURE S4 | Distribution of population heterogeneity in *Arabidopsis* rosette area grown in different MS media with or without 75 mM NaCl for 7 days.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplement II.

FIGURE S1 | Green area (pixels) of 9 DAG *Arabidopsis* seedlings grown in independent 12-well plates (replicates, R1 and R2) at 12:00 and at 14:00 H.

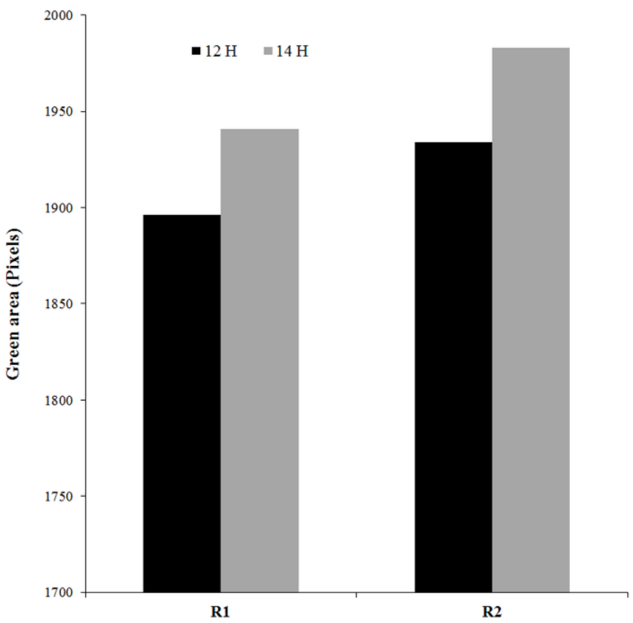

FIGURE S2 | Green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates (n = 36) with 1× MS medium with different sucrose concentrations (0, 0.1 or 1%) for 8 days. Mean ± SE.

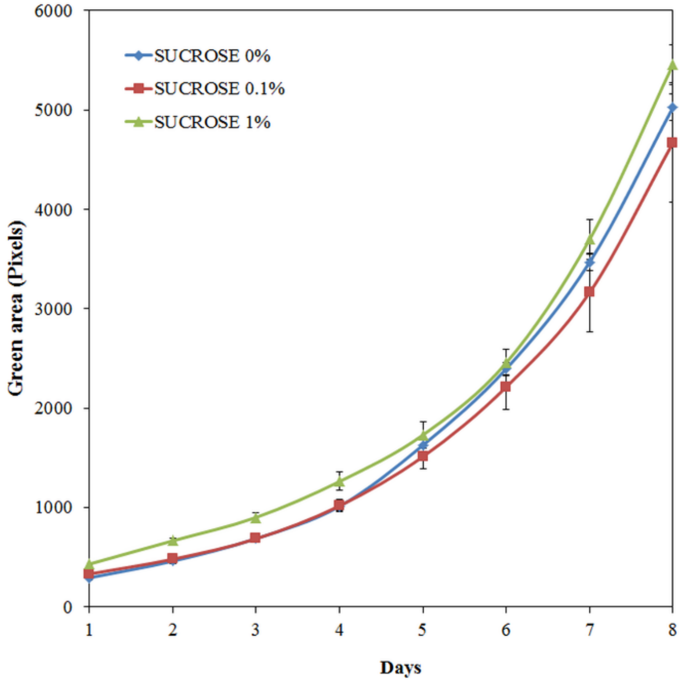

FIGURE S3 | Variation among replicates in *Arabidopsis* rosette growth in different MS media with salt. Box plots representing the green area (pixels) of 4 DAG *Arabidopsis* seedlings grown in 12-well plates containing different MS media with or without 75 mM NaCl for 9 days. Statistical analysis was performed using Kruskal–Wallis' test. $*p < 0.05$; ns, non-significant.

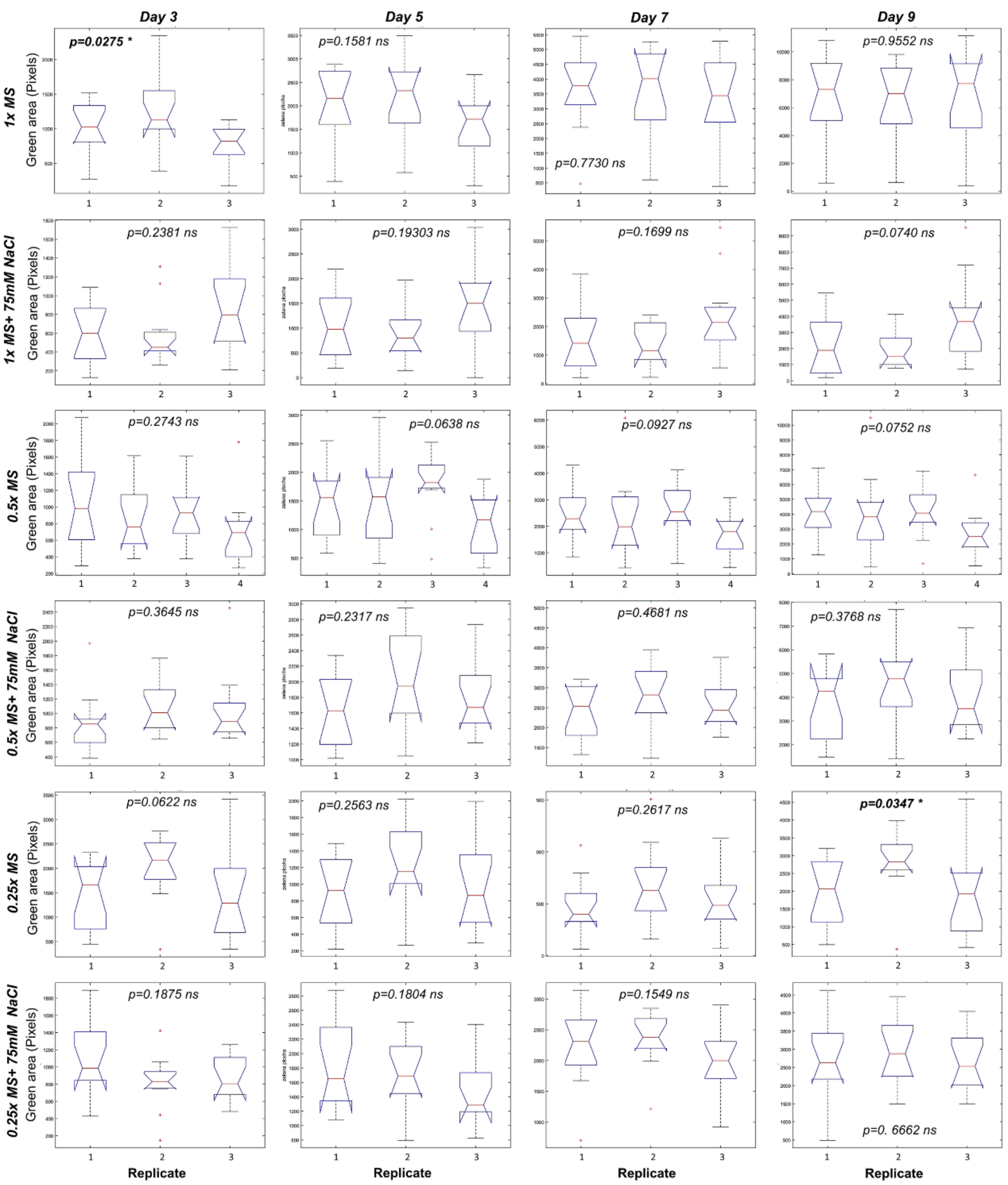

FIGURE S4 | Distribution of population heterogeneity in *Arabidopsis* rosette area grown in different MS media with or without 75 mM NaCl for 7 days.

The Supplementary Figure S4 for this article can be found online at:

http://journal.frontiersin.org/article/10.3389/fpls.2017.01702/full#supplementary-material

Lydia Ugena*, Adéla Hýlová*, Kateřina Podlešáková, Jan F. Humplík, Karel Doležal, Nuria De Diego and Lukáš Spíchal. "Characterization of biostimulant mode of action using novel multi-trait high-throughput screening of *Arabidopsis* germination and rosette growth". *Frontiers in Plant Science*, vol.9, Art. 1327, 2018.

❖ *Supplement III*

ORIGINAL RESEARCH
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Characterization of Biostimulant Mode of Action Using Novel Multi-Trait High-Throughput Screening of *Arabidopsis* Germination and Rosette Growth

Lydia Ugena[1†], Adéla Hýlová[1†], Kateřina Podlešáková[1], Jan F. Humplík[1,2], Karel Doležal[1], Nuria De Diego[1*] and Lukáš Spíchal[1]

[1] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czechia, [2] Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czechia

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Giuseppe Colla,
Università degli Studi della Tuscia, Italy

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Università degli Studi di Milano, Italy
Barbara De Lucia,
Università degli Studi di Bari Aldo Moro, Italy

****Correspondence:***
Nuria De Diego
nuria.de@upol.cz

†These authors have contributed equally to this work

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Environmental stresses have a significant effect on agricultural crop productivity worldwide. Exposure of seeds to abiotic stresses, such as salinity among others, results in lower seed viability, reduced germination, and poor seedling establishment. Alternative agronomic practices, e.g., the use of plant biostimulants, have attracted considerable interest from the scientific community and commercial enterprises. Biostimulants, i.e., products of biological origin (including bacteria, fungi, seaweeds, higher plants, or animals) have significant potential for (i) improving physiological processes in plants and (ii) stimulating germination, growth and stress tolerance. However, biostimulants are diverse, and can range from single compounds to complex matrices with different groups of bioactive components that have only been partly characterized. Due to the complex mixtures of biologically active compounds present in biostimulants, efficient methods for characterizing their potential mode of action are needed. In this study, we report the development of a novel complex approach to biological activity testing, based on multi-trait high-throughput screening (MTHTS) of *Arabidopsis* characteristics. These include the *in vitro* germination rate, early seedling establishment capacity, growth capacity under stress and stress response. The method is suitable for identifying new biostimulants and characterizing their mode of action. Representatives of compatible solutes such as amino acids and polyamines known to be present in many of the biostimulant irrespective of their origin, i.e., well-established biostimulants that enhance stress tolerance and crop productivity, were used for the assay optimization and validation. The selected compounds were applied through seed priming over a broad concentration range and the effect was investigated simultaneously under control, moderate stress and severe salt stress conditions. The new MTHTS approach represents a powerful tool in the field of biostimulant research and development and offers direct classification of the biostimulants mode of action into three categories: (1) plant growth promotors/inhibitors, (2) stress alleviators, and (3) combined action.

Keywords: biostimulants, multi-trait high-throughput screening assay, proline, polyamines, plant biostimulant characterization index, salinity

INTRODUCTION

Agricultural crop production will be extremely challenging in the coming decades. Due to the increase in population, a 50% (maximum) increase in the demand for food is expected by 2030. During the growing season, crops around the world are subjected to environmental stresses that affect plant germination, metabolism, growth and yield. Breeders worldwide have therefore focused on quantitative analyses of plant traits in order to accelerate the development of appropriate strategies for improving lines or varieties which are adaptable to resource-limited environments (Rahaman et al., 2017). Soil salinity is an important environmental factor that results in decreased crop productivity on a global scale. In fact, owing to this factor, an estimated 1.5 million hectares of land is taken out of production each year and by 2050 a 50% loss of cultivable lands is expected (Ibrahim, 2016).

The application of biostimulants represents one of the most innovative and promising strategies for minimizing stress impact, including salinity. A plant biostimulant is defined as a material of biological origin which includes bacteria, fungi, seaweeds, higher plants, animals and humate-containing raw materials (Sharma et al., 2014; Yakhin et al., 2016; Cristiano et al., 2018). This material induces beneficial plant processes (including nutrient uptake, nutrient use efficiency, tolerance to abiotic stress and crop quality), independently of its nutrient content (Calvo et al., 2014; Yakhin et al., 2016). Exposure of seeds to abiotic stresses, such as salinity among others, results in lower seed viability, reduced germination, and poor seedling establishment (Savvides et al., 2016). Increasing the salt concentration of the soil leads to a decrease in the germination percentage and delays the germination starting point (Kaveh et al., 2011; Thiam et al., 2013; Ibrahim, 2016). Seed-priming might improve seed 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). Seeds primed with biostimulants from varied origins trigger fast seed germination (Zeng et al., 2012; Colla et al., 2014; Garcia-Gonzalez and Sommerfeld, 2016). Besides, priming seeds with certain biostimulants can promote tolerance to adverse environmental conditions during the imbibition and germination stages (Mahdavi, 2013; Sharma et al., 2014; Pichyangkura and Chadchawan, 2015; Van Oosten et al., 2017).

Recently, the global biostimulant market has grown rapidly and, to satisfy crop requirements, many companies are actively introducing various innovative products and ingredients (Calvo et al., 2014; Sharma et al., 2014). However, in general, the raw materials used by the biostimulant manufacturers exhibit considerable compositional variations which may impact on the composition and concentration of major components (Povero et al., 2016; Sharma et al., 2016). The origin of biostimulants is diverse, and can range from single compounds to complex matrices with different groups of bioactive components that have only been partly characterized (du Jardin, 2015). Irrespective of their complexity, biostimulants are known to contain different groups of plant signaling compounds such as plant hormones, amino acids, and polyamines among others (Craigie, 2011; du Jardin, 2015). The exogenous application of these signaling molecules has been reported to ameliorate the adverse effect of stress through a sophisticated crosstalk among them leading to the activation of conserved pathways [reviewed in Podlešáková et al. (2018)].

In this work we present a novel approach for biostimulant mode of action characterization based on multi-trait high-throughput screening (MTHTS) of *Arabidopsis* germination and rosette growth under salinity. The analyzed traits included the germination rate, rosette growth rate and color. The potential of the approach was demonstrated by applying (via seed priming) representatives of the most common compounds present in biostimulants (i.e., polyamines and amino acids). In addition, we optimized the principles of two previously described protocols for implementation into the MTHTS approach. These included (i) the fast scoring of the germination rate based on a standardized 96-well plate test coupled with spectrophotometric reading of tetrazolium salt reduction (Pouvreau et al., 2013) and (ii) an automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates (De Diego et al., 2017). A highly efficient and reliable method for characterizing biostimulant efficacy at various salt stress levels was realized by developing and combining a high-throughput seed germination assay in *Arabidopsis* with the improved *Arabidopsis* rosette growth assay.

MATERIALS AND METHODS

HTS of *Arabidopsis in vitro* Seed Germination

Arabidopsis thaliana (L.) Heynh seeds (accession Col-0) were surface-sterilized by soaking in 70% Ethanol plus 0.01% Triton X-100 for 10 min. After that, the seeds were washed with sterilized water and then resuspended at a density of 10 g L^{-1} in 1 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (Carl Roth GmbH + Co. KG., Germany) (pH 7.5). Seeds were stratified at 4°C in the dark for 72 h. To investigate the effect of biostimulants on *Arabidopsis in vitro* seed germination, four single active compounds commonly present in many commercial biostimulant products were selected for seed priming; three polyamines: putrescine (Put) (1,4-butanediamine dihydrochloride), spermidine (Spd) (*N*-(3-aminopropyl)-1,4-butanediamine trihydrochloride), spermine (Spm) [N-(3-Aminopropyl)-1,4-butanediamine trihydrochloride] and the amino acid *L*-proline (Pro) [(*S*)-Pyrrolidine-2-carboxylic acid], all purchased from Sigma-Aldrich, Inc., (Germany). These compounds were added before the stratification, reaching final concentrations of 0.001, 0.01, 0.1, or 1 mM. After the cold stratification, seed suspension was washed three times with 20 mL sterile water to remove the biostimulants. In the last wash half of the water volume was removed and an additional 10 mL solution of sterilized 0.1% agarose with 1 mM of HEPES

Abbreviations: GLI, green leaf index; MTHTS, multi-trait high-throughput screening; NGRDI, normalized green red difference index; Pro, *L*-proline; Put, putrescine; Spd, spermidine; Spm, Spermine; VARI, visible atmospherically resistant index.

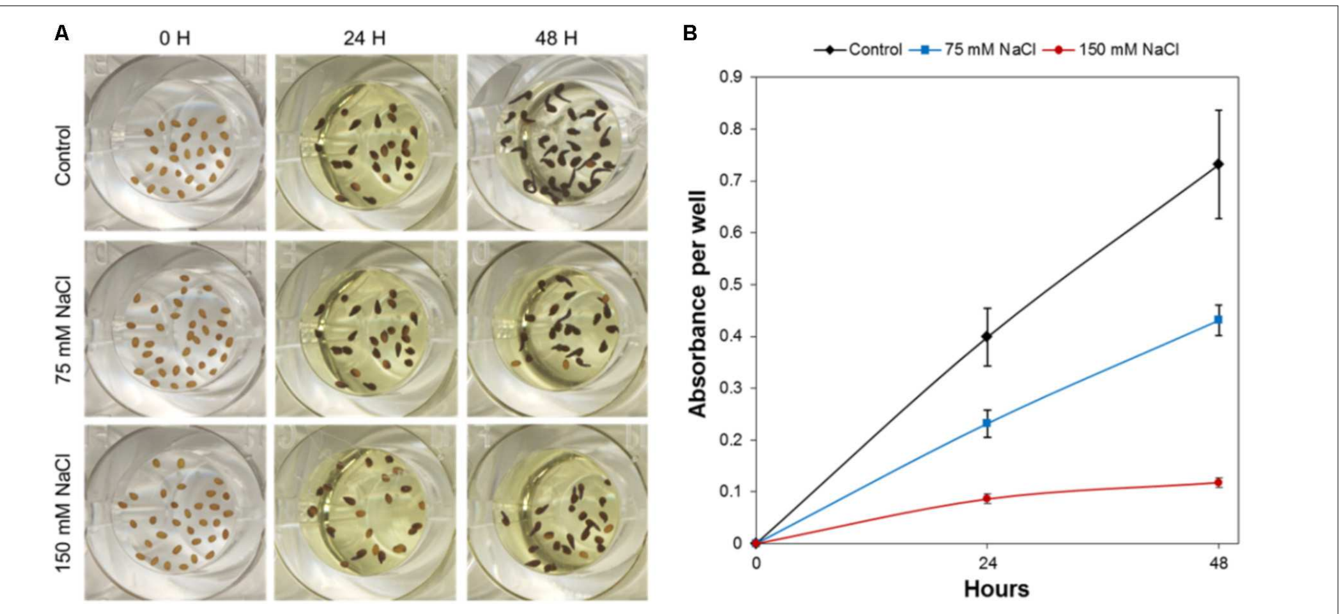


FIGURE 1 | HTS of *Arabidopsis* germination under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions. **(A)** Characteristics of *Arabidopsis* seeds in one well of the 96-well plate before germination (0 h), and after 24 and 48 h, respectively, of germination with subsequent MTT treatment. **(B)** Absorbance of MTT after solubilization of formazan from *Arabidopsis* seeds germinated under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions. The values represent *Mean* ± *SE*.

buffer was added. This is because seeds do not sediment in 0.05% agarose and are suspended in an adequate homogeneous solution for pipetting. The 96-well plate was filled with the seed suspension, 50 μL per well, representing ∼20–30 seeds per well. The final volume was adjusted to 100 μL per well with demineralized water or, in the case of the salt stress treatments, a NaCl solution that yields a final concentration of 75 or 150 mM NaCl in the well. Plates were sealed and incubated for seed germination at 21°C in darkness.

For the quantification of the *Arabidopsis* germination rate, the methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, Inc.) assay was performed in accordance with Pouvreau et al. (2013). In this process, 10 μL of 0.5% MTT solution per well was added after 24 or 48 h under germination conditions. Plates were placed in the culture chamber for an additional 24 h in darkness and a redox reaction, which is a reduction of MTT to formazan, lasted for 24 h (**Figure 1**). After MTT addition, the formazan salt deposit was solubilized by adding 100 μL of lysis buffer (10% Triton X-100, 0.04 mol L^{-1} HCl in isopropanol) to each well, and holding at 21°C in darkness for another 24 h. Subsequently, the absorbance was read with a BioTek^{TM} Synergy^{TM} H4 Hybrid Microplate Reader (BioTek Instruments, Inc., United States). For each well, the final absorbance was calculated by subtracting the absorbance at a reference wavelength of 690 nm from a test absorbance of 570 nm (A570–690 nm).

Image Acquisition and Data Analysis

Images were acquired by scanning each plate twice (HP ScanJet 5300c; resolution 1200 DPI; HP Development Company, L.P., United States), immediately after placing the seeds in the 96 multi-well plates (0 h) and after 24 or 48 h under seed-germination conditions with the subsequent 24 h MTT treatment. The images were saved as TIFF format. For seed counting, the images of *Arabidopsis* seeds at 0 h (immediately after cold stratification) were used and the number of seeds per well was estimated using an in-house software routine implemented in MATLAB R2015. The free of charge access to the software application for academical purposes is described in the next section.

HTS of *Arabidopsis* Rosette Growth

Experimental Setup and Assay Conditions

The protocol for analysis of *Arabidopsis* rosette growth described by De Diego et al. (2017) was modified as follows. Seeds of *A. thaliana* (ecotype Col-0) were surface- sterilized and sown on 12 cm × 12 cm square plates containing a 0.5× MS medium (Murashige and Skoog, 1962) (pH 5.7) supplemented with a gelling agent (0.6% Phytagel; Sigma-Aldrich, Germany). The seeds were kept for 4 days at 4°C in the dark (in the case of primed variants, the growth medium contained the tested biostimulant described below). The plates were then positioned vertically in a growth chamber under controlled conditions (22°C, 16/8 h light/dark cycle with the light cycle starting at 5 a.m., photon irradiance: 120 μmol photons of PAR m^{-2} s^{-1}). 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 μL 1× MS medium (pH 5.7; supplemented with 0.6% Phytagel), with NaCl added for different salt stress

intensities (75 and 150 mM NaCl) and the plates were sealed with perforated transparent foil allowing gas and water exchange. The 48-well plates containing the transferred *Arabidopsis* seedlings were placed the OloPhen platform[1] that 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 regime of at 22°C/20°C in a 16/8 h light/dark cycle, an irradiance of 120 mmol 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 and growing tables with a total area of approximately 7 m^2. To increase the throughput of the assay, the capacity of the growing area was improved to accommodate in total 572 multi-well. 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. RGB images (resolution 2500 × 2000 pixels) of a single plate with a file size of approximately 10 MB in the PNG compression format were 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 data were automatically stored in PlantScreen XYZ database, exported by PlantScreen Data Analyzer software and analyzed using an in-house software routine implemented in MATLAB R2015.

The software application for *Arabidopsis* rosette growth analysis (same as for above described *Arabidopsis* seed counting) can be used without any charge upon obtaining a license from the author. The license can be obtained by e-mail to Palacky University upon agreeing not to use the application for commercial purpose. After obtaining the license, the enduser will be provided (free of charge) with the MCRInstaller.exe. MCRInstaller simulates the MATLAB environment on computers where MATLAB is not installed and enables to execute the applications. To obtain the application executable files, please contact the author Tomas Furst by email tomas.furst@upol.cz. The email must contain the following statement: "Neither the application nor the MCRInstaller will be used for any commercial purpose."

Seed Priming With Biostimulants

The biostimulant effect was determined using Put, Spd, Spm, and Pro for seed priming. After sterilization, the aforementioned seeds were placed on 12 cm × 12 cm square plates containing a 0.5× MS medium (pH 5.7) supplemented with the tested compounds at four concentrations (0.001, 0.01, 0.1, or 1 mM). After 4 days in the dark and 3 days of germination, seedlings were transferred into 48 multi-well plates filled with a 1× MS with/without salt (75 or 150 mM NaCl solution) addition. Two plates per growth condition, compound and concentration (96 seedlings) were used as replicates for the control and 75 mM NaCl. Due to the high mortality of seedlings under severe salt stress conditions, three plates for the seedling in 150 mM NaCl were used to obtain sufficient reproducible data and an adequate number of measurable individuals.

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

Biometrical Parameters

The changes in green area (Pixels) were measured twice per day in each *Arabidopsis* seedling using the aforementioned automatic system. From the obtained data, the relative growth rate (RGR) per hour or day was estimated for each replicate and variant as follows:

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

Where t*i* is the *i* time (h or days).

Determination of the Leaf Color in *Arabidopsis* Rosette Under Control and Salt Stress Conditions

For non-invasive estimation of the changes in leaf color, we calculated three vegetative indices (NGRDI, GLI, and VARI) which have exhibited correlation with the plant biomass, nutrient status or tolerance to abiotic stress (Gitelson et al., 2002; Perry and Roberts, 2008; Hunt et al., 2013). 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). Afterward, the values corresponding to particular color channels (red = R, green = G, and blue = B) were extracted for each pixel within the plant mask, and the vegetative indices were calculated as follows:

Normalized green red difference index

$$NGRDI = (G\text{-}R)/(G + R) \quad (2)$$

Green leaf index

$$GLI = (2G\text{-}R\text{-}B)/(2G + R + B) \quad (3)$$

Visible atmospherically resistant index

$$VARI = (G\text{-}R)/(G + R - B) \quad (4)$$

Subsequently, indices representing particular seedlings were determined by calculating the mean values for each plant mask. The mean value for each 48-well plate was then calculated.

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to assess the differences between the projected areas (Pixels) or seed germination (absorbance) of two or more plant groups at a particular time-point. The test compares the variance (or variation) between the data samples to variation within each particular sample. When ANOVA was significant the differences among groups was determined using Dunn & Sidák's approach.

The relationship among traits was analyzed via Pearson's correlation. Furthermore, the significance of the regression was determined by applying a Student's *t*-test to the linear curves and after linearization of non-linear curves.

RESULTS

Development of HTS of *Arabidopsis* Seed Germination Under Control and Salt Stress Conditions

To efficiently determine the effect of biostimulant priming on the seed germination rate, we developed a HTS assay for seed germination using the MTT method proposed by Pouvreau et al. (2013). In this method, the MTT is used as a marker of metabolic activity in the embryo and its reduction to purple formazan can be quantified spectrophotometrically in a microtiter plate. We optimized this assay for *Arabidopsis* seeds and validated the assay for determining the effect of salinity at two time points (i.e., 24 and 48 h; see **Figure 1**). The severity of the salinity was expected to exert a dose-dependent negative effect on seed germination (seed staining and radicle length decrease; **Figure 1A**), leading to a decrease in the absorbance values measured (**Figure 1B**). During optimization of the assay, we observed a strong correlation between the absorbance values and the number of seeds per well under all three growth conditions (**Figure 2A**). Thus, a stable number of seeds per well was critical to reducing the variability in the experiment. This is, however, technically difficult under HTS conditions when a high number of wells/plates must be rapidly filled. To solve this problem we used 0.05% agarose solution allowing homogeneous suspension of seeds through vortexing. This way using multi-step pipette the average number of 21 ± 5.4 (mean ± SD) seeds per well was achieved. Besides, we handled the relatively high variability (∼25%) by developing an automatic simple software that counts the exact number of seeds per well (rather than finding a technical solution that allows precise and repeated filling of the plate wells with the same number of seeds). Using this software routine, the measured absorbance per well can be recounted to the absorbance per seed. In the first step of this process, the software identifies the wells in the plate. The seeds are then identified via simple thresholding in the R, G, and B channels and single seeds or clusters of seeds are subsequently separated from the background. Afterward, single seeds are distinguished from clusters by computing the solidity (i.e., the ratio of the area of the convex hull of an object to the area of an object) of each object. Single seeds have a high solidity (usually >0.9), whereas clusters of seeds are larger and have lower solidity. The number of seeds in a cluster is estimated by dividing the area of the cluster by the average seed area which is determined from previous runs of the software. The accuracy of the software was determined by manually counting the seeds on several plates, and a high correlation was obtained between the real number and the software-estimated number of seeds (**Figure 2B**). As shown in **Table 1**, the counting of the seeds allowed us to reduce the dispersion of the absorbance per variant, with an at least three times lower standard deviation (28 vs. 9%) in the two analyzed points at 24 and 48 h. Thus, we observed a significant correlation ($p < 0001$) between the absorbance per seed and the percentage of *Arabidopsis* seeds germinated under control and salt stress conditions (**Figure 2C**).

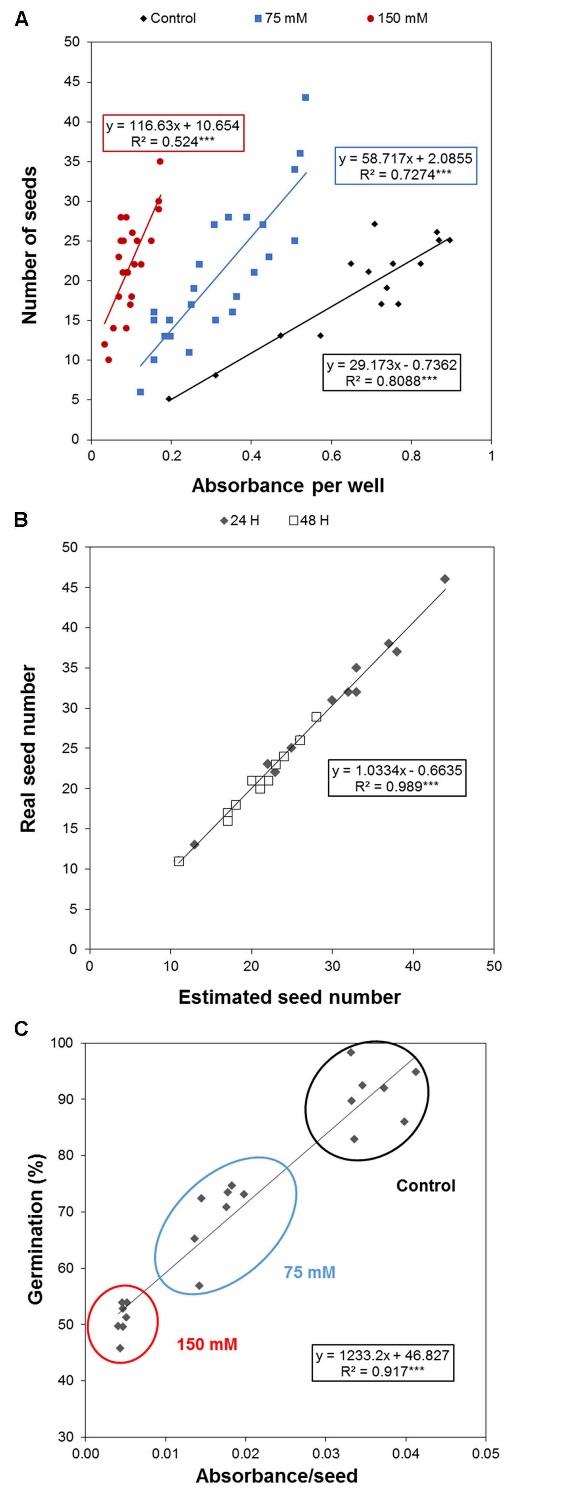


FIGURE 2 | Optimization of *Arabidopsis* germination HTS. Correlation between the **(A)** number of seeds per well and the absorbance under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions, **(B)** manually counted number of seeds and the software-estimated number of seeds per well, and **(C)** germination rate (%) and the estimated number of seeds. The equation of the curve and the Pearson's correlation coefficient with significance according to ANOVA were calculated. ***$p < 0.001$.

TABLE 1 | Comparison of measured overall formazan absorbance and the absorbance recounted per seed after 24 or 48 h of germination.

	24 h		48 h	
	Absorbance	**Absorbance/seed**	**Absorbance**	**Absorbance/seed**
Mean	0.399	0.019	0.73	0.041
SD	0.112	0.002	0.21	0.003
%	28.08	9.14	28.52	8.28

Mean ± SD and contribution of the SD to each mean.

Effect of Biostimulant Seed Priming on *Arabidopsis* Seed Germination

We used the above-described optimized protocol to evaluate the effect of biostimulant (Put, Spd, Spm, and Pro) seed priming on seed germination under salt stress conditions. After 24 h, the tested variants differed only slightly (**Figure 3**). However, 1 mM Spd inhibited seed germination under control conditions and after 48 h of exposure to 75 mM NaCl, but exerted no effect under severe salt conditions (**Figure 3**). The same holds true for 1 mM Spm which also inhibited seed germination in 75 mM NaCl. The most visible effect was obtained for seeds primed with 0.01 and 0.1 mM Put and (to a lesser extent) 1 mM Pro, which yielded a significant increase in the germination in 150 mM NaCl (**Figure 3**).

Seed Size Conditions Associated With *Arabidopsis* Rosette Growth

To determine the effect of biostimulants on the early seedling development of *Arabidopsis* plants under salt stress conditions, we further optimized our previously published protocol (De Diego et al., 2017) for HTS of the rosette growth. For rapid characterization of the plant biostimulants, the protocol was improved as follows: the response of 4-day-old *Arabidopsis* seedlings grown in 1× MS was evaluated using 48 well plates with four biological replicates randomly distributed in the platform. Due to the rapid image acquisition of our system (∼250 plates per hour) the seedlings were imaged twice per day (at 10:00 and at 16:00) for seven consecutive days (**Supplementary Figure S1**). The time-dependent increase in the rosette area (represented by the green region) and RGR were determined for each replicate. The green area differed negligibly among the replicates according to ANOVA (**Figure 4A**), which also exhibited similar RGR. Using this approach, we could record fluctuations in the RGR (per hour) between the 2 days sessions, thereby increasing the sensitivity and applicability of the assay to analysis of circadian rhythms. Higher RGR occurred in the period from 10:00 a.m. to 4:00 p.m. (**Figure 1B**) than in other sessions.

The effect of seed size on the variability of early seedling development via rosette growth was evaluated to further increase the technical precision of the assay. Using sieves, the seed batch was separated into three different size categories: 250–280, 280–300, and >300 μm. Seeds larger than 280 μm produced seedlings with similar rosette area (see **Figure 4C**), whereas seeds with sizes of 250–280 μm yielded significantly smaller rosettes (**Supplementary Table S1**). Although seeds with sizes of 280–300 μm were quite abundant, seeds larger than 300 μm were rare. Thus, due to their abundance and good growth performance, we selected the 280–300 μm seeds as the standard for subsequent experiments.

HTS of *Arabidopsis* Rosette Growth as a Suitable Assay for the Characterization of Biostimulants Under Control and Salt Stress Conditions

Our OloPhen platform has sufficient capacity for the simultaneous testing of numerous variants (De Diego et al., 2017). To demonstrate the capacity for large-scale stress-response studies, we performed an experiment analogous to the germination assay using a 1× MS medium supplemented with two concentrations of NaCl (75 or 150 mM). The seeds were primed with Put, Spd, Spm, and Pro over the same concentration range (0.001, 0.01, 0.1, and 1 mM) described in the Methods section. The 4-day old seedlings were transferred for continued growth under three different conditions: control, moderate salt (75 mM NaCl) and severe salt (150 mM NaCl). In this experimental design, 119 units of 48 well plates containing a total of 5,712 plants were analyzed in a single run. As shown in **Figure 5**, seed priming with biostimulants induced significant differences in the rosette growth of individual variants (**Supplementary Table S2**). All concentrations of Put and Spd improved rosette growth and RGR, in both control and salt stress conditions, acting as plant growth promotors and stress alleviators (**Figure 5**). The best results were obtained with Put and Spd (**Figures 5**, **6**), especially under the severe salt condition (150 mM NaCl). In this case, exponential growth of the plants was maintained (**Figure 5**) through more efficient RGR per day (**Figure 7**) than that associated with other conditions. Spm priming promoted concentration-dependent growth under control and moderate salt stress conditions, although this growth stimulation was less than that induced by Put or Spd (**Figure 5**). Although Spm application improved rosette growth under severe stress conditions, maximum growth of the Spm-primed seedlings occurred earlier than that of seedlings grown only with 150 mM NaCl (**Figure 5**). Spm can therefore be classified as a plant growth promotor rather than a stress alleviator. In the case of stress-related amino acid Pro, we observed that low concentrations of Pro inhibited plant growth, whereas the highest concentrations stimulated growth in control and 75 mM NaCl conditions (**Figure 5** and **Supplementary Table S2**). Under the moderate stress induced by 75 mM NaCl, high concentrations of Pro exerted a stress-alleviating effect, but had

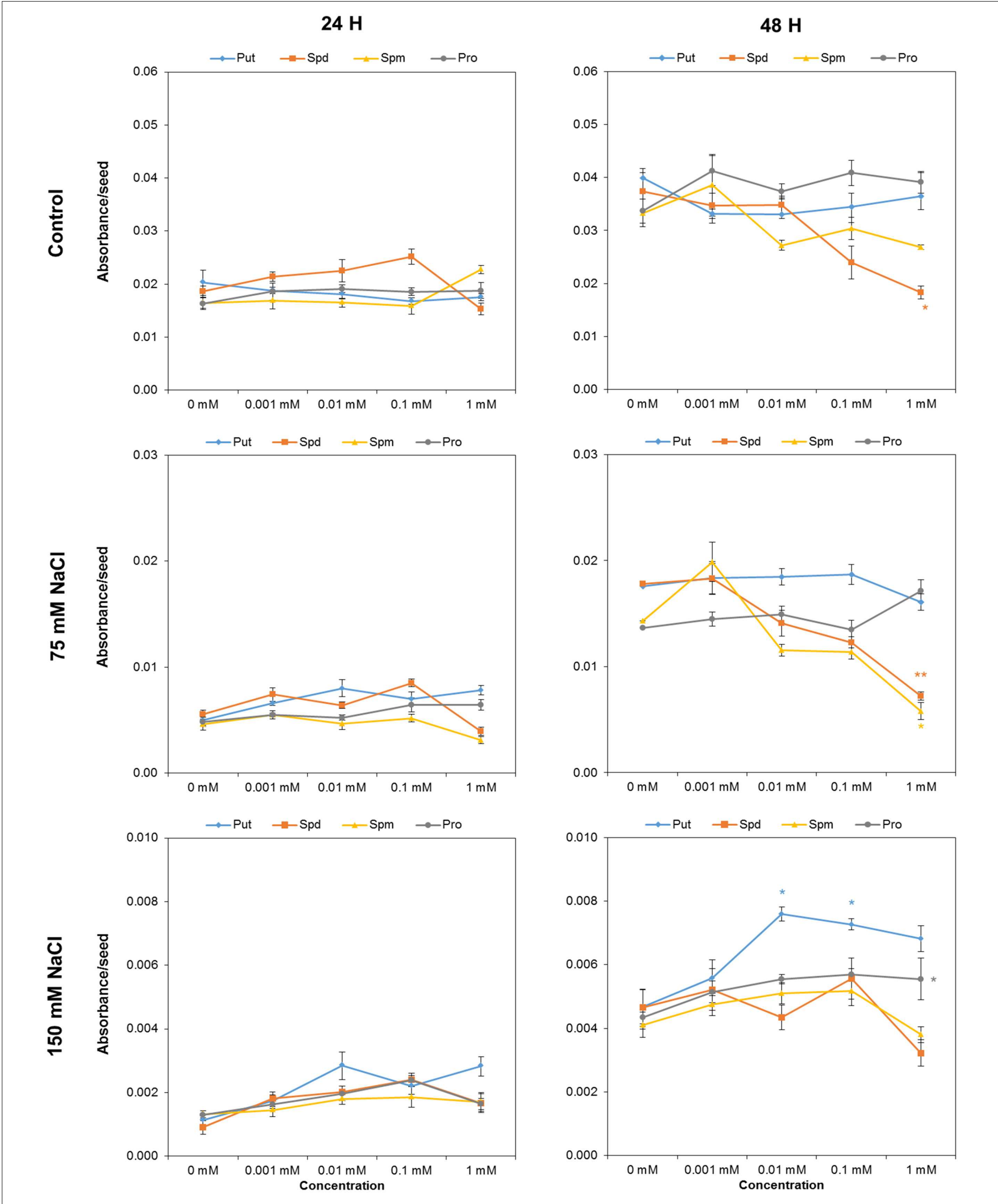


FIGURE 3 | Absorbance per seed of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM), after 24 or 48 h of germination. *Mean* ± *SE*. Statistical analysis was performed via the Kruskal-Wallis test. Asterisks indicate differences relative to the non-treated variant $^{**}p < 0.01$; $^{*}p < 0.05$.

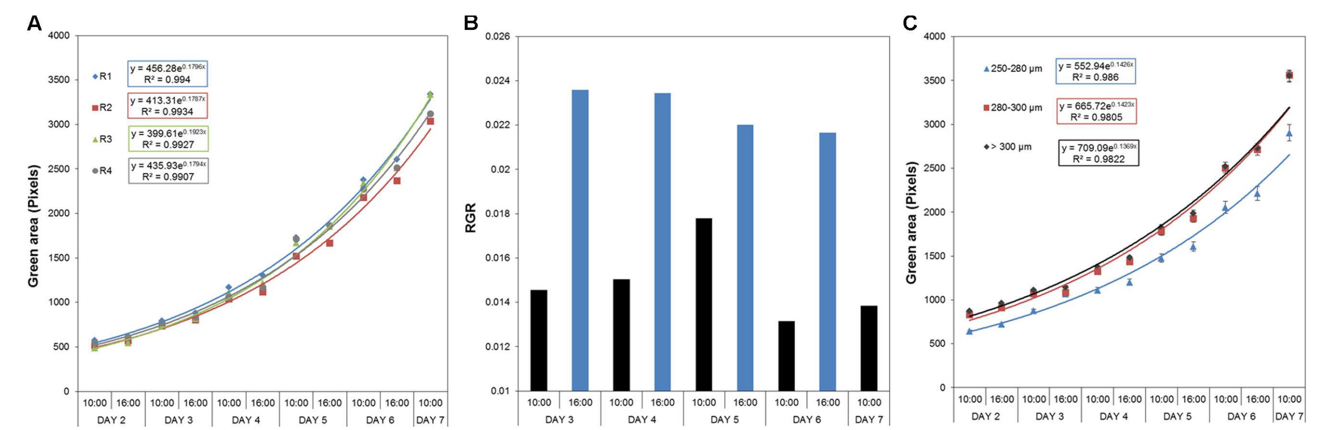


FIGURE 4 | Natural variation in *Arabidopsis* rosette growth in 48 multi-well plates under control conditions. **(A)** Green area (pixels) associated with the growth of four DAG *Arabidopsis* seedlings in independent 48-well plates (replicates; R1–R4) for 7 days. *Mean* ± *SE.* **(B)** Relative growth ratio (RGR, pixel pixel^{-1} hour^{-1}) of four DAG *Arabidopsis* seedlings grown in 48-well plates (n = 192). **(C)** Effect of the seed size on the green area (pixels) associated with the growth of four DAG *Arabidopsis* seedlings in independent 48-well plates. Three different size categories of seeds were considered: 250–280, 280–300, and >300 µm. The equation of the curve and the Pearson's correlation coefficient were calculated. 250–280 µm seeds were significantly smaller than 280–300 and >300 µm ones, according to the multiple comparisons after ANOVA.

FIGURE 5 | Variation in *Arabidopsis* rosette growth from primed seeds with moderate and severe salt stress. Green area (pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown for 7 days in 48-well plates under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions. *Mean* ± *SE.* The equation of the curve and the Pearson's correlation coefficient were calculated.

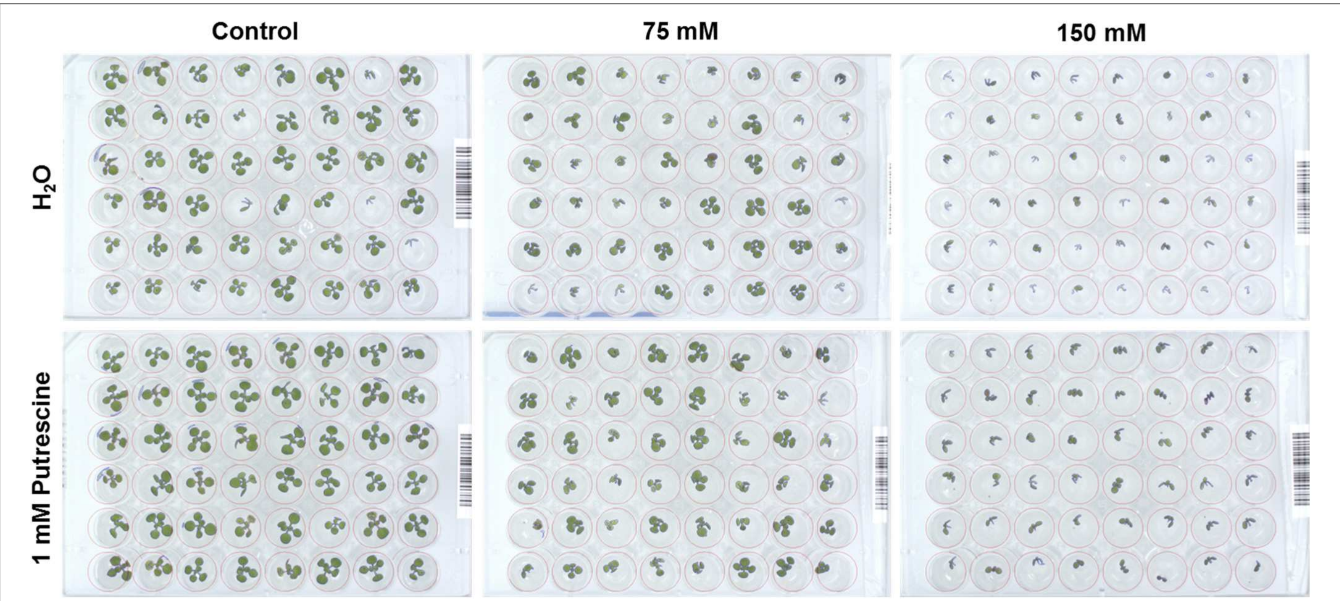


FIGURE 6 | RGB image of an individual 48-well plate containing four DAG *Arabidopsis* seedlings primed with 1 mM Put grown for 7 days under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

a rather negative effect under the severe salt stress condition (**Figure 5** and **Supplementary Table S2**).

Effect of Biostimulant on *Arabidopsis* Seedling Establishment

Analysis of the dataset recorded from the above-described HTS of rosette growth revealed the effect of seed priming on early-seedling establishment. In this case, we analyzed the green area of the *Arabidopsis* seedlings immediately after the transfer to 48 well plates, corresponding to time zero of the HTS focused on *Arabidopsis* rosette growth as a suitable assay. Without salt stress, the sizes of seedlings established from primed seeds differed significantly from the sizes of seedlings resulting from non-primed seeds (**Figure 8**). For the entire range of concentrations, the priming by Put and Spd resulted in significantly larger rosettes compared to those seedlings from non-primed seeds. Except for the highest (1 mM) concentration, all Spm concentrations lead to a significant increase in the green area of the seedlings, whereas for Pro a considerable increase was observed only at the highest concentration (**Figure 8**). These results showed that our method can record traits in a complex manner that describes the effect of priming on all important stages of early development (e.g., germination, early seedling establishment and rosette growth).

Effect of Biostimulants on the Leaf Color of *Arabidopsis* Rosettes Under Control and Salt Stress Conditions

The degradation of chlorophyll, manifested as a change in leaf color, represents one of the most important symptoms of stresses in plants. This change in color may serve as an important marker in stress-related plant studies, especially in those employing salinity. To obtain this information, we introduced another trait into our method describing the effect of seed priming on the plant stress response. As described in the Methods section, the leaf color of the *Arabidopsis* rosettes was determined. We also evaluated the potential of three vegetation indices (VI) calculated using all three mixed visible bands (i.e., R, G, and B bands) which included the NGRDI, GLI, and VARI as indicators of leaf color change. These indices were strongly correlated with changes in the rosette area of the *Arabidopsis* seedlings and the values thereof depended on the seed priming treatment and salt intensity (**Figures 9A,B**). Of the three indices, GLI exhibited the highest sensitivity to salt stress (R^2: 0.97; R^2 for NGRDI and VARI: 0.95). However, when the three VI were separately evaluated for the seedling with 150 mM NaCl, a significant positive correlation with the green area of the *Arabidopsis* rosette (**Figures 9D–F**) was obtained only for GLI. The seed priming with Put and Spd generated *Arabidopsis* rosettes with the highest greenness under control and salt stress conditions. The highest values were observed for GLI where 1 mM Put and Spd yielded 22 and 31%, respectively, higher levels of greenness than that of the non-treated seeds (**Figure 9E**).

PBC Index for Estimating the Biostimulant Mode of Action

We developed a Plant Biostimulant Characterization (PBC) index aimed at integrating both HTS methods into a pipeline that yields straight-forward information allowing simple selection of the best treatment under each condition. The index can represent up to four analyzed traits: seed germination rate (%), seedling establishment (green pixels after transfer to 48 well plates), growth capacity (Pixels) and the leaf color

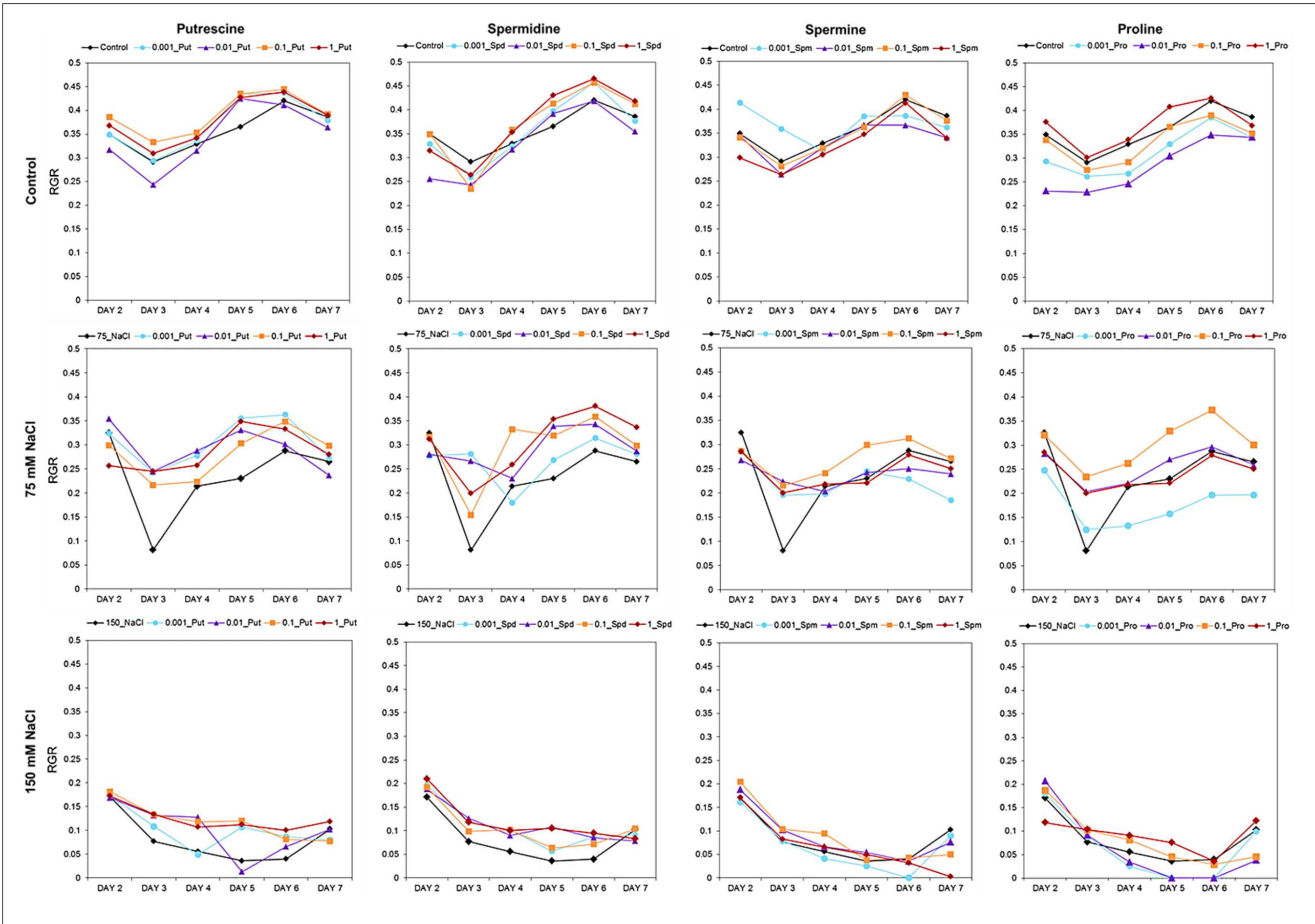


FIGURE 7 | Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown for 7 days in 48-well plates under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

index (GLI) for the primed and non-primed seeds. For the index calculation first the differences between the controls of the different growth conditions and variants (compound and concentration) under the same conditions were calculated as the log2 of the ratio. The number represented by the independent traits and treatment constituting the PBC index can be then represented in a parallel coordinate plot (**Figures 10**, **11**). This type of representation allows a better visualization (than that provided by other representations) of the variant-induced changes in each trait. In addition, the connection between the traits can be quickly identified. For example, under control conditions, it is easier to visualize that the seed priming with Put and Spd mainly improved *Arabidopsis* growth capacity, and to less extend the early seedling establishment and leaf color index, whereas the germination remained unchanged or was even inhibited by these agents (**Figure 10**). Under salt stress condition, seed priming with polyamines improved *Arabidopsis* growth capacity and leaf color index under both intensities tested (75 and 150 mM) (**Figures 11A,B**). Nevertheless, only under severe conditions, the priming with polyamines improved seed germination in almost all cases compared with their respective control (**Figure 11A**).

The concentration effect of the tested compound under three different growth conditions (control, 75 mM NaCl or 150 mM NaCl) was then determined by summing the relative changes (log2) obtained for the parallel coordinate plot ending with a single number as shown in **Figure 12**. This sum yielded a total that can reach a positive (biostimulant- blue) or negative (inhibitor-red) value. The resulting numbers were then plotted in a multidimensional graphic "radar chart" using the concentrations as quantitative variables (**Figure 12**). From these results we confirmed that Put was the most efficient plant growth promotor and stress alleviator with higher values in each concentration and growth condition, compared with the controls. The remaining compounds exhibited a concentration- and growth-condition-dependent response. For example, Spd and Spm yielded the highest index values at low concentrations, whereas Pro acted as plant biostimulants at high concentrations only, and its effectiveness increased with increasing salt stress intensity (**Figures 12B,C**). These results confirm that the presented MTHTS approach is an adequate tool for a fast and simultaneous analysis of various concentrations and growth conditions for identification and, especially, characterization of the operation mode associated with new biostimulants.

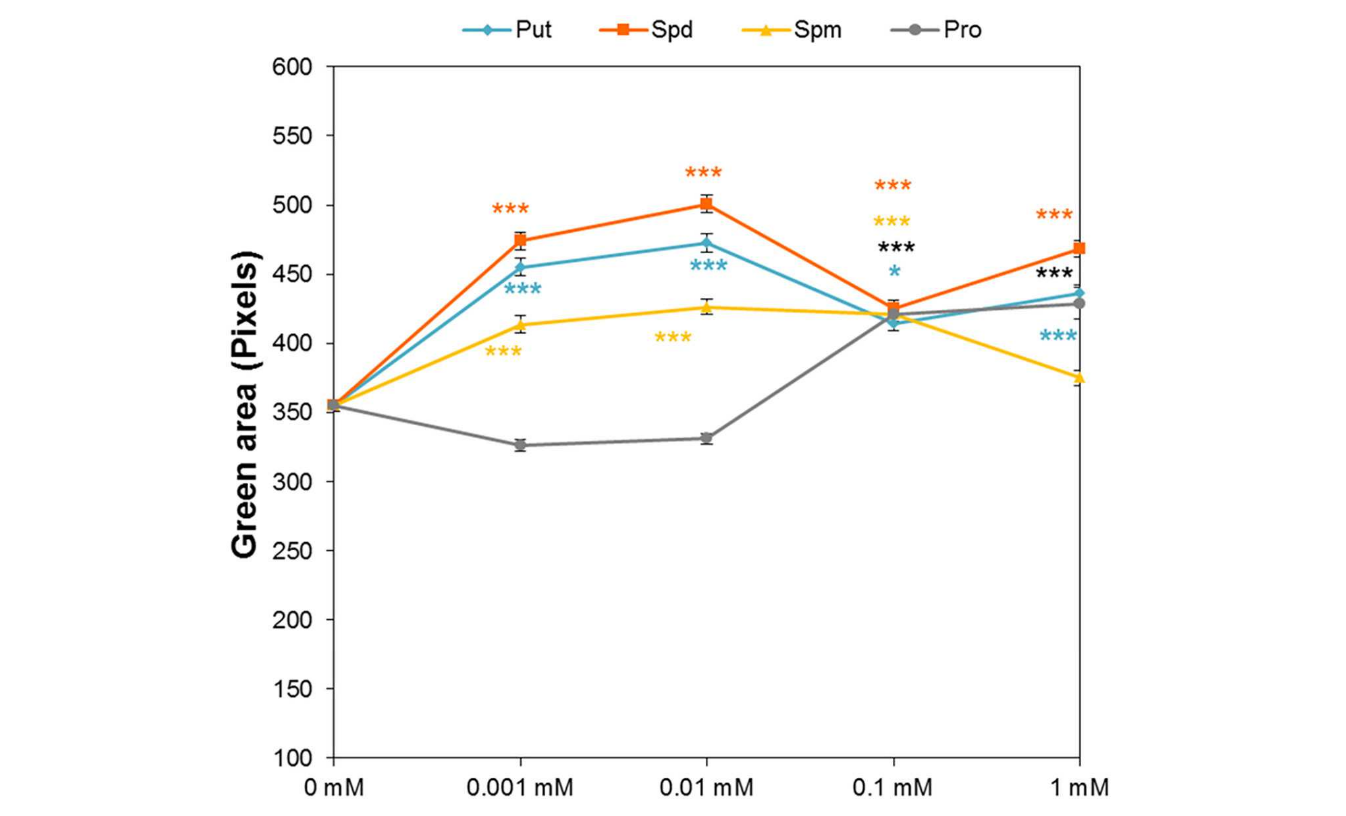


FIGURE 8 | Effect of biostimulant seed priming on the seedling establishment of *Arabidopsis*. Green area (pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control conditions after the transfer to 48-well plates. Statistical analysis was performed via the Kruskal-Wallis test. Asterisks indicate differences relative to the non-treated variant. ***$p < 0.001$; *$p < 0.05$.

DISCUSSION

Uniform and efficient seed germination and establishment of early seedlings are crucial for agricultural crop production under stress conditions, especially drought and/or salinity (Savvides et al., 2016). Seed priming, where seeds are pre-sown with certain compounds with the aim of increasing the uniformity and vigor of seedlings, represents an innovative alternative to coping with the negative stress effects. In addition, the use of natural compounds or biostimulants as priming agents can improve the efficiency of crop production and yield under suboptimal conditions. The use of these substances is more sustainable and environmentally friendly compared with the use of other materials. The priming with single compounds such as polyamines and amino acids can be a good technology against different abiotic stresses (Savvides et al., 2016). However, despite the fact that most of the complex biostimulants of several origins (i.e., protein hydrolysis from agroindustrial by-product from both plant sources and animal waste, and seaweed extracts) contain these types of compounds (du Jardin, 2015), their biostimulant activity potential hasn't been fully evaluated. For this reason, we used in this study the stress related amino acid Pro and polyamines' representatives as priming agents to bring additional information about their possible biostimulant mode of action. Therefore, biostimulant manufacturers require tools for identifying new biostimulants, characterizing and quantifying their biological effects and describing the corresponding mode of action. Moreover, during biostimulant preparation, the tools for rapid control of the quality during the extraction processes and production of different batches are needed. Taking into account the mentioned facts, we suggest that Put, Spd, Spm, and Pro have potential to be used as positive controls in the biostimulant research and manufacturing.

Screening platforms based on the semi-automated or automated bioassaying of simple traits based on *in vitro Arabidopsis* assays might be useful to accelerate the process for preliminary screening of stability, composition and effect of raw material. This testing allows for a rapid first-step screening on plants, eliminating the influence of soil and other environmental parameters (Povero et al., 2016). The testing of biostimulants using bioassays has been traditionally performed with Petri dishes, thus having low-throughput requiring posterior manual quantification (Durand et al., 2003; Colla et al., 2014; Povero et al., 2016). Recently, Rodriguez-Furlán et al. (2016) published an *in vitro* bioassay using *Arabidopsis* for the testing of several compounds. However, the use of scanners for image analysis yields an analysis rate of 20 min per plate and the analysis is performed only at one time-point (Rodriguez-Furlán et al., 2016). Several other manual and semi-automated HTS protocols using RGB imaging for phenotyping of *Arabidopsis*

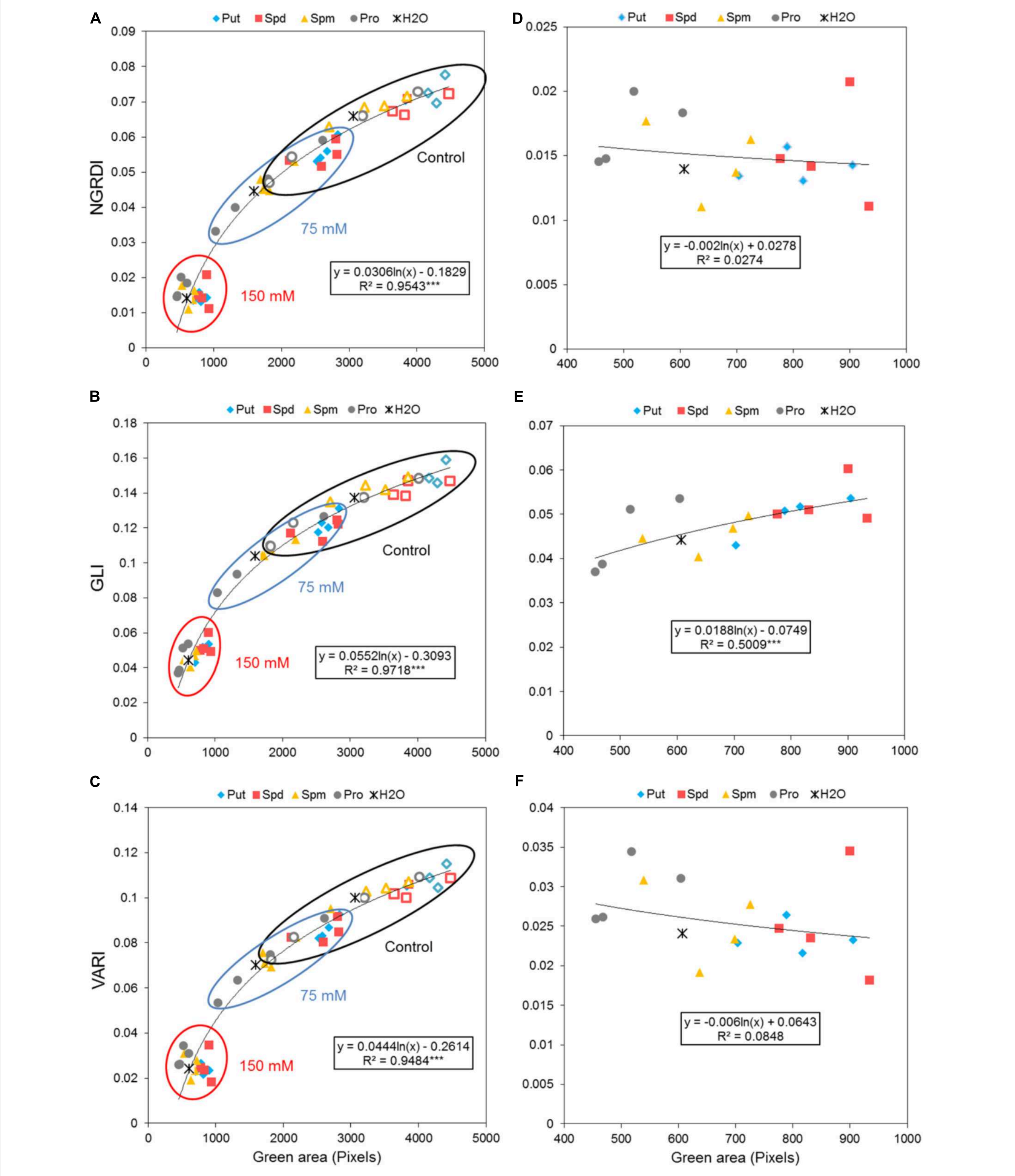


FIGURE 9 | Effect of biostimulant seed priming on the stress response of *Arabidopsis*. **(A)** Correlation between the color index; NGRDI **(A)**, GLI **(B)**, or VARI **(C)** and the green area (Pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control (empty symbols), moderate (75 mM NaCl), and severe (150 mM NaCl) (filled symbols) salt stress conditions for 7 days. **(B)** Correlation between NGRDI **(D)**, GLI **(E)**, or VARI **(F)** and the green area of the *Arabidopsis* seedlings grown only under severe stress conditions. The equation of the curve and the Pearson's correlation coefficient with significance, according to ANOVA, were calculated after linearization. ***$p < 0.001$.

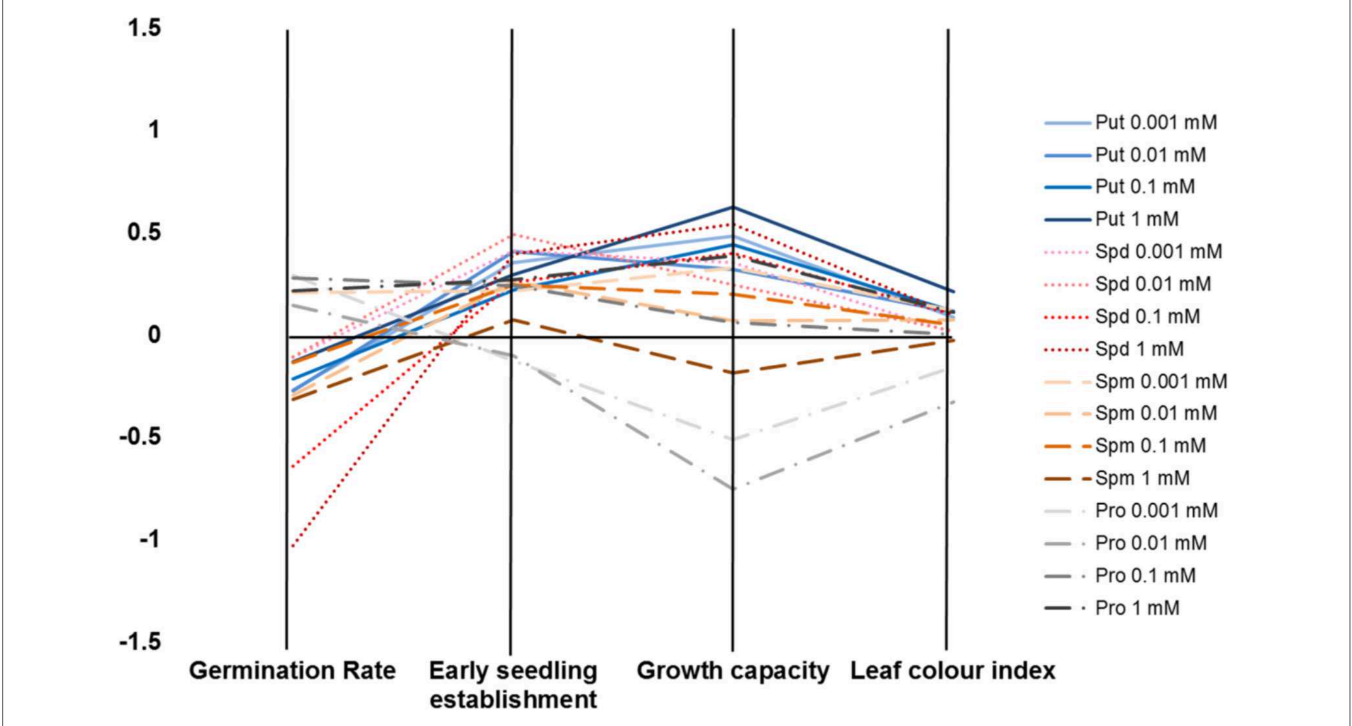


FIGURE 10 | Characterization of plant biostimulants under control conditions. Parallel coordinate plot of the traits (germination, seedling establishment, plant growth capacity, and leaf color index) obtained from the Multi-trait HTS of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

in the controlled conditions have been published with different throughputs and (dis)advantages. The method of Granier et al. (2006) showed possibilities solving potential complications and methodological difficulties with the spatial and temporal variability of micrometeorological conditions within a growth chamber, reaching throughput of 500 plants per hour. Recently, simple HTS protocol based on *in vitro* growth of *Arabidopsis* using square plates with 16 seedlings and manual image acquisition followed by analysis of plant size and color was published by Faragó et al. (2018). The protocol presented by us is based on our previous report of an automated method for HTS of *Arabidopsis* rosette growth in multi-well plates accessible at OloPhen facility (De Diego et al., 2017). The potential of this method was in our recent protocol improved in several ways through (1) increase of the number of plates per run from 480 to 572; (2) significant increase of the total number of plants analyzed by use of 48-well plates, instead of 24-well plates that increased the number of analyzed plants to more than 27.000 in less than 3 h; and (3) through increase of the resolution of the growth analysis by automated measurement twice a day within 1 week. As presented here, our new method allows a simultaneous study of different growth conditions without compromising the number of variants, replicates and plants per treatment. Moreover, compared to Faragó et al. (2018), the growth analysis of each plant is done for the whole cycle by imaging of the same plant individual. Further, the use of independent wells per plant permits an easier detection of the single plant so they are located in a concrete XY position. Thus, there is no requirement of any manual adjustment to separate individual plants. As clear example illustrating the potential of our method, in this work we automatically recorded the rosette growth of 5,712 *Arabidopsis* (119 plates × 48 seedling). The imaging of each well-plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days, ending with 14 data points per plant in very short time. Altogether, we developed a very fast *in vitro* bioassay to analyze simultaneously a huge amount of treatments and plants.

The improved HTS of rosette growth under control and stress salinity was integrated in a pipe-line for the screening of biostimulants together with the HTS of *Arabidopsis* seed germination. For that, we developed a simple and fast bioassay for *Arabidopsis* seed germination based on (Pouvreau et al., 2013) using spectrophotometric analysis of MTT reduction in microtiter plates. With the classical method using a microscope, the distinction between non-germinated seeds and germinated seeds with a very short protruded radicle is very difficult, increasing the risk of germination rate underestimation (Pouvreau et al., 2013). However, the MTT method is simple and accurate and can be easily adapted for high-throughput germination bioassays. The HTS method is performed in 96 well plates. These plates allow many variants per plate (compounds, concentrations, and/or germination conditions) using a spectrophotometric MTT method with a simple read out of the germination rate per variant (**Figure 1**). In addition, we developed a simple in-house software routine to automatically count the seed number per well. This reduced the time consuming counting of the seed number per well necessary

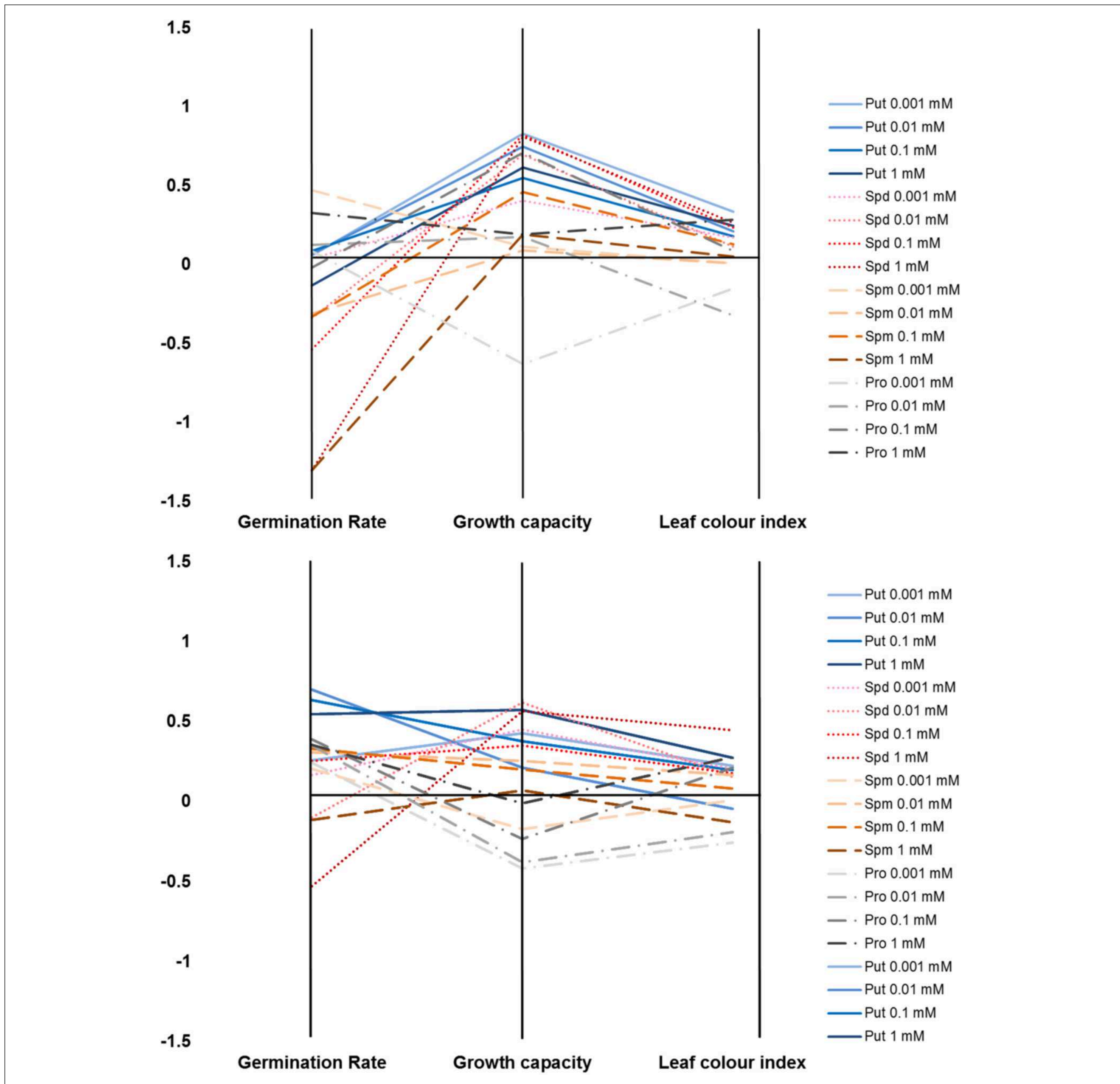
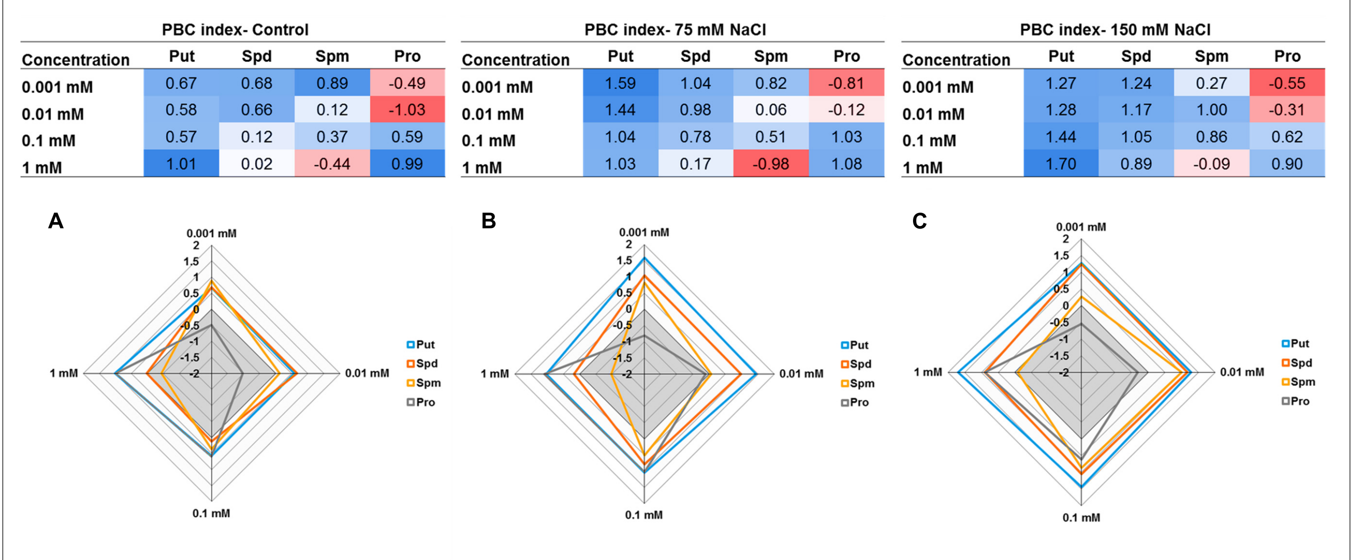


FIGURE 11 | Characterization of plant biostimulants under moderate **(A)** or severe **(B)** salt stress conditions. Parallel coordinate plot of the traits (germination, plant growth capacity, and leaf color index) obtained from the Multi-trait HTS of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under moderate (75 mM NaCl) or severe (150 mM NaCl) salt stress conditions.

for increasing the accuracy of the method by reducing the variability within treatments (**Figure 2A**). Although free software applications exist for image-based analysis of seeds allowing automated definition of the seed shape and size (Tanabata et al., 2012), for our purpose we created a very simple software routine in MATLAB suitable for detecting and counting objects (seeds) in multi-well plates at 0 h (immediately after cold stratification and before seed germination). The obtained number is then used to recalculate the total absorbance of the well recorded by spectrophotometer to the absorbance per seed that represents the germination rate. This trait together with those obtained from the HTS of Arabidopsis rosette growth (plant establishment, plant growth capacity under different conditions and leaf color index), constitute the MTHTS for biostimulant characterization achievable within 1 week.

Many biostimulants contain various groups of components including complex mixtures of biologically active compounds



PBC index- Control

Concentration	Put	Spd	Spm	Pro
0.001 mM	0.67	0.68	0.89	-0.49
0.01 mM	0.58	0.66	0.12	-1.03
0.1 mM	0.57	0.12	0.37	0.59
1 mM	1.01	0.02	-0.44	0.99

PBC index- 75 mM NaCl

Concentration	Put	Spd	Spm	Pro
0.001 mM	1.59	1.04	0.82	-0.81
0.01 mM	1.44	0.98	0.06	-0.12
0.1 mM	1.04	0.78	0.51	1.03
1 mM	1.03	0.17	-0.98	1.08

PBC index- 150 mM NaCl

Concentration	Put	Spd	Spm	Pro
0.001 mM	1.27	1.24	0.27	-0.55
0.01 mM	1.28	1.17	1.00	-0.31
0.1 mM	1.44	1.05	0.86	0.62
1 mM	1.70	0.89	-0.09	0.90

FIGURE 12 | Tables and Radar chart of the Plant Biostimulant Characterization (PBC) index by summing the relative changes (log2) obtained for the parallel coordinate plot for each compound (Put, Spd, Spm, or Pro) concentration (0.001, 0.01, 0.1, and 1 mM) and growth condition; control **(A)**, 75 mM NaCl **(B)**, or 150 mM NaCl **(C)**.

and, hence, the testing should be performed over a broad concentration range allowing evaluation of concentration-dependent effects. We selected individual molecules as a first step in optimizing our bioassays for biostimulant characterization. The polyamines Put, Spd, and Spm, and the amino acid Pro, which also have been identified in the raw material of complex formulations from different natural origins, were selected (Colla et al., 2014; du Jardin, 2015). Moreover, we selected salinity as a stressor, owing to its negative impact on seed germination and plant growth. Using our approach, each compound can be simultaneously tested at different concentrations and plant growth conditions in both HTS methods. The results revealed differences in the mode of action for the four compounds applied to *Arabidopsis* seed germination and rosette growth (**Figures 3–9**). Put and Spd were identified as plant growth promotors and stress alleviators, whereas Spm and Pro were less efficient and their positive effect was concentration dependent (**Figures 5**, **10**, and **11**). The exogenous application of polyamines yields improved salt tolerance in many crops via enhanced germination and/or plant productivity (Roychoudhury et al., 2011; Li et al., 2015; Shekari et al., 2015). For example, exogenous application of Spd in *Cucumis sativus* L. induces accumulation of endogenous polyamines that act as free radical scavengers, thereby stabilizing cellular membranes and maintaining cellular ionic balance under salinity (Shu et al., 2012). This was attributed to a relatively high Put/(Spd+Spd) ratio that rendered seed priming with Put the most efficient treatment. As confirmation, Shu et al. (2015) demonstrated that Put application regulates protein expression at transcriptional and translational levels by increasing endogenous polyamine levels in thylakoid membranes which may stabilize the photosynthetic apparatus under a salt stress. In addition, changes in polyamines biosynthesis and catabolism influence plant tolerance and recovery capacity though a sophisticated crosstalk with plant hormones, which induces changes in primary metabolism such as the synthesis of amino acids, and improves photosynthesis and nutrient uptake under stress conditions (review in Podlešáková et al., 2018). Therefore, priming with polyamines could be a cheap, healthy, and easy solution for mitigating adverse salinity-induced stresses occurring during the initial developmental phases of crops.

The priming with Pro was less effective than with polyamines, and the most positive effect was in the germination rate under a severe salt stress. This may have resulted from the fact that enhanced Pro levels in plants occur in the first phases of seed germination and the seed-to-seedling transition (Silva et al., 2017). Similar results were obtained in rice, where the seeds pre-treated with Pro provided significant evidence for assessing the salt tolerance at the germination stage (Deivanai et al., 2011). However, the effect was variety dependent. In sugar cane grown *in vitro*, the anti-stress effect was also genotype dependent (Medeiros et al., 2015), but both dependences increased the stress tolerance by activating the plant antioxidative response. Other studies consider the Pro mode of action to be long-term, when the plant accumulated high levels of Pro, and attributed this action to plant recovery and hardening (De Diego et al., 2015; Sabagh et al., 2015). This could be explained by the fact that stress-tolerance improvement in many other crops required relatively high concentrations (Talat et al., 2013; Dawood et al., 2014). However, contradictory results regarding the Pro effect have been obtained for the same crop under the same stress conditions. For example, Teh et al. (2016) reported that 5 or 10 mM Pro improved salt stress tolerance of rice, but Deivanai et al. (2011) considered

the 10 mM concentration toxic. This contradiction resulted mainly from the different intensities of salinity considered. Therefore, integrating a wide range of concentrations in the same bioassay combined with different stress levels for the testing of biostimulants constitutes a viable strategy for biostimulant mode of action characterization.

CONCLUSION

In this work we present a complex pipe-line for a fast characterization of plant biostimulants suitable for seed-priming application giving straight-forward information for simple selection of the best treatments under control, moderate and severe salt stress conditions, using treatment evaluation through newly introduced index. The MTHTS approach based on the semi-automated analysis of *Arabidopsis* germination and rosette growth analyses four traits: *in vitro* germination rate, early seedling establishment capacity, growth capacity under stress and stress response based on plant greenness. The approach allows the acceleration of the biostimulant characterization through a simultaneous spanning of a broad number of biostimulants in a wide range of concentrations and stress conditions. Further, the method helps to define a biostimulant made of action based on its contribution to the plant development and stress tolerance such as plant growth promotor/inhibitor and/or stress alleviator. The presented approach (i) represents a useful tool for biostimulant research and development, and (ii) when combined with chemical-composition analysis and biological-activity measurements can help to identify the specific mode of action characterizing the biostimulants and their main bioactive ingredients.

AUTHOR CONTRIBUTIONS

LU, AH, JH, KD, LS, and NDD designed the experiments. LU, AH, JH, and KP performed the experiments. NDD and LS supervised the study and formulated the concept of the project. LU, AH, and NDD performed the data analysis. All authors discussed the results. LU, AH, JH, NDD, and LS wrote the manuscript.

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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.2018.01327/full#supplementary-material

FIGURE S1 | *Arabidopsis* rosette growth in 48 multi-well plates for 7 days under control conditions.

TABLE S1 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings from three different size categories of seeds (250–280, 280–300, and >300 μm) grown in 48-well plates (three biological replicates per treatment) for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

TABLE S2 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control, moderate (75 mM NaCl) and severe (150 mM NaCl) salt stress conditions for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplement III.

FIGURE S1 | *Arabidopsis* rosette growth in 48 multi-well plates for 7 days under control conditions.

The Supplementary Figure S1 for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01327/full#supplementary-material

TABLE S1 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings from three different size categories of seeds (250–280, 280–300, and >300 mm) grown in 48-well plates (three biological replicates per treatment) for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

	Day 2		Day 3		Day 4		Day 5		Day 6		Day 7
	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h
ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p = 0.0301$ *	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
250-280 µm	b	b	b	b	b	b	b	b	b	b	b
280-300 µm	a	a	a	ab	a	a	a	a	a	a	a
>300 µm	a	a	a	a	a	a	a	a	a	a	a

Note: multiple comparison according Dunn & Sidák's approach after ANOVA

Blue colour	Significantly higher size compared to 250-280 µm

TABLE S2 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control, moderate (75 mM NaCl) and severe (150 mM NaCl) salt stress conditions for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

- **Control Conditions**

		Day 1		Day 2	Day 3		Day 4		Day 5		Day 6		Day 7
		10:00 h	16:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h
Putrescine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	ab	a	a	a	ab	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a
Spermidine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	ab	ab	ab	ab	ab	ab	ab	a	a	a	ab	ab
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a
Spermine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	bc	bc	bc	bc	bc	bc
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	ab	ab	ab	ab
	0.1 mM	a	a	a	a	a	a	ab	ab	ab	a	ab	ab
	1 mM	b	b	b	b	b	b	c	c	c	c	c	c
Proline	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	a	b	b	b	b	b	b	b
	0.001 mM	b	b	c	c	b	c	c	c	c	c	c	c
	0.01 mM	b	b	c	c	b	c	c	c	c	c	c	c
	0.1 mM	a	a	a	a	ab	ab	ab	ab	ab	ab	ab	ab
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a

Note: multiple comparison according Dunn & Sidák's approach after ANOVA

Blue colour	Significantly higher size compared to control
Red colour	Significantly lower size compared to control

75 mM NaCl

		Day 1		Day 2	Day 3		Day 4		Day 5		Day 6		Day 7
		10:00 h	16:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h
Putrescine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	a	ab	ab	a	a	a	a	a	a	a	a	a
	1 mM	ab	ab	ab	a	a	a	a	a	a	a	a	a
Spermidine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	a	a	a	a	ab	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a
Spermine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p = 0.1118$	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	a	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	ab	ab	ab
	0.01 mM	ab	a	a	a	a	ab	ab	ab	ab	ab	ab	ab
	0.1 mM	a	a	a	a	a	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	ab	ab	ab	ab	ab
Proline	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	a	b	b	b	b	b	b	b	b	b
	0.001 mM	c	c	b	b	b	bc	bc	bc	bc	bc	bc	bc
	0.01 mM	bc	bc	a	b	b	c	c	c	c	c	c	c
	0.1 mM	ab	ab	a	ab	ab	ab	ab	ab	ab	ab	ab	ab
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a

Note: multiple comparison according Dunn & Sidák's approach after ANOVA

Blue colour	Significantly higher size compared to control
Red colour	Significantly lower size compared to control

- **150 mM NaCl**

		Day 1		Day 2	Day 3		Day 4		Day 5		Day 6		Day 7
		10:00 h	16:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h	16:00 h	10:00 h
Putrescine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	ab	ab	ab	ab	ab	a	ab	ab	ab	a	ab	ab
	0.1 mM	ab	ab	ab	ab	a	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a
Spermidine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	a	a	a	a	a	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	a	a	a	a	a
Spermine	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	b	b	b	b	b	b	b	b	b	b	b	b
	0.001 mM	b	b	b	b	b	b	b	b	b	b	b	b
	0.01 mM	a	a	a	a	a	a	a	a	a	a	a	a
	0.1 mM	a	a	a	a	a	a	a	a	a	a	a	a
	1 mM	a	a	a	a	a	a	a	a	a	a	a	ab
Proline	ANOVA	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***	$p < 0.001$ ***
	H_2O	a	a	a	a	a	a	a	a	a	a	a	a
	0.001 mM	b	b	b	b	b	b	b	b	b	b	b	b
	0.01 mM	ab	ab	b	ab	ab	b	b	b	b	b	b	b
	0.1 mM	b	b	b	b	b	b	ab	ab	ab	ab	ab	b
	1 mM	ab	ab	b	b	ab	ab	ab	ab	ab	ab	ab	ab

Note: multiple comparison according Dunn & Sidák's approach after ANOVA

Blue colour	Significantly higher size compared to control
ed colour	Significantly lower size compared to control

❖ *Supplement IV*

Lydia Ugena, Jan F. Humplík, Tomáš Fürst, Nuria De Diego, Lukas Spichal. “CroSeEm: a high-throughput emergence assay for screening maize seedlings under salinity” (*Under revision*).

1 **CroSeEm: a high-throughput emergence assay for screening maize seedlings under**
2 **salinity**

3 **Running title: HTS of maize seedling emergence under salinity**

4 Lydia Ugena[1], Jan F Humplík[1,2], Tomáš Fürst[1,3], Nuria De Diego[1*], and Lukáš Spíchal[1]

5 [1] Department of Chemical Biology and Genetics, Centre of the Region Haná for
6 Biotechnological and Agricultural Research, Faculty of Science, Šlechtitelů 27, Palacký
7 University, Olomouc, CZ-78371, Czech Republic.

8 [2]Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and
9 Agricultural Research, Institute of Experimental Botany, Czech Academy of Sciences,
10 Olomouc, Czech Republic.

11 [3]Department of Mathematical Analysis and Applications of Mathematics, Faculty of Science,
12 Palacky University, Olomouc, Czech Republic.

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14

15 lydia.ugena@upol.cz; jan.humplik@upol.cz; tomas.furst@upol.cz;

16 lukas.spichal@upol.cz

17

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19 [*]**Corresponding author:** Nuria De Diego**; email:** nuria.de@upol.cz**; Phone**:+420585634940

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27 **Keywords:** high-throughput bioassay, **Cro**p **Se**edling **Em**ergence, polyamines, priming agents,
28 seedlings emergence, synchronicity, salt stress.

29

Abstract

Seedling emergence is a key phenological stage that strongly influences the success of an annual plant. As a part of early plant development, seedling emergence represents the moment of transformation from a heterotrophic to an autotrophic organism. This trait thus provides not only information about the ability of seeds to germinate, but also about the ability of a seedling to establish, reach the light and develop photosynthetic apparatus before the seed reserves are exhausted. Its informative value and the potential for a simple measurement makes seedling emergence a perfect target trait for high-throughput screening in real crop models such as maize. Development of standardised screening protocols helps to validate the true activity and mode of action of potential biostimulants. In this work, we designed and tested a robust high-throughput assay for analysing **Cro**p **Se**edling **Em**ergence using maize (*Zea mays* L.) as plant model grown under salt stress in controlled conditions. We further validated the assay to be used as a tool for characterising priming agents to mitigate the adverse salinity effect in maize seedling emergence. For this validation, the plant stress related compounds, the polyamines putrescine, spermidine and spermine, were used as priming agents. In our approach, the time of seedling emergence was analysed using regular, automated RGB image acquisition and semi-automated image analysis and data post-processing. Several traits, such as percentage of emerged seedling, difference in the time of the start of emergence and synchronicity of the emergence, were defined and automatically determined. In addition, we demonstrated that seed priming with polyamines improved synchronicity of the maize emergence under salt stress conditions. This suggests that priming with polyamines can represent a simple technology to improve stress tolerance in crops.

Introduction

Plants are sessile organisms exposed to a rapidly changing environment without the means to escape. In order to cope with this, plants have to respond to numerous external stimuli resulting in plant acclimation to specific growing conditions. When growing conditions are unfavourable and plants are not able to acclimate to them, growth becomes inhibited and the plant may die. Seedling emergence is the most fragile stage in the life cycle of annual plants. This stage strongly influences the success of growth and represents the moment of transformation from a heterotrophic to an autotrophic organism (Arsovski et al., 2012; Mercer et al., 2011). Seed germination, seedling emergence and, therefore, crop establishment are the major determinants for achieving optimal crop growth and better productivity especially under stress conditions. 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 (Carpıcı et al., 2009). Most crops are highly susceptible to saline stress, which obstructs or at least delays seed germination and seedling emergence. Soil salinity has a detrimental effect on plants, often observed in two phases: the first, the osmotic phase, is characterised by a rapid response to the elevated osmotic pressure, followed by a second, the so-called ionic phase, that represents a slower response due to the accumulation of Na^+ in leaves (Munns and Tester, 2008).

To cope with salinity and other abiotic stresses, intensive stress-tolerance breeding and biotechnology programmes are necessary. However, different methodologies have been employed aimed at enhancing multiple stress tolerance: some are particularly time-consuming (e.g. conventional breeding) and others are currently unacceptable in many countries around the world (e.g. plant genetic modification). Modern breeding, genetic and molecular approaches are focused on the identification of genes that can improve plant abiotic stress tolerance. Despite the improvement in the stress tolerance achieved in certain crops, all these approaches are expected never to compromise on features such as productivity and other traits important for agriculture (Vijayakumari et al., 2016). As an alternative, plants can be ‘prepared’ to tolerate future biotic and abiotic stress conditions more successfully through priming (also known as sensitisation or hardening) (Gebremedhn and Berhanu, 2013; Maiti and Pramanik, 2013; Savvides et al., 2016). Seed priming stimulates many of the metabolic processes involved in the early phases of germination, resulting in improved seed performance and providing faster, synchronised germination and more vigorous seedlings with higher level of abiotic stress tolerance compared to seedlings originating from non-primed seeds. For example, simple compounds such as phytohormones, amino acids and polyamines or complex biostimulants have been shown to have a “priming” action for inducing defence responses in tested plants (Ellouzi et al., 2017; Ibrahim, 2016; Iqbal et al., 2006; Zheng et al., 2016). Thus, seed priming is an easy, low cost and low risk technique used to overcome the problem of saline agricultural land (Maiti and Pramanik, 2013). The positive effects of seed priming under salinity conditions have been reported for many crops (Ibrahim, 2016). Seed priming improves seed performance and provides faster and synchronised germination by modifying physiological, biochemical, cellular and molecular processes in the plant, which can increase the rate of crop emergence, and thus increase rates of crop development, reduce crop duration and increase production as well as productivity (Maiti and Pramanik, 2013). For that, the development of more sophisticated high-throughput bioassays for testing diffent priming agents is needed to provide information about developmental and physiological status of the treated plants. Such understanding may contribute

to accelerate the selection of the most adequate compound and concentration per crop and stress condition and intensity.

Scoring of seedling emergence represents a very informative phenotyping trait that fulfils the criteria of a first-step screening approach, such as rapid response, low cost, simple readout, high-throughput, quantitative and low false negative prediction rate of a screened trait. However, manual emergence scoring is very laborious and ineffective. Automated screening, based on image analysis, thus represents a clear alternative for large-scale campaigns. However, to the best of our knowledge, no study has been carried out so far describing the use of such a method for high-throughput indoor screening in crops. Several reports on field scoring of seedling emergence with various sensors have been published recently (Liu et al., 2017; Sankaran et al., 2015; Yu et al., 2013), but the methods are not applicable to initial indoor high-throughput screening. The selection of potentially tolerant genotypes based on seed germination often leads to false positive “hits” that are not consistent with salinity tolerance in the subsequent selection steps carried out in later developmental stages (Munns and James, 2003). Conversely, seedling emergence as a trait provides information not only about the ability of seeds to germinate, but also about the ability of a seedling to grow and reach the light and develop photosynthetic apparatus before the seed reserves are exhausted.

In this work, we report the development of a high-throughput bioassay that monitors **Cro**p **Se**edling **Em**ergence (CroSeEm) under saline conditions by automated detection of the first appearance of a coleoptile (first green pixel). We further describe identification of three different aspects of the emergence trait: the final germination rate, time lag and emergence synchronicity. Finally, using example of three related stress compounds such polyamines as priming agent, we demonstrate that our multidimensional analysis of the emergence curves allows for the mapping of the response of maize populations treated with potentially bioactive compounds, over a wide range of concentrations under different intensities of salt stress.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of the maize (*Zea mays* L.) hybrid Koblens (KWS Osiva s.r.o., Czech Republic) were imbibed with distilled water for 16 hours at 4ºC in the dark (Figure 1). Meanwhile, nursery trays TEKU JP 3050/160 T were filled with soil substrate (Substrat 2, Klassmann Deilmann, Geeste, Germany) and cut to fit into hydroponic inserts for standard PlantScreen™ measuring trays (Photon Systems Instruments, Brno, Czech Republic). The cut trays had 110 cells (volume of one cell equals to 21.5 mL), allowing for the sowing of the same number of seeds (110) (Figure 1). The trays were also cut in the corners to allow for watering using a funnel. Thereafter, one seed per cell was sown 1 cm deep into the substrate. Each tray was watered to its full capacity with tap water or with a solution of NaCl at two concentrations: 75 mM NaCl and 150 mM NaCl. Afterwards, all trays were watered using 0.5 L of tap water (average conductivity around 56 mS/m) every third day until the end of the experiment. The trays were assigned to the control, moderate salt stress, and severe salt stress groups randomly at the beginning of the experiment.

To develop the high-throughput emergence assay **CroSeEm,** firstly we performed the assay twice using non-priming maize seeds. They were emerged under control condition and

151 with the two salt intensities 75 mM NaCl and 150 mM NaCl. The experiment was repeated twice
152 (**1st** and **2nd experiment**– Table 1) over different days to evaluate the reproducibility of the
153 bioassay.
154

155 The power of the bioassay for the characterisation of priming agents as stress alleviators
156 reducing the negative salt stress in maize emergence was also evaluated (**3rd experiment**- Table
157 1). Maize seeds were primed with three polyamines; putrescine (Put) (1,4-butanediamine
158 dihydrochloride), spermidine (Spd) (*N*-(3-aminopropyl)-1,4-butanediamine trihydrochloride),
159 and spermine (Spm) (N-(3-Aminopropyl)-1,4-butanediamine trihydrochloride), all purchased
160 from Sigma-Aldrich, Inc. These compounds were added in three concentrations (0.01, 0.1 and
161 1 mM). The treatment was applied during the imbibition phase described above. The solutions
162 were removed before seed sowing. Each treatment was evaluated for the control and in moderate
163 and severe salt stress conditions. In each of two independent experiments, one tray per variant
164 (treatment and/or concentration) was analysed, with a total of 21 trays including controls. One
165 tray contained 110 seeds that were evaluated for seedling emergence.
166

167 Phenotyping platform, experimental setup and assay conditions

168 The trays with the sowed maize seeds were placed onto an OloPhen platform
169 (http://www.plant-phenotyping.org/db_infrastructure#/tool/57) that uses the PlantScreen^{TM}
170 XYZ system installed in a growth chamber with a controlled environment and LED lighting
171 (Photon Systems Instruments, Brno, Czech Republic) (Figure 1). The conditions were set to
172 simulate a long day with a regime of 22ºC/20ºC in a 16/8 h light/dark cycle, an irradiance of 120
173 µmol photons of PAR m^{-2} s^{-1} and a relative humidity of 60%. The PlantScreen^{TM} XYZ system
174 consists of a robotically driven arm holding an RGB camera, with a customised lighting panel
175 and growing tables, covering a total area of approximately 7 m^2 with a capacity of 60 customised
176 trays (Figures 1 and 2). The XYZ robotic arm automatically moved the camera and took RGB
177 images of individual trays from overhead. Each tray was photographed every 2 hours over 5
178 days. The RGB images were stored in a database server and analysed using an in-house software
179 routine implemented in MatLab R2015 developed and validated by the authors of this study.
180 The application can be used without any charge upon obtaining a license from the author. The
181 license can be obtained by e-mail to Palacky University upon agreeing not to use the application
182 for commercial purpose. After obtaining the license, the end user will be provided (free of
183 charge) with the MCRInstaller.exe. MCRInstaller simulates the MatLab environment on
184 computers where MatLab is not installed and enables to execute the application. To obtain the
185 application executable files, please contact the author Tomas Furst by email
186 tomas.furst@upol.cz. The email must contain the following statement: "Neither the application
187 nor the MCRInstaller will be used for any commercial purpose".
188

189 Statistical analysis and representation

190 For each maize seed, the time of emergence (i.e. the moment of the coleoptile
191 appearance) was recorded (Figure 2b-d). Since the images were taken once every two hours, the
192 time of emergence was set as the first imaging time when the seedling was already visible
193 (Supplementary Figure S1). Some of the seedlings may not have emerged at all until the end of
194 the experiment. For these, the total duration of the experiment was recorded and they were

195 denoted as "censored". The resulting data set consisted of several predictor variables (severity
196 of the salt stress, presence and concentration of the priming agent) and of the outcome variable
197 (the time to emergence) which may be "censored". Such data are often encountered in medicine
198 (survival times) and engineering (failure times). The statistical method commonly used to
199 analyse such time-to-event data is called survival analysis, or failure time analysis. A detailed
200 description of the data processing, analysis, and visualisation is given in the Results section.
201

202 **RESULTS**

203 **A- Septup for *CroSeEm* analysis**

204 *Maize emergence curves and their estimation*

205 A population of seeds tends to germinate in a characteristic pattern over time. Under the
206 same growth conditions, some seeds in the population will complete emergence very quickly,
207 most will do so around the average time, and the remainder will be much slower than the
208 average. Some may not germinate and emerge at all. Thus, the number of already emerged
209 seedlings (i.e. the cumulative distribution function of the emergence times) forms a sigmoid
210 shape over time. However, seedling emergence and, similarly, seed germination data differ from
211 other types of data usually encountered in biology. For example, the data are typically collected
212 by following cohorts of seeds, so the cumulative percentages of seeds that have germinated in
213 successive days exhibit serial autocorrelation. Also, some seeds remain non-germinated when
214 the experiment ends, and there is no way to know when these seeds would have germinated if
215 the experiment had continued indefinitely.
216

217 For testing the difference in seeding emergence among various subgroups, the non-
218 parametric log-rank test was used. The test is used to compare two or more emergence curves
219 and find whether the difference among them is significant. The first step was to compare the
220 emergence curves obtained from non-primed maize seeds grown in control conditions in two
221 independent experiments; **1st experiment** and **2nd experiment** (Table 1 and Figure 3). The
222 starting point of the picture capturing is the moment when the trays are placed into the platform.
223 This can differ among independent experiments, e.g. due to the different sizes of the experiments
224 and the time demands for their preparations. Hence, the initial delay in onset of emergence was
225 one of the main problems for an adequate comparison. For better comparison among different
226 experiments, the time axis of each experiment was shifted so that the medians of the controls
227 (i.e. the times when 55 seedlings had emerged) occurred at the same time (Figure 4A). This time
228 was denoted by 0. Thus, the resulting emergence curves are defined for both positive and
229 negative time in the rest of the variants (e.g. seedlings emerged under 75 mM or 150 mM NaCl)
230 (Figure 4B and C). No significant differences were found among the control curves from both
231 experiments (p= 0.1). The effect of different salinity levels in maize emergence was also
232 evaluated and compared to controls using the log-rank test. There was no significant difference
233 (p= 0.06) between the seedlings emerging under the control condition or grown in 75 mM NaCl
234 in both experiments (Figure 4B). However, the severe salinity of 150 mM NaCl significantly
235 affected seedling emergence in all cases compared to the control (p= 0) (Figure 4C). Thus, when
236 almost all seedlings under the control and 75 mM NaCl conditions emerged, the coleoptiles of
237 those in 150 mM NaCl had only just started to appear in both experiments (Figure 3).
238 The log-rank test compares two or more emergence curves but it is not suitable for
239 capturing differences in various aspects of the emergence process. Parametric methods are more

240 suitable for this purpose. In our case, the emergence of maize seedlings was analysed by fitting
241 the Gompertz curve to the empirical cumulative distribution function. The Gompertz curve is
242 given by
243
244
$$y(t) = A \exp(-x(t)) \quad (1)$$
245
246 where
247
$$x(t) = \exp(-k(t - t_m)) \quad (2)$$
248
249 The Gompertz curve was chosen because, unlike the more familiar sigmoid (also known
250 as logistic) curve, it is not symmetrical and better fits to the empirical emergence curves. This
251 is necessary because the convex part of the emergence curve (around the time of emergence of
252 the fastest seeds) tends to proceed faster than the concave part of the emergence curve (around
253 the time of emergence of the slowest seeds). In other words, the time required for the first half
254 of the seeds to emerge tends to be shorter than the time for the second half.
255
256 *The emergence conditions affect various aspects of the emergence curve*
257 The Gompertz curve obtained from maize seedling emergence times consists of an initial
258 exponential phase, an approximately linear phase (which contains the inflection point at which
259 the rate of emergence is maximal), and finally an asymptotic phase, in which the curve
260 approaches a constant (the final number of seeds emerged). Thus, three traits can be extracted
261 from the fitted emergence curves:

262 1. The **final emergence rate** (i.e. the total number of seedlings that had emerged at the end
263 of the experiment, divided by 110), corresponding to the coefficient A in equation **(1).**
264 2. The **time lag** (i.e. the difference between the positions of the peak of the derivative of
265 the fitted Gompertz curve for the control and the treatment).
266 3. The **emergence synchronicity** (i.e. a measure of the width of the peak of the derivative
267 of the fitted Gompertz curve) also denoted as emergence “speed”. In this case,
268 synchronicity is measured by the coefficient k in equation (2).

269 For better visualisation, we proposed a new type of graph to capture the three traits
270 simultaneously (Figure 5). We plotted the analytical derivative of the fitted Gompertz curve and
271 rescaled it so that the value of its maximum (the value at peak) is equal to the final germination
272 rate. Thus, the curve is not a probability distribution function (as the area under the curve is not
273 equal to one) but a rescaled emergence density. All the three traits are readable from the graph:
274 the maximum value corresponds to the final emergence rate, the time lag corresponds to the
275 position of the maximum, and the synchronicity corresponds to the width of the peak (Figures
276 5 and 6). Further, all the traits can be captured by means of a radar chart as shown in the right
277 panels of Figure 6. In the radar charts (right panel), the difference among variants was estimated
278 as the logarithm of base 2 ($\log_2$) of the ratio between control seeds and those grown under saline
279 conditions for the values obtained from final emergence rate and emergence synchronicity. For
280 the time lag, the difference was estimated by subtracting the time when 55 seedlings had
281 emerged under control conditions (normally 0) from the time the seedlings under moderate or
282 severe stress conditions emerged. For example, in experiment 1, the time lag was 0 for controls

283 but 0.27 for seeds emerged in 150 mM (Table 2), with a final result of - 0.27 represented in
284 Figure 6 (right panel).

285 By means of this visualisation, we observed that the final emergence rate was not a very
286 informative trait because there was almost no difference between the seeds grown under control
287 conditions and severe salt stresses conditions (Figure 6). However, seeds sowed in 75 mM NaCl
288 exhibited lower synchronicity of emergence in two independent experiments, on average 7%
289 less than the values of the controls (Figure 6 and Table 2). The use of 150 mM NaCl decreased
290 the emergence synchronicity by at least 50%, and produced a time lag of 8 hours on average
291 compared to control conditions (Figure 6). The results showed that this model has the capacity
292 to distinguish the changes in the seedling emergence under moderate and severe stresses. The
293 high throughput screening (HTS) using a time series of images is thus adequate to measure all
294 the useful traits of seedling emergence in a maize population under defined growth conditions.

295 **B- CroSeEm as HTS of maize emergence for characterising priming agents in control**
296 **and salt stress conditions-** A case study of polyamines as stress alleviators

297 To evaluate the use of HTS of maize emergence for the characterisation of priming
298 agents, we analysed the maize emergence using seeds primed with three polyamines Putrescine
299 (Put), Spermidine (Spd), and Spermine (Spm) at three concentrations (0.01, 0.1 and 1 mM) in
300 control and salt stress conditions (**3rd experiment**-Table 1). Overall, we analysed in this
301 experiment 27 experimental variants counting in total almost 3.000 maize seedlings in one run.
302 The resulting rescaled emergence densities (see above for explanation) are shown in Figure 7.
303 Several aspects of the cross-talk between the compounds and the growth conditions can be
304 observed. None of the polyamines seem to affect the final germination rate under any growth
305 conditions (Figure 7). Under control conditions (i.e. no salt stress), the application of polyamines
306 does not yield particularly obvious benefits. All the three traits remain largely unaffected with
307 the possible exception of time lag –primed seeds tend to emerge slightly sooner than the control
308 (Figures 7). However, under salt stress, the effect of the polyamines becomes visible. Under
309 moderate salt stress, Spd tends to increase the time lag and decrease synchronicity in all three
310 tested concentrations, whereas priming with Put at high concentrations (1 and 0.1 mM) increased
311 the emergence speed without modifications in the time lag. Under severe salt stress, the effect
312 of the tested seed priming agents becomes even more pronounced. Spd in both 0.1 and 1 mM
313 concentrations decreases the time lag (with respect to untreated seeds in severe salt stress
314 conditions). The lowest concentration 0.01 mM does not seem to be enough to produce this
315 effect. On the other hand, Put in all the three tested concentrations increased the time lag and in
316 0.01 mM concentration improved the emergence synchronicity (Figure 7). Altogether, we
317 showed that CroSeEm is good approach for characterizing priming agents in maize populations.

318 **DISCUSSION**

319 Seed priming has been shown to improve maize germination under optimal (Colla et al.,
320 2014) as well as stress (salinity) (Gebremedhn and Berhanu, 2013) conditions. The application
321 may accelerate seed germination and synchronicity by activating a tolerance-related response
322 which leads to seed enhancement. However, although the use of priming to enhance plant
323 tolerance of multiple abiotic stresses is highly promising, there are still many questions to

324 answer, such as in what species is priming most effective and has the biggest economic impact,
325 or which is the most effective priming agent against abiotic stresses.

326 Recent advances in high-throughput phenotyping have allowed simultaneous screening
327 of multiple quantitative traits of plants. Several automated high-throughput assays dealing with
328 plant germination and emergence have been recently published. However, they were mainly
329 based on the use of *Arabidospsis* germinated under *in vitro* conditions (Joosen et al., 2010;
330 Rodriguez-Furlán et al., 2016). To test whether the application of some compounds can
331 condition maize seed germination, measurements of the germination rate and the coleoptile
332 elongation in seeds placed on filter paper have been the most widely used (Colla et al., 2014;
333 Savy et al., 2015), with the germination induced in petri dishes with solutions to simplify the
334 measurements. At the end, this method results in tedious manual work with a limited number of
335 seeds and variants. In this work, we developed a high-throughput assay for *ex vitro* maize
336 emergence to be used as an effective screening of seed priming compounds under saline
337 conditions. To obtain a reproducible and accurate method, the most important step was the data
338 analysis. Time-to-event analysis (also known as survival analysis, failure time analysis and
339 reliability analysis) is very flexible and can be used with all kinds of germination and emergence
340 studies, but has only rarely been applied to germination data and is poorly documented in
341 biological literature (McNair et al., 2012). Such analysis meets all the requirements in terms of
342 error distribution and independence and can also account for censored observations and late
343 germination ⁄ emergence flushes, which may be problematic for more traditional techniques
344 (Onofri et al., 2010). For example, seed germination and emergence assays pose problems for
345 data analysis, due to non-normal error distribution and serial correlation between the numbers
346 of seeds counted on different dates from the same experimental unit (Petri dish, pot, plot).
347 Furthermore, it is necessary to consider viable seeds that have not germinated ⁄ emerged at the
348 end of an experiment (censored observations), as well as late germination ⁄ emergence flushes,
349 that relate to genotypic differences within naturally occurring seed populations. To solve all
350 these problems, we compared the emergence curves of the maize seeds under control conditions
351 obtained from two independent experiments using the non-parametric log-rank test (Figure 4A).
352 In both experiments, the curves for seeds under control conditions did not have significant
353 differences among them and only differed with those seeds that emerged under severe salt
354 conditions (Figure 4C). This may be because the log-rank test compares two or more emergence
355 curves but it is not suitable for capturing differences in various aspects of the emergence process
356 (McNair et al., 2012). Thus, we chose the Gompertz curve because it better fitted the empirical
357 emergence curve. In addition, the Gompertz curve allowed us to extract three different traits:
358 final emergence rate, time lag and emergence synchronicity (Figure 5). As a result, we observed
359 a delay in the speed of maize seedling emergence compared to the control in two independent
360 experiments when they were sown with 75 mM NaCl (Figure 6). This trait, together with the
361 time lag, was particularly affected when 150 mM NaCl was used. However, the final
362 germination rate was almost unchanged, suggesting that this trait was less of a stress indicator.
363 These results showed that the three extracted traits should be independently analysed because
364 the growth conditions altered them to different extents. Thus, analysing them separately is a
365 more sensitive and reproducible approach for the characterisation of maize emergence under
366 salinity.
367

368 Finally, to optimize our HTS method for analysis seed priming agents, we treated the
369 maize seeds with the same polyamines and concentrations used in the HTS described for
370 *Arabidopsis* (Ugena et al., 2018). The exogenous application of polyamines has improved
371 abiotic stress tolerance in many crops (Duan et al., 2008; Puyang et al., 2015; Shu et al., 2015;
372 Zhang et al., 2016). However, there are no almost studies using priming with these types of
373 compounds and the known aspects of their mode of action strongly suggest that they can be
374 potentially used against multiple abiotic stress phenomena (Savvides et al., 2016). For example,
375 priming with polyamines synchronized germination, increased the seedling emergence, and
376 early seedling growth in hot pepper (Aziz Khan et al., 2012). Under stress conditions, priming
377 with Spd improved chilling tolerance in rice (Sheteiwy et al., 2017). The exogenous application
378 of Put and Spd had also positive effect on the germination and early seedling growth of
379 sunflower under salt stress (Farooq et al., 2009). In this case, the priming with Spd was the most
380 effective treatment to improve the germination synchronicity and other germination indexes.
381 The soaking of *Atropa belladonna* seeds with Put also improved and accelerated the germination
382 (Ali, 2000). In our study we observed that the priming of maize seeds with Put was the most
383 efficient treatment improving the emergence synchronicity under moderate and severe salt stress
384 (Figure 6). Interestingly, similar positive effects of priming with Put we also found using
385 *Arabidopsis* (Ugena et al., 2018). The use of polyamines as seed priming agents thus seems to
386 be interesting strategy to improve the stress tolerance of plants. However, the most effective
387 combination of the compounds and their concentrations can be species dependent. Here we
388 would like to emphasize the importance of the fact that seedlings developing from seeds
389 germinating under the soil are facing two problems - first, there is little or no light to drive its
390 life-sustaining photosynthesis; and, second, it must generate sufficient mechanical force to push
391 aside whatever is covering it (Briggs, 2016). Until the seedling reaches the light, it must rely on
392 stored energy reserves to sustain it. In this regard, recent work performed in cucumber subjected
393 to salt stress observed that the exogenous application of Put modified C metabolism making the
394 plant energetically more efficient. The same way, priming with polyamines increased α-amylase
395 activity, soluble sugars and soluble protein contents and enhance the antioxidative response of
396 rice under chilling stress (Sheteiwy et al., 2017). In addition, both germination and seedling
397 emergence are triggered by plant hormones, C and N metabolism and NO content (Osuna et al.,
398 2015), that can be influenced by changes in polyamine content (Podlešáková et al., 2019).
399 Specifically in maize the interaction between ethylene and polyamine pathway has already been
400 shown to be related to salt stress tolerance (Freitas et al., 2018). This result points to one of the
401 possible mechanisms of polyamines' action which can be amelioration of the negative effects
402 of ethylene during the seedling emergence especially under stress conditions.
403
404 **CONCLUSION**
405
406 In this study, we presented an accurate high-throughput assay for screening seedling
407 emergence "CroSeEm" suitable for characterising different maize lines and/or seed priming
408 agents against salinity. The relatively new statistical analyses used here are appropriate tools for
409 the discrimination and characterisation of the emergence of maize seeds exposed to various
410 salinity conditions. For example, using the entire survivor function for maize seedling
411 emergence in different salinities exposed clearly different response patterns over time, which
412 ended with variations in the starting point and synchronicity of the emergence. However, in our
413 hybrid all treatments used (salt stress intensity and priming) did not vary the final emergence

414 rate (Figures 6 and 7), making it an adequate internal control for further studies of maize lines.
415 However, it is clear that estimating only the cumulative percentage of maize seedling emergence
416 at the end of the experiment would have led to a conclusion of there being no evidence of a
417 significant salinity effect. Besides, we demonstrated that polyamines can be used as seed
418 priming agents to improve seedling emergence in maize under salt stress conditions.
419

420 **AUTHOR CONTRIBUTIONS**

421 LU, JFH, TF, NDD and LS designed the experiments. LU and JFH performed the experiments.
422 NDD and LS supervised the study and the concept of the project. JFH, TF and NDD performed
423 the data analysis. All authors discussed the results and contributed to the final version of the
424 manuscript.

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537

Table 1| Scheme of the three performed experiments.

Experiment	Growth conditions	Seed priming	
1st and 2nd	Control 75 mM NaCl 150 mM NaCl	Non-primed seeds	
3rd	Control 75 mM NaCl 150 mM NaCl	Put, Spd or Spm	0.01, 0.1 or 1 mM

Table 2| Values for the three emergence traits: final emergence rate (number of emerged seedlings), time lag (days), and speed (synchronicity of emergence represented as the curve slope) in untreated seeds under control, moderate (75 mM NaCl) and severe (150 NaCl) salt stress conditions.

	Growth Conditions	Emergence rate	Speed	Time lag
Experiment 1	Control	108.8	7.61	0.00
	75 mM NaCl	109.7	6.99	0.00
	150 mM NaCl	109.5	5.58	0.27
Experiment 2	Control	108.0	8.85	0.04
	75 mM NaCl	109.0	8.33	0.04
	150 mM NaCl	105.5	6.02	0.44
AVERAGE	Control	108.5	8.11	0.02
	75 mM NaCl	109.3	7.62	0.02
	150 mM NaCl	108.4	5.80	0.35

FIGURES

Figure 1| A schematic of the protocol used for high-throughput screening of maize seedlings emergence "CroSeEm" under salt stress conditions. (a) 110 maize seeds were stratified for 16 h and sowed into soil in standardized PlantScreen^{TM} measuring trays. The trays were transferred to an XYZ PlantScreen^{TM} chamber for automatic image acquisition. (b) The seedling emergence was determined as first "green pixel". **(c)** The emergence was analysed using an in-house software routine implemented in MatLab R2015, and three independent traits; final emergence rate (number of emerged seedlings), time lag (days), and speed (synchronicity of emergence represented as the curve slope) were extracted from empiric emergence curve fitted to the Gompertz curve.

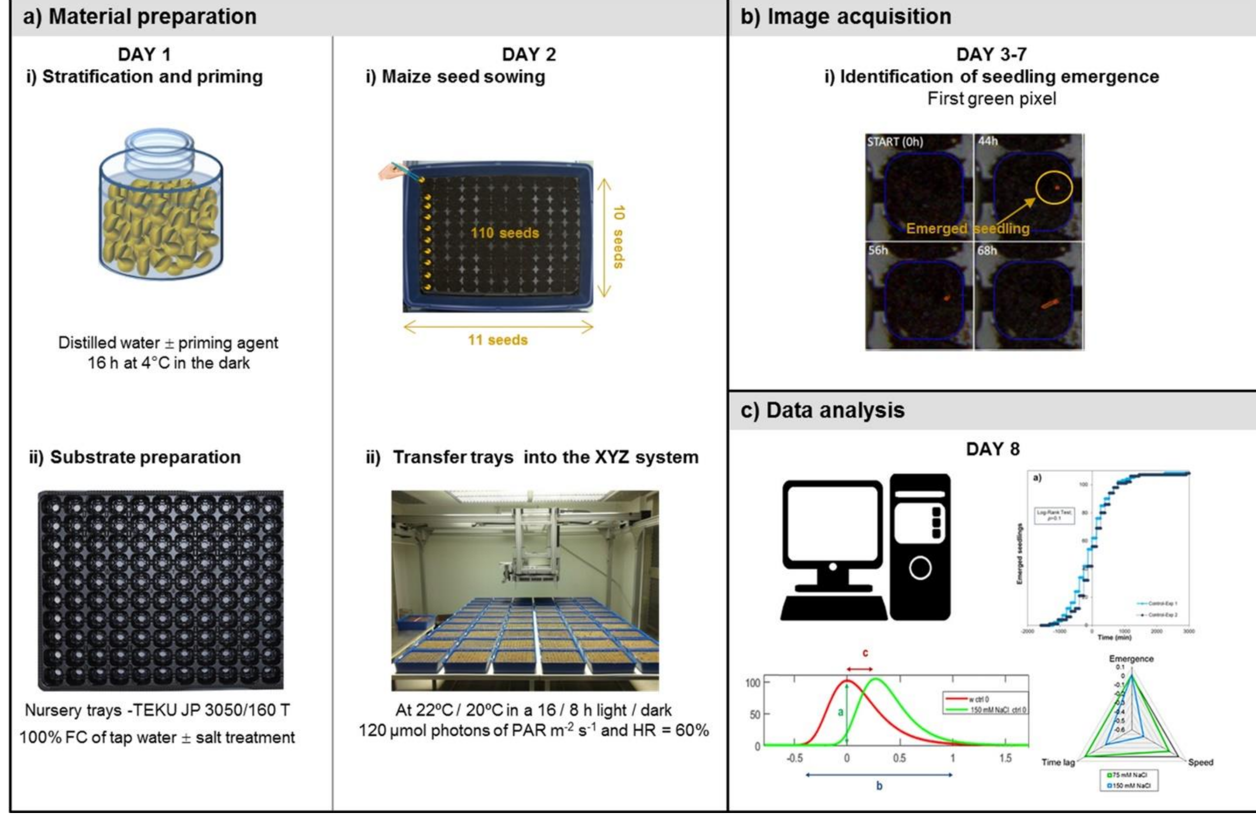


Figure 2| The maize emergence assay using the PlantScreen^{TM} XYZ system on an OloPhen platform at full capacity of 60 trays (a). A detail of one of the 110 cells of a tray before seedling emergence (b), first identified pixel of maize coleoptile marked with a red circle (c), fully emerged seedling (d).

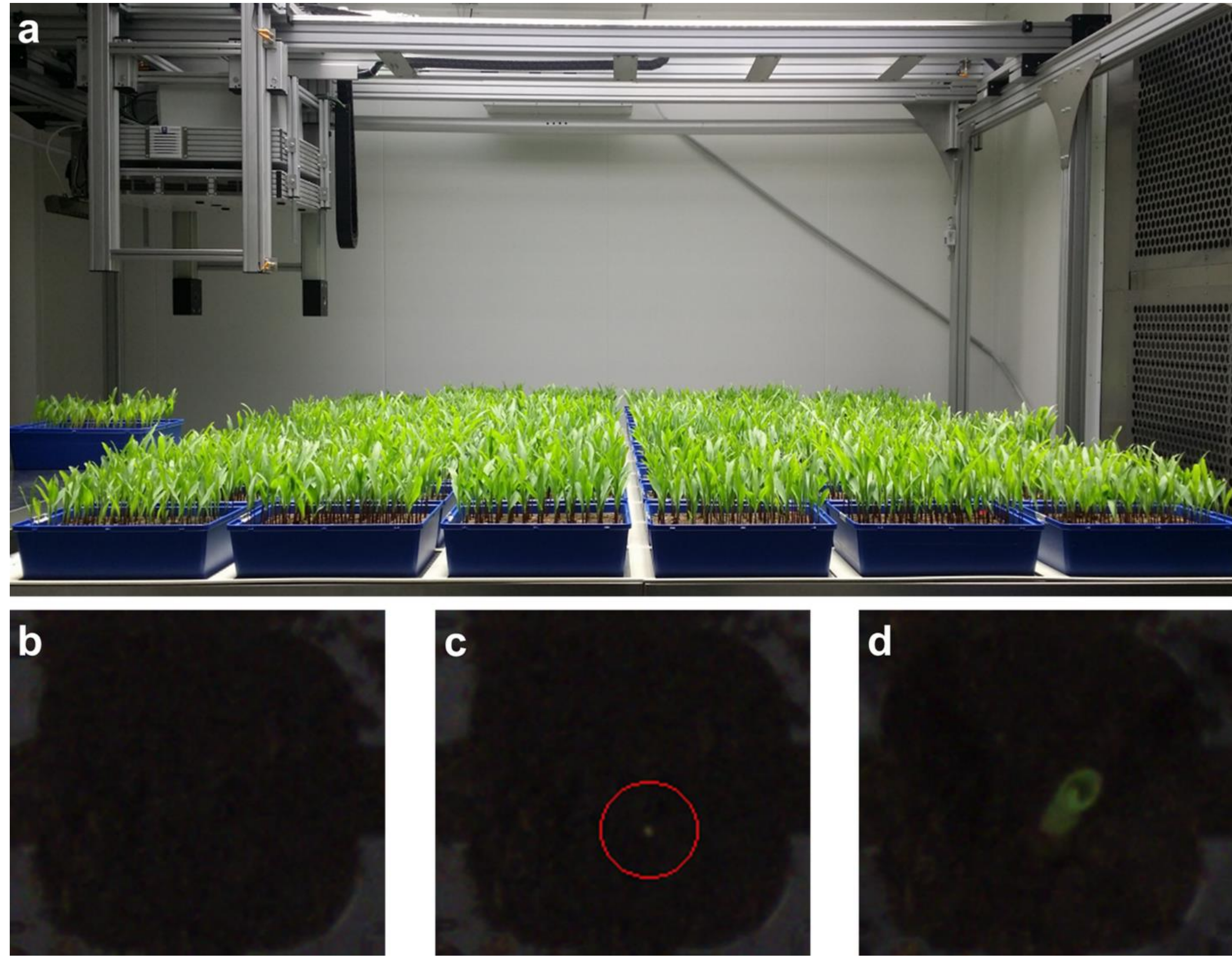


Figure 3| RGB images of trays from two independent experiments showing the emerged seedlings under control, moderate (75 mM NaCl) and severe (150 NaCl) salt stress conditions at the point when the control seedlings are fully emerged.

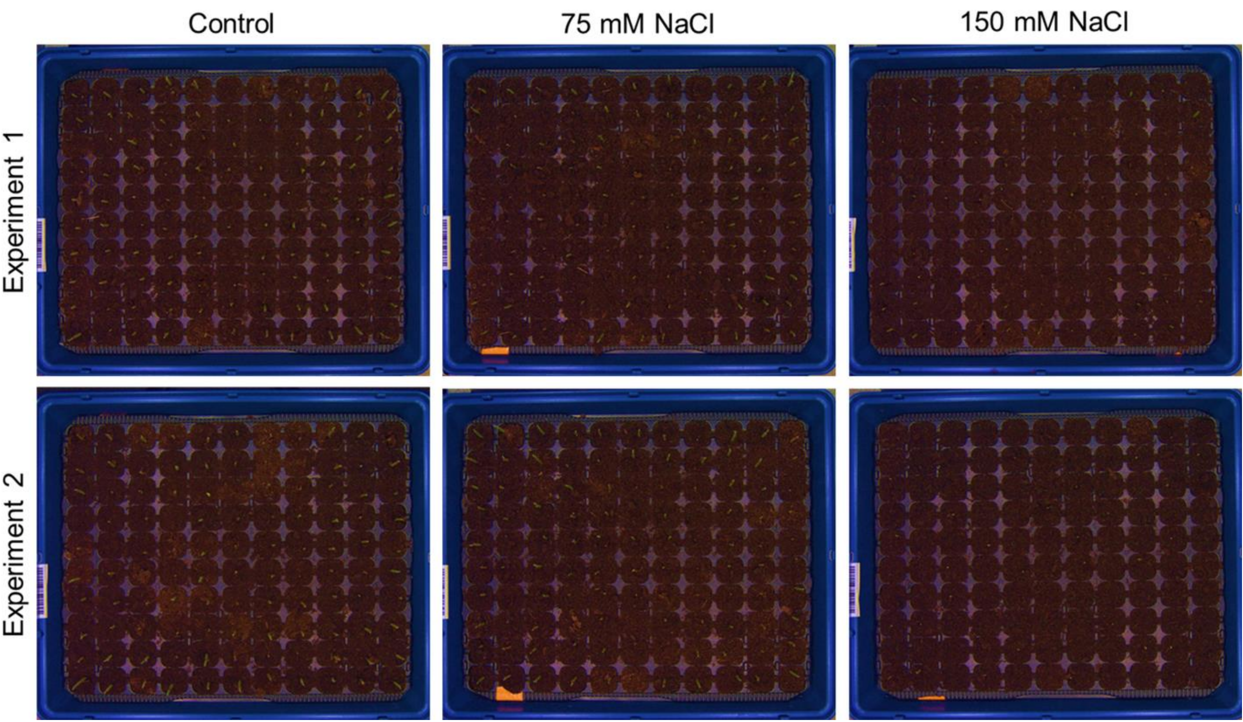


Figure 4| Sigmoidal emergence curves. Comparison between the emergence curves in untreated maize seeds under control conditions from two independent experiments (exp 1 and exp 2) (a), between untreated seeds under control and 75 mM NaCl conditions (b), and between controls and 150 mM NaCl (c) using log-rank test in Matlab2015.

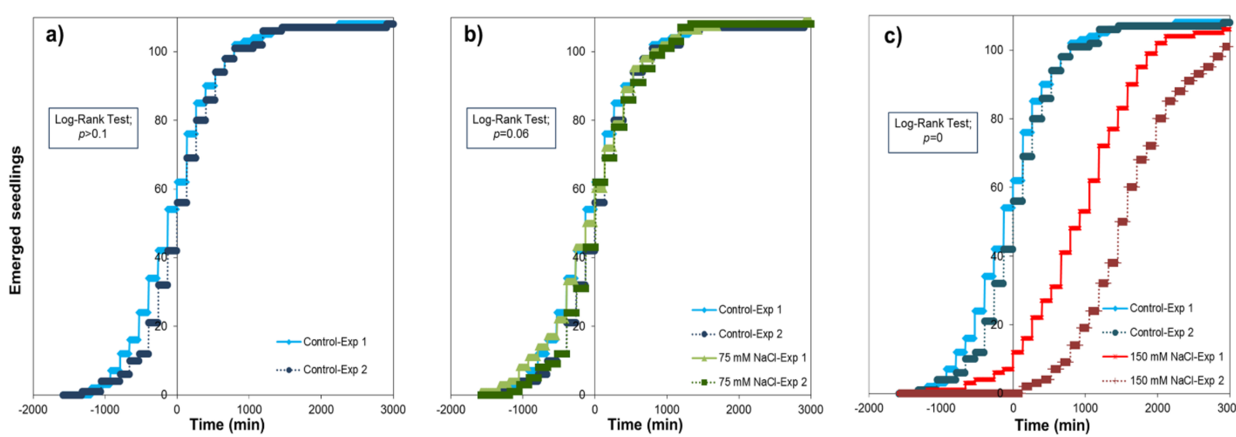


Figure 5| Empiric emergence curves. The three aspects of the emergence curves of maize seedlings under control and severe (150 NaCl) salt stress conditions. The maximum value (a) corresponds to the final **emergence** rate, the **time lag** (b) corresponds to the position of the maximum, and the emergence synchronicity or **speed** (c) corresponds to the width of the peak.

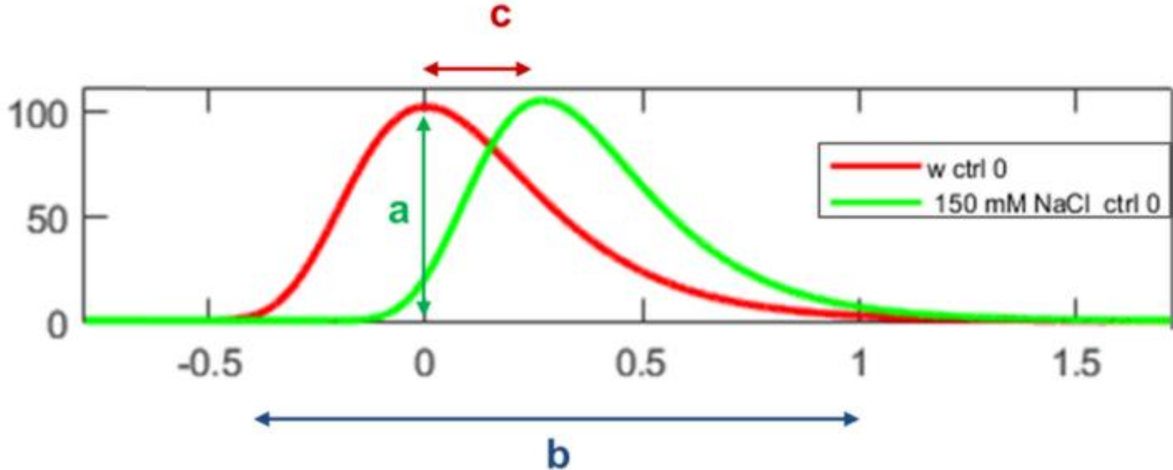


Figure 6| The three aspects of the empiric emergence curves of biostimulant-untreated seeds under control, moderate (75 mM NaCl) and severe (150 NaCl) salt stress conditions. Left panels: The rescaled emergence distribution curves. The maximum value corresponds to the final **emergence** rate, the **time lag** corresponds to the position of the maximum, and the synchronicity (**speed**) corresponds to the width of the peak. Right panel: Radar charts of the three traits (emergence, time lag and speed).

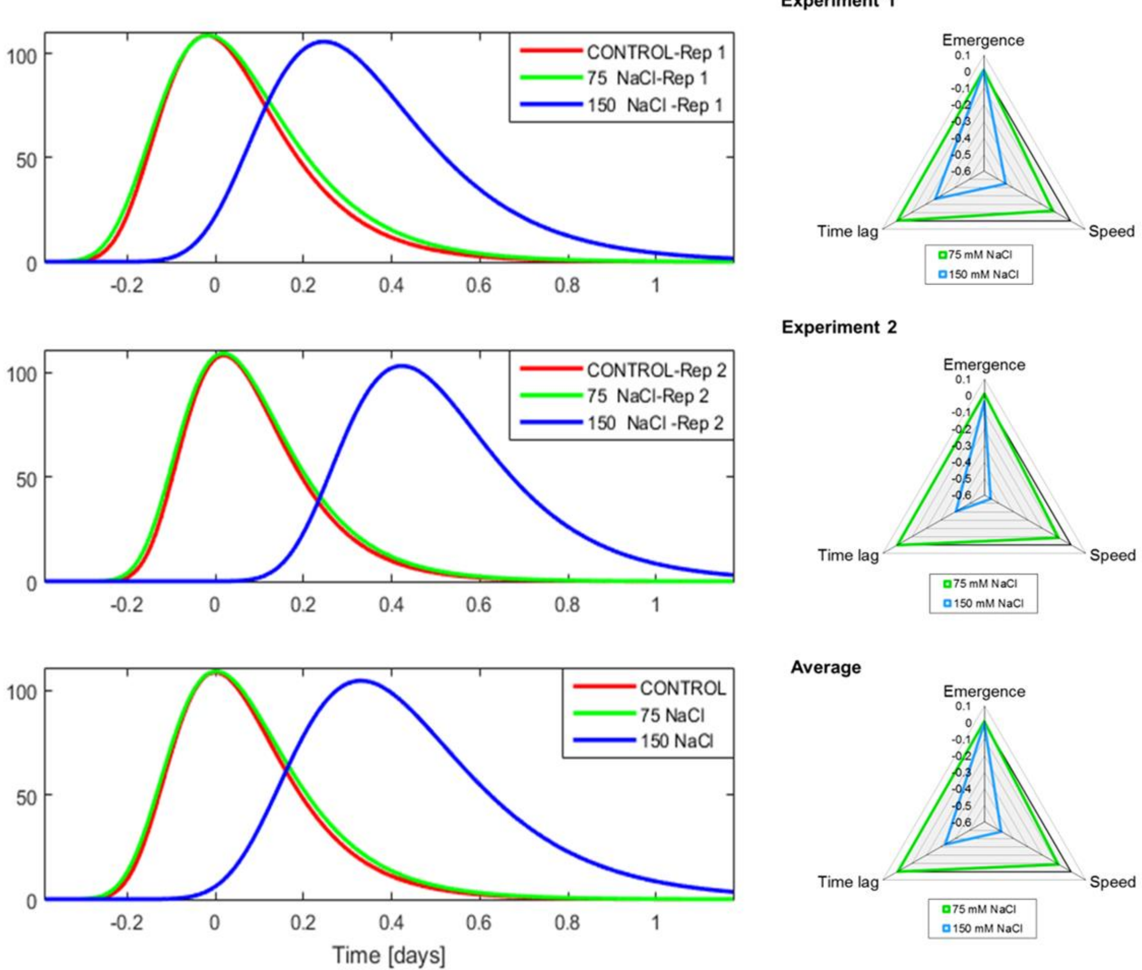
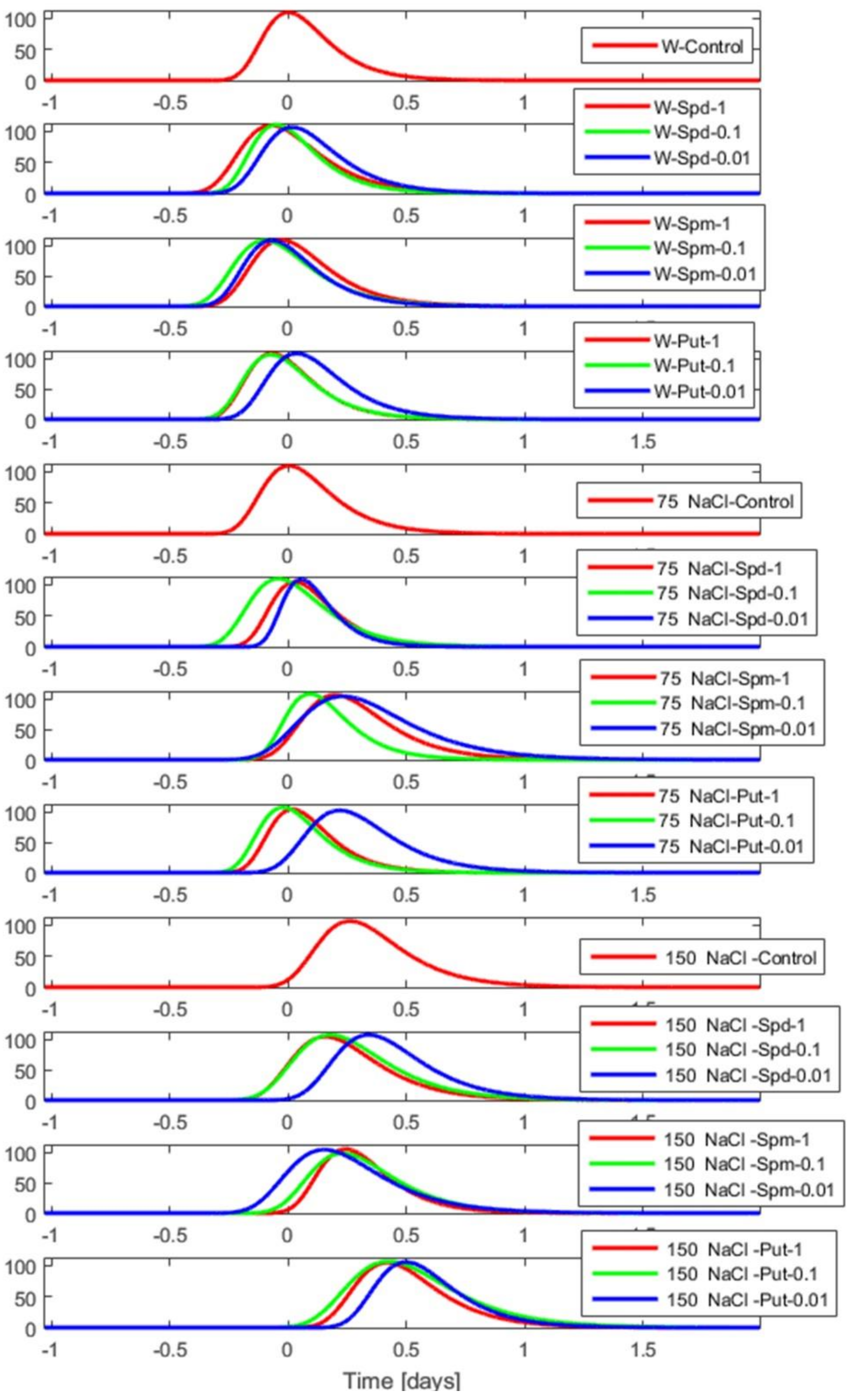


Figure 7| The three aspects of the empiric emergence curve in seeds primed with Put, Spd and Spm in three concentrations (0.01, 0.1 and 1 mM) under control, moderate (75 mM NaCl) and severe (150 NaCl) salt stress conditions. The rescaled emergence distribution curves show the final **emergence** rate (the maximum value of the curve), the **time lag** (the position of the maximum), and the emergence synchronicity or **speed** (the width of the peak).



Supplemental Figure S1| RGB images of trays showing the emergence of maize seedlings under control conditions.

The Supplementary Figure S1 for this article can be found online at:

https://drive.google.com/open?id=1dzp6nrmCDA0U9pck-YyAWCCQ1yttB2BJ