PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Characterization of new chemicals for mitigating the effect of different abiotic stresses on *Arabidopsis* plants.

DIPLOMA THESIS

Author:	**Bc. Andrea Hybenová**
Study program:	B1406 Biochemistry
Field of study:	Biochemistry
Form of study:	full-time
Supervisor:	**Ing. Nuria De Diego Sanchez, Ph.D.**
Year:	2020

I declare that I have done the whole diploma thesis independently under the supervision of Ing. Nuria De Diego Sanchez, Ph.D.
I agree with the publication of my diploma thesis according to Act no. 111/1998 Coll., On Higher Education Institutions, as amended. I have been informed about the fact that my work is subject to the rights and obligations of the Act no. 121/2000 Coll., Copyright and Related Rights, as amended.

In Olomouc 2020

.....

Bc. Andrea Hybenová

Acknowledgement

First of all, I would like to thank to my supervisor Ing. Nuria De Diego Sanchez, Ph.D. for professional guidance, patience, all the valuable advice and help with the elaboration of my diploma thesis. My thanks also go to Department of Growth Regulators (LRR) for allowing me to perform a part of my diploma thesis in their laboratories as well as for the analysis of phytohormones.

Bibliografická identifikácia:

Meno a priezvisko autora:	Bc. Andrea Hybenová
Názov práce:	Charakterizácia nových chemikálii znižujúcich vplyv rôznych abiotických stresových faktorov na rastliny *Arabidopsis*.
Typ práce:	Diplomová
Pracovisko:	Oddelenie chemickej biologie a genetiky
Vedúci práce:	Ing. Nuria De Diego Sanchez, Ph.D
Rok obhajoby práce:	2020

Abstrakt:
Táto diplomová práca je zameraná na štúdium rastu rozét rastliny *Arabidopsis thaliana* v rôznych stresových podmienkach po aplikácii troch rozličných skupín látok považovaných za stresové aleviátory.
Všetky analýzy boli vykonané pomocou vysoko výkonného fenotypingu (HTS).
Metabolický profil rastlín zameraný na zmeny v hladinách rôznych fytohormónov bol taktiež študovaný a to pomocou hmotnostnej spektrometrie.

Kľúčové slová:	*Arabidopsis thaliana*, stresový aleviátor, rastový stimulátor, abiotický a biotický stres, seed priming, rastlinné hormóny, cytokiníny, auxíny, ABA, high-throughput phenotyping
Počet strán:	76
Jazyk:	anglický

Bibliographical identification:

Name of the author:	Bc. Andrea Hybenová
Title:	Characterization of new chemicals for mitigating the effect of different abiotic stresses on *Arabidopsis* plants.
Type:	Diploma thesis
Department:	Department of Chemical Biology and Genetics
Supervisor:	Ing. Nuria De Diego Sanchez, Ph.D
The year of presentation:	2020

Abstract:
This diploma thesis pays a particular attention in the study of *Arabidopsis thaliana* rosette growth in different stress conditions after the application of three different groups of compounds as possible stress alleviators.
All analyses were performed using high-throughput phenotyping.
The metabolome profile of the plants focused on the changes in the levels of different phytohormones was also studied using mass spectrometry approaches.

Keywords:	*Arabidopsis thaliana*, stress alleviator, growth promotor, abiotic and biotic stress, seed priming, plant hormones, cytokinins, auxins, ABA, high-throughput phenotyping
Number of pages:	76
Language:	English

Content

Objectives

The primary purpose of this diploma thesis is the characterization of three different groups of cytokinin analogs as possible plant growth promotors and/or stress alleviators.

The theoretical part is focused on the integration and understanding of the literature associated with the topic of the thesis, especially on the effect of seed priming with different chemicals on plant development under different growth conditions.

In the experimental part, a germination protocol for *Arabidopsis* plants was developed and optimized for priming. High-throughput phenotyping platform was then used in order to obtain reliable and reproducible data about different plant growth related traits.

Furthermore, a quantitative analysis of different phytohormones was performed to study the mode of action by determining the endogenous changes induced by selected compounds and the correlation of these changes with the growth traits under different growth conditions.

Finally, all obtained data were processed, evaluated, described and discussed to give a clear formulation of conclusions about this diploma thesis.

1 Introduction

During the life, plants are subjected to many abiotic and biotic stress factors that may negatively affect their morphology and physiology leading to the reduction of crop quality and productivity. In order to avoid these effects, plants have to develop various defense mechanisms (Egamberdieva *et al.*, 2017).

Among others, abiotic stresses such as drought and soil salinity trigger many physiological, biochemical and morphological changes regulating plant growth processes (Sah *et al.*, 2016). Since seed germination and seedling establishment are the most essential steps of plant growth, the exposure of plants may affect the efficiency of the growth and also final quality (Ibrahim, 2016). According to The Food and Agricultural Organization (FAO), human population is predicted to reach 8 or 9 billion by 2030 (Egamberdieva *et al.*, 2017). Therefore, development of plants tolerant to these challenging growth conditions is one of the main purposes to meet in order to satisfy the demand of food production (Uddin *et al.*, 2016).

Recently, seed priming has become one of the methodological approaches used in order to improve stress tolerance of plants. This method is a pre-sowing treatment in which seeds are being partially prepared for the germination using biological or chemical, natural or synthetic compounds. Therefore, pre-treatment of seeds by priming do not only improve the germination and overall performance of crop plants but also helps to alleviate negative impact of many environmental stress factors by faster defense responses and enhanced resistance to various abiotic stresses (Jisha *et al.*, 2013; Nakaune *et al.*, 2012; Yadav *et al.*, 2011).

In order to obtain more information and better knowledge about the mode of action of seed priming and the plant response to stress conditions, *Arabidopsis thaliana* seeds were firstly primed with eight different compounds and subsequently exposed to two kinds of abiotic stress.

Recently, high-throughput screening (HTS) of plants have become an indispensable tool in screening of several different plant traits at the same time. Unlike other destructive and time-consuming methods, HTS offers fast, non-invasive, automated and simultaneous analysis of plant morphology, growth and collection of many phenotyping traits, including size, shape, color, etc (Faragó *et al.*, 2018).

Besides that, in order to investigate possible involvement in plant responses to different abiotic stresses, quantitative analysis of different plant hormones, mainly cytokinins (CKs), auxins and abscisic acid (ABA) were performed.

2 Present state of knowledge

2.1 Plant stress - definition, classification and plant responses

As long as plants are sessile organisms, they have to deal with many environmental changes in order to growth and survive (Suzuki *et al.*, 2014). Unlike animals, plants are not able to escape from these unfavorable conditions that in many cases negatively affect their growth and development processes. For this reason, plants had to develop sophisticated defense mechanisms that define their tolerance capacity and durability (Liu *et al.*, 2015).

Despite the fact that original idea of plant stress was firstly described by Larcher (1987), the most widely used classification of stress in plants is the one defined by Lichtenthaler (Lichtenthaler, 1996). He stated that the stress is any unfavorable condition/substance that affects or blocks a plant's metabolism, growth and/or development. Furthermore, he also divided the term stress into two different types: eu-stress and di-stress. While eu-stress is a stress with positive, activating and stimulating effects on plant development, di-stress is a harmful type with negative effects on plants (Kranner *et al.*, 2010; Lichtenthaler, 1996).

Of course, the effect of different stresses on plants also depends on the type of the stressor, duration (long- and short-term), ability of plants to deal with all these stress factors, their sensitivity, tolerance, vitality and growth stage (Kranner *et al.*, 2010).

Another way of stress classification is its division into two most frequently used categories: abiotic and biotic stress. Regarding abiotic stress, this type of stress is caused by any chemical or physical effect. This category of plant stress include drought, flooding, temperature (heat and cold), UV radiation, salinity or toxicity of many chemicals and heavy metals, among others (Bajguz & Hayat, 2009). On the other hand, biotic stress in plants is different from abiotic. It is caused by any living organism that interact with the plant, such as bacteria, fungi, viruses, nematodes, weeds, etc (Bhardwaj *et al.*, 2014). All these stress factors have a direct impact on its host plant, mainly competing for the nutrients or vitality, which may lead to the death itself (Gull *et al.*, 2012; Suzuki *et al.*, 2014).

Before the plants are exposed to any stressful condition, they are in a steady state - optimal living conditions (e.g. sufficient mineral supply, light). After the application of a stressor, the plants respond in four phases (**Figure 1**):

1. alarm phase leading to reduction of vitality,

2. stage of resistance, adaptation process starts,
3. stage of exhaustion when the intensity of stress is too high and
4. regeneration phase after the removal of the stressor (if the stressor did not have damaging and too severe effect)

Regarding direct responses to environmental stress, when the plants are under the stress, they have to develop several defense mechanisms to overcome all these unfavorable conditions. As first, by accepting the external stimuli from the environment, they sense the stress factor. Receiving the signal leads to many different transcriptional translational, post-translational and metabolic changes that consequently cause higher tolerance against plant stress (Gull *et al.*, 2012; Liu *et al.*, 2015; Popko *et al.*, 2010; Suzuki *et al.*, 2014).

The most important in this process is a signaling pathway that ensures the connection between stress sensing and subsequent biochemical and physiological changes in plant body. Using the sensors located on the cell surface, mainly on plasma membrane, the signal from outside is transferred to the nucleus of the cell using secondary messengers (ROS, calcium, etc.) (Fahad *et al.*, 2015). This transduction pathway causes the activation of different processes on transcription level that makes the plant more tolerant against stress (Liu *et al.*, 2015).

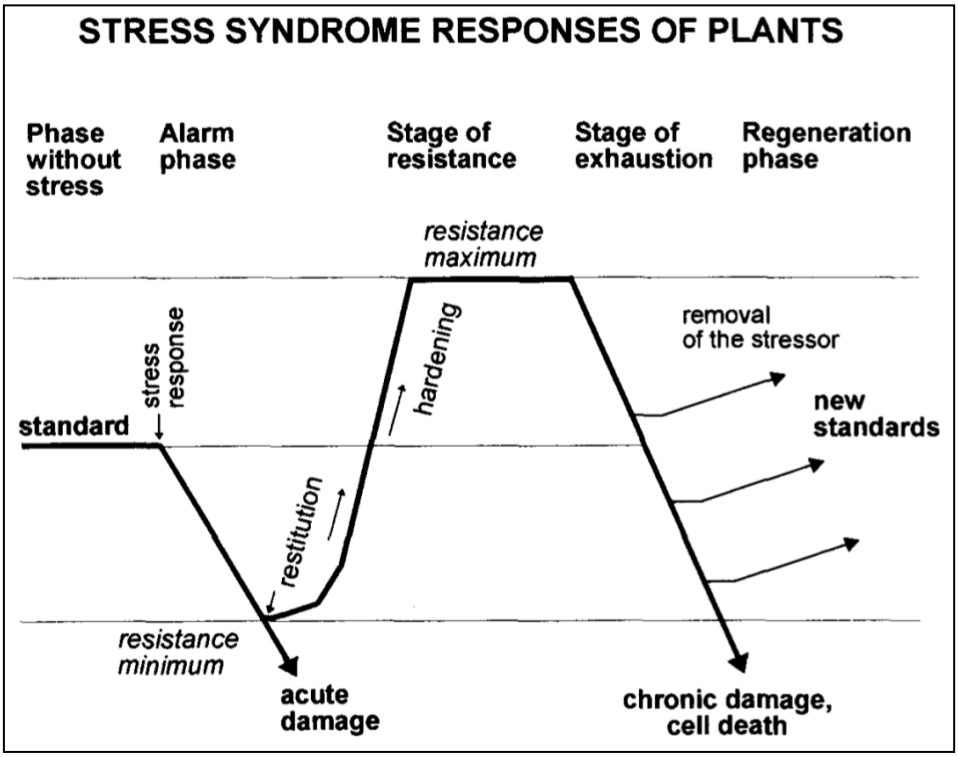

Figure 1: Four different phases of plant response to stress (Lichtenthaler, 1998).

2.2 Effect of abiotic stress on plants

Due to the climatic change, growth, productivity and yield of many crop plants is often affected by different abiotic stress (**Figure 2**). Among the different abiotic stressors, salinity and drought are probably the most frequently occurring and most serious once worldwide. It is believed that the areas affected with these two types of stress are going to expand and so result in reduction of productivity of many plant species (Pavlů *et al.*, 2018; Savvides *et al.*, 2016; Uddin *et al.*, 2016).

2.2.1 Salinity

Soil salt stress or salinity is one of the biggest problems worldwide, causing the reduction in productivity and yield of agriculturally important plants. Owing to this fact, more than 230 million hectares of world's irrigated land (approximately 20%) is affected by soil salinity (Munns & Tester, 2008). This type of abiotic stress causes either the increase of osmotic pressure so the plants are being limited with water and nutrient uptake from the soil, or sodium and chloride ions have a toxic effect on early germination of the seeds, its delay or reduction (Jafar *et al.*, 2012; Jamil *et al.*, 2005; Yongyin Wang *et al.*, 2001).

2.2.2 Drought

Due to the increasing temperature average around the world, plants are exposed to another type of abiotic stress - drought. Drought is basically a long period of time during which a specific land area suffers water deficit. Water supply in soil is still being limited more and more. For this reason, agriculture around the world is significantly affected. It is assumed that the production of crop plants will fall by 30% until 2025. When exposed to this type of abiotic stress, plants start to response by decreasing their growth and reducing the metabolic processes which results in lower yields and crop production (Jisha *et al.*, 2013; Popko *et al.*, 2010; Siddique *et al.*, 2016; Suzuki *et al.*, 2014).

2.2.3 Interaction between different abiotic stresses

A combination of various abiotic stresses may have several effects on plant growth. These may act synergistically or additively. It has been shown that various stress occurring at the same time, for example salinity and high temperature, drought and

salinity, or combination of heavy metals and high temperature, have bigger damaging impact on plant growth than the effect of these stresses separately (Colmenero-Flores & Rosales, 2014; Savvides *et al.*, 2016; Zhu, 2002).

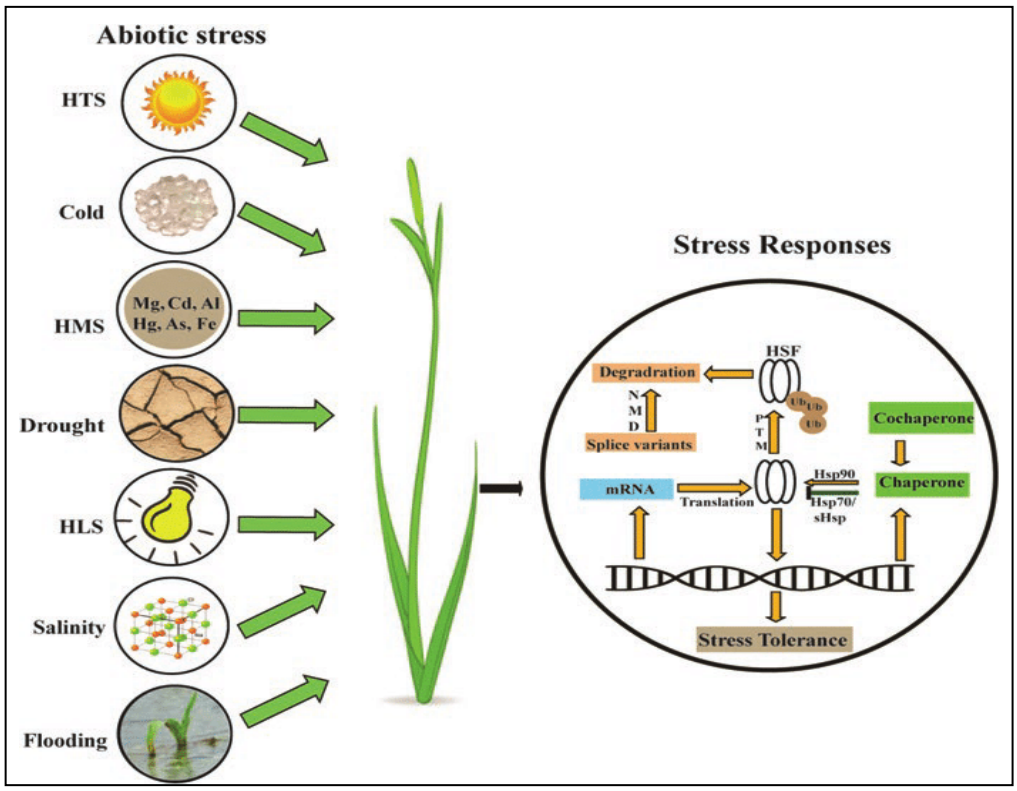

Figure 2: Different types of abiotic stress on plant (Mishra *et al.*, 2018)**.**

2.3 Defense priming of plants

Regarding the fact that the world population is continuously increasing and the climate changes are affecting crop production and yield, the need to enhance the production of agriculturally important products has become a priority necessary to satisfy a food demand (Gill & Tuteja, 2010).

There are many different methodological approaches employed in enhancement of stress tolerance. Unfortunately, some of these techniques are highly time-consuming (such as breeding) or ethically unacceptable (genetic modification). That is why seed priming became a very suitable alternative to all these methodologies. In addition, seed priming became one of the most promising solutions to face the problem with the quality and quantity of the crops (Savvides *et al.*, 2016).

Seed priming is an effective pre-sowing technology in which seeds are firstly treated with small doses of chemical or biological, synthetic or natural agents just before the germination. Priming procedure itself helps plants to prepare their metabolic and defense responses to be more effective and accurate in a given growing conditions. This state of the primed seed is called "primed state". In general, priming of the seeds improves seed performance, ensure higher uniformity among the seeds, faster and synchronized germination, enhance plant growth, etc (Anosheh *et al.*, 2011; Beckers & Conrath, 2007; Gamir *et al.*, 2014; Ibrahim, 2016; Lutts *et al.*, 2016).

Moreover, the individual steps of seed priming, such as soaking or re-dehydration, may also create a mild abiotic stress. This fact indicates that priming does not only contribute to better germination and development-related processes but also include other mechanisms that help the seeds to better tolerate harmful environmental conditions and co-operate with damaging effects of different stresses (Kubala *et al.*, 2015).

Improvement of seed tolerance to stress and better germination is a cross tolerance caused by a process of priming. Firstly, priming starts certain biochemical, physiological, molecular and cellular changes, energy mobilization and storage, growth of the embryo and preparation for the germination. Secondly, abiotic stress caused by priming postpones the radicle protrusion. Thanks to these two processes, seeds develop so-called "priming memory" which in fact improve stress-tolerance of seeds in case that they are exposed to any stress during the germination process (Chen & Arora, 2013; Ibrahim, 2016; Ugena *et al.*, 2018; Yadav *et al.*, 2011).

Unlike un-primed seeds, primed seeds are able to respond to a very low levels of a certain stimuli which results in higher resistance to biotic or abiotic stress factors (Conrath, 2011; Paparella *et al.*, 2015). For this purpose, seed industry has a huge interest in defining and finding suitable agents that could be used for priming.

2.4 Seed germination and the effect of priming on plant development

Uniform and efficient seed germination is an essential event for creating a new generation of plants (Ibrahim, 2016). During the process of germination, the seed has to undergo several processes of its development from maturation to germination and early establishment of a seedling. After the seed is fully mature, it has only about 5 to 15% of water content. In agriculture, the most important process for prosperous plant production is an efficient seed germination, successful early seedling establishment, and root development. During these first stages of development, seeds and seedlings are very sensitive to any small change in the environment (Gallardo *et al.*, 2001; Kubala *et al.*, 2015).

To start the germination, the mature seeds have to be firstly hydrated and located in suitable growing conditions. The general process of seed germination involves three phases: In the first phase (I), process of rapid seed hydration occurs for the first time. This phase is also known as imbibition. Second phase (II), also known as activation or lag phase, is related with the activation of metabolic and repair processes at cell levels. In this phase, the uptake of water is minimal because the ability of seed to accept water is near to its equilibrium. In the last phase (III), growing processes such as cell elongation and radicle protrusion are induced (Lemmens *et al.*, 2019; Silva *et al.*, 2017).

All stages of seed germination and growth of the seedling are especially sensitive to any abiotic stress factor, which may be delayed, reduced or even fully inhibited. For this reason, seed priming is a very effective pre-sowing treatment of the seeds. Most of the priming methods are based on soaking the seeds in water or PEG solution with specific priming agents. Water content of the seeds is strictly controlled and kept at level below water level normally needed for the germination. On the contrary, this moisture of the seeds is sufficient to start initial physiological processes initiated with the first germination phase but keeps the seeds towards full germination. That means that the seeds undergo first and second phase of the germination but are prevented from the beginning of the third phase (growth). Instead of fully maturing, seeds are kept at lag

phase (II) until the removal of the priming agent and dried to the original water content (**Figure 3**) (Ashraf & Foolad, 2005; Sadeghi & Robati, 2015).

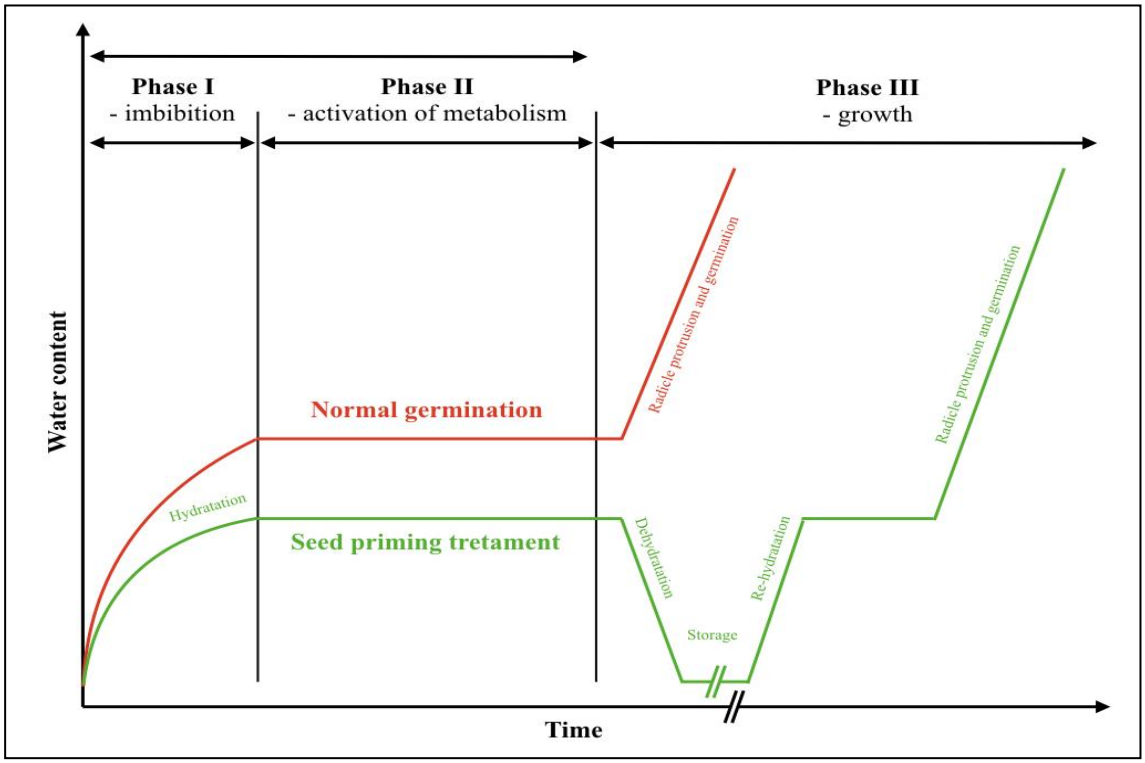

Figure 3: Seed hydration curves and germination phases in unprimed and primed seeds.

2.5 Priming methods

To reinforce the seeds and to alleviate different environmental stresses, several methods of seed priming such as (1) hydropriming, (2) osmopriming, (3) hormopriming, (4) biopriming or (5) chemical priming have been developed (Jisha *et al.*, 2013; Paparella *et al*, 2015). We have to take in account that even though the priming has many positive effects on seedling emergence, every priming method and priming agent has a different effectiveness among plant species and under different stress conditions.

The simplest method of seed priming is hydropriming in which seeds are soaked in sterilized distilled water and then dried again to the original water content. It is very important to keep dried seeds in appropriate conditions, including temperature and duration of hydropriming, during the germination. As long as this method does not use any other chemical compounds, this type of priming is very easy and cheap method of seed priming. The biggest problem of this method is that the seeds may have a different affinity to water and so unequal uptake of water may occur.

Another method of seed priming is osmopriming or also osmoconditioning in which seeds are soaked in osmotic solution with low water potential. This low osmotic potential causes slow water intake by seeds and so continuous seed imbibition and also activation of seed germination. There is a variety of compounds widely used as osmotic agents, for example glycerol, mannitol or some inorganic salts such as sodium chloride (NaCl) or potassium chloride (KCl), but the most widely used is polyethyleneglycol (PEG) because its large molecular size limit its penetration into seeds and so lowers the water potential.

In hormopriming, the presence of plant growth regulators and hormones causes seed imbibition and can also affect seed metabolism. The most often used growth regulators are abscisic acid (ABA), gibberellins, cytokinins (CKs), auxins, ethylene or polyamines.

Biopriming is also one of priming method in which seeds are infected with some pathogenic organism and at the same time hydrated and kept at warm and favorable conditions. This technique does not only increase seed imbibition and uniformity but also protects the seeds against soil pathogens and contribute to better plant health.

Regarding chemical priming, this method uses different chemical solutions, either synthetic or natural, as priming agents in which seeds are pre-soaked (Jisha *et al.*, 2013; Lutts *et al.*, 2016; Nawaz *et al.*, 2013; Paparella *et al.*, 2015).

In this diploma thesis, we evaluated hormopriming of *Arabidopsis* seeds with three different groups of cytokinin analogs.

2.6 Phytohormones

Since the plants are constantly being exposed to different types of stresses, during the decades they have developed complex and unique responses. These responses include many morphological, anatomical, physiological and biochemical changes (Sah *et al.*, 2016).

Phytohormones play the most important role in all these changes and the ability of plants to acclimatize to stress conditions. Although plant response to a different stresses depends on several other factors, plant hormones are probably the most crucial substances for plant survival. By definition, these hormones are small molecules, also known as plant growth regulators, derived from biosynthetic pathway with diverse structures that regulate all plant developmental stages including growth and death. Even though that they are usually produced in a very low concentrations, being signal molecules they are able to regulate many cell processes, such as germination, cell proliferation, differentiation and elongation, formation of organ and even responses to various stressors (Du *et al.*, 2012). Plant hormones serve as messengers of internal and also external stimuli and so coordinate many transduction signals. They can act locally (at their synthesis site) or can be transported to the other sites of the plant body (Fahad *et al*., 2015; Wani *et al.*, 2016).

Plant hormones are divided into various classes including cytokinins (CKs), auxins, abscisic acid (ABA), giberellins, ethylene, salicylic acid, jasmonates and brassinosteroids. Although each group of these hormones has their own specific biological effects on plant (e.g. ABA as stress related compound or CKs as cell division regulator), they crosstalk each other regulating almost all-biological processes, including plant stress response (Pan *et al.*, 2008; Pan & Wang, 2009).

These hormones are also being synthesized in a particular plant organs at specific developmental stages of the plants controlling many metabolic pathways (Egamberdieva *et al.*, 2017).

2.7 Analysis of plant hormones

As long as plant hormones as signal molecules are usually present only in a very small levels (usually in $pmol.g^{-1}$ of fresh weight), it is often difficult to analyze and identify them (Du *et al.*, 2012; Pan & Wang, 2009). Furthermore, except hormones, plants contain many other substances occurring in much bigger amounts which may interfere during the hormone analysis itself (Tarkowská *et al.*, 2014).

Recently, there have been several methodological approaches developed in order to extract, purify, identify and determine phytohormones in various plant samples (Pan & Wang, 2009).

2.7.1 Preparation, extraction and purification of sample

Collection of plants for sample preparation is one of the main steps before extraction and subsequent hormone analysis. Plant material needs to be firstly homogenized in order to disrupt the cell wall and release all intracellular substances into selected extraction solution (Tarkowská *et al.*, 2014).

During extraction, any kind of degradation (oxidative, enzymatic, light or heat induced degradation) must be avoided to obtain, ideally, sample containing specific substances with the highest possible purity and yield (Du *et al.*, 2012; Nordström *et al.*, 2004).

Among others, solid phase extraction (SPE) is one of the most commonly used methods for purification of various plant samples. By using SPE, analyzed substances are trapped in the column via polar, ion or hydrophobic interactions created between hormones and sorbent, while other interfering substances are washed away. After washing the SPE column with appropriate solvent, hormones are eluted and prepared for further analysis (Ljung *et al.*, 2010; Tarkowská *et al.*, 2014).

Moreover, one of the main problems in the quantification of hormones is the collection of enough plant material, which in many cases is a tedious work. In this regard, in the last years new technics are appearing to reduce these needs. StageTip purification (STop And Go Extraction Tip) is one example of the purification methods, which we used in this diploma thesis. StageTips are made out of standard pipette tips which are filled with specific sorbents and used for sample purification and analyte capture (Ishihama *et al.*, 2006; Svačinová *et al.*, 2012).

2.7.2 Methods of plant hormone determination

Nowadays, chromatographic methods are the most widely used methods for final quantitative and qualitative analysis of plant hormones. These methods are based on the interaction between stationary and mobile phase containing solutes. Depending on the type of a mobil phase, there are several types of chromatographic method, such as gas or liquid chromatography (Du *et al.*, 2012). Liquid chromatography (LC), High-performance liquid chromatography (HPLC) or Ultra-High-performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry (MS/MS) is the most commonly used method for hormone profiling (Novák *et al.*, 2014; Novák *et al.*, 2008). Mass spectrometry itself (MS) is a very sensitive analytical method for separation of molecules according to their mass to charge ratio (*m/z*). Prior to separation itself, sample is firstly loaded into the ion source via the inlet system, then ionized and so converted into gaseous state. Lastly, mass detector converts the ion beam into electric signal (Ljung *et al.*, 2010).

Moreover, MS/MS analysis improves specificity and selectivity of the analysis. The first MS analyzer removes all potentially interfering molecules and subsequently, the second analyzer detects the ions fragmented from the parent ion (Ljung *et al.*, 2010).

2.8 Plant hormones

2.8.1 Cytokinins

Cytokinins (CKs) are one of the most important plant hormones that are involved in regulation of many growth and development aspects, such as cell proliferation, differentiation, seed dormancy, germination, senescence and also in reaction to different biotic and abiotic stressors (Davies, 2010; Egamberdieva *et al.*, 2017; Hare *et al.*, 1997; Werner & Schmülling, 2009).

Naturally occurring CKs are N^6-substituted adenine derivates with aromatic or aliphatic side chains. Based on the side chain, there are two types of CKs - aromatic and isoprenoid CKs (**Figure 4A**). The most abundant CKs have isoprenoid side chain, commonly *trans*-zeatin. Zeatin occurs in both configurations, *cis*- and *trans*-. Unlike *trans*-zeatin which is highly biologically active, its *cis*- isomer has much lower activity. (Antoniadi *et al.*, 2015; Haberer & Kieber, 2002; Kakimoto, 2003; Kieber & Schaller, 2014).

Generally, plant tissues contain several types of CKs - free bases, nucleotides (with phosphate group attached to ribose), nucleosides (with attached ribose) and glycosides (*O*- or *N*-glycosides). However, all CK derivates differ in their biological and metabolic functions and their ability to travel across the plant body (Antoniadi *et al.*, 2015; Kieber & Schaller, 2014).

Figure 4B summarize key biosynthetic pathways of CKs. The first step of isoprenoid CK synthesis starts after the transfer of isoprenoid chain to adenine from its donor, either dimethylallyl pyrophosphate (DMPP) or (E)-4-hydroxy- 3-methyl-but-2-enyl diphosphate (HMBDP). By this way, the major intermediate of CK biosynthesis, iP nucleotide, is being made. In *Arabidopsis*, cytochrome P450 monooxygenases hydrolyze isopentenyladenin and converts it into *t*Z nucleotides. Furthermore, enzyme family LONELY GUY (LOG) transforms iP and *t*Z nucleotides into its biologically active forms firstly by dephosphorylation and ten deribosylation (Bielach *et al.*, 2017; Frébort *et al.*, 2011; Hirose *et al.*, 2008).

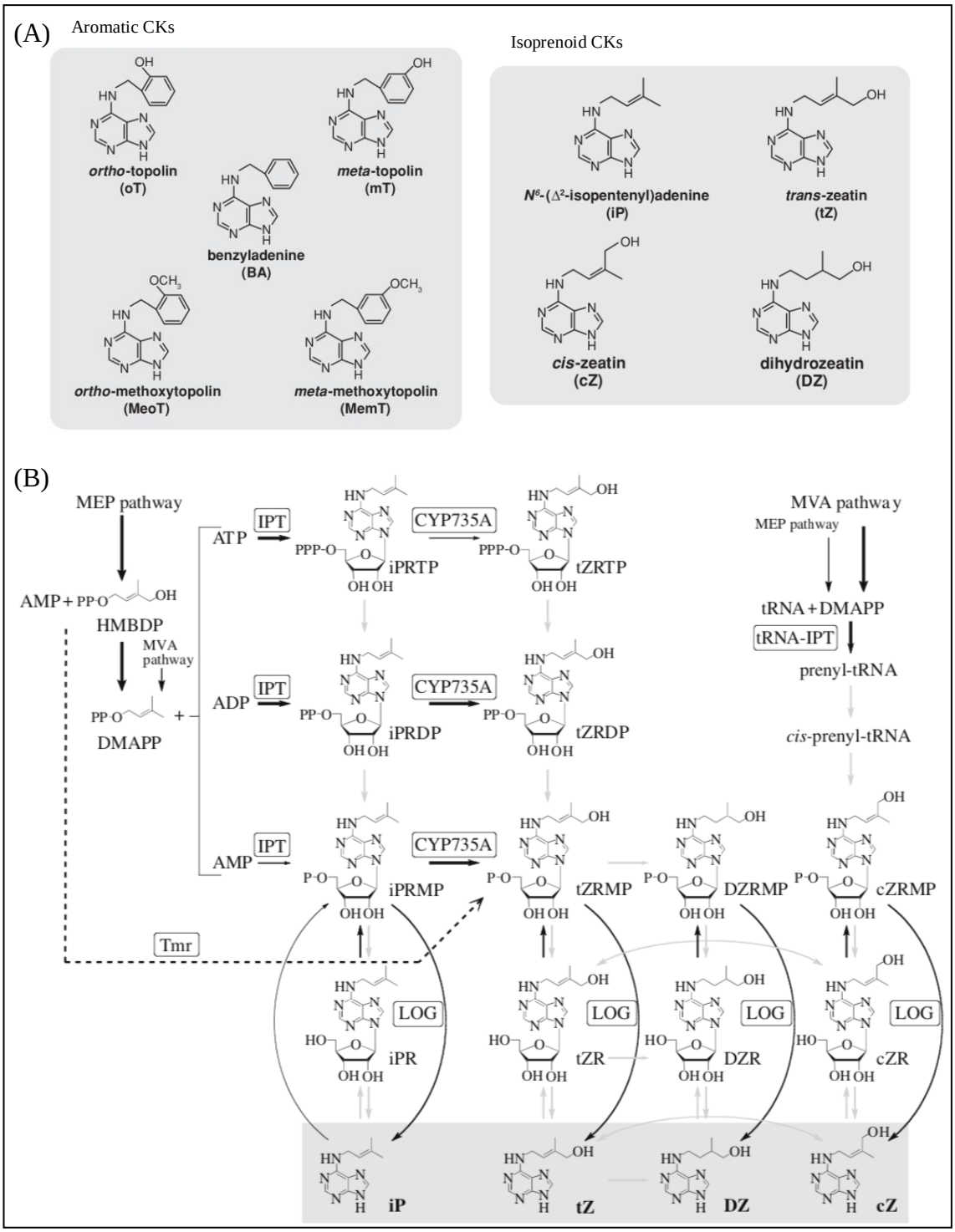

Figure 4: (A) Types of cytokinins based on their side chain - aromatic and isoprenoid cytokinins (Sakakibara, 2006); **(B)** Two pathways of cytokinin biosynthesis (Kamada-Nobusada & Sakakibara, 2009)

2.8.2 Auxins

Likewise CKs, auxins are also one of the important groups of phytohormones that are essential in developmental and growth processes of the plants (Kazan, 2013). Even though that this group of hormones have been firstly mentioned almost 100 years ago, their biosynthetic, transport and signaling mechanisms have not been clearly described (Wani *et al.*, 2016).

The most commonly and naturally occurring auxin in plants is indole-3-acetic acid (IAA). It is produced mainly in meristematic tissues (roots, cotyledons and leaves) and so involved in growth and developmental processes such as cell expansion and differentiation, root initiation, etc (Davies *et al.*, 2005; Egamberdieva *et al.*, 2017; Ljung *et al.*, 2010).

Speaking of the biosynthesis of auxins, there are two ways: *de novo* synthesis or synthesis from auxin conjugates. There are likely to by several pathways of *de novo* biosynthetic pathways of auxins, two of which are tryptophan-dependent and tryptophan-independent pathway. Unfortunately, tryptophan-independent pathway is not well described but tryptophan-dependent pathway starts from the essential amino acid tryptophan (Trp) (Mano & Nemoto, 2012; Zhao, 2014). In Trp-dependent biosynthetic pathway, Trp is converted to IAA through two steps and one intermediate. In the first reaction, Trp is transaminated (removal of the amino group) to indole-3-pyruvate (IPA) and then finally converted to IAA by oxidative decarboxylation (**Figure 5**) (Popko *et al.*, 2010; Zhao, 2014).

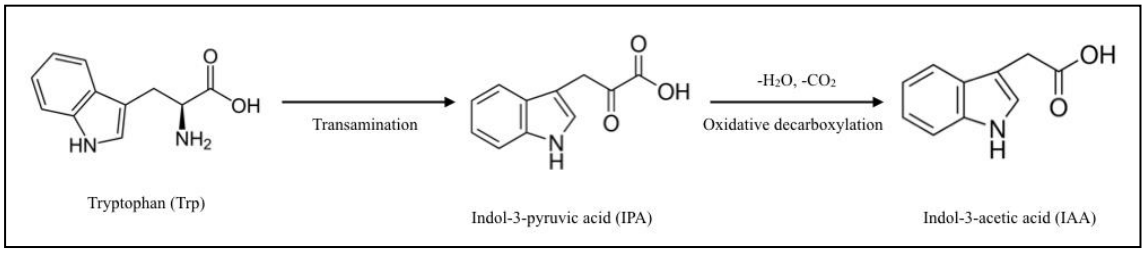

Figure 5: Scheme of tryptophan-dependent pathway of IAA synthesis.

2.8.3 Abscisic acid (ABA)

As many other plant hormones, ABA is a phytohormone involved in the process of embryogenesis, growth and development of a plant body. What is more important, ABA is a key regulator of physiological responses to many environmental stimuli, such as pathogens, drought or salinity (Davies *et al.*, 2005; Finkelstein, 2013; Seo & Koshiba, 2002).

ABA is a 15-C acid (sesquiterpenoid) which is composed of one aromatic system with -*cis*,-*trans* side chain. This active form of S-(+)-ABA is occurring mostly naturally (Zaharia *et al.*, 2005). On the other side, -*trans*,-*trans* form of ABA is biologically inactive (Finkelstein, 2013). There are two possible ways of ABA synthesis: direct and indirect. As indicated in **Figure 6**, the indirect, or also non-mevalonate pathway of ABA synthesis, starts from 5-C compound isopentenyl pyrophosphate (IPP). Through several intermediates (farnesyl pyrophosphate - FPP, geranylgeranyl pyrophosphate - GGPP), IPP is converted to 40-C phytoen and to carotenoids (lycopene, β-carotene) followed by the conversion to zeaxanthin, violaxanthyn, xanthoxin and finally to ABA (Popko *et al.*, 2010; Seo & Koshiba, 2002).

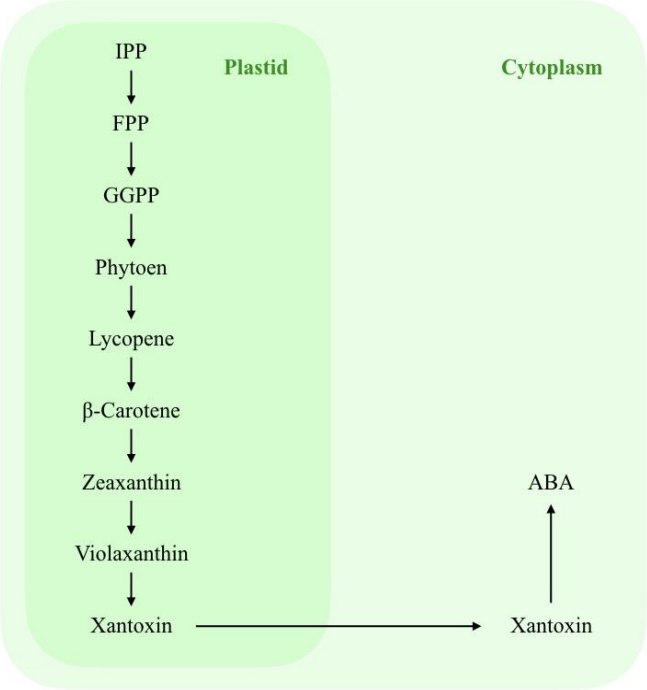

Figure 6: Diagram of ABA biosynthetic pathways in a plant cell.

2.8.4 Alternations in plant hormone levels under abiotic stress

Plants have adopted many various defense mechanisms in order to resist environmental changes. Despite the fact that different plants have different ways of protection from negative stressors, there are many basic patterns common for all species. One of the most important adaptation response of plants is the regulation using secondary messengers including several phytohormones (Shi *et al.*, 2014).

ABA is one of the major plant hormones considered as an important messenger in response to abiotic stresses, mainly in adaptation to drought, salinity and high temperature. It is mostly synthesized and accumulated as a reaction to these stresses in leaves and roots from where it is transported through xylem to the places where needed. As a first response to stress, ABA travels into leaves and closes stomata in order to prevent loss of water and reduce transpiration. Reduction in leaf size also occurs. This plant hormone has also an important role in sufficient and effective uptake and further transport of water, minerals and nutrients. Among others, ABA is also indispensable in control of seed germination and plant development (Popko *et al.*, 2010; Shi *et al.*, 2014; Siddique *et al.*, 2016; Wani *et al.*, 2016).

Similarly, auxins are known to be a significant controller of various plant processes such as cell differentiation, tropism of a plant or plant development. It is well-established that high levels of auxins are associated to a high growth rate. Due to this fact, down-regulation of a plant growth together with lower levels of auxins serves as a response to a stress conditions (Popko *et al.*, 2010; Shi *et al.*, 2014).

Auxins and ABA both interact in plant defense mechanisms that are necessary for plant's adjustment to all negative environmental effects. It can be assumed that there is a potential interaction between these two but also other plant hormones (Shi *et al.*, 2014).

Considering CKs, they are commonly considered as ABA antagonists (Hare *et al.*, 1997; Kaya *et al.*, 2006; Nishiyama *et al.*, 2011). Compared with ABA, CKs prevent plants from leaf senescence, stomatal closure and may also delay seed germination. Under water stress, synthesis of CKs in roots and their transport to shoots and leaves is typically decreased. However, increased CK levels may support transpiration via opening the stomata (Ha *et al.*, 2012; Nishiyama *et al.*, 2011; Pospisilova, 2002).

2.9 Plant phenotyping techniques

Better understanding of characteristics (phenotype) and genetic composition (genotype) of plants is necessary in order to improve productivity of agriculturally important crops, to ensure increased tolerance to various stressors, diseases and changing environmental conditions (Yang *et al.*, 2017).

During last decades, analytical methodologies of plant genotype, such as DNA sequencing, has been widely used (Yang *et al.*, 2017).Unfortunately, identification of plant traits related to development, growth, architecture, resistance and tolerance to abiotic and biotic stress factors needs detailed phenotypical analyses (Faragó *et al.*, 2018). Such analyses require simultaneous monitoring of multiple traits of hundreds or even thousands of plants. Typically, standard phenotyping analyses are based on a visual evaluation of different quantitative and qualitative characteristics, such as measurement of size and shape of the plant or its photosynthetic capacity. However, these techniques are extremely tedious and laborious and impossible to perform in large-scale experiments (Berger *et al.*, 2012; Faragó *et al.*, 2018; Granier *et al.*, 2006). Moreover, because of destroying the plant material, the use of such methods is often a final step of the experimental setup (De Diego *et al.*, 2017; Faragó *et al.*, 2018; Rahaman *et al.*, 2015).

For this reason, another, non-destructive, plant phenotyping methods which offer easier, faster, reliable, high-throughput and automated analysis have been developed (Rodriguez-Furlán *et al.*, 2016; Zhao *et al.*, 2019). These methods are based mainly on simultaneous measurement of various parameters (physiological and morphological) of a large number of plant samples cultured *in vitro* in green houses or growth chambers under controlled environmental conditions (humidity, temperature, light intensity, etc.) (De Diego *et al.*, 2017; Yang *et al.*, 2017). The analysis of growth parameters is done by imaging of an individual plant samples in regular intervals. Subsequently, all data are being automatically recorded and stored in a database (Ugena *et al.*, 2018).

3 List of chemicals and devices

3.1 Plant material

- *Arabidopsis thaliana* ecotype Columbia (Col-0)

3.2 Chemicals

- acetic acid (0.1% AcA), Merck KGaA
- acetone, Merck KGaA
- ammonia solution (25% NH_3), Suprapur
- deuterium labeled internal standards:

  $^{13}C_6$-IAA, $^{13}C_6$-oxIAA, $^{13}C_6$-IAAsp, $^{13}C_6$-IAGlu, D_6-ABA, $^{13}C_5$-*t*Z, D_5-*t*ZR, D_5-*t*Z7G, D_5-*t*Z9G, D_5-*t*ZOG, D_5-*t*ZROG, D_5-*t*ZRMP, $^{13}C_5$-*c*Z, D3-DHZ, D_3-DHZR, D_3-DHZ9G, D_9-DHZOG, D_3-DHZRMP, D_6-iP, D_6-iPR, D_6 iP7G, D_6-iP9G, D_6-iPRMP
- diethyldithiocarbamic acid (DEDTCA; $C_5H_{10}NS_2Na$), Sigma
- ethanol (96% EtOH; H_5C_2OH), Lach-Ner s.r.o.
- formic acid (98% HCOOH), Honeywell Fluka
- chloric acid (HCl), Lach-Ner s.r.o.
- liquid nitrogen – N_2
- mannitol, Merck KGaA
- methanol (100% MeOH), Merck KGaA
- methanol, HiPerSolv CHROMANORM® gradient for HPLC – H_3COH, VWR CHEMICALS
- MES – low moisture content, Sigma Aldrich
- milli-Q water, Direct-Q, Merck KGaA
- Murashige & Skoog (MS -1962) including vitamins, Duchefa Biochemie
- nitric acid (65% HNO_3), Lach-Ner s.r.o.
- phytagel, Sigma Aldrich
- potassium hydroxide (1M KOH), Lach-Ner s.r.o.
- sodium chloride (NaCl), Sigma Aldrich
- triton X-100 (0.01%), Sigma Aldrich

3.3 Culture media

1x MS medium:

MS (4.4 $g.l^{-1}$), MES (0.5 $g.l^{-1}$), KOH (1M) – pH 5.7, phytagel (6 $g.l^{-1}$)

0.5x MS medium:

MS (2.2 $g.l^{-1}$), MES (0.5 $g.l^{-1}$), KOH (1M) – pH 5.7, phytagel (6 $g.l^{-1}$)

3.4 Solutions

Sterilization solution:

70% EtOH with 0.01% Triton X-100

Bieleski buffer (BS):

187.5 ml MeOH, 12.5 ml HCOOH, 50 ml Milli-Q water

Phosphate Buffer Solution (PBS), pH 7.00:

50 mM PBS, 0.1% DEDTCA

Ammonium hydroxide - NH_4OH (0.35M):

6.25 ml 25% NH_3OH, 243.75 ml Milli-Q water

Ammonium hydroxide - (NH_4OH 0.35M) in 60% MeOH:

6.25 ml 25% NH_3OH, 93.75 ml Milli-Q, 150 ml MeOH

Nitric acid – HNO_3 (50%):

76.9 ml 65% HNO_3, 23.1 ml Milli-Q

1 M HCOOH:

9.625 ml 98% HCOOH, 240.375 ml Milli-Q water

0.1% Acetic acid (AcA):

30 µl 100% AcA, 29.7 ml Milli-Q water

3.5 Devices

- 48 Multi-well plates, Jetbiofil, Guangzhou, China
- analytical balance XA110/2X, RADWAG
- autoclave STERIVAP HP IL, BMT Medical technology s.r.o.
- automatic pipette, Eppendorf Research Plus
- Benchtop Vaccum Concentrators, Labconco
- ceramic beats (ZrO_2)
- column for LC/MS - Kinetex C18 (1.7 μm) EVO, Phenomenex
- FluorCam, Photon Systems Instruments, Czech Republic
- grow chamber CMP6010, CONVIRON ADAPTIS
- High Speed Compact Centrifuge Beckman AvantiR 30, Beckman Coulter, Inc
- incubator KBF 240, BINDER
- laminar flow cabinet, MERCI s.r.o.
- Liquid chromatography - 1260 Infinity II LC System coupled with Mass spectrometer 6495C Triple Quadrupole, Agilent Technologies
- magnetic mixer, LAVAT
- mixer mill Retsch MM400, Retsch GmbH
- nanosep MF centrifugal devices with Bio-Inert® membrane, Pall Corporation
- parafilm M, Sigma Aldrich
- pH meter ORION STAR A111, ThermoScientific
- phenotyping platform Olophen (FYTOSCOPE FS-WI-XYZ), PlantScreen™ PSI
- rotator Stuart SB3, Stuart-Equipment
- SPE columns - MCX (300 mg/1 ml), OASIS®, Waters Corporation
- square Petri dish (12 x 12 cm), P-lab a.s.
- ultrasonic bath TRANSSONIC T310, Elma®
- vials with inserts, Sigma Aldrich
- Visiprep Spe Vacuum manifold SUPELCO, Sigma Aldrich
- vortex mixer WIZARD IR, VELP® SCIENTIFICA
- vortexer HEATHROW SCIENTIFIC, P-lab a.s.

4 Methods

Figure 7 schematize a general overview of the experimental protocol. This protocol shows several steps ranging from seed sterilization, seed priming and sowing, germination and further transfer of young seedlings into multi-well plates, RGB imaging up to the final data processing and analysis. This protocol is based on De Diego *et al.*, 2017 and was modified for the purposes of the experiments included in this diploma thesis.

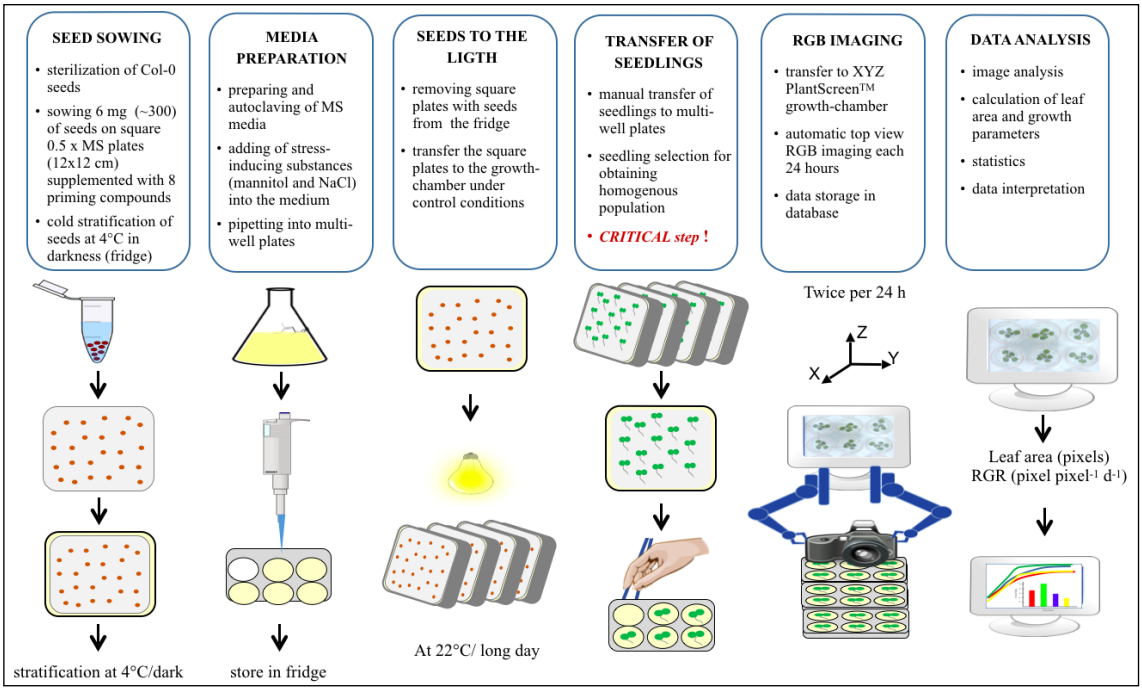

Figure 7: General overview of high-throughput phenotyping protocol optimized for screening of *Arabidopsis* rosette growth (De Diego *et al.*, 2017).

4.1 Plant growth conditions

Seeds of *A. thaliana* ecotype Col-0 were surface-sterilized by soaking in 200 µl of 70% ethanol with 0.01% Triton X-100 for 10 min. Afterwards, seeds were washed three times in sterilized water and sown on a square Petri dish (12 x 12 cm) fulfilled with 0.5x Murashige-Skoog (MS) medium (2.2 g of MS, 0.5 g of MES to keep the pH at 5.7, 0.6% of gelling agent phytagel; pH 5.7 was adjusted using KOH). In the case of primed variants, the growth medium was complemented with eight compounds from three groups of CK analogs as listed below (**Table 1**).

The seeds were kept for 4 days at 4°C in dark to synchronize the germination. Thereafter, plates were transferred into a growth-chamber with controlled growth conditions (22°C, 60% humidity, photoperiod 16/8h, photon irradiance - 110 µmol photons of PAR m^{-2} s^{-1}) for another 3 days and kept at vertical position.

After three days of germination, seedlings of a similar size were transferred under sterile conditions into multiple well plates (with 48 wells). One seedling was transferred into each well filled with 850 µl of 1x MS medium (4.4 g MS, 0.5 g MES, 0.6% gelling agent phytagel; pH 5.7) supplemented with 100 mM NaCl for induction of salt stress or 100 mM mannitol for osmotic stress or drought. Subsequently, plates were sealed with a perforated transparent foil avoiding condensation and allowing gas and water exchange.

Group 1	Compound 1	2-F-2-deOBAPA
	Compound 2	3-OH-2-F-2-deOBAPA
	Compound 3	3-MeO-2-F-2-deOBAPA
	Compound 4	iP-2-F-2-deOBAPA
Group 2	Compound 5	3-MeOBAPA
	Compound 6	3-OHBAPA
Group 3	Compound 7	3-MeO-2-dPR
	Compound 8	3-OH-2-dPR

Table 1: Three tested groups of CK analogs.

4.2 Seed priming

To test the effect of hormopriming, after the sterilization, the seeds of *Arabidopsis thaliana* were sown on 12 cm x 12 cm square plates containing 0.5x MS medium (pH 5.7) with the addition of eight CK analogs from three different groups of compounds in four concentrations (0.1, 1, 10 and 100 μM) dissolved in 0.5% DMSO, or without compound addition with only 0.5% DMSO (MOCK), in which seeds go through stratification and germinate.

The first group was composed by four compounds (1-4) formed by different N^6-substituted-2′-deoxy-2′-fluoro-9-(*β*)-D-arabinofuranosylpurine. The second group of compounds (5 and 6) was formed by two D-arabinofuranosylpurine derivated compounds, and the third group consists of two ribofuranosylpurin derivates (**Table 1**).

Furthermore, three days old seedlings were transferred into 48-well plates filled with 1x MS medium without or with the addition of salt (100 mM NaCl) or mannitol (100 mM) as treatments. Two plates per growth condition, compound and concentration were used as replicates for the control (in total 96 seedlings). In case of the tested compounds, one plate (48 seedlings) per growth condition, compound and concentration were used for 100 mM NaCl and 100 mM mannitol.

4.3 High-throughput phenotyping analysis

The 48-well plates with the transferred *Arabidopsis* seedlings were placed to the OloPhen platform with 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, Czech Republic) (De Diego *et al.* 2017). The growth conditions were set to simulate a long day (22ºC/20ºC in a 16/8 h light/dark cycle, an irradiance of 120 µmol photons of PAR $m^{-2}s^{-1}$ and 60% of a relative humidity).

This PlantScreen system is composed of a robotically driven arm with RGB camera. This RGB arm was automatically driven over every single multi-well plate in order to make RGB image (**Figure 8**). RGB images with the resolution 2,500 x 2,000 pixels and with a file size of approximately 10 MB (in the PNG compression format) were stored in a database, having a filename carrying a specific information about the acquisition time and the coordinates (x, y) of the camera. All data were subsequently stored in PlantScreen XYZ database and analyzed using a MatLab R2015 software (Ugena *et al.*, 2018).

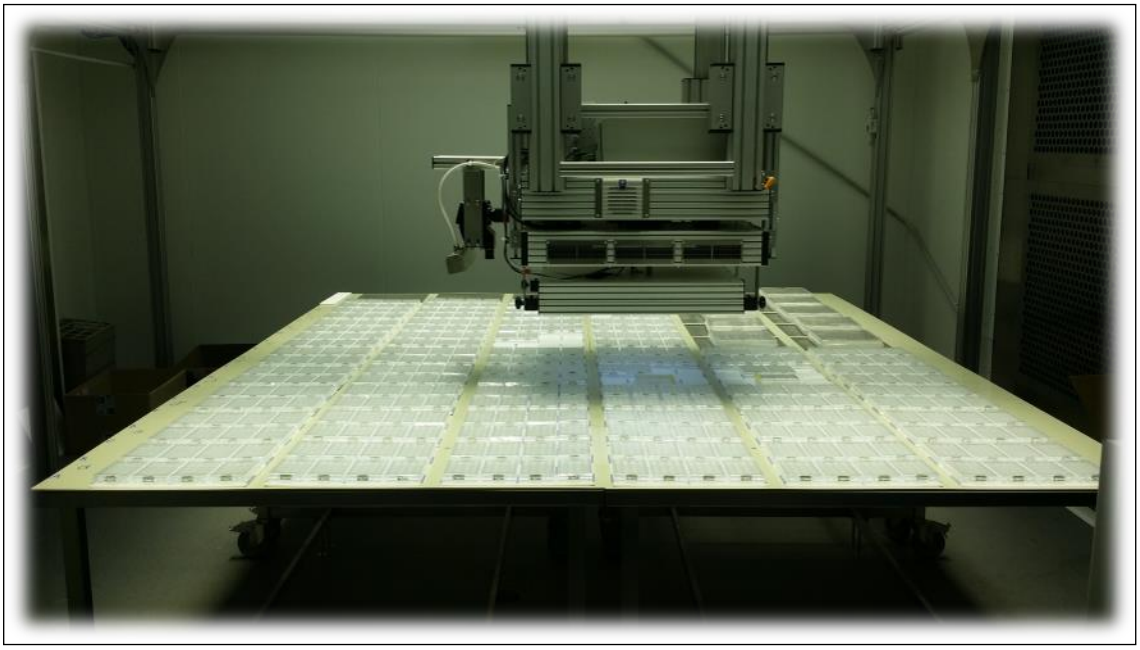

Figure 8: OloPhen platform with PlantScreen^{TM} XYZ system installed in a growth chamber with a controlled environment and with robotic arm with RGB camera driven over the multi-well plates with *Arabidpsis thaliana* plants (Hybenová, 2018).

4.4 Analysis of phytohormones

The aerial part of the *Arabidopsis* plants from each hormopriming and growth condition were collected for further analysis of the endogenous levels of CKs, auxins and ABA, using the established protocols by the department of Plant Growth Regulators (LRR) of Palacký University described below.

4.4.1 Preparation of samples, extraction and purification for LC-MS/MS analysis of CKs

All samples were grinded using liquid nitrogen and subsequently divided into Eppendorf tubes containing 5 mg fresh weight (FW) of plant material (*Arabidopsis* rosettes). Four different pools of plants (with 24 plants each) from each hormopriming and growth condition were used as biological replicates. Into each sample, three ZrO_2 beads with a diameter of 2 mm, 15 µl of a mixture of deuterium labelled-internal standards (IS) of CKs and 0.5 ml of extraction solution (Bieleski buffer) were added. Thereafter, the content of the Eppendorf tubes was homogenized for 5 min using a MM 400 ball mill and frequency of 27 Hz. Prepared and homogenized samples were further sonicated for 3 minutes in ultrasonic bath and in the next step, they were left for 30 min on a laboratory rotator operating at 4°C and afterwards centrifuged at 15,000 *g* at 4°C for 15 min. After this procedure, all the supernatants were collected and placed into the new Eppendorf tubes.

The procedure of CK purification consists of the following steps. First of all, MCX columns (30 mg sorbent / 1 ml cartridge volume) were conditioned and then used for the purification of the supernatants obtained from the homogenized plant material as follows:

1. Activation of sorbent: 1 ml of 100% MeOH
   1 ml Milli-Q H_2O
   1 ml 50% HNO_3
   1 ml Milli-Q H_2O
   1 ml 1 M HCOOH
2. Application of supernatant: 3 ml sample
3. Washing: 1 ml 1 M HCOOH
   1 ml Milli-Q H_2O

4. Elution (into borosilicate tubes): 1 ml 0.35 M NH_4OH
   1 ml 0.35 M NH_4OH in 60% MeOH

Finally, the purified extracts were evaporated to dryness using a vacuum concentrator. The resulting sample residue was reconstructed in a volume of 40 µl of 5% MeOH, filtered through a centrifuge Micro Spin Filter (0.2 µm) for 5 min at 7,500 *g* and transferred into a conical vial.

4.4.2 Preparation of samples, extraction and purification for quantitative analysis of auxins and ABA

Similarly, as in the protocol of CKs, 5 mg of a pool with *Arabidopsis* rosettes was used for the analysis of auxins and ABA. For the procedure of extraction, 1 ml of Phosphate Buffer Solution (PBS) (0.05 M), 10 µl of a mixture of deuterium labelled-internal standards (IS) of auxins and ABA and three ZrO_2 beads (2 mm in diameter) were added in an Epperdorf tube together with the sample. Then, samples were homogenized for 10 min using a MM 400 ball mill at a frequency of 27 Hz. Afterwards, homogenized samples were left for 10 min on a laboratory rotator operating at 4°C for further extraction and lastly they were centrifuged at 20,000 *g* and at 4°C for 15 min. Supernatants were subsequently collected, replaced into new Eppendorf tubes and the pH was adjusted to value 2.7 by adding 140 µl of 0.1 M HCl.

The process of purification of auxins and ABA was performed by stage tips prepared from the SDB-XC and C18 Solid Phase Extraction disks as follows:

1. Activation of sorbent: 50 µl of acetone
   50 µl of 100% MeOH
   50 µl of Milli-Q H_2O
2. Application of supernatant: 200 µl of sample extract
3. Wash: 50 µl of 0.1% AcA
4. Elution: 50 µl of 80% MeOH (into a clean Eppendorf tubes)

Purified tissue extracts were then transferred into a conical vials, evaporated to dryness by means of a vacuum concentrator, and the resulting residue was reconstructed using 30 µl of 10% MeOH.

After the final preparation of conical vials of all samples, individual residues were injected onto the liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS) system in order to perform the quantitative analysis of CK, auxin and ABA levels.

4.5 Leaf color determination and other biometrical parameters

The green area (Pixels) of each plant in multi-well plates were measured twice per day (at 10 a.m. and at 4 p.m.) during seven days of experiment. On the last day, the images of *Arabidopsis* rosettes were used for the segmentation and for further extraction of values corresponding to specific color channels (R - red, G - green, B - blue) in order to determine changes in leaf color. Afterwards, green leaf index (GLI) was calculated as follows:

$$GLI = \frac{(2G-R-B)}{(2G+R+B)}$$

Using the aforementioned high-throughput phenotyping automatic system, the changes in green area (Pixels) were recorded and subsequently used for the determination of the growth curve and the estimation of the relative growth rate (RGR) and absolute relative growth (ARG) per day for each hormopriming and growth condition.

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

$$ARG = (\text{green area})_{ti} – (\text{green area})_{ti-1} / ti - ti\text{-}1$$

Where t*i* means initial (*i*) time (days).

4.6 Statistical analysis

The growth curves and their trendlines were performed in EXCEL 2010. The parallel plot and the correlations between phenotypical traits and the metabolites, their equations and Pearson's R square were also carried out in the same program.

5 Results

5.1 Effect of seed pre-sowing treatment on early seedling establishment

Seeds of *Arabidopsis thaliana* were cultured on 0.5x MS medium supplemented with three different groups of CK analogs (in total 8 compounds) (**Table 1**) in four concentrations (0.1, 1, 10 and 100 μM). After the germination, three days old seedlings were transferred to 48-well plates. The green area of *Arabidopsis* seedings was analyzed immediately after the transfer to these plates (referred as time zero) using HTS method as a suitable assay for analysis of a rosette growth (green area).

Figure 9 summarize early seedling establishment of *Arabidopsis* seeds after the priming with above mentioned compounds.

Regarding the first group of compounds, the best results of priming were achieved with the use of compound 4. In this case, the lowest concentrations of the compound 4, specifically 0.1 and 1 μM, caused a significant increase in the green area of the *Arabidopsis* seedlings (1.3 and 1.35-fold relative to control). On the other hand, the higher concentrations of compound 4 had a strong negative effect leading to a severe decrease of a rosette growth and eventually may also lead to death of the seedlings. Similarly, very satisfactory results occurred after the use of compound 2 and also compound 3. These compounds promoted rosette growth with 1.3 to 1.36-fold increase in the green area.

Considering second group of compounds, the results obtained after seed priming with compound 5 and 6 are comparable. In both cases, the entire range of concentrations caused an increase in rosette size, from 1.25 to 1.54-fold, compared to those non-primed seeds.

From the last group of compounds, the most significant results were observed with the compound number 8. While concentrations 1, 10 and 100 μM caused only a small change in seed size, 1.58-fold increase in the green area of seeds primed with this compound was achieved with its lowest concentration corresponding to 0.1 μM.

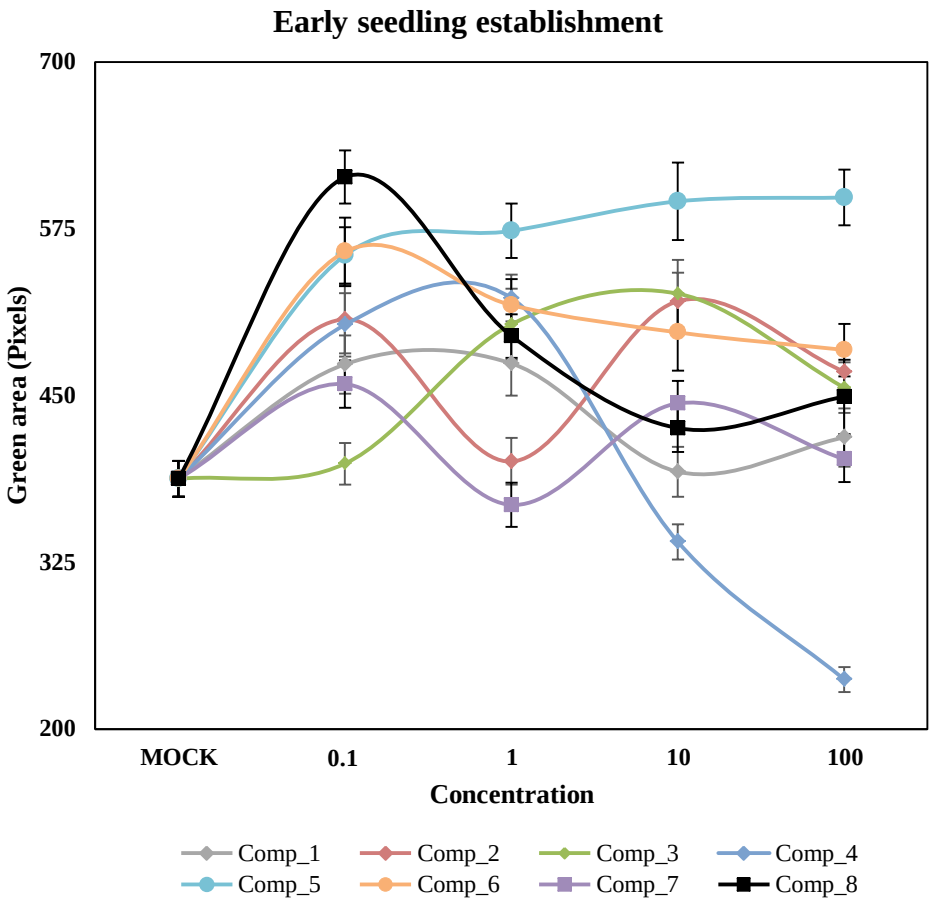

Figure 9: The effect of seed hormopriming on early seedling establishment of *Arabidopsis* plants. Green area (Pixels) of 3 DAG seedlings primed with compounds 1-8 in concentrations 0.1, 1, 10, 100 μM and grown in control conditions.

5.2 Rosette growth of primed and unprimed *Arabidopsis* seeds under control and stress conditions

After the transfer to 48-well plates, *Arabidopsis* seedlings were cultured in 1x MS medium with or without the presence of stress factor. Sodium chloride (100 mM NaCl) was used to invoke salt stress and mannitol (100 mM) was used as osmotic stressor.

As indicated in **Figure 10**, seed priming with all eight compounds resulted in differences in the rosette growth. After the comparison of the data obtained for first group of compounds, compound 1, 2 and 3 showed a similar rate of rosette growth in all culture conditions (control, salt and osmotic stress). The most remarkable results were obtained after priming with compound 4. In case of control and osmotic conditions, the highest increase in growth of *Arabidopsis* plants was achieved with the concentration 1 μM (6-fold and 2.9-fold increase relative to first day of treatment).

While other concentrations of this compound showed similar exponential growth of rosettes, concentration 100 μM had an inhibitory effect in all treatment conditions. Under salt conditions induced by 100 mM NaCl, the lowest concentration of compound 4 (0.1 μM) caused the highest increase in growth, specifically 2.6-fold compared to first day.

In case of compound 5 and 6, all concentrations of these compounds in control and both stress conditions showed almost the same growth profile. After 7 days of cultivation in control conditions, green area of all *Arabidopsis* plants primed with compound 5 increased by five to six-fold, whereas compound 6 exhibited 3.7 to 4.6-fold increase compared to the first day of treatment. In salt and osmotic stress conditions, rosette size of the plants increased two to three-fold, respectively.

Concerning the compound 7, data presented in **Figure 10** show that in all conditions the use of this compound resulted in the maintenance of exponential growth of the plants during all six days of treatment.

The most significant results after priming with compound 8 were observed in salt and osmotic conditions with low concentrations of this compound (0.1 and 1 μM). The size of *Arabidopsis* plants increased in this case by 2.3-fold (under salt stress) and by 3-fold (under osmotic stress).

As **Figure 11** indicate, more efficient Relative Growth Rate (RGR) per day was maintained in plants primed with all 8 CK analogs and cultured under stress conditions induced by 100 mM NaCl and 100 mM Mannitol.

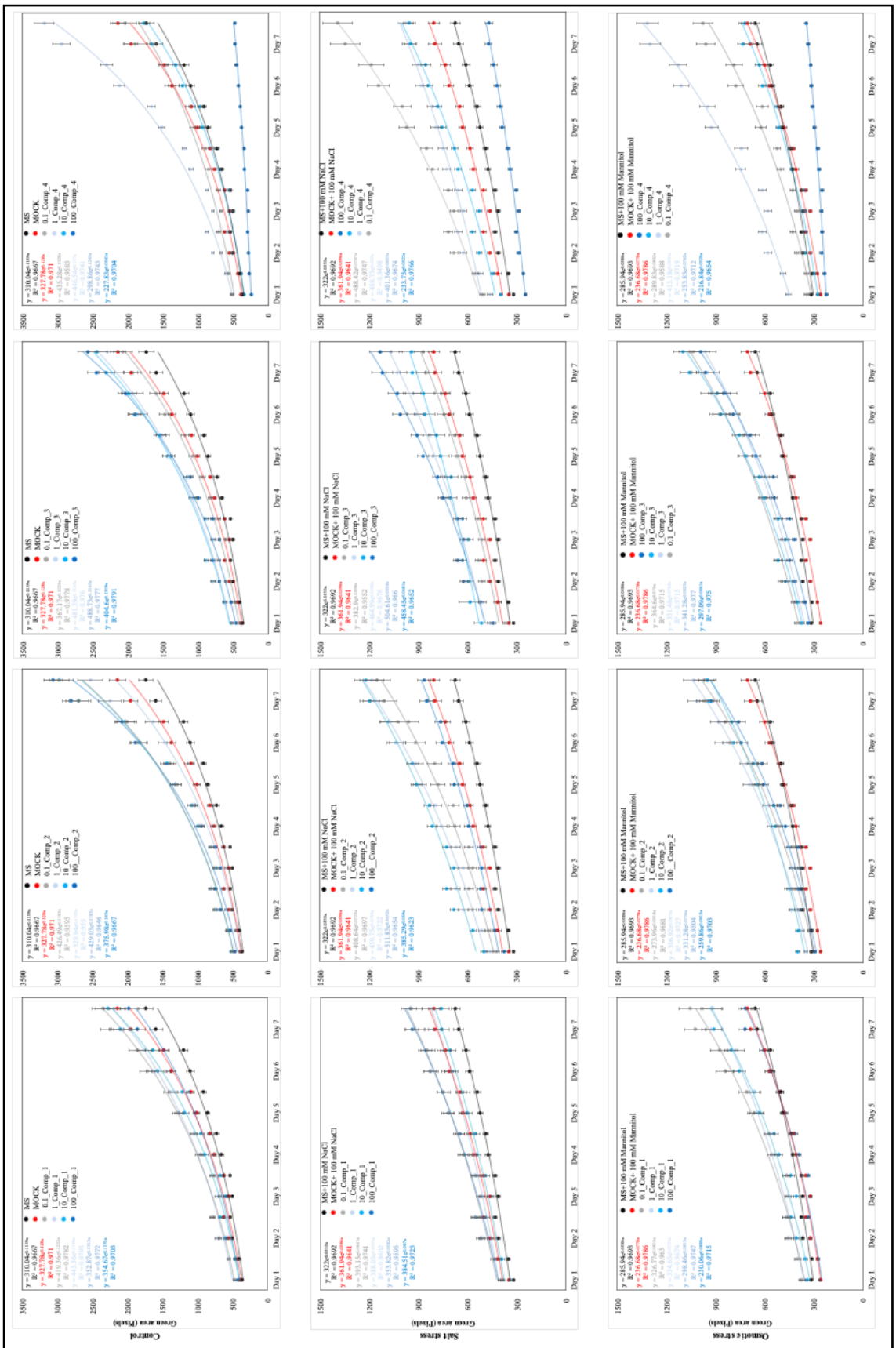

Figure 10: Growth of *Arabidopsis* rosettes after seed priming with 8 different compounds in control and stress conditions. Green area (Pixels) of 3 DAG seedlings primed with these compounds in concentrations 0.1, 1, 10, 100 µM and grown in 48-well plates under control, salt (100 mM NaCl) and osmotic (100 mM mannitol) stress conditions.

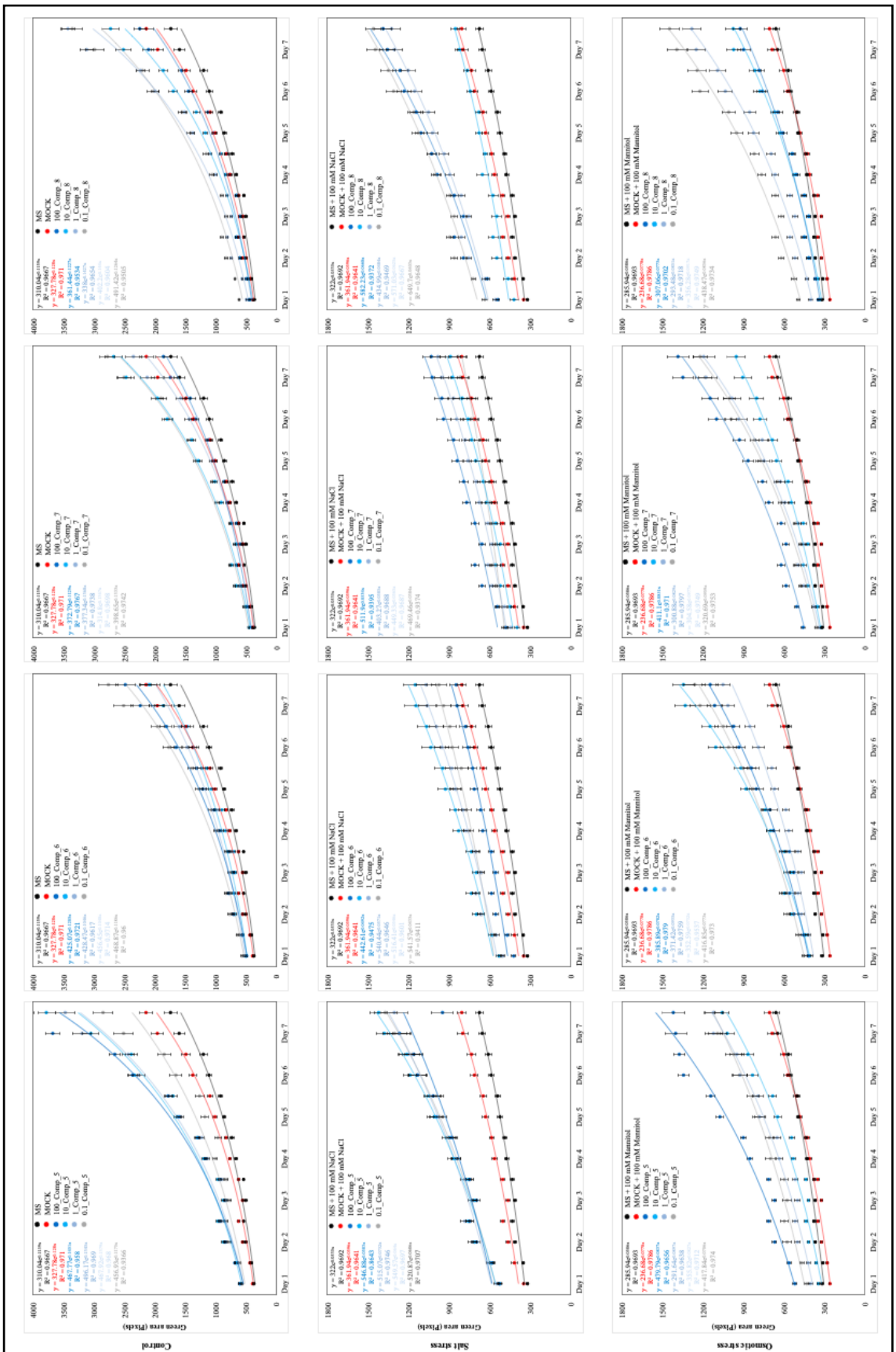

Figure 10: (Continued).

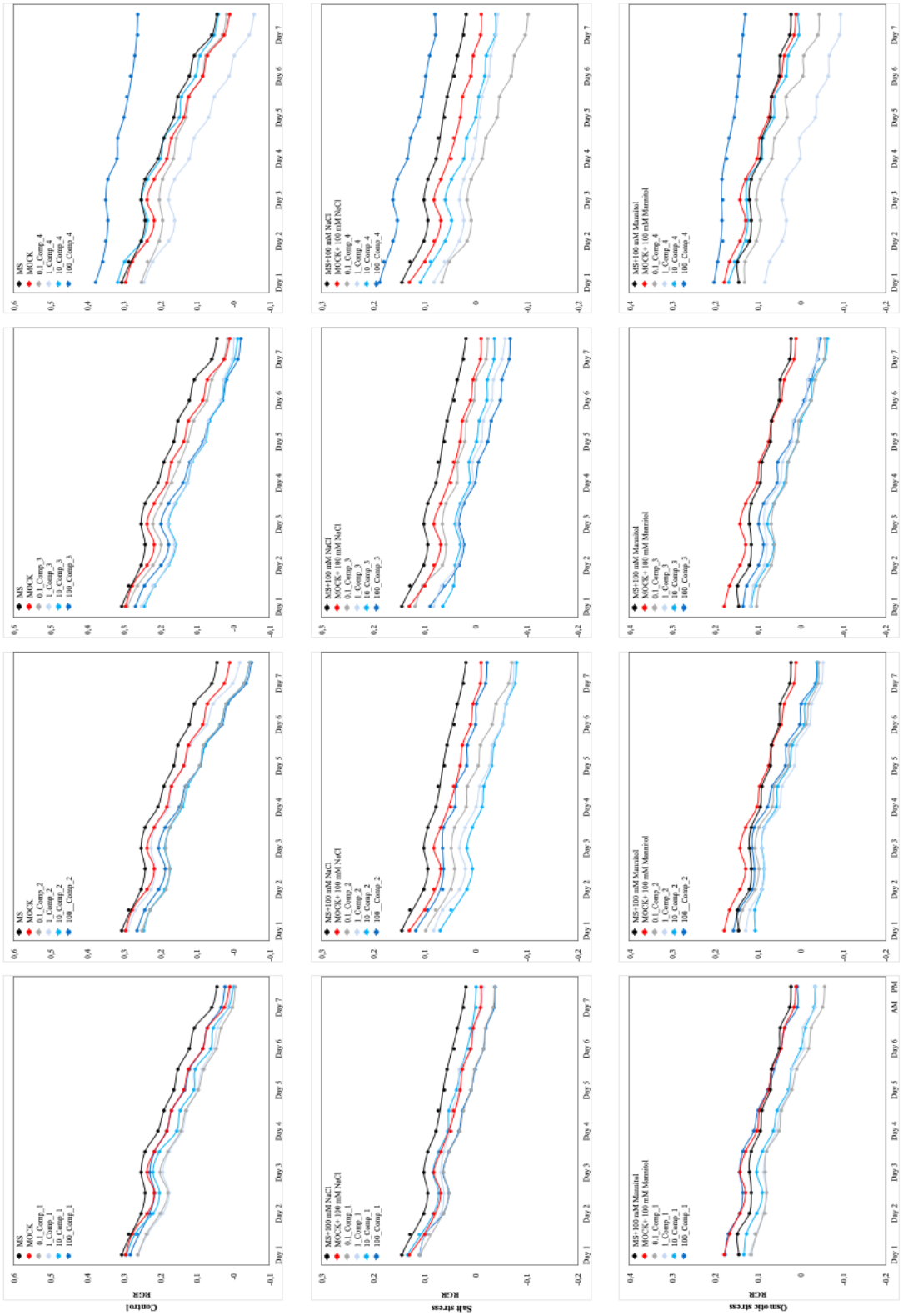

Figure 11: Relative growth ratio (RGR) of *Arabidopsis* seedlings after seed priming with 8 different CK analogs cultured in control and stress conditions (100 mM NaCl and 100 mM Mannitol).

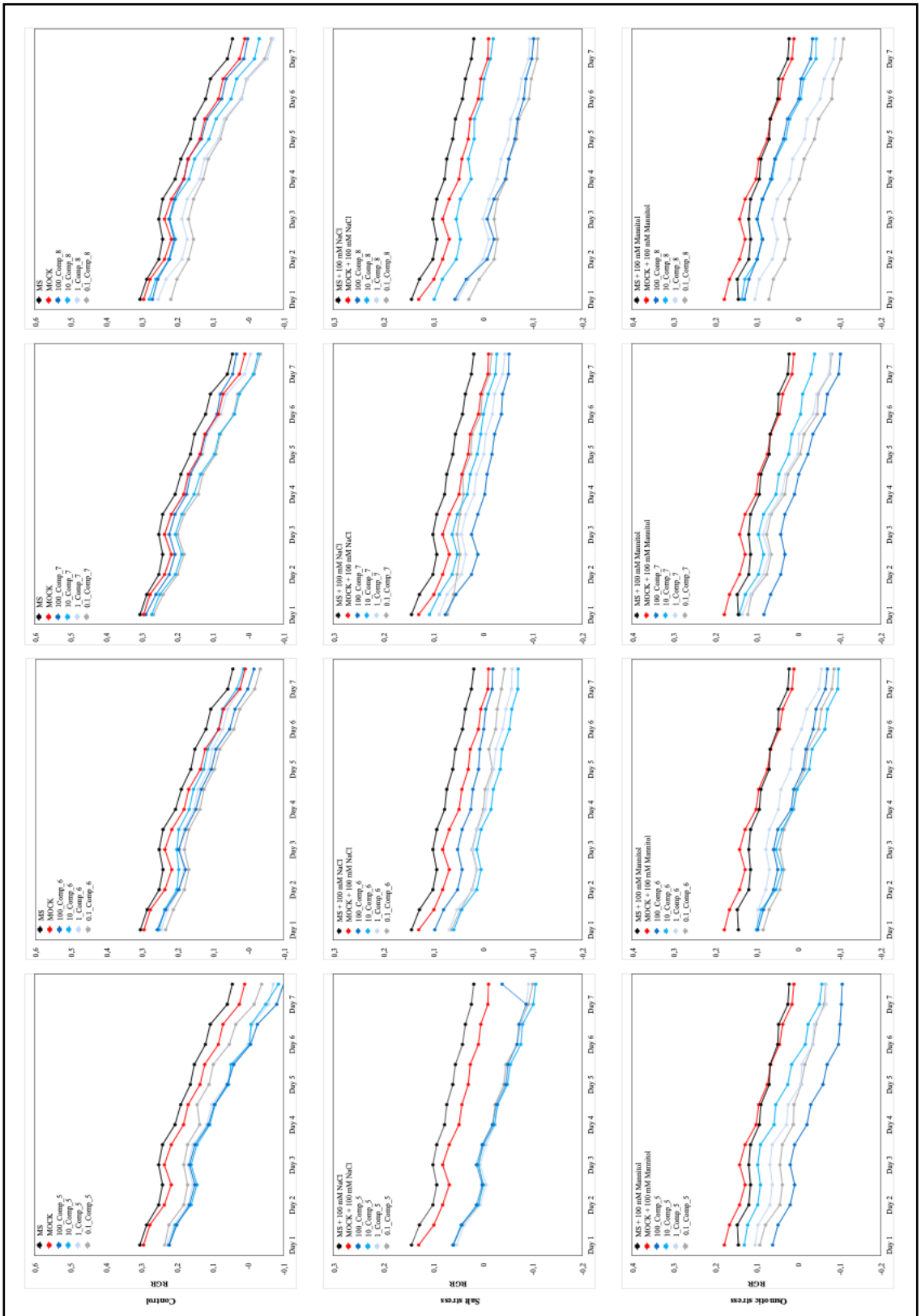

Figure 11: (Continued).

5.3 Final growth of *Arabidopsis* plants

After 7 days of cultivation in control and both stress conditions, final measurement of all *Arabidopsis* plants primed with 8 different CK analogs in four concentrations was performed.

While compounds 1 and 3 did not have any significant effect on plants grown under control conditions, compound 3 in salt stress and both compounds (1 and 3) in osmotic environment improved the growth (1.3 to 1.52-fold increase). Regarding compound 2 and its effect in control conditions, concentrations 0.1, 10 and 100 μM promoted the growth of rosettes about 1.4 to 1.46-fold, relative to control. Similarly, all concentrations of this compound caused an increase in the size of *Arabidopsis* plants cultured under salt and osmotic stress conditions. Among the first group of compounds, the most interesting results were obtained with compound 4 which in control and osmotic stress conditions caused a rapid increase in plants size with the concentration 1 μM. After the seed pre-treatment with this compound, the biggest growth under 100 mM NaCl was recorded with the concentration 0.1 μM (1.7-fold increase relative to control). On a contrary, highest concentration (100 μM) of compound 4 generated much smaller plants in all treatment conditions indicating its negative effect on the growth processes.

Whereas in control conditions, priming with compound 5 resulted in concentration-dependent growth, under stress conditions all concentrations caused approximately similar increase in *Arabidopsis* plant growth. Furthermore, 100 μM concentration of this compound in osmotic conditions doubled the size of rosettes. Concerning compounds 6 and 7, size of plants after 7 days in control conditions did not change. Rather different results were acquired in salt stress in which concentrations 1 and 10 μM of compound 7 produced slightly bigger plants. Under osmotic conditions, whole range of concentrations of compounds 6 and 7 positively affected the size of plants.

In case of compound 8, low concentrations (0.1 and 1 μM) had the best impact on the rosette growth in all treatment conditions. These concentrations increased the size of *Arabidopsis* plants compared to control by 1.62 to two-fold, respectively. Concentration 10 μM in salt environment and concentrations 10 and 100 μM in control and osmotic conditions almost did not affect the growth of the plants but concentration 100 μM increased the size of plants under 100 mM Mannitol.

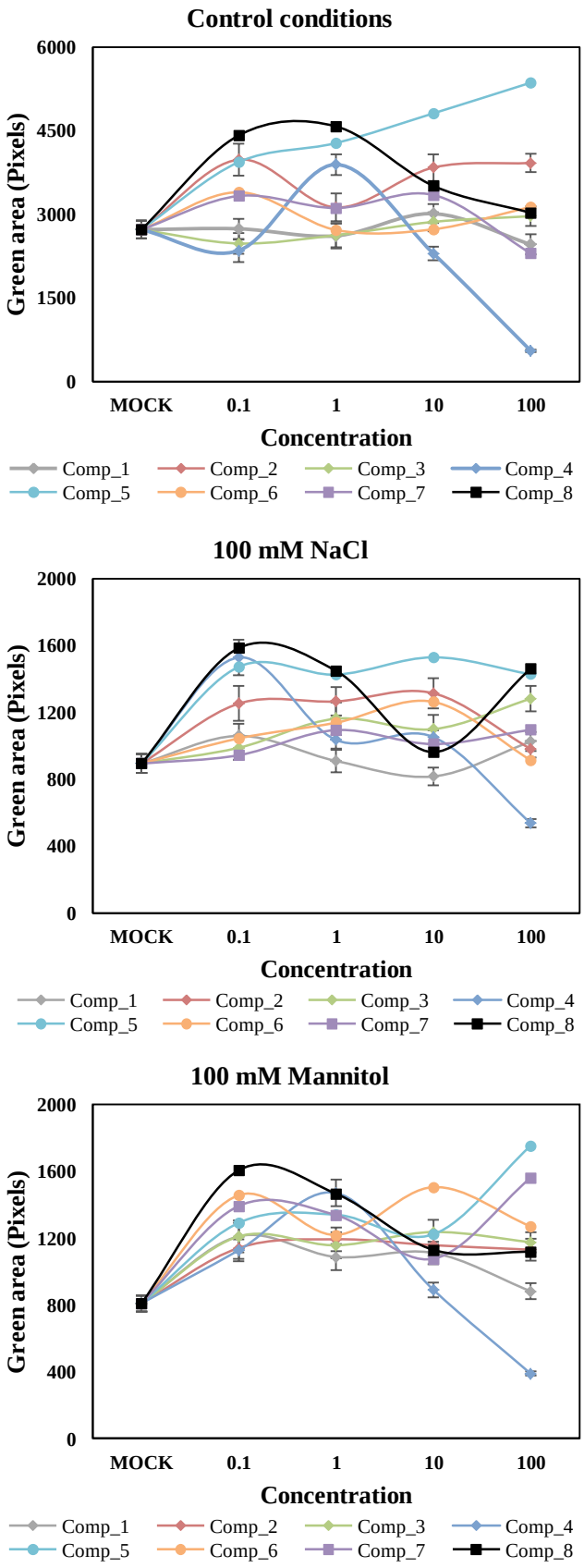

Figure 12: Final growth of *Arabidopsis* rosettes after 7 days of cultivation under control and two stress conditions after initial pre-sowing seed treatment with 8 cytokinin analogs.

5.4 Effect of *Arabidopsis* seed priming on leaf color under different culture conditions

One of the most important characteristics of plant stress is a change in leaf color associated with the degradation of the chlorophyll. For this reason, the change in *Arabidopsis* leaf color was determined as described in section Methods.

After the 7 days of *Arabidopsis* cultivation under control and stress conditions (either salt or osmotic stress), all plants were measured in order to obtain R, G and B data necessary to calculate the color change of the rosettes.

As illustrated in **Figure 13**, priming with first group of compounds caused a decrease of leaf greenness by half in control culture conditions, except the concentrations 10 and 100 μM of compound 1 which caused no change in *Arabidopsis* leaf color. Unlike first group of compounds, second and third group of compounds did not show any remarkable changes in color index under control conditions, only a slight increase.

Regarding salt stress conditions, any of the compounds caused a significant change in color index, relative to non-primed seed. In this case, individual concentrations of these compounds resulted in moderate increase or decrease of index values.

The most significant results were obtained after the cultivation of *Arabidopsis* plants under osmotic stress conditions induced by 100 mM Mannitol. While color index after the seed priming with compounds 5 to 8 improved only very slightly, compounds 1 to 4 have generated plants with higher greenness. Specifically, compound 1 with concentrations 0.1 and 1 μM; compound 2 with concentrations 1, 10 and 100 μM; all concentrations of compound 3 and concentrations 0.1, 10 and 100 μM of compound 4 (**Figure 13**).

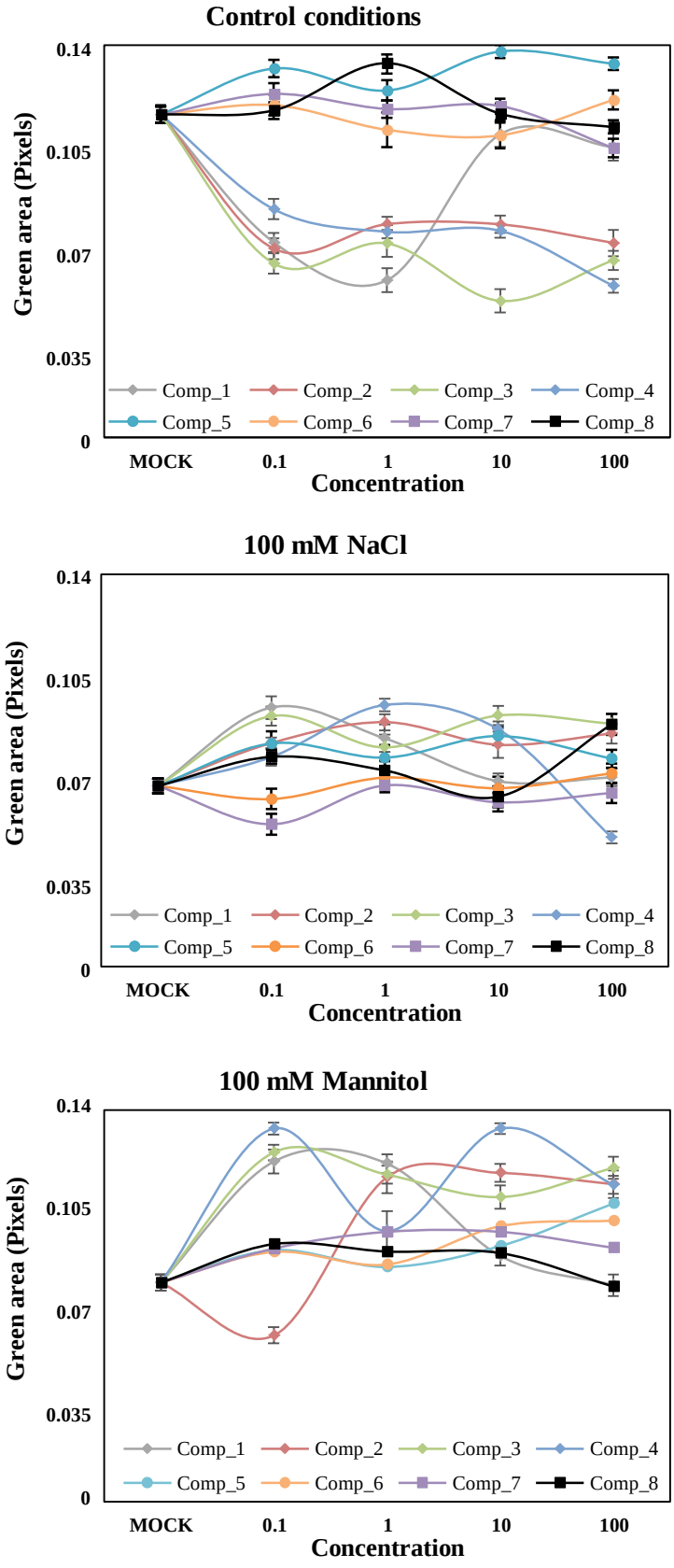

Figure 13: Effect of seed priming on *Arabidopsis* stress responses. Correlation between the color index GLI and the concentrations (0.1, 1, 10, 100 µM) of 8 different compounds used as a priming agents on *Arabidopsis* seeds and their subsequent one week cultivation under control and stress conditions.

5.5 Evaluation of Biostimulant Mode of Action by PBC Index

Plant Biostimulant Characterization (PBC) Index is a method developed and previously described by Ugena *et. al.*, 2018, focused on the integration of HTS methods into a pipe-line in order to allow easier selection of the best treatment under several distinct culture conditions. As presented in **Figure 14**, PBC index is able to render up to 4 traits of treated and untreated plant seeds, such as early seedling establishment (green area in pixels at time zero), rosette growth (Pixels) and color index (GLI).

Under control conditions, while early seedling establishment of compounds 1-4 was mostly improved or remained unchanged, these agents had an inhibitory effect on color index (GLI) values. In contrast, each of these four compounds had a different effect on rosette growth. Whereas compound 2 promoted the growth, compound 3 suppressed development of this trait. As already stated in previous sections, concentration 100 μM of compound 4 had a negative effect on plant growth and development. Regarding compounds 5 to 8, these compounds positively affected all studied plant traits (**Figure 14A, D**). Moreover, growth and leaf color index after seed priming with all eight compounds improved or did not change under both stress conditions (salt and osmotic) (**Figure 14B, C, E, F**) except highest concentration (100 μM) of compound 4 which inhibited growth of rosette area

These results were obtained after the calculation of differences between the controls and individual variants under all treatment conditions and subsequent log2 of the ratio. The effect of the concentration of the compounds under control, salt and osmotic stress conditions was further examined by summing log2 data obtained previously, reaching positive (red) or negative (blue) values. Radar chart was then used to plot these sums (**Figure 15**). From these results it is easy to evaluate that whole concentration range of compound 5 and low concentrations of compound 8 are the most efficient plant growth promotors and stress alleviators under control and salt stress conditions. However, the efficiency of compounds 1 to 4 increased with the presence of stress factor, especially in presence of osmotic stress induced with 100 mM Mannitol. Under salt stress, highest increase was achieved with low concentrations of compounds 2 and 4. Concerning osmotic conditions, the highest effectiveness was reached with compounds 2 and 3.

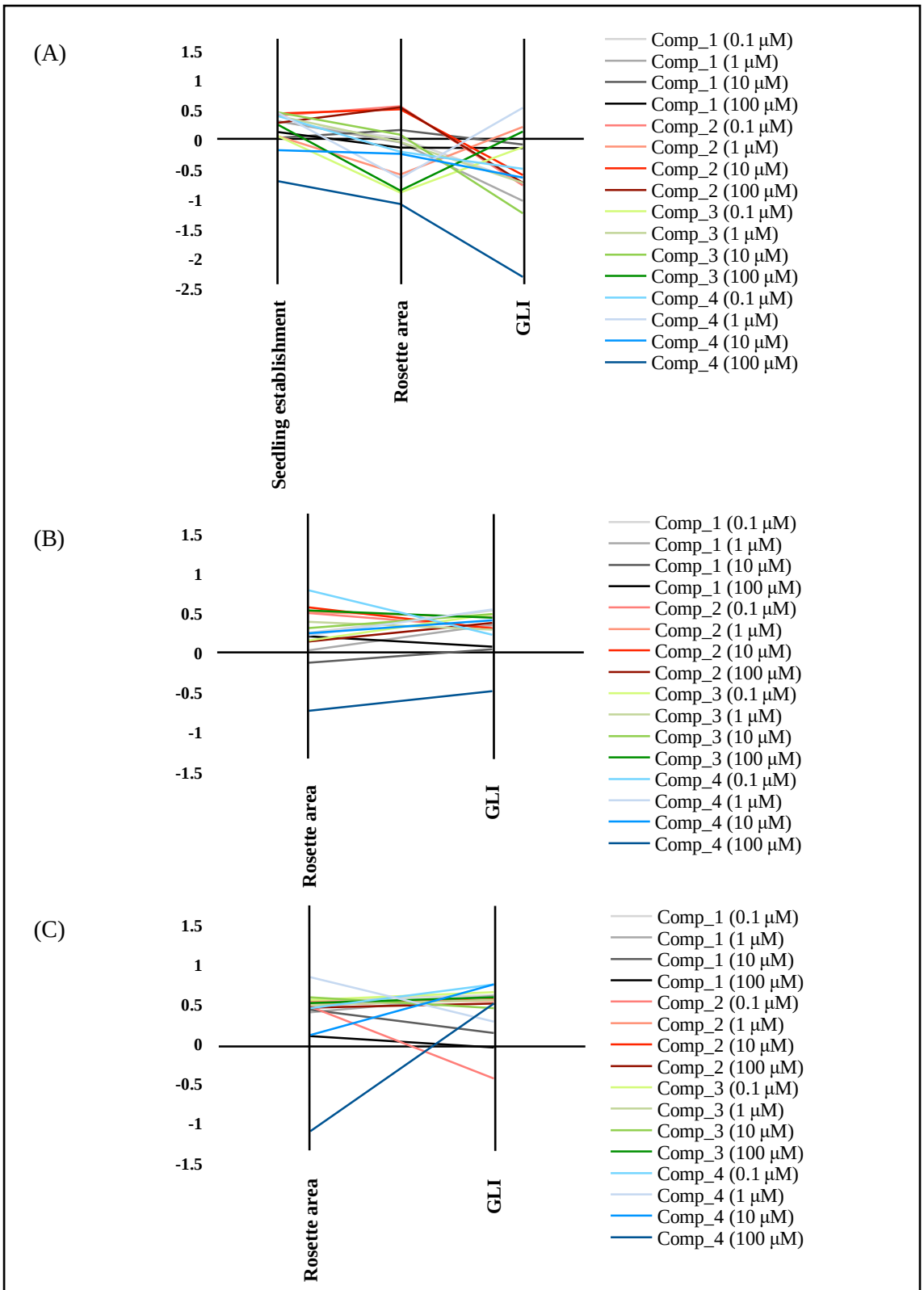

Figure 14: Parallel plot of plant traits (seedling establishment, rosette area and GLI) obtained from HTS analysis of *Arabidopsis* plants primed with compounds 1 to 4 (**A-C**) and 5 to 8 (**D-F**) at four different concentrations (0.1, 1, 10, 100 µM) and cultured under control (**A, D**), salt (**B, E**) and osmotic (**C, F**) stress conditions.

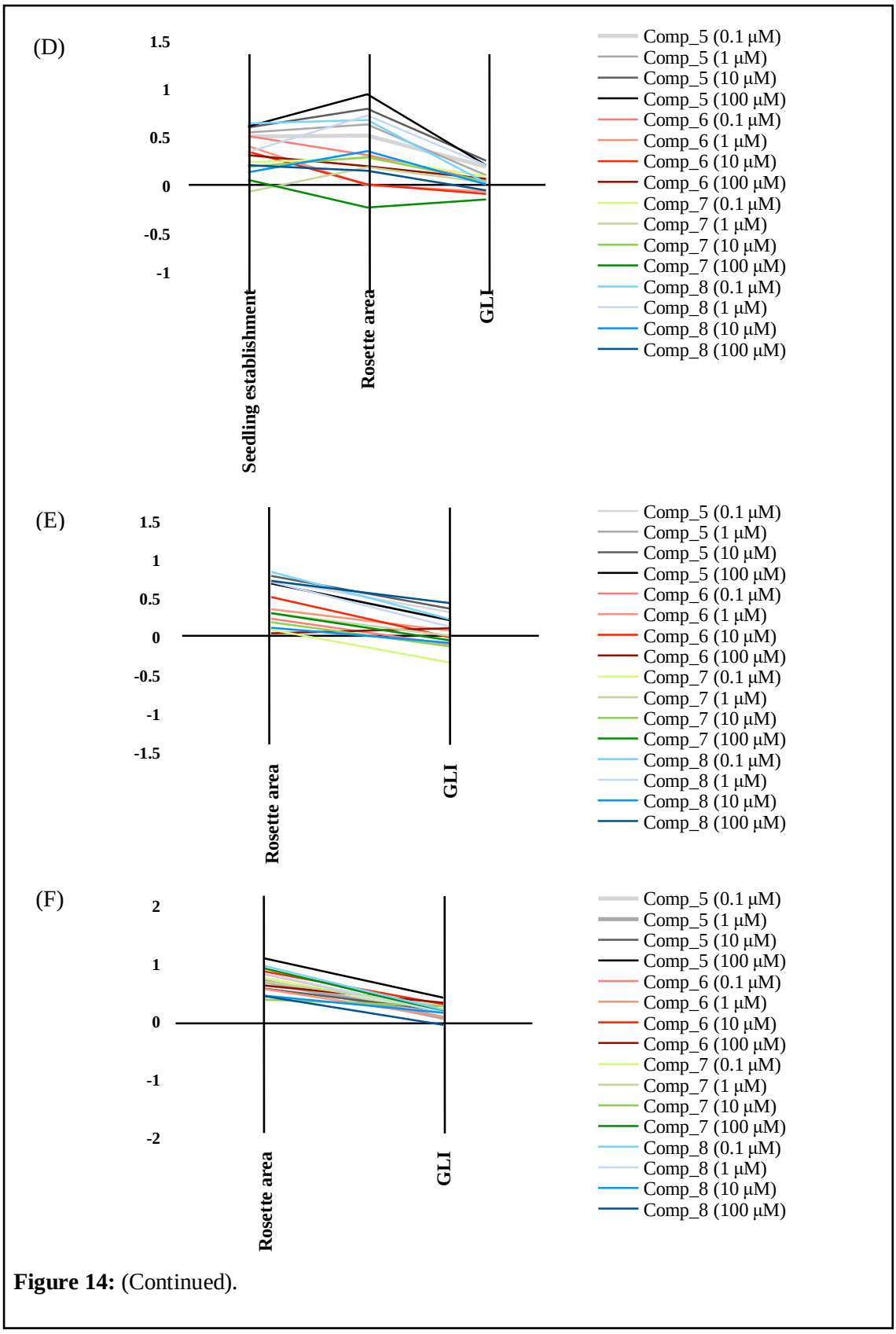

Figure 14: (Continued).

	Control condition				100 mM NaCl				100 mM Mannitol			
	Concentration (µM)				Concentration (µM)				Concentration (µM)			
	0.1	**1**	**10**	**100**	**0.1**	**1**	**10**	**100**	**0.1**	**1**	**10**	**100**
Comp_1	-0.43	-2.82	0.22	-0.49	1.08	0.09	-0.29	0.63	1.85	0.82	1.73	1.81
Comp_2	0.70	0.03	0.83	0.76	1.73	1.76	1.60	0.58	1.22	1.85	1.42	2.19
Comp_3	-1.11	-0.56	-0.76	-0.22	0.76	1.06	0.20	1.69	0.99	1.21	1.88	0.85
Comp_4	-0.82	0.98	-1.42	-8.30	2.20	0.74	0.99	-2.64	0.14	1.80	1.80	-3.60
Comp_5	1.56	2.12	2.67	2.99	2.11	1.66	2.34	0.24	-1.33	-1.25	-1.21	-0.21
Comp_6	1.12	-0.08	-0.13	0.71	-0.28	0.57	0.80	-0.24	-0.88	-1.62	-0.37	-1.07
Comp_7	1.10	0.44	0.95	-0.78	-1.35	0.44	0.10	0.38	-0.71	-0.63	-1.51	-0.50
Comp_8	2.02	2.24	1.02	0.26	1.88	1.61	-0.30	2.20	-0.41	-0.65	-1.54	-1.99

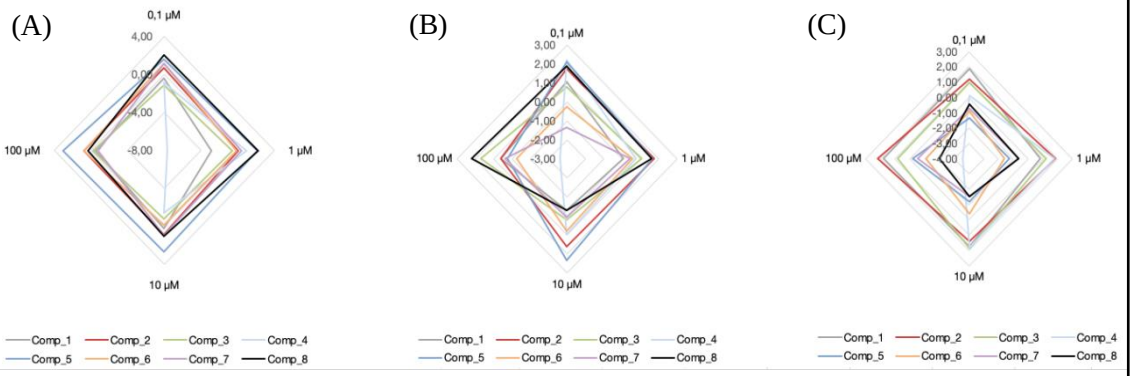

Figure 15: Plots of data obtained from parallel plot for each compound (1 to 8), concentration (0.1, 1, 10, 100 µM) and growth condition **(A)** control, **(B)** 100 mM NaCl, **(C)** 100 mM Mannitol. Red color indicates promotion and blue color inhibition.

5.6 Hormones

The extraction, purification and finally quantification of different groups of phytohormones were performed in four biological replicates of selected samples. The biological replicate consisted in a pool of 24 *Arabidopsis* rosettes. The selected samples were those seedlings primed with different concentrations of compound 2 and 5 in three growth conditions, control, salt stress and osmotic stress. For better comparison, the controls (MOCK) per each situation were also evaluated.

5.6.1 Cytokinins

In the experiment with compound 2, the levels of isopentenyl adenine (iP), *cis*-zeatin (*c*Z) and dihydroxy zeatin (DHZ) free bases were too low for detection except *trans*-zeatin (*t*Z) which under the control conditions did not show any changes. Although, under salt stress there was 3.76 and 3.54-fold increase in its levels with the lowest concentrations (0.1 and 1 μM) of compound 2 and increase by one half with concentrations 10 and 100 μM, compared to controls. Moreover, under the osmotic stress conditions the levels of *t*Z increased 1.7-fold and with the highest concentration (100 μM) they were even four times higher.

Regarding iP and DHZ ribosides (iPR and DHZR) under control conditions, there were almost no changes observed, while under the effect of salt stress conditions, the content of iPR increased with the concentration 10 μM of compound 2 by one half and under osmotic conditions, concentrations 10 and 100 μM increased its levels 1.4 and 1.52-fold. Interestingly, DHZR content under salt stress conditions increased with concentration 1 and 10 μM (1.75 and 1.6-fold) and decreased 1.8-fold with the highest concentration of compound 2 but under osmotic stress, low concentrations of this compound (0.1 and 1 μM) caused 1.36 and 2.14-fold decrease and concentration 100 μM increased its levels by half. Similar results were obtained for precursors of iP, *t*Z and *c*Z ribosides (iPRMP, *t*ZRMP, *c*ZRMP). While the levels of DHZRMP were immeasurable low, levels of iPRMP under control conditions were maintained with no significant changes. Conversely, iPRMP levels under salt conditions increased with the increasing concentration of compound 2. Under osmotic stress, these levels also increased from 1.8 to 3.25-fold. In case of *t*Z ribosides (*t*ZR), its levels increased with decreasing concentration of compound 2 up to 2-fold under control conditions and 3.6-fold under salt stress, relative to control. However, under osmotic stress, the trend of

*t*ZR content increase was opposite. Furthermore, all concentrations of compound 2 caused an increase in *t*ZRMP levels ranging from 1.45 to 1.91-fold under control conditions and 1.56 to 3.46-fold under both stress conditions.

Concerning MVA derived *c*Z ribosides and *c*Z nucleotides (*c*ZR, *c*ZRMP), compound 2 caused a decrease in their levels from 1.4 up to two-fold under control conditions. The most interesting changes were observed with the lowest concentration of this compound which caused decrease in *c*ZR levels 3.7-fold under salts and 2.4-fold under osmotic stress. Conversely, concentrations 1, 10 and 100 μM under salt stress and all concentrations under osmotic stress increased levels of *c*ZRMP 1.4 to 2.9-fold, relative to controls.

As illustrated in **Table 2**, whereas the content of inactive *N*- and *O*-glycosylated CK forms of iP (iP7G, iP9G) did not radically change under control and osmotic conditions, under the effect of salt stress these levels increased ranging from 1.3 to 2-fold (**Table 2B**). The most notable changes in levels were observed in glycosylated forms of tZ. While levels of *t*ZOG under control and osmotic stress conditions increased 1.3 to 2-fold, under salt stress these levels reached even two to three fold increase. In case of *t*ZROG under control conditions, its levels dropped up to 3.6 times, relative to control. Conversely, under both stress conditions there was no change in *t*ZROG levels, except the highest concentration of compound 2 (100 μM) which caused 2.4-fold decrease under salt stress. Additionally, whole concentration range of compound 2 led to increase of *t*Z7G (1.3 to 1.7-fold) and *t*Z9G (2 to 3.3-fold) levels under control conditions. On a contrary, under salt stress, levels of these two glycosylated *t*Z forms increased only with the concentrations 0.1 and 1 μM. Under osmotic stress conditions levels of *t*Z7G and *t*Z9G were negatively affected by too high concentration of compound 2 (3-fold decrease). Regarding *c*Z glucosides, *c*ZOG levels under control conditions dropped 1.7 to 2-fold but under stress conditions, these levels increased 1.6 fold with concentration 10 μM (salt stress) and 0.1 μM (osmotic stress) of compound 2. **Table 2** also indicates that the levels of *c*ZROG changed only under stress culture conditions (1.5 to 2.2-fold increase). Interesting is that while levels of *c*Z7G decreased and *c*Z9G levels did not change under control conditions, under salt and osmotic stress these levels increased. However, concentration 100 μM of compound 2 under osmotic stress caused a decrease of these levels almost 2-fold.

Unfortunately, the levels of DHZOG, DHZROG were unable to measure due to their low concentration under control conditions. Considering DHZ7G and DHZ9G,

levels of these glycosylated CK forms increased with concentrations 0.1, 1 and 10 μM of compound 2 from 2 to 2.78. Similarly, under osmotic stress conditions, the same concentrations caused an increase in DHZ7G and DHZ9G levels (1.55 to 2.2-fold), but the highest concentration (100 μM) decreased these levels to half.

Likewise in case of compound 2, free bases of iP, *c*Z and DHZ were not detected after the *Arabidopsis* seed priming with compound 5, except *t*Z whose levels under control conditions increased four times with the highest concentration (100 μM).

Different results were obtained under stress conditions. While in salt stress, content of *t*Z did not radically change, in osmotic stress conditions concentrations 0.1 and 100 μM caused a decrease in these levels 3.7 and 1.6-fold, and with concentration 1 μM increased 2.7-fold.

The most notable changes occurred in levels of CK ribosides (iPR, *c*ZR, DHZR) under all culture conditions. In all cases, levels of iPR and *c*ZR decreased with decreasing concentration of compound 5. Similar results were observed also in case of DHZR (**Table 2**). Regarding *t*ZR, concentration 100 μM caused 1.7-fold increase in its levels under control and salt stress conditions but concentration 0.1 μM under control and osmotic stress conditions decreased *t*ZR levels 1.8 and 3-fold, respectively.

Just like compound 2, highest concentration of compound 5 slightly increased content of iP nucleotides (iPRMP) under control conditions and under the effect of osmotic stress whole concentration range of compound 5 increased these levels up to 2.5-fold. However, salt stress had a negative effect on iPRMP levels and so caused its 1.4-fold decrease. In case of *t*ZRMP, under control and osmotic conditions its levels increased together with increasing concentration of compound 5 which was used as a priming agent. On the other hand, content of *t*ZRMP almost did not change under salt stress. Concerning *c*Z nucleotides (*c*ZRMP), while all four concentrations of compound 5 highly decreased its levels under control and salt stress conditions, under osmotic stress its levels remained same except the concentration 0.1 μM which decreased the content of *c*ZRMP by half.

Concerning *N*-glucosides of iP, while all four concentrations of compound 5 decreased the levels of iP9G under control and both stress conditions, iP7G levels remained unchanged, except small decrease under salt and osmotic conditions with the lowest concentration of compound 5 (1.3 to 1.5-fold). After the seed pre-treatment with compound 5 and further cultivation under control conditions, levels of *N*-glucosides *t*Z7G and *t*Z9G increased, ranging from 1.4 to 6-fold. However, under salt

stress conditions only the highest concentration of compound 5 (100 μM) increased these levels. Furthermore, osmotic stress caused even their slight decrease. In case of *O*-glucosides, while levels of *t*ZOG increased only with 100 μM concentration (control conditions) and concentrations 1 and 100 μM (both stressors) by 1.5 to 1.7-fold, levels of *t*ZROG under control and salt stress conditions dropped, mainly with the concentration 0.1 μM. As in the previous experiment with compound 2, DHZ *O*-glucosides were not detected. Regarding *N*-glucosides DH7G and DHZ9G, after priming with all concentrations of compound 5 their levels increased 1.5 to 3.5-fold under control and 1.4 to 2.7-fold under osmotic conditions, relative to control. Under salt stress conditions, increase in these levels was observed mainly with higher concentrations (10 and 100 μM). The greatest impact of compound 5 on levels of *c*Z glycosylated forms was observed under control conditions in which levels of *c*ZROG, *c*Z7G, *c*ZOG and *c*Z9G (except concentration 100 μM) dropped (**Table 2A**). In case of salt stress, decrease in these levels was maintained for most of the concentrations but similarly as in control conditions, concentration 100 μM of compound 5 increased levels of *c*ZROG and *c*Z9G 1.4 and 2.15-fold, respectively. On the other hand, there were not many changes under osmotic conditions in *c*Z glucoside levels. Specifically, *c*ZOG levels dropped 1.4 fold with concentrations 0.1 and 100 μM, *c*ZROG levels decreased with concentration 0.1 μM but increased in rest of the concentrations, levels of *c*Z9G decreased 1.7-fold with concentration 10 μM and increased 1.5-fold with 100 μM concentration.

Table 2: Changes in CK levels (pmol.g^{-1}) in *Arabidopsis thaliana* plants primed with compound 2 and compound 5 and subseguently cultured under **(A)** control, **(B)** salt stress and **(C)** osmotic stress conditions.

(A) Control conditions

		MOCK	Compound 2				Compound 5			
			0.1 µM	1 µM	10 µM	100 µM	0.1 µM	1 µM	10 µM	100 µM
	Total CKs	**104.73 ± 18.265**	**108.33 ± 14.54**	**97.85 ± 14.04**	**114.00 ± 21.56**	**114.54 ± 16.29**	**74.68 ± 8.43**	**82.63 ± 10.57**	**87.36 ± 7.50**	**132.96 ± 35.20**
	Total CK Bases	0.081 ± 0.014	0.071 ± 0.023	0.080 ± 0.023	0.070 ± 0.016	0.070 ± 0.018	0.078 ± 0.020	0.072 ± 0.008	0.104 ± 0.032	0.33 ± 0.09
	Total CK Ribosides	18.645 ± 3.525	19.96 ± 4.08	16.98 ± 4.72	20.10 ± 5.28	16.77 ± 2.88	5.77 ± 1.07	11.90 ± 3.28	9.33 ± 1.54	16.69 ± 3.71
	Total CK Nucleotides	15.35 ± 5.895	12.25 ± 0.97	12.98 ± 1.44	14.73 ± 2.41	18.51 ± 3.29	9.36 ± 1.54	11.66 ± 2.14	12.25 ± 2.48	19.41 ± 4.33
	Total CK *O*-glucosides	7.53 ± 1.3	7.94 ± 1.21	6.65 ± 1.56	7.63 ± 2.02	6.49 ± 0.91	3.98 ± 0.64	4.97 ± 0.57	5.26 ± 1.10	8.35 ± 1.67
	Total CK *N*-glucosides	63.12 ± 9.19	68.11 ± 9.45	61.15 ± 7.43	71.46 ± 14.07	72.70 ± 9.93	55.52 ± 5.69	54.05 ± 7.70	60.41 ± 4.01	88.19 ± 26.25
MEP- pathway (plastid) derived CKs	**Total iP-types**	**47.395 ± 8.845**	**47.29 ± 5.40**	**45.92 ± 6.30**	**54.05 ± 9.58**	**56.09 ± 8.43**	**34.19 ± 3.08**	**41.96 ± 4.47**	**40.54 ± 4.47**	**55.23 ± 9.93**
	iP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	11.92 ± 2.64	12.30 ± 2.99	10.81 ± 3.08	13.30 ± 3.71	11.49 ± 2.23	3.99 ± 0.84	7.24 ± 2.29	6.20 ± 1.24	11.33 ± 2.83
	iPRMP	6.43 ± 1.43	6.05 ± 1.04	7.01 ± 0.62	7.23 ± 1.42	10.22 ± 1.72	6.38 ± 1.07	7.93 ± 1.27	6.62 ± 1.07	9.44 ± 1.91
	iP7G	25.02 ± 4.29	25.48 ± 3.05	24.99 ± 2.85	29.69 ± 5.79	29.84 ± 4.42	20.97 ± 1.66	24.32 ± 2.41	24.86 ± 3.16	30.07 ± 6.91
	iP9G	4.03 ± 0.675	3.46 ± 0.59	3.12 ± 0.39	3.83 ± 0.67	4.54 ± 0.49	2.85 ± 0.27	2.47 ± 0.29	2.86 ± 0.30	4.39 ± 0.96
	Total *t*Z-types	**25.405 ± 4.515**	**32.79 ± 6.04**	**28.94 ± 3.01**	**32.33 ± 6.57**	**30.42 ± 6.23**	**23.77 ± 3.34**	**22.30 ± 4.00**	**27.25 ± 2.14**	**49.68 ± 18.34**
	***t*Z**	0.081 ± 0.014	0.071 ± 0.023	0.080 ± 0.023	0.070 ± 0.016	0.070 ± 0.018	0.078 ± 0.020	0.072 ± 0.008	0.104 ± 0.032	0.33 ± 0.09
	***t*ZR**	2.265 ± 0.325	4.41 ± 1.32	4.07 ± 1.03	4.09 ± 0.89	3.06 ± 0.61	1.27 ± 0.21	2.48 ± 0.56	2.25 ± 0.32	3.81 ± 0.76
	***t*ZRMP**	1.445 ± 0.245	2.10 ± 0.47	2.26 ± 0.22	2.12 ± 0.39	2.76 ± 0.65	1.57 ± 0.20	2.69 ± 0.60	2.65 ± 0.64	5.11 ± 1.45
	***t*ZOG**	1.885 ± 0.33	2.92 ± 0.42	2.37 ± 0.54	2.94 ± 0.66	2.17 ± 0.37	1.80 ± 0.18	2.00 ± 0.51	2.19 ± 0.28	3.26 ± 0.57
	***t*ZROG**	0.725 ± 0.195	0.32 ± 0.05	0.20 ± 0.03	0.35 ± 0.09	0.23 ± 0.02	0.16 ± 0.05	0.24 ± 0.04	0.20 ± 0.01	0.32 ± 0.07
	***t*Z7G**	7.79 ± 1.82	10.17 ± 0.97	12.45 ± 2.59	13.53 ± 2.30	10.68 ± 2.86	9.07 ± 1.87	10.97 ± 0.51	17.07 ± 4.45	13.13 ± 2.11
	***t*Z9G**	3.125 ± 0.605	9.79 ± 1.07	10.32 ± 2.66	8.60 ± 2.44	5.99 ± 1.37	5.77 ± 1.18	8.89 ± 1.76	19.79 ± 11.57	9.83 ± 2.65
	Total DHZ-types	**2.96 ± 0.505**	**4.36 ± 0.74**	**3.46 ± 0.94**	**4.09 ± 0.90**	**4.42 ± 0.53**	**3.68 ± 0.57**	**3.73 ± 2.13**	**4.00 ± 0.37**	**5.55 ± 1.43**
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.145 ± 0.035	0.17 ± 0.07	0.12 ± 0.04	0.15 ± 0.05	0.09 ± 0.01	0.03 ± 0.00	0.16 ± 0.05	0.18 ± 0.04	0.09 ± 0.02
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	1.5 ± 0.37	3.20 ± 0.90	3.80 ± 0.83	4.18 ± 0.48	1.49 ± 0.44	3.53 ± 2.15	3.81 ± 0.45	5.25 ± 1.42	4.03 ± 0.70
	DHZ9G	0.065 ± 0.015	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.07 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.21 ± 0.06	0.16 ± 0.01
MVA-pathway (cytosol) derived CKs	**Total *c*Z-types**	**28.97 ± 7.725**	**23.89 ± 3.33**	**19.53 ± 4.80**	**23.52 ± 4.90**	**23.61 ± 2.97**	**13.03 ± 1.73**	**14.65 ± 2.21**	**15.57 ± 2.70**	**22.49 ± 6.35**
	***c*Z**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	***c*ZR**	4.325 ± 1.235	3.07 ± 0.47	1.98 ± 0.64	2.57 ± 0.66	2.16 ± 0.42	0.49 ± 0.06	2.06 ± 0.69	0.80 ± 0.20	1.45 ± 0.20
	***c*ZRMP**	7.48 ± 4.995	4.10 ± 1.36	3.71 ± 1.04	5.37 ± 0.67	5.53 ± 1.02	1.40 ± 0.63	1.03 ± 0.47	2.98 ± 1.13	4.86 ± 3.12
	***c*ZOG**	1.02 ± 0.22	0.58 ± 0.11	0.52 ± 0.10	0.59 ± 0.14	0.55 ± 0.13	0.47 ± 0.05	0.62 ± 0.17	0.40 ± 0.08	1.32 ± 0.35
	***c*ZROG**	3.895 ± 0.86	4.12 ± 0.69	3.57 ± 1.02	3.76 ± 1.21	3.54 ± 0.77	1.55 ± 0.43	2.12 ± 0.11	2.47 ± 0.76	3.45 ± 0.86
	***c*Z7G**	14.455 ± 3.81	9.16 ± 2.50	10.69 ± 2.64	11.32 ± 1.35	13.79 ± 3.38	8.51 ± 1.88	8.46 ± 0.93	10.37 ± 1.87	11.19 ± 1.44
	***c*Z9G**	0.57 ± 0.14	0.59 ± 0.16	0.55 ± 0.14	0.51 ± 0.09	0.64 ± 0.20	0.31 ± 0.10	0.45 ± 0.11	1.04 ± 0.32	0.83 ± 0.27

Table 2: (Continued).

(B) Salt stress conditions

		MOCK	Compound 2				Compound 5			
			0.1 µM	1 µM	10 µM	100 µM	0.1 µM	1 µM	10 µM	100 µM
	Total CKs	**157.34 ± 35.63**	**173.13 ± 27.90**	**208.79 ± 19.85**	**244.29 ± 61.86**	**164.18 ± 22.60**	**78.41 ± 5.24**	**99.04 ± 16.83**	**89.65 ± 18.39**	**120.97 ± 21.33**
	Total CK Bases	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06	0.14 ± 0.03	0.18 ± 0.04	0.15 ± 0.04	0.10 ± 0.01
	Total CK Ribosides	75.345 ± 17.82	53.94 ± 16.41	76.50 ± 16.27	95.05 ± 28.28	44.51 ± 11.92	14.13 ± 2.34	20.89 ± 5.87	23.60 ± 4.67	36.07 ± 3.62
	Total CK Nucleotides	4.505 ± 1.055	4.25 ± 1.43	6.80 ± 0.96	9.36 ± 5.12	8.35 ± 2.36	2.46 ± 0.91	4.10 ± 0.62	2.46 ± 0.36	3.34 ± 0.68
	Total CK *O*-glucosides	8.48 ± 1.47	14.33 ± 3.63	15.66 ± 2.17	16.32 ± 3.80	14.50 ± 3.38	6.22 ± 0.37	7.18 ± 1.19	6.06 ± 1.41	9.23 ± 2.22
	Total CK *N*-glucosides	68.9 ± 17.88	100.11 ± 7.04	109.36 ± 13.89	123.37 ± 25.39	96.63 ± 18.40	55.47 ± 3.75	66.70 ± 11.84	57.38 ± 12.76	72.28 ± 15.20
MEP- pathway (plastid) derived CKs	**Total iP-types**	**67.33 ± 15.565**	**73.08 ± 7.08**	**95.70 ± 11.80**	**117.44 ± 25.00**	**80.28 ± 8.09**	**36.02 ± 5.17**	**46.91 ± 8.88**	**40.58 ± 9.31**	**61.23 ± 11.77**
	iP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	30.555 ± 5.825	27.47 ± 8.71	36.77 ± 10.13	47.19 ± 13.93	26.68 ± 8.57	8.36 ± 1.74	12.71 ± 3.92	12.81 ± 3.61	20.51 ± 2.53
	iPRMP	1.88 ± 0.49	1.70 ± 0.47	2.59 ± 0.56	3.12 ± 0.82	4.32 ± 0.51	1.71 ± 0.56	1.34 ± 0.30	1.29 ± 0.37	1.74 ± 0.36
	iP7G	30.485 ± 8.87	39.03 ± 5.18	51.16 ± 10.73	61.20 ± 9.55	45.16 ± 8.81	23.29 ± 4.19	29.55 ± 5.14	23.63 ± 5.89	35.71 ± 8.55
	iP9G	4.41 ± 1.085	4.88 ± 1.29	5.18 ± 0.19	5.93 ± 1.33	5.20 ± 1.42	2.66 ± 0.38	3.30 ± 0.53	2.85 ± 0.69	3.27 ± 0.72
	Total *t*Z-types	**24.3 ± 6.3**	**57.99 ± 10.36**	**42.58 ± 9.15**	**41.62 ± 11.39**	**19.86 ± 4.25**	**24.32 ± 3.20**	**26.00 ± 4.46**	**25.56 ± 5.12**	**27.04 ± 5.99**
	***t*Z**	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06	0.14 ± 0.03	0.18 ± 0.04	0.15 ± 0.04	0.10 ± 0.01
	***t*ZR**	4.185 ± 1.095	15.19 ± 4.67	7.14 ± 1.73	7.93 ± 2.54	2.25 ± 0.66	3.89 ± 0.99	3.65 ± 1.03	5.13 ± 0.60	6.94 ± 2.18
	***t*ZRMP**	0.5 ± 0.115	0.43 ± 0.08	1.18 ± 0.26	1.73 ± 0.53	0.78 ± 0.18	0.45 ± 0.14	0.36 ± 0.10	0.41 ± 0.13	0.54 ± 0.14
	***t*ZOG**	1.72 ± 0.51	5.50 ± 1.38	3.48 ± 0.97	3.65 ± 1.13	1.58 ± 0.44	1.79 ± 0.29	2.34 ± 0.44	1.93 ± 0.46	2.28 ± 0.64
	***t*ZROG**	0.765 ± 0.21	0.70 ± 0.18	0.65 ± 0.13	0.68 ± 0.17	0.32 ± 0.11	0.24 ± 0.04	0.28 ± 0.06	0.30 ± 0.09	0.37 ± 0.11
	***t*Z7G**	10.865 ± 2.14	19.13 ± 4.16	17.41 ± 4.46	9.97 ± 1.76	10.52 ± 2.24	10.54 ± 2.06	11.59 ± 2.91	10.28 ± 2.20	20.44 ± 1.53
	***t*Z9G**	6.035 ± 1.355	10.54 ± 2.45	10.03 ± 2.59	4.76 ± 1.54	6.81 ± 1.89	8.65 ± 1.26	6.14 ± 1.43	6.58 ± 1.85	15.24 ± 3.57
	Total DHZ-types	**2.035 ± 0.51**	**3.46 ± 0.81**	**4.06 ± 0.69**	**4.33 ± 1.06**	**2.22 ± 0.50**	**1.68 ± 0.13**	**3.29 ± 1.07**	**2.46 ± 0.61**	**4.25 ± 0.95**
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.58 ± 0.105	0.57 ± 0.12	1.02 ± 0.26	0.93 ± 0.25	0.32 ± 0.09	0.13 ± 0.02	0.23 ± 0.07	0.25 ± 0.07	0.54 ± 0.10
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	2.59 ± 0.54	2.90 ± 0.54	3.27 ± 0.84	1.76 ± 0.52	2.65 ± 0.66	3.02 ± 1.05	2.11 ± 0.54	3.64 ± 0.95	2.72 ± 0.65
	DHZ9G	0.115 ± 0.01	0.14 ± 0.02	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.04	0.11 ± 0.03	0.10 ± 0.03	0.09 ± 0.01	0.17 ± 0.06
MVA-pathway (cytosol) derived CKs	**Total *c*Z-types**	**63.67 ± 15.175**	**38.60 ± 10.84**	**66.44 ± 9.76**	**80.90 ± 25.69**	**61.83 ± 10.99**	**16.39 ± 0.73**	**22.84 ± 4.26**	**21.05 ± 4.24**	**28.45 ± 4.22**
	***c*Z**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	***c*ZR**	40.02 ± 12.19	10.72 ± 3.10	31.58 ± 6.52	39.00 ± 12.40	15.25 ± 3.84	1.75 ± 0.29	4.36 ± 1.22	5.41 ± 1.35	8.08 ± 1.24
	***c*ZRMP**	2.125 ± 0.545	2.12 ± 1.02	3.03 ± 0.57	4.51 ± 3.81	4.34 ± 1.22	0.58 ± 0.31	2.40 ± 0.88	0.85 ± 0.21	1.06 ± 0.34
	***c*ZOG**	1.895 ± 0.59	2.02 ± 0.53	1.87 ± 0.19	3.08 ± 0.76	2.43 ± 0.75	1.25 ± 0.38	0.93 ± 0.36	0.83 ± 0.18	0.77 ± 0.18
	***c*ZROG**	4.095 ± 0.895	6.12 ± 1.58	9.67 ± 1.38	8.91 ± 1.95	10.17 ± 2.52	2.94 ± 0.62	3.63 ± 0.76	3.00 ± 0.84	5.81 ± 1.57
	***c*Z7G**	14.485 ± 2.53	19.28 ± 2.58	24.63 ± 7.60	28.43 ± 8.26	16.72 ± 4.28	11.08 ± 2.86	9.96 ± 2.39	12.52 ± 3.02	16.05 ± 4.53
	***c*Z9G**	0.73 ± 0.165	1.03 ± 0.16	0.78 ± 0.25	1.21 ± 0.39	0.86 ± 0.28	0.45 ± 0.11	1.00 ± 0.32	0.21 ± 0.03	1.57 ± 0.48

Table 2: (Continued).

(C) Osmotic stress conditions

		MOCK	Compound 2				Compound 5			
			0.1 µM	1 µM	10 µM	100 µM	0.1 µM	1 µM	10 µM	100 µM
	Total CKs	**83.87 ± 18.5**	**112.08 ± 8.93**	**103.89 ± 9.47**	**148.80 ± 15.57**	**138.51 ± 10.90**	**55.69 ± 2.99**	**89.12 ± 12.66**	**78.28 ± 12.38**	**106.55 ± 21.87**
	Total CK Bases	0.056 ± 0.0125	0.10 ± 0.02	0.095 ± 0.024	0.098 ± 0.026	0.23 ± 0.08	0.014 ± 0.004	0.15 ± 0.03	0.049 ± 0.014	0.035 ± 0.007
	Total CK Ribosides	16.83 ± 3.275	15.71 ± 2.19	13.91 ± 3.03	21.62 ± 2.88	25.41 ± 4.07	3.19 ± 0.59	9.99 ± 1.20	7.42 ± 0.50	15.48 ± 2.98
	Total CK Nucleotides	5.635 ± 1.515	10.41 ± 1.25	9.43 ± 1.44	15.15 ± 2.53	16.63 ± 0.98	5.60 ± 0.81	9.05 ± 1.03	8.71 ± 1.09	11.23 ± 2.71
	Total CK *O*-glucosides	6.44 ± 1.265	11.41 ± 1.34	8.71 ± 1.13	11.30 ± 1.15	12.15 ± 1.75	4.59 ± 0.58	8.96 ± 2.15	7.20 ± 1.82	9.46 ± 1.48
	Total CK *N*-glucosides	54.915 ± 14.035	74.45 ± 6.65	71.77 ± 6.45	100.63 ± 10.93	84.09 ± 10.49	42.30 ± 2.28	61.04 ± 9.81	54.91 ± 10.20	70.35 ± 16.44
MEP- pathway (plastid) derived CKs	**Total iP-types**	**41.23 ± 9.84**	**49.60 ± 2.20**	**46.33 ± 3.91**	**70.72 ± 6.57**	**61.58 ± 6.30**	**23.60 ± 2.20**	**39.86 ± 6.20**	**34.60 ± 6.56**	**50.23 ± 10.45**
	iP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	10.83 ± 2.35	11.25 ± 1.62	9.02 ± 1.97	15.12 ± 1.64	16.54 ± 2.72	2.05 ± 0.35	6.95 ± 0.92	4.84 ± 0.65	10.64 ± 2.44
	iPRMP	2.095 ± 0.63	4.93 ± 1.25	3.80 ± 0.75	6.81 ± 1.36	6.04 ± 1.26	2.91 ± 0.49	4.56 ± 0.53	4.20 ± 0.51	5.39 ± 1.56
	iP7G	25.375 ± 7.075	30.05 ± 1.13	30.24 ± 1.85	44.51 ± 4.67	35.41 ± 5.77	17.02 ± 2.73	25.96 ± 5.85	23.14 ± 5.66	31.11 ± 6.45
	iP9G	2.935 ± 0.815	3.38 ± 0.53	3.28 ± 0.72	4.28 ± 0.74	3.58 ± 0.33	1.62 ± 0.16	2.40 ± 0.14	2.43 ± 0.36	3.09 ± 1.03
	Total *tZ*-types	**16.33 ± 3.335**	**28.25 ± 3.95**	**24.96 ± 2.68**	**31.01 ± 6.53**	**28.95 ± 3.19**	**15.20 ± 1.67**	**23.23 ± 3.84**	**18.61 ± 2.36**	**25.40 ± 6.34**
	tZ	0.056 ± 0.0125	0.10 ± 0.02	0.095 ± 0.024	0.098 ± 0.026	0.23 ± 0.08	0.015 ± 0.004	0.15 ± 0.03	0.049 ± 0.014	0.035 ± 0.007
	tZR	2.04 ± 0.46	2.78 ± 0.61	2.29 ± 0.32	3.21 ± 0.69	4.15 ± 0.26	0.70 ± 0.22	1.89 ± 0.26	1.35 ± 0.12	2.38 ± 0.46
	tZRMP	0.565 ± 0.15	1.47 ± 0.41	1.08 ± 0.27	1.66 ± 0.28	1.96 ± 0.38	0.72 ± 0.17	1.68 ± 0.14	1.51 ± 0.22	2.70 ± 0.66
	tZOG	1.595 ± 0.31	2.69 ± 0.52	2.25 ± 0.44	3.18 ± 0.56	2.58 ± 0.36	1.28 ± 0.06	2.55 ± 0.64	1.63 ± 0.34	2.47 ± 0.39
	tZROG	0.32 ± 0.045	0.41 ± 0.06	0.37 ± 0.07	0.33 ± 0.08	0.41 ± 0.11	0.14 ± 0.02	0.37 ± 0.09	0.34 ± 0.08	0.34 ± 0.12
	tZ7G	11.815 ± 2.555	11.59 ± 2.08	13.41 ± 2.63	11.21 ± 1.53	4.01 ± 1.07	9.56 ± 2.13	8.96 ± 1.23	10.32 ± 3.11	11.31 ± 1.49
	tZ9G	6.26 ± 1.505	7.30 ± 1.15	9.12 ± 2.94	8.42 ± 1.37	2.19 ± 0.65	7.12 ± 1.13	4.80 ± 0.84	7.16 ± 1.88	9.49 ± 1.60
	Total DHZ-types	**2.08 ± 0.5**	**3.14 ± 0.24**	**3.32 ± 0.79**	**4.33 ± 1.08**	**4.58 ± 0.66**	**2.16 ± 0.34**	**3.35 ± 0.62**	**3.33 ± 1.22**	**5.45 ± 0.80**
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.15 ± 0.035	0.11 ± 0.03	0.07 ± 0.00	0.15 ± 0.05	0.22 ± 0.07	0.06 ± 0.02	0.03 ± 0.00	0.01 ± 0.00	0.18 ± 0.04
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	1.915 ± 0.325	3.17 ± 0.76	4.00 ± 1.01	4.20 ± 0.67	1.06 ± 0.34	3.21 ± 0.63	3.18 ± 1.24	5.19 ± 0.79	2.87 ± 0.26
	DHZ9G	0.11 ± 0.025	0.11 ± 0.02	0.17 ± 0.05	0.17 ± 0.03	0.05 ± 0.01	0.12 ± 0.02	0.15 ± 0.04	0.12 ± 0.02	0.15 ± 0.01
MVA-pathway (cytosol) derived CKs	**Total *cZ*-types**	**24.23 ± 5.095**	**31.09 ± 2.90**	**29.28 ± 2.93**	**42.74 ± 6.71**	**43.40 ± 1.98**	**14.72 ± 1.08**	**22.67 ± 2.80**	**21.73 ± 4.02**	**25.47 ± 5.22**
	cZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	cZR	3.825 ± 0.97	1.57 ± 0.17	2.56 ± 0.75	3.14 ± 0.91	4.50 ± 1.23	0.39 ± 0.04	1.14 ± 0.29	1.23 ± 0.38	2.32 ± 0.43
	cZRMP	2.98 ± 0.935	4.01 ± 0.52	4.55 ± 0.63	6.68 ± 1.68	8.63 ± 1.23	1.97 ± 0.27	2.81 ± 0.41	3.00 ± 0.60	3.13 ± 1.10
	cZOG	0.865 ± 0.19	1.40 ± 0.26	0.75 ± 0.16	0.96 ± 0.30	1.12 ± 0.25	0.62 ± 0.14	0.98 ± 0.18	0.78 ± 0.16	0.59 ± 0.10
	cZROG	3.67 ± 0.835	6.91 ± 0.72	5.34 ± 0.99	6.84 ± 1.37	8.04 ± 1.15	2.48 ± 0.47	5.07 ± 1.37	4.46 ± 1.40	6.06 ± 1.02
	cZ7G	14.2 ± 2.68	15.53 ± 2.02	24.35 ± 4.67	20.14 ± 2.40	7.87 ± 2.07	12.09 ± 1.15	11.62 ± 2.26	12.98 ± 3.80	16.22 ± 2.40
	cZ9G	0.67 ± 0.18	0.55 ± 0.16	0.79 ± 0.23	0.96 ± 0.14	0.34 ± 0.07	0.57 ± 0.16	0.64 ± 0.15	0.39 ± 0.13	0.98 ± 0.16

5.6.2 Auxins and ABA

As indicated in **Table 3**, after the evaluation of the results obtained from the analysis of phytohormones it has been observed that there are two different fractions of auxins [fractions of indol-3-acetic acid (IAA) and fractions of 2-oxoindol-3-ylacetic acid (oxIAA)] and an auxin-related metabolite [conjugate of IAA with Aspartic acid (IAAsp)] occurring in all treatment conditions.

In the experiment with compound 2, fractions of IAA were identified in control and salt stress conditions. Unfortunately, the levels of IAA in osmotic stress conditions were too low for the detection so in this case it was not possible to evaluate the results. The highest impact on the levels of IAA was observed after the use of the concentration 0.1 μM of compound 2. In control conditions, there was 1.44-fold increase in IAA levels and under salt stress the increase reached 1.65-fold. Furthermore, the concentration 1 μM under salt stress has also caused a slight increase in IAA levels (1.37-fold).

Regarding oxIAA, the most significant results were obtained after the seed priming with the lowest concentration (0.1 μM) of compound 2. In control and salt stress conditions, there was 1.87 and 2.49-fold increase in the levels of oxIAA, respectively.

After the use of other tree concentrations (1, 10 and 100 μM) of this compound in control and salt stress, the levels of oxIAA did not change radically. On the other hand, the best results were obtained after the use of this compound in osmotic stress conditions. Based on these results it can be assumed that the lower the concentration of compound 2 in osmotic stress conditions, the higher the increase of oxIAA levels, ranging from 1.36 to 3.32-fold increase relative to control (**Figure 15**).

Similarly, concentration 0.1 μM of compound 2 had the highest impact on the levels of IAA conjugates with Aspartic acid (IAAsp) in all treatment conditions. These levels increased by 1.27-fold in control conditions, 1.95-fold in salt and 1.43-fold in osmotic stress.

As presented, the highest increase in auxin levels happens under the effect of the lowest concentration of compound 2, whereas change in growth of the plants under the same culture conditions was not that visible.

These results show that the main changes in auxins levels occurred with the lowest concentrations of compound 2 (**Table 3**). However, in comparison with phenotyping data, the growth of the rosettes under the same culture conditions was not that visible

indicating that the changes in auxin levels was not the main reason of stress alleviation and growth promotion of *Arabidopsis* plants.

The most notable results were obtained in case of ABA. While in control conditions the lowest concentration of compound 2 caused decrease of ABA levels more than three times, in salt and osmotic stress conditions these levels increased 4.01-fold and 1.95-fold relative to control. What is more, all other concentrations of compound 2 in osmotic stress conditions have also affected the levels of ABA, the lower the concentration of this compound, the higher the increase (**Figure 15**).

Concerning compound 5, the increase in IAA levels was observed mainly in control conditions with concentrations 0.1 and 1 μM. These two concentrations of compound 5 caused 1.71 and 2.54-fold increase of IAA. In salt stress conditions, the change in the levels of IAA was not that significant.

In case of oxIAA levels, similar results were obtained with the same concentrations (0.1 and 1 μM) of compound 5 in control conditions. These levels increased 1.80 and 1.62-fold. While concentration 0.1 and 1 μM did not affect the levels of oxIAA in any of stress conditions, concentrations 1 and 10 μM in salt stress conditions caused a slight drop in oxIAA levels and in osmotic stress conditions increased the levels by 1.31 and 1.69-fold.

As illustrated in **Figure 15**, while in control conditions only the concentration 10 μM of compound 5 significantly increased the levels of IAAsp, in both stress conditions different results were achieved. In salt stress conditions concentrations 0.1, 1 and 100 μM lowered the IAAsp levels from 1.33 to 1.60-fold. Similar results were also observed in case of osmotic culture conditions where mainly concentration 0.1 and 100 μM decreased IAAsp levels.

Likewise in compound 2, the most interesting effect of plant pre-treatment with compound 5 was observed in ABA level changes. Regarding the control conditions, ABA levels did not change so significantly related to control, except with the lowest concentration (0.1 μM) which caused a slight increase (1.27-fold). Conversely to this, concentration 0.1 and 1 μM of compound 5 in salt stress conditions caused 1.65 and 1. 38-fold decrease in ABA levels. Different trend was observed in osmotic stress in which concentration 1 and 100 μM increased ABA levels by 1.55-fold.

Conditions	Compound	Concentration (µM)	IAA	oxIAA	IAAsp	ABA
Control	MOCK	-	37.74 ± 8.11	193.35 ± 4.89	28.07 ± 2.88	18.75 ± 2.97
	Compound 2	0.1	54.20 ± 14.73	360.77 ± 30.85	35.64 ± 3.32	26.6. ± 1.16
		1	41.38 ± 6.08	157.65 ± 20.96	31.29 ± 0.39	13.44 ± 2.10
		10	36.64 ± 6.81	169.72 ± 22.36	28.49 ± 3.00	14.32 ± 2.84
		100	43.47 ± 5.64	185.33 ± 9.97	28.38 ± 1.16	26.18 ± 6.20
	Compound 5	0.1	64.62 ± 2.37	348.07 ± 6.87	31.36 ± 2.97	23.81 ± 3.66
		1	44.68 ± 6.83	204.35 ± 48.49	29.62 ± 2.58	16.23 ± 3.49
		10	95.71 ± 7.54	312.92 ± 66.86	46.69 ± 3.25	19.81 ± 0.60
		100	47.02 ± 1.55	176.41 ± 0.16	32.00 ± 4.30	19.53 ± 1.24
Salt stress	MOCK	-	46.41 ± 8.07	126.15 ± 16.48	28.13 ± 1.25	15.40 ± 2.75
	Compound 2	0.1	76.60 ± 4.32	314.01 ± 126.16	54.88 ± 17.09	61.79 ± 23.97
		1	63.70 ± 13.15	147.77 ± 15.34	30.02 ± 1.89	18.31 ± 1.66
		10	42.59 ± 9.86	116.40 ± 14.55	31.24 ± 1.56	19.89 ± 0.10
		100	50.74 ± 3.64	115.71 ± 0.90	24.73 ± 2.18	15.95 ± 1.02
	Compound 5	0.1	54.38 ± 7.38	126.38 ± 3.97	19.52 ± 0.52	9.36 ± 0.30
		1	50.55 ± 4.40	96.87 ± 6.85	17.63 ± 0.73	11.15 ± 0.84
		10	52.44 ± 6.37	121.15 ± 12.94	31.90 ± 2.99	17.98 ± 4.47
		100	46.88 ± 1.89	108.06 ± 11.81	21.14 ± 0.00	15.80 ± 2.57
Osmotic stress	MOCK	-	< LOD	120.82 ± 18.88	20.45 ± 1.79	14.95 ± 0.73
	Compound 2	0.1	< LOD	400.68 ± 69.15	29.18 ± 0.59	29.11 ± 2.22
		1	< LOD	194.59 ± 29.65	18.80 ± 1.10	26.87 ± 3.45
		10	< LOD	166.10 ± 33.80	21.74 ± 2.38	24.50 ± 3.43
		100	< LOD	164.46 ± 2.51	20.18 ± 1.00	21.03 ± 0.20
	Compound 5	0.1	< LOD	115.37 ± 12.55	15.68 ± 0.51	13.68 ± 0.57
		1	< LOD	204.50 ± 37.00	20.84 ± 0.98	23.20 ± 3.19
		10	< LOD	130.19 ± 5.51	18.39 ± 2.32	16.90 ± 1.35
		100	< LOD	157.95 ± 29.27	13.85 ± 2.30	23.22 ± 0.44

Table 3: Content of Auxins and ABA (pmol.g^{-1} FW) in *Arabidopsis thaliana* plants primed with different CK analogs in four concentrations (0.1, 1, 10 and 100 µM) and cultured in control or stress conditions.

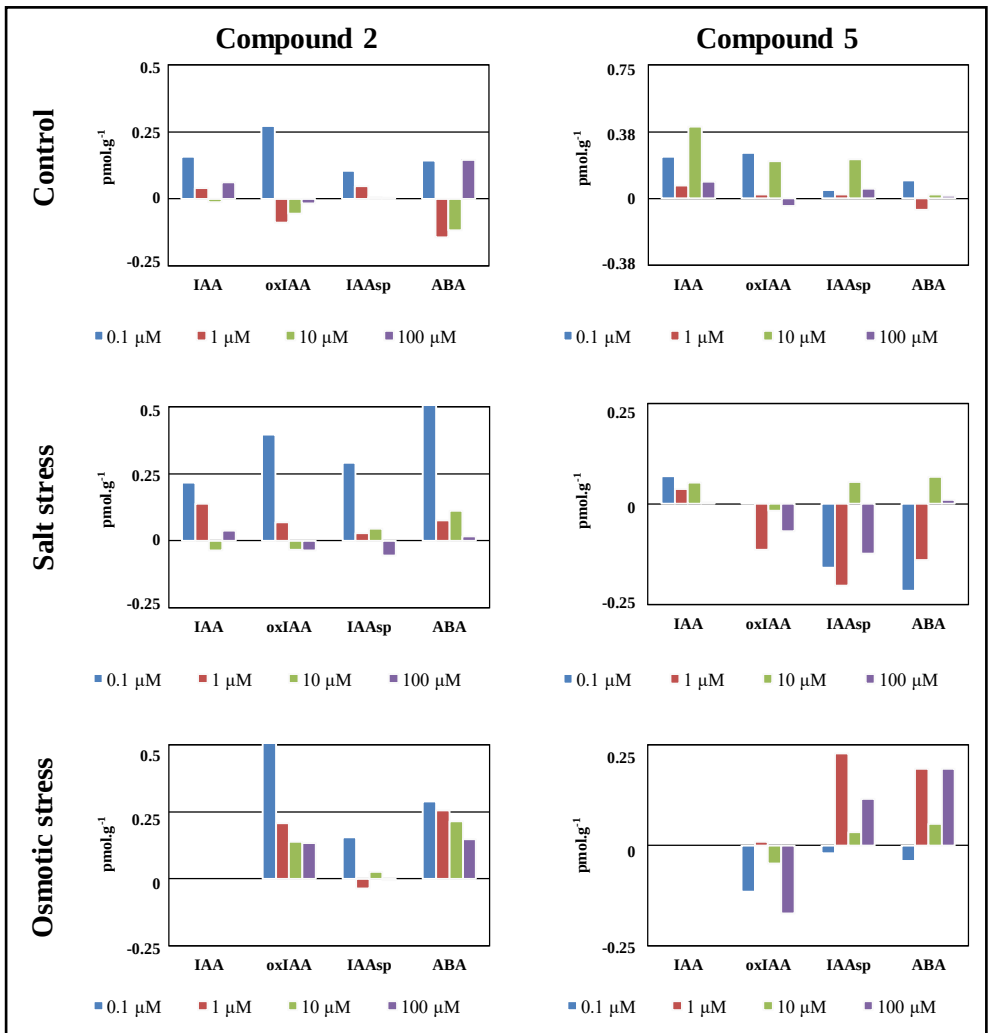

Figure 15: Changes in the levels of IAA, oxIAA, IAAsp and ABA due to the priming compounds and culture conditions.

5.7 Correlations between growth parameters and plant hormone levels

In additions, correlations between various growth parameters, such as rosette growth (Pixels), absolute growth rate (AGR), color index (GLI) and PBC index, obtained after HTS analysis were evaluated.

From the results presented in **Figure 16** and **Figure 17,** it is clear that after the treatment with compound 2 and compound 5, the most correlated in all treatment conditions (control, salt and osmotic stress) is DHZ-type of CKs. What is more, in case of compound 5, there were relatively high correlation between phenotyping traits and DHZ ribosides (DHZR) under both stress conditions and with iP-type of CKs under control and salt conditions (**Figure 17**).

Regarding auxins and ABA, no significant correlations were demonstrated except after priming with compound 2 under control conditions, in which a negative relationship was found between the GLI color index and the content of ABA and IAA. Besides that, the same primed plants also showed negative correlation between oxIAA and GLI when they were grown under osmotic stress conditions (**Figure 16**). Another exception was observed when seeds were primed with compound 5 and then grown under salt stress conditions induced with 100 mM NaCl (**Figure 17**). However, in this case, higher levels of IAA correlated with higher AGR (more growth) and better PBC index. Altogether, it is clear that the priming with CK analogs modified *Arabidospsis* hormonal homeostasis, changing the endogenous levles of CKs, auxins and ABA, and these changes depend on both the concentration of the compound applied and the plant growth conditions.

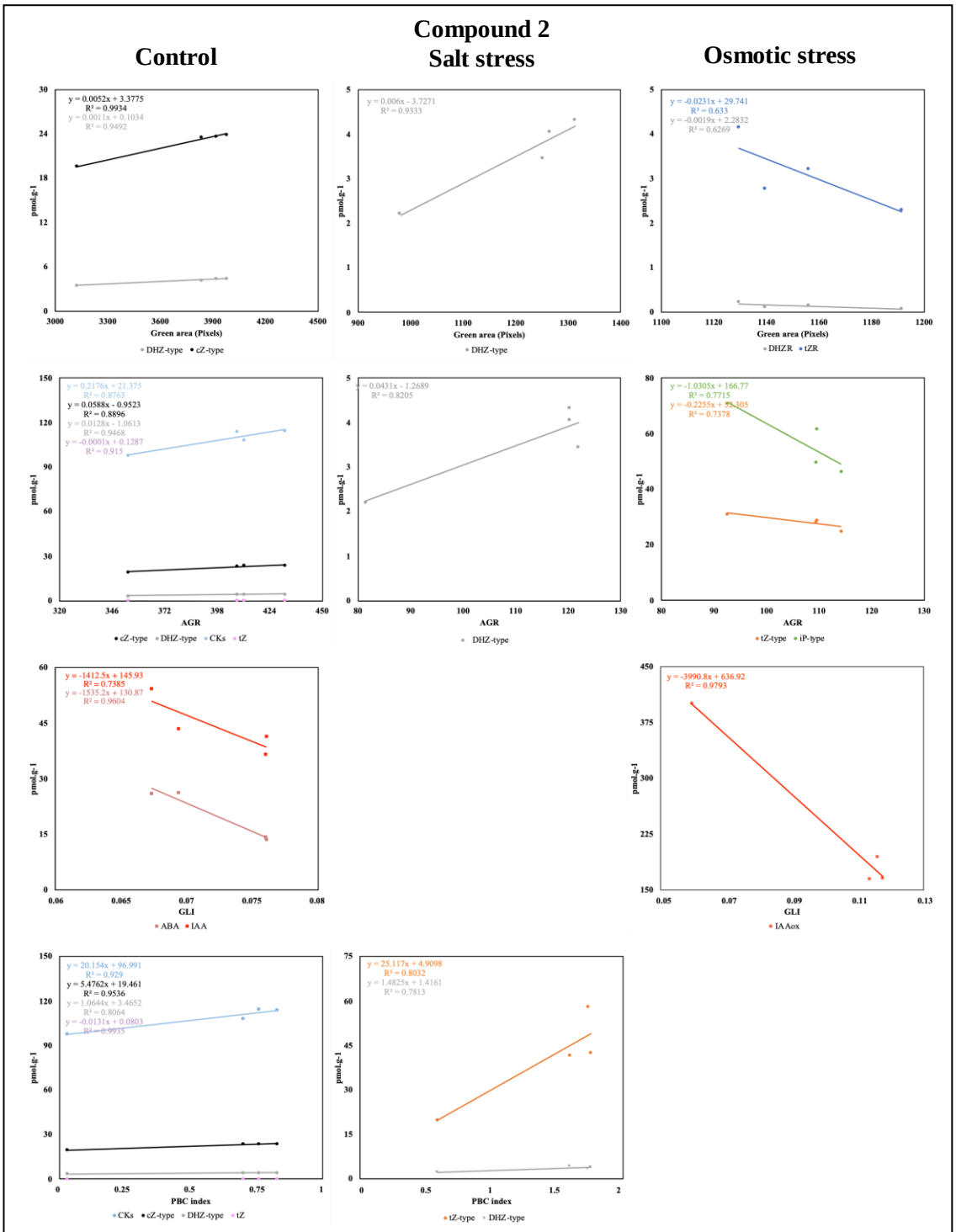

Figure 16: Correlations between different growth parameters and changes in plan hormone levels after *Arabidopsis* seed priming with compound 2.

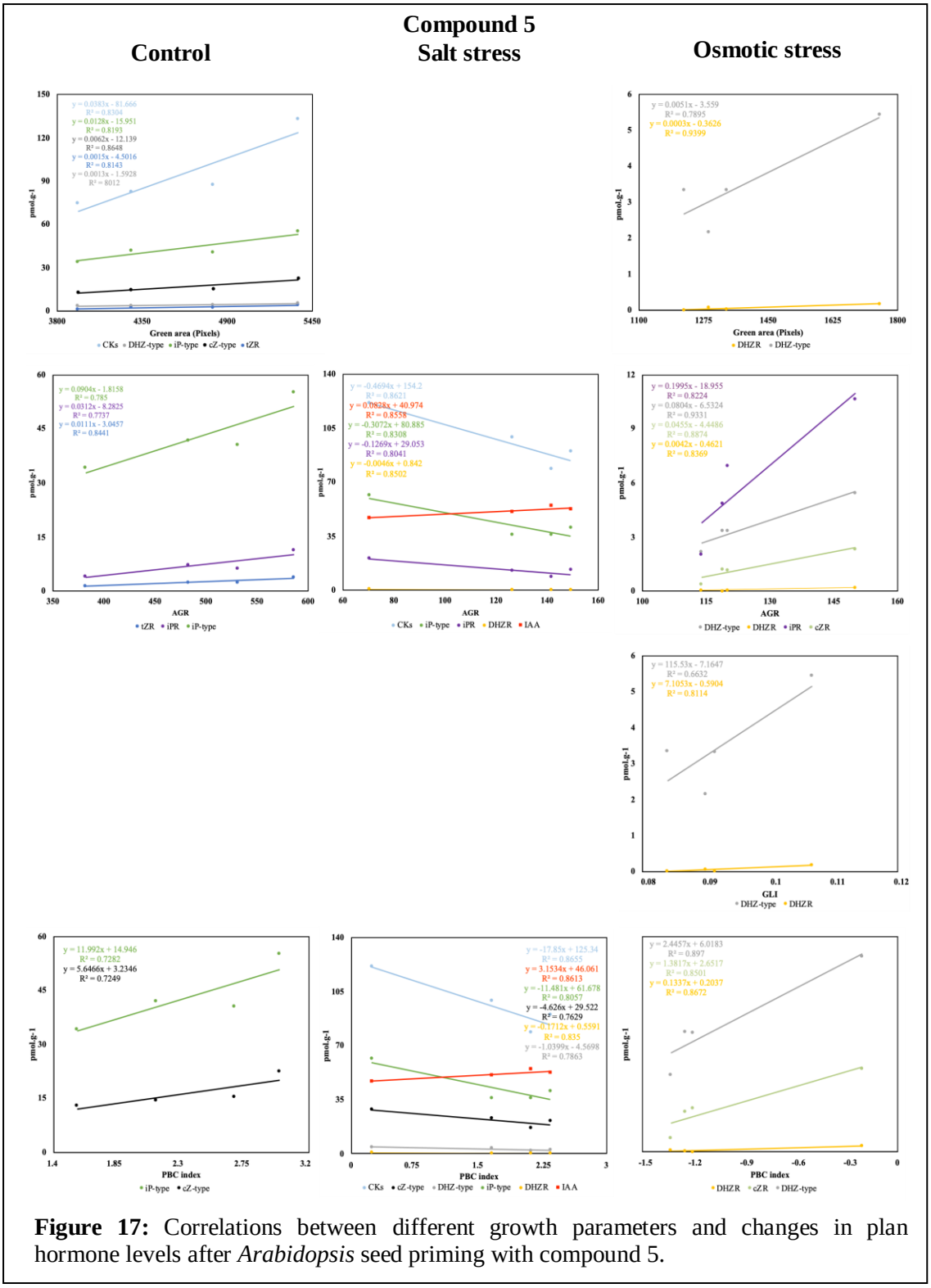

Figure 17: Correlations between different growth parameters and changes in plan hormone levels after *Arabidopsis* seed priming with compound 5.

6 Discussion

6.1 High-throughput phenotyping screening as efficient tool for chemical testing

Throughout the last years, a non-destructive image based high-throughput screening methods have become a powerful tool for obtaining and further analysis of different plant growth parameters (De Diego *et al.*, 2017; Granier *et al.*, 2006; Hartmann *et al.*, 2011; Rahaman *et al.*, 2015; Tanabata *et al.*, 2012; Walter *et al.*, 2007). Our research group is not an exception, and so developed a multi-trait high-throughput screening (MTHTS) method (Ugena *et al.*, 2018) which evaluate several traits of *Arabidopsis* plants (seedling establishment, RGR, AGR, rosette growth and changes in leaf color) in a high-throughput manner (thousand plants) under different growth conditions in only a single run.

6.2 Priming with different CK analogs promoted early seedling establishment and *Arabidopsis* rosette growth

As long as seedling emergence and uniform seed germination are essential stages in plants life cycle, the actual agriculture is attempting to employ various techniques with the aim to enhance growth, vitality, quality and yield. One of such methods is seed priming, in which seeds are treated with specific compounds prior to their sowing (Ibrahim, 2016; Savvides *et al.*, 2016; Ugena *et al.*, 2018). It has been previously approved that exogenous application of specific compounds (such as polyamines or amino acids) enhance plant tolerance to stress conditions and increase germination (Li *et al.*, 2014; Ugena *et al.*, 2018). Many other plant species has showed that the seed priming with certain hormones or other technologies can also improve seed germination and plant fitness (Hussain *et al.*, 2016; Van Hulten *et al.*, 2006) This is because seed priming has been proved to 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 & Arora, 2013). In this work, we demonstrate that the hormonal priming with different CK analogs induce early seed establishment and plant growth promotion in *Arabidopsis thaliana*.

Eight CK analogs from three different groups of compounds in four different concentrations were used as priming agents for the analysis of their mode of action and connection with plant response to control, salt and osmotic stress conditions.

With regards to PBC index (**Figure 15**), the best growth promoting effect was observed with the second group of compounds, concretely with the compound number 5. However, when the young *Arabidopsis* seedlings were grown under stress conditions, only compounds from the first group behaved as stress alleviators for the both, salt and osmotic stress conditions. Specifically, the best results were obtained when the compound 2 was used as seed priming agent (**Figure 15**).

The beneficial effect of seed priming with CKs has been previously described in many plant species and growth conditions such as spring wheat (*Triticum aestivum* L.) (Iqbal *et al.*, 2006; Iqbal & Ashraf, 2005) or basil (*Ocimum basilicum* L.) (Bagheri *et al.*, 2014). Despite that, the positive effect of the priming is not always clear or may also have a negative effect (Miyoshi & Sato, 1997; Sneideris *et al.*, 2015; Willams *et al.*, 2016). This must be due to the type of compound or the concentration used for priming. As corroboration, many compounds (e.g. compound 4) at high concentration (100 μM) showed a strong growth inhibitory effect. This is why it is so important to test these types of compounds in high-throughput manner in model plants such as *Arabidopsis*, and later on in the targeted species and growth conditions.

6.3 Seed priming affects *Arabidopsis thaliana* greenness

As already published in Ugena *et al.*, 2018, MTHTS method is suitable for obtaining not only different growing parameters but also for the analysis of leaf color changes. The extraction of the contribution to the red (R), blue (B) and green (G) color and their use in previously tested index allow us to estimate indirectly the chlorophyll content in leaves of *Arabidopsis thaliana* after each priming treatment and growth conditions.

In control and salt conditions, the hormonal priming did not affect GLI index or had a negative effect (**Figure 13A**). However, under osmotic stress conditions induced by 100 mM mannitol the primed seeds with the first group of CK analogs highly increased their greenness (compounds 1 to 4) (**Table 1**). Taking in account that the GLI index makes reference to the greenness (indirect chlorophyll content) and the nitrogen nutritional levels of the plant (Ugena *et al.*, 2018), we can say that this group contain a good anti-senescence compounds, protecting plant cells from the oxidative damage and hence delaying the degradation of the chlorophyll under osmotic stress conditions (Hönig *et al.*, 2018).

6.4 Seed priming alters plant hormone homeostasis

It is well known that phytohormones are necessary regulators of plant growth and stress responses. Under abiotic stress, it is well-known that both alternations of plant hormones and changes in the growth occur (Kaya *et al.*, 2006; Llanes *et al.*, 2016). In this regard, many studies have shown that several groups of plant hormones (e.g. auxins, ABA and CKs) in plants grown under various stress conditions (Hai *et al.*, 2020; Popko *et al.*, 2010; Youning Wang *et al.*, 2009). In addition, it has been proved that exogenous application of plant growth promoting compounds improves the plant tolerance to drought and salt stress (Li *et al.*, 2014; Podlešáková *et al.*, 2019; Prerostova *et al.*, 2018; Rulcová & Pospíšilová, 2001; Ugena *et al.*, 2018).

Here, two selected CK analogs (compound 2 and 5) and their effect on changes in endogenous levels of plant hormones as a response to stressful environmental conditions were tested. From our results, it is clear that hormonal priming induced changes in the endogenous hormonal levels of *Arabidopsis* plants, and the changes varied between concentrations and/or growth conditions. For example, priming with low concentrations of compound 2 caused an increase in levels of IAA, oxIAA and IAAsp under salt stress conditions. Unfortunately, IAA levels under osmotic stress were not detected (**Figure 14**). On the contrary, levels of oxIAA in these conditions increased with all concentrations of compound 2 indicating conversion of IAA into its oxidized, and these changes were correlated with a better GLI index (**Figure 16**). However, the changes of other auxin-related metabolites did not show any correlation with the phenotypical changes of the plants primed with compound 2.

Priming with compound 5 increased the endogenous levels of IAA under control and salt stress conditions. Besides, a clear positive correlation between IAA changes and ARG and PBC index was also detected in *Arabidopsis* seedling grown under salt stress conditions (**Figure 17**). The accumulation of IAA in some plant species under stress conditions is still unclear. One possible explanation to this accumulation can be that some plants increase the endogenous IAA levels in their leaves under stress conditions to activate a defense mechanism called epinasty (leaf curvature) that can help the plant to reduce the light capturing leaf surface (De Diego *et al.*, 2012).

The most remarkable results were between the changes of the endogenous CK levels and the analyzed phenotyping traits. Thus, in almost all the cases, the hormonal priming induced changes in the DHZ-type, which were positively related to the plant growth. However, the role of the DHZ-type CKs in plants is still unclear. Only when the seeds

were primed with the compound 5, the increase of iP-type CKs was negatively correlated to plant growth under salt stress conditions. This was mainly due to the iPR accumulation. In this regard, it has been reported that the accumulation of the ribosides forms under stress conditions can be a defense mechanism to deal against the stress (De Diego *et al.*, 2015; Man *et al.*, 2011; Veerasamy *et al.*, 2007).

In summary, the hormopriming with CK analogs is shown to be a good biotechnological approach to improve early seedling establishment and plant growth under control and stress conditions. However, the compounds they must be tested in high-throughput manner to define the better range of concentration and the growth conditions in which they are working more efficient.

7 Conclusion

The main purpose of this diploma thesis was the analysis of different growth parameters of *Arabidopsis thaliana* plants after their pre-treatment with eight CK analogs and after their further cultivation under various culture conditions. Changes in plant hormone levels were also subject of the interest.

The results acquired from the experiments proved that seed priming with CK analogs may induce plant growth promotion under control conditions and may be a good biotechnological approach to alleviate the negative effects caused by salt or osmotic stress.

Considering all three groups of compounds, compound 2 (first group) showed the best stress alleviating effect under salt and also osmotic stress conditions. On the other side, compound 5 (second group) had the best plant growth promoting effect on *Arabidopsis* plants under control conditions.

Moreover, seed priming with eight different CK analogs modify hormone homeostasis in a complex crosstalk between the three types of hormones analyzed (CKs, auxins and ABA) in order to regulate plant growth.

Based on the results it can be assumed that the use of previously mentioned compounds 2 and 5 as hormopriming agents on agriculturally important plants may have a huge perspective in a potential general use with the aim to better productivity, vitality and yield of these plants.

List of abbreviations

- 2D — two-dimensional
- ABA — abscisic acid
- Asp — aspartic acid
- CKs — cytokinins
- DAG — days after germination
- DMPP — dimethylallyl pyropho
- EtOH — ethanol
- FAO — The Food and Agricultural Organization
- FW — fresh weight
- *g* — G-force
- GGPP — geranylgeranyl pyrophosphate
- GLI — green leaf index
- HMBDP — (E)-4-hydroxy- 3-methyl-but-2-enyl diphosphate
- HPLC — high-performance liquid chromatography
- HTS — high-throughput screening
- IAA — indol-3-acetic acid
- iP — isopentenyl adenine
- IPP — isopentenyl pyrophosphate
- IS — internal standards
- LC — liquid chromatography
- LED — light emitting diode
- LOD — limit of detection
- LOG — LONELY GUY
- LRR — Department of Plant Growth Regulators
- MeOH — methanol
- MEP — 2-C-methyl-D-erythriol 4-phosphate, non-mevalonate pathway
- MES — 2-(N-Morpholino)ethanesulfomic acid
- MS — Murashige and Skoog
- MS — mass spectrometry
- MVA — mevalonate pathway
- *m/z* — mass to charge ration
- PAR m^{-2} s^{-1} — photosynthetically activated radiation per second and square meter
- PBC index — plant biostimulant characterization index
- PBS — Phosphate Buffer Solution
- PEG — polyethylene glycol
- pmol.g^{-1} — picomol per gram
- RGB — red-green-blue
- ROS — reactive oxygen species
- RGR, AGR — relative growth rate, absolute growth rate
- SPE — solid phase extraction
- Trp — tryptophan
- *t*Z/ *c*Z/ DHZ — *trans*-zeatin/ *cis*-zeatin/ dihydroxy-zeatin

Bibliography

Anosheh, H. P., Sadeghi, H., & Emam, Y. (2011): Chemical priming with urea and KNO3 enhances maize hybrids (*Zea mays L.*) seed viability under abiotic stress. *Journal of Crop Science and Biotechnology*, ***14*** (4), 289–295.

Antoniadi, I., Plačková, L., Simonovik, B., Doležal, K., Turnbull, C., Ljung, K., & Novák, O. (2015): Cell-type-specific cytokinin distribution within the arabidopsis primary root apex. *Plant Cell*, **27** (7), 1955–1967.

Ashraf, M., & Foolad, M. R. (2005): Pre-sowing seed treatment—A shotgun approach to improve germination, plant growth, and crop yield under saline and non-saline conditions. *Advances in Agronomy*, ***88***, 223–271.

Bajguz, A., & Hayat, S. (2009): Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiology and Biochemistry*, **47**, 1-8.

Beckers, G. J., & Conrath, U. (2007): Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biology*, **10** (4), 425–431.

Berger, B., de Regt, B., & Tester, M. (2012): High-Throughput Phenotyping of Plant Shoots. *Methods of molecular biology*, ***918,*** 9-20.

Bhardwaj, R., Ohri, P., Kaur, R., Rattan, A., Kapoor, D., Bali, S., Kaur, P., Khajuria, A., & Singh, R. (2014): Gene Silencing: A Novel Cellular Defense Mechanism Improving Plant Productivity under Environmental Stresses. In: *Emerging Technologies and Management of Crop Stress Tolerance*. Vol. 1, (Ahmad P. ed.), Elsevier Inc., 209–228.

Bielach, A., Hrtyan, M., & Tognetti, V. B. (2017): Plants under stress: Involvement of auxin and cytokinin. *International Journal of Molecular Sciences*, **18** (7).

Chen, K., & Arora, R. (2013). Priming memory invokes seed stress-tolerance. *Environmental and Experimental Botany*, **94**, 33–45.

Colmenero-Flores, J. M., & Rosales, M. A. (2014): Interaction between salt and heat stress: When two wrongs make a right. *Plant, Cell and Environment*., **37** (5), 1042–1045.

Conrath, U. (2011): Molecular aspects of defence priming. *Trends in Plant Science*, **16** (10), 524–531.

Davies, P. J. (2010): The Plant Hormones: Their Nature, Occurrence, and Functions. *Plant Hormones*, 1–15.

Davies, W. J., Kudoyarova, G., & Hartung, W. (2005): Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant's response to drought. *Journal of Plant Growth Regulation*, **24** (4), 285–295.

De Diego, N., Pérez-Alfocea, F., Cantero, E., Lacuesta, M., & Moncaleán, P. (2012): Physiological response to drought in radiata pine: Phytohormone implication at leaf level. *Tree Physiology*, **32** (4), 435–449.

De Diego, N., Saiz-Fernández, I., Rodríguez, J. L., Pérez-Alfocea, P., Sampedro, M. C., Barrio, R. J., Lacuesta, M., & Moncaleán, P. (2015): Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. *Journal of Plant Physiology*, **188**, 64–71.

De Diego, Nuria, Fürst, T., Humplík, J. F., Ugena, L., Podlešáková, K., & Spíchal, L. (2017): 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*, **8**, 1–16.

Du, F., Ruan, G., & Liu, H. (2012): Analytical methods for tracing plant hormones. *Analytical and Bioanalytical Chemistry*, **403** (1), 55–74.

Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd-Allah, E. F., & Hashem, A. (2017): Phytohormones and beneficial microbes: Essential components for plants to balance stress and fitness. *Frontiers in Microbiology*, **8**, 1-14.

Fahad, S., Hussain, S., Matloob, A., Khan, F. A., Khaliq, A., Saud, S., Hassan, S., Shan, D., Khan, F., Ullah, N., Faiq, M., Khan, M. R., Tareen, A. K., Khan, A., Ullah, A., Ullah, N., & Huang, J. (2015): Phytohormones and plant responses to salinity stress: a review. *Plant Growth Regulation*, **75**, 391-404.

Faragó, D., Sass, L., Valkai, I., Andrási, N., & Szabados, L. (2018): Plantsize offers an

affordable, non-destructive method to measure plant size and color in vitro. *Frontiers in Plant Science*, **9**, 219.
Finkelstein, R. (2013): Abscisic Acid synthesis and response. *The Arabidopsis Book*, **11**, e0166.
Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., & Galuszka, P. (2011): Evolution of cytokinin biosynthesis and degradation. *Journal of Experimental Botany*, **62** (8), 2431–2452.
Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., & Job, D. (2001): Proteomic Analysis of *Arabidopsis* Seed Germination and Priming 1. *Plant Physiology*, **126**, 835-848.
Gamir, J., Sánchez-Bel, P., & Flors, V. (2014): Molecular and physiological stages of priming: how plants prepare for environmental challenges. *Plant Cell Reports*, **33** (12), 1935–1949.
Gill, S. S., & Tuteja, N. (2010): Polyamines and abiotic stress tolerance in plants. *Plant Signaling and Behavior*, **5** (1), 26–33.
Granier, C., Aguirrezabal, L., Chenu, K., Cookson, S. J., Dauzat, M., Hamard, P., Thioux, J. J., Rolland, G., Bouchier-Combaud, S., Lebaudy, A., Muller, B., Simonneau, T., & Tardieu, F. (2006): PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. *New Phytologist*, **169** (3), 623–635.
Gull, A., Wani, N. U. I., & Lone, A. A. (2019): Biotic and Abiotic Stresses in Plants. In: *Abiotic and Biotic Stress in Plants*. (Oliviera A. B. ed.), Intech.
Ha, S., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L. S. P. (2012): Cytokinins: Metabolism and function in plant adaptation to environmental stresses,. *Trends in Plant Science*, **17**, 172-179.
Haberer, G., & Kieber, J. J. (2002): Cytokinins. New insights into a classic phytohormone. *Plant Physiology*, **128** (2), 354–362.
Hai, N. N., Chuong, N. N., Tu, N. H. C., Kisiala, A., Hoang, X. L. T., & Thao, N. P. (2020): Role and regulation of cytokinins in plant response to drought stress. *Plants*, **9** (4), 10–12.
Hare, P. D., Cress, W. A., & Van Staden, J. (1997): The involvement of cytokinins in plant responses to environmental stress. *Plant Growth Regulation*, **23** (1–2), 79–103.
Hartmann, A., Czauderna, T., Hoffmann, R., Stein, N., & Schreiber, F. (2011): HTPheno: An image analysis pipeline for high-throughput plant phenotyping. *BMC Bioinformatics*, **12** (1), 148.
Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., & Sakakibara, H. (2008): Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, **59** (1), 75–83.
Hönig, M., Plíhalová, L., Husičková, A., Nisler, J., & Doležal, K. (2018): Role of cytokinins in senescence, antioxidant defence and photosynthesis. *International Journal of Molecular Sciences*, **19** (12), 1–23.
Hussain, S., Khan, F., Cao, W., Wu, L., & Geng, M. (2016): Seed priming alters the production and detoxification of reactive oxygen intermediates in rice seedlings grown under sub-optimal temperature and nutrient supply. *Frontiers in Plant Science*, **7**, 1–13.
Hybenová, A. (2018): Study about possible positive interaction between plants and microbes. bachelor thesis, *Palacký University Olomouc*, Czech Republic.
Ibrahim, E. A. (2016): Seed priming to alleviate salinity stress in germinating seeds. *Journal of Plant Physiology*, **192**, 38–46.
Iqbal, M., & Ashraf, M. (2005): Presowing seed treatment with cytokinins and its effect on growth, photosynthetic rate, ionic levels and yield of two wheat cultivars differing in salt tolerance. *Journal of Integrative Plant Biology*, **47** (11), 1315–1325.
Iqbal, M., Ashraf, M., & Jamil, A. (2006): Seed enhancement with cytokinins: Changes in growth and grain yield in salt stressed wheat plants. *Plant Growth Regulation*, **50** (1), 29–39.
Ishihama, Y., Rappsilber, J., & Mann, M. (2006): Modular stop and go extraction tips with stacked disks for parallel and multidimensional peptide fractionation in proteomics. *Journal of Proteome Research*, **5** (4), 988–994.
Jafar, M. Z., Farooq, M., Cheema, M. A., Afzal, I., Basra, S. M. A., Wahid, M. A., Aziz, T., &

Shahid, M. (2012): Improving the performance of wheat by seed priming under saline conditions. *Journal of Agronomy and Crop Science*, **198** (1), 38–45.
Jamil, M., Lee, C., Rehman, S., Lee, D., Ashraf, M., & Rha, E. (2005): Salinity (NaCl) tolerance of *Brassica* species at germination and early seedling growth. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, **4** (4), 970–976.
Jisha, K. C., Vijayakumari, K., & Puthur, J. T. (2013): Seed priming for abiotic stress tolerance: An overview. *Acta Physiologiae Plantarum*, **35** (5), 1381–1396.
Kakimoto, T. (2003): Biosynthesis of cytokinins. *Journal of Plant Research*, **116** (3), 233–239.
Kaya, M. D., Okçu, G., Atak, M., Çikili, Y., & Kolsarici, Ö. (2006): Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus L.*). *European Journal of Agronomy*, **24** (4), 291–295.
Kazan, K. (2013): Auxin and the integration of environmental signals into plant root development. *Annals of Botany*, **112** (9), 1655–1665.
Kieber, J. J., & Schaller, G. E. (2014): Cytokinins. *The Arabidopsis Book*, **12**, e0168.
Kranner, I., Minibayeva, F. V., Beckett, R. P., & Seal, C. E. (2010): What is stress? Concepts, definitions and applications in seed science. *New Phytologist*, **188** (3), 655–673.
Kubala, S., Garnczarska, M., Wojtyla, Ł., Clippe, A., Kosmala, A., Zmieńko, A., Lutts, S., & Quinet, M. (2015): Deciphering priming-induced improvement of rapeseed (*Brassica napus L.*) germination through an integrated transcriptomic and proteomic approach. *Plant Science*, **231**, 94–113.
Lemmens, E., Deleu, L. J., De Brier, N., De Man, W. L., De Proft, M., Prinsen, E., & Delcour, J. A. (2019): The Impact of Hydro-Priming and Osmo-Priming on Seedling Characteristics, Plant Hormone Concentrations, Activity of Selected Hydrolytic Enzymes, and Cell Wall and Phytate Hydrolysis in Sprouted Wheat (*Triticum aestivum L.*). *ACS Omega*, **4** (26), 22089-22100.
Li, L., Zhang, Q., & Huang, D. (2014): A review of imaging techniques for plant phenotyping. *Sensors*, **14** (11), 20078–20111.
Lichtenthaler, H. K. (1996): Vegetation Stress: an Introduction to the Stress Concept in Plants. *Journal of Plant Physiology*, **148** (1–2), 4–14.
Liu, J.-H., Wang, W., Wu, H., Gong, X., & Moriguchi, T. (2015): Polyamines function in stress tolerance: from synthesis to regulation. *Frontiers in Plant Science*, **6**, 827.
Ljung, K., Sandberg, G., & Moritz, T. (2010): Methods of Plant Hormone Analysis. *Plant hormones*, 717-740.
Llanes, A., Andrade, A., Alemano, S., & Luna, V. (2016): Alterations of Endogenous Hormonal Levels in Plants under Drought and Salinity. *American Journal of Plant Sciences*, **7** (9), 1357–1371.
Lutts, S., Benincasa, P., Wojtyla, L., Kubala, S., Pace, R., Lechowska, K., Quinet, M., & Garnczarska, M. (2016): Seed Priming: New Comprehensive Approaches for an Old Empirical Technique. In: *New Challenges in Seed Biology - Basic and Translational Research Driving Seed Technology*. (Arajuno S. ed.), Intech.
Man, D., Bao, Y. X., Han, L. B., & Zhang, X. (2011): Drought tolerance associated with proline and hormone metabolism in two tall fescue cultivars. *HortScience*, **46** (7), 1027–1032.
Mano, Y., & Nemoto, K. (2012): The pathway of auxin biosynthesis in plants. *Journal of Experimental Botany*, **63** (8), 2853–2872.
Mishra, D., Shekhar, S., Singh, D., Chakraborty, S., & Chakraborty, N. (2018): Heat Shock Proteins and Abiotic Stress Tolerance in Plants. *Regulation of Heat Shock Protein Responses*, 41–69.
Miyoshi, K., & Sato, T. (1997): The effects of kinetin and gibberellin on the germination of dehusked seeds of indica and japonica rice (*Oryza sativa L.*) under anaerobic and aerobic conditions. *Annals of Botany*, **80** (4), 479–483.
Munns, R., & Tester, M. (2008): Mechanisms of Salinity Tolerance. *Annual Review of Plant Biology*, **59** (1), 651–681.
Nakaune, M., Hanada, A., Yin, Y. G., Matsukura, C., Yamaguchi, S., & Ezura, H. (2012): Molecular and physiological dissection of enhanced seed germination using short-term low-concentration salt seed priming in tomato. *Plant Physiology and Biochemistry*, **52**,

28–37.
Nawaz, J., Hussain, M., Jabbar, A., Nadeem, G. A., Sajid, M., Subtain, M., & Shabbir, I. (2013): Seed Priming A Technique. *International Journal of Agriculture and Crop Sciences*, **6** (20), 1373–1381.
Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D. T., Kojima, M., Werner, T., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K., Kakimoto, T., Sakakibara, H., Schmülling, T., & Tran, L. S. P. (2011): Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *Plant Cell*, **23** (6), 2169–2183.
Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., & Sandberg, G. (2004): Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. *Proceedings of the National Academy of Sciences of the United States of America*, **101** (21), 8039–8044.
Novák, O., Pěnčík, A., & Ljung, K. (2014): Identification and Profiling of Auxin and Auxin Metabolites. *Auxin and Its Role in Plant Development*, 39–60.
Novák, Ondřej, Hauserová, E., Amakorová, P., Doležal, K., & Strnad, M. (2008): Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry*, **69** (11), 2214–2224.
Pan, X., & Wang, X. (2009): Profiling of plant hormones by mass spectrometry. *Journal of Chromatography B*, **877** (26), 2806–2813.
Pan, X., Welti, R., & Wang, X. (2008): Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography–electrospray tandem mass spectrometry. *Phytochemistry*, **96** (8), 1773-1781.
Paparella, S., Araújo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D., & Balestrazzi, A. (2015): Seed priming: state of the art and new perspectives. *Plant Cell Reports*, **34** (8), 1281–1293.
Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., & Černý, M. (2018): Cytokinin at the crossroads of abiotic stress signalling pathways. *International Journal of Molecular Sciences*, **19** (8), 1–36.
Podlešáková, K., Ugena, L., Spíchal, L., Doležal, K., & De Diego, N. (2019): Phytohormones and polyamines regulate plant stress responses by altering GABA pathway. *New Biotechnology*, **48**, 53–65.
Popko, J., Hänsch, R., Mendel, R. R., Polle, A., & Teichmann, T. (2010): The role of abscisic acid and auxin in the response of poplar to abiotic stress. *Plant Biology*, **12** (2), 242–258.
Pospisilova. (2002): Partiticaption of phytohormones in regulation of leaf stomatal conductance. *Biologia Plantarum*, **46**, 491–506.
Prerostova, S., Dobrev, P. I., Gaudinova, A., Knirsch, V., Körber, N., Pieruschka, R., Fiorani, F., Brzobohatý, B., Cerný, M., Spichal, L., Humplik, J., Vanek, T., Schurr, U., & Vankova, R. (2018): Cytokinins: Their impact on molecular and growth responses to drought stress and recovery in arabidopsis. *Frontiers in Plant Science*, **9**, 1–14.
Rahaman, M. M., Chen, D., Gillani, Z., Klukas, C., & Chen, M. (2015): Advanced phenotyping and phenotype data analysis for the study of plant growth and development. *Frontiers in Plant Science*, **6**, 619.
Rodriguez-Furlán, C., Miranda, G., Reggiardo, M., Hicks, G. R., & Norambuena, L. (2016): High throughput selection of novel plant growth regulators: Assessing the translatability of small bioactive molecules from Arabidopsis to crops. *Plant Science*, **245**, 50–60.
Rulcová, J., & Pospíšilová, J. (2001): Effect of benzylaminopurine on rehydration of bean plants after water stress. *Biologia Plantarum*, **44** (1), 75–81.
Sadeghi, H., & Robati, Z. (2015): Response of Cichorium intybus L. to eight seed priming methods under osmotic stress conditions. *Biocatalysis and Agricultural Biotechnology*, **4** (4), 443–448.
Sah, S. K., Reddy, K. R., & Li, J. (2016): Abscisic acid and abiotic stress tolerance in crop plants. *Frontiers in Plant Science*, **7**, 1–26.
Savvides, A., Ali, S., Tester, M., & Fotopoulos, V. (2016): Chemical Priming of Plants Against Multiple Abiotic Stresses: Mission Possible? *Trends in Plant Science*, **21** (4), 329–340.

Seo, M., & Koshiba, T. (2002): Complex regulation of ABA biosynthesis in plants. *Trends in Plant Science*, **7** (1), 41–48.

Shi, H., Chen, L., Ye, T., Liu, X., Ding, K., & Chan, Z. (2014): Modulation of auxin content in Arabidopsis confers improved drought stress resistance. *Plant Physiology and Biochemistry*, **82**, 209–217.

Siddique, Z., Jan, S., Imadi, S., Gul, A., & Ahmad, P. (2016): Drought stress and photosynthesis in plants. In: *Water Stress and Crop Plants: A Sustainable Approach.* Vol. **1**, (Ahmad P. ed.), John Wiley & Sons, Ltd, 1–11.

Silva, A. T., Ligterink, W., & Hilhorst, H. W. M. (2017): Metabolite profiling and associated gene expression reveal two metabolic shifts during the seed-to-seedling transition in Arabidopsis thaliana. *Plant Molecular Biology*, **95** (4–5), 481–496.

Sneideris, L. C., Gavassi, M. A., Campos, M. L., D'amico-Damião, V., & Carvalho, R. F. (2015): Effects of hormonal priming on seed germination of pigeon pea under cadmium stress. *Anais Da Academia Brasileira de Ciencias*, **87** (3), 1847–1852.

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E., & Mittler, R. (2014): Abiotic and biotic stress combinations. *New Phytologist*, **203** (1), 32–43.

Svačinová, J., Novák, O., Plačková, L., Lenobel, R., Holík, J., Strnad, M., & Doležal, K. (2012): A new approach for cytokinin isolation from *Arabidopsis* tissues using miniaturized purification: pipette tip solid-phase extraction. *Plant Methods*, **8** (17).

Tanabata, T., Shibaya, T., Hori, K., Ebana, K., & Yano, M. (2012): SmartGrain: High-throughput phenotyping software for measuring seed shape through image analysis. *Plant Physiology*, **160** (4), 1871–1880.

Tarkowská, D., Novák, O., Floková, K., Tarkowski, P., Turečková, V., Grúz, J., Rolčík, J., & Strnad, M. (2014): Quo vadis plant hormone analysis? *Planta*, **240** (1), 55–76.

Uddin, M. N., Hossain, M. A., & Burritt, D. (2016): Salinity and drought stress: Similarities and differences in oxidative responses and cellular redox regulation. In: *Water Stress and Crop Plants: A Sustainable Approach.* Vol. **1**, (Ahmad P. ed.), John Wiley & Sons, Ltd, 86–101.

Ugena, L., Hýlová, A., Podlešáková, K., Humplík, J. F., Doležal, K., Diego, N. De, & Spíchal, L. (2018): Characterization of Biostimulant Mode of Action Using Novel Multi-Trait High-Throughput Screening of *Arabidopsis* Germination and Rosette Growth. *Frontiers in Plant Science*, **9**.

Van Hulten, M., Pelser, M., Van Loon, L. C., Pieterse, C. M. J., & Ton, J. (2006): Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, **103** (14), 5602–5607.

Veerasamy, M., He, Y., & Huang, B. (2007): Leaf senescence and protein metabolism in creeping bentgrass exposed to heat stress and treated with cytokinins. *Journal of the American Society for Horticultural Science*, **132** (4), 467–472.

Walter, A., Scharr, H., Gilmer, F., Zierer, R., Nagel, K. A., Ernst, M., Wiese, A., Virnich, O., Christ, M. M., Uhlig, B., Jünger, S., & Schurr, U. (2007): Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: A setup and procedure designed for rapid optical phenotyping of different plant species. *New Phytologist*, **174** (2), 447–455.

Wang, Yongyin, Mopper, S., & Hasenstein, K. H. (2001): Effects of salinity on endogenous ABA, IAA, JA, and SA in Iris hexagona. *Journal of Chemical Ecology*, **27** (2), 327–342.

Wang, Youning, Li, K., & Li, X. (2009): Auxin redistribution modulates plastic development of root system architecture under salt stress in *Arabidopsis thaliana*. *Journal of Plant Physiology*, **166** (15), 1637–1645.

Wani, S. H., Kumar, V., Shriram, V., & Sah, S. K. (2016): Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal*, **4** (3), 162–176.

Werner, T., & Schmülling, T. (2009): Cytokinin action in plant development. *Current Opinion in Plant Biology*, **12** (5), 527–538.

Willams, F. S. B., Fábio, S., Leandro, C. M. de O., Paulo, H., & Menezes, das C. (2016): Comparison of seed priming techniques with regards to germination and growth of watermelon seedlings in laboratory condition. *African Journal of Biotechnology*, **15** (46), 2596–2602.

Yadav, P. V., Kumari, M., & Ahmed, Z. (2011): Seed priming mediated germination improvement and tolerance to subsequent exposure to cold and salt stress in Capsicum. *Research Journal of Seed Science*, **4** (3), 125-136.

Yang, G., Liu, J., Zhao, C., Li, Z., Huang, Y., Yu, H., Xu, B., Yang, X., Zhu, D., Zhang, X., Zhang, R., Feng, H., Zhao, X., Li, Z., Li, H., & Yang, H. (2017): Unmanned Aerial Vehicle Remote Sensing for Field-Based Crop Phenotyping: Current Status and Perspectives. *Frontiers in Plant Science*, **8**.

Zaharia, L. I., Walker-Simmon, M. K., Rodríguez, C. N., & Abrams, S. R. (2005): Chemistry of Abscisic Acid, Abscisic Acid Catabolites and Analogs. *Journal of Plant Growth Regulation*, **24** (4), 274–284.

Zhao, C., Zhang, Y., Du, J., Guo, X., Wen, W., Gu, S., Wang, J., & Fan, J. (2019): Crop phenomics: Current status and perspectives. *Frontiers in Plant Science*, **10**

Zhao, Y. (2014): Auxin Biosynthesis. *The Arabidopsis Book*, **12**, e0173.

Zhu, J.-K. (2002): Salt and Drought Stress Signal Transduction in Plants. *Annual Review of Plant Biology*, **53** (1), 247–273.