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Study about possible positive interaction between plants and microbes

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In Olomouc 2018

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Andrea Hybenová

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Abstrakt:	Táto bakalárska práca je zameraná na interakciu rastlín s prchavými zlúčeninami vylučovanými fytopatogénnym mikroorganizmamom a tiež na interakciu rastlín s exsudátmi tohto organizmu. Analýzy rastu rastlín boli vykonané pomocou vysoko výkonného fenotypingu. Ďalším cieľom tejto štúdie bolo preukázanie vysokých korelácií medzi projektovanou plochou a klasickým určením rastlinnej biomasy. Kvantifikácia hladín rôznych druhov fytohormónov zapojených do odlišných procesov, ktoré pozitívne ovplyvňujú rast rastlín bola taktiež predmetom tejto štúdie.
Kľúčové slová:	biostimuláty, *Alternaria alternata*, patogénne mikroorganizmi, *Arabidopsis thaliana*, biotický stres, volatilné látky, exudáty, fytohormóny, cytokiníny, auxíny, vysoko-výkonný fenotyping
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Abstract:	This bachelor thesis pays a particular attention in the interaction between plants and volatile compounds emitted by phytopathogenic microorganism, as well as the interaction of plants with the exudates of this organism. The analyses were performed by a high-throughput phenotyping. Another of the purposes of this research was the demonstration of the high correlations between the projected area and the classical biomass determination. The quantification of the levels of different phytohormones involved in different processes of the plant growth was also an object of interest.
Keywords:	biostimulants, *Alternaria alternata*, pathogenic microorganisms, *Arabidopsis thaliana*, biotic stress, volatile compounds, exudates, phytohormones, cytokinins, auxins, high-throughput phenotyping
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Contents

List of abbreviations

- 2D two-dimensional
- AA *Alternaria alternata*
- AACP amino acid containing products
- ABA abscisic acid
- Al aluminium
- Asp aspartic acid
- BF Bieleski buffer
- BRs brassinosteroids
- CFIM chlorophyll fluorescence imaging
- CKs cytokinins
- Co cobalt
- DMAPP dimethylallyl pyrophosphate
- EBIC European Biostimulant Industry Council
- ET ethylene
- EtOH ethanol
- FAO Food Association Organization
- FW/ DW fresh weight/dry weight
- g G-force
- GA gibberellins
- GGPP geranylgeranyl pyrophosphate
- Glu glutamic acid
- HCP hormone containing products
- HPLC high-pressure liquid chromatography
- HS humic substances
- HTP/HTPP high-throughput phenotyping/ high-throughput phenotyping platform
- IAA indol-3-acetic acid

- iP — isopentenyl adenine
- IPP — isopentenyl pyrophosphate
- IS — internal standards
- ISR/ SAR — induced systematic resistance/ systemic acquired resistance
- JA — jasmonic acid
- LED — light emitting diode
- LOD — limit of detection
- 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
- MIVOISAP — Microbial Volatiles Induced Starch Accumulation Process
- MS — Murashige and Skoog
- MVA — mevalonate pathway
- Na — sodium
- PAR m^{-2} s^{-1} — photosynthetically activated radiation per second and square meter
- PBS — Phosphate Buffer Solution
- PGPR/ PGPF — plant-growth promoting rhizobacteria/ plant-growth promoting fungi
- RGB — red-green-blue
- SA — salicylic acid
- Se — selenium
- Si — silicon
- SL — strigolactones
- Trp — tryptophan

- *t*Z/ *c*Z/ DHZ — *trans*-zeatin/ *cis*-zeatin/ dihydroxy-zeatin
- UHPLC-MS/MS — Ultra-high performance liquid chromatography tandem mass spectrometry
- USSR — Union of Soviet Socialist Republics
- VCs — volatile compounds

Objectives

The main objective of this bachelor thesis was the development and optimization of a growing protocol for *Arabidopsis thaliana* plants focus on the positive effects of interaction between plants and exudates and plants and fungal volatile compounds (VCs) emitted by *Alternaria alternata* (AA).

In the theoretical part, literary sources related to the topic of the bachelor thesis were compiled, specifically on biostimulants and their interactions in plants.

For the performance of the experiments, high-throughput phenotyping platform Olophen was used as a tool to give reliable and reproducible data about the biostimulant effects on plant performance.

In addition, quantification of levels of some phytohormones was performed in order to investigate their possible involvement in plant responses to AA exudates and volatiles and to study the crosstalk between them.

Afterwards, the evaluation of the obtained results and their comparison with those published in the literature was carried out. Finally, the discussion and a clear formulation of conclusions about the work were performed.

1. Introduction

Plants are sessile organisms exposed to numerous external stimuli caused by many environmental changes. That is why the growth of plants has to be regulated and adjusted to their needs. Adaptation of crops to these stresses is necessary for enhancement of the plant growth and productivity. One of the most innovative and promising solutions to address these important challenges consists of the use of plant biostimulants.

Biostimulants are defined as a materials that contain substances and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate plant growth, increase their quality, productivity, and yield and tolerance to different biotic and abiotic stresses, independently of its nutrient content. (Yakhin *et al.*, 2016). There are several sources of biostimulants depending on their biological origin, e.g. bacteria, fungi, seaweeds, higher plants, animals, etc.

Microbes synthesize different substances including carbohydrates, proteins, lipids, amino acids and hormones. Many of these microorganisms, including non-pathogenic and pathogenic fungi and bacteria, may also emit VCs and exudates, which participate in some interactions promoting plant growth. They may activate plant immune system, enhance plants photosynthetic capacity, help to increase the uptake of water and nutrients from the soil or either lower negative effects of biotic and abiotic stress (Sánchez-López *et al.*, 2016a).

In this study, in order to obtain a higher knowledge of the mechanisms and plant responses during the plant-microbe interactions, *Arabidopsis thaliana* plants were exposed to continuous presence of VCs and exudates emitted by the opportunistic fungal plant pathogen AA.

High - throughput phenotyping (HTP) approaches have become an essential tool in plant physiology providing a chance not only to investigate several traits collectively in one run but also to understand the mechanisms of action of biostimulants.

The study and development of a protocol for the screening of biostimulants has been established using previously mentioned technique to determine the overall *Arabidopsis* plants performance in response of different treatments. HTP combines various methods of automated, non-destructive and simultaneous analyses of plant growth, morphology and photosystem efficiency using RGB and chlorophyll fluorescence imaging (CFIM). The advances in this integrative system enable to perform large mapping populations and

collect a huge amount of data reducing time-consuming processes and rising the understanding of quantitative plant phenotyping traits.

Furthermore, quantification of levels of phytohormones, namely cytokinins (CKs), auxins and abscisic acid (ABA) were performed in order to investigate the potential involvement of these hormones in the plant responses to AA exudates and VCs and to be able to highlight possible patterns of interactions between them.

2. Present state of knowledge

2.1 Stress in plants

Plants are sessile organisms exposed to rapidly changing environment without any possibility to escape. In order to cope with these changes, plants have to respond to numerous external stimuli leading to plant adaptation to specific growing conditions. When growing in unfavourable conditions to which plants are not able to acclimate and resist them, their growth may be inhibited. The most important stage in plants life cycle is seedling emergence that strongly influences the success of growth and represents the moment of transformation from heterotrophic to autotrophic organism (Mercer *et al.*, 2011; Arsovski *et al.*, 2012). All stress factors may affect plant growth, yield and other physiological, biochemical and morphological processes (Fahad *et al*., 2015; Sharma *et al.*, 2016). One of the challenges for agriculture worldwide is to enhance the quality of crops, improve plant yield and productivity in unfavourable growth conditions. (Gill and Tuteja, 2010).

The term plant stress defined by Lichtenthaler (Lichtenthaler, 1996) refers to any unfavourable condition or substance that affects or blocks plant metabolism, growth or development.

Based on the origin, stress conditions are divided into two main groups - abiotic and biotic stress. Abiotic stressors are all non-living environmental factors that can have a negative impact on a plants ability to grow and thrive in a given environment. These stress factors include temperature stress (both extreme heat and extreme cold), water stress, aridity or salinity (Bajguz and Hayat, 2009). Similarly, biotic stress involving beneficial or harmful insects, pathogens, nematodes and weeds may also influence plant developmental and signalling processes (Bhardwaj *et al.*, 2014).

Another factor to take in account is the regeneration phase of plants, when the stressors are removed, differentiating them between eu-stress and dis-stress (Lichtenthaler, 1998). Eu-stress is an activating, stimulating stress and a positive element for plant development, whereas dis-stress is a severe stress that negatively affects the plant and causes damage.

In order to survive, plants have developed many sophisticated mechanisms as a response to all these kinds of environmental stimuli. They are continuously adjusting their physiology throughout the lifespan. Depending of the species, the lifespan of a plant can take several months or even hundreds of years. The stress tolerance threshold depends not only on the plant species, but also on the type of stressors applied and on the

predisposition of the plant (Lichtenthaler, 1998). As illustrated in **Figure 1**, there are four phases in plant development induced by stress:

1. Response phase: alarm reaction (beginning of stress), reduction of vitality.
2. Restitution phase: stage of resistance (continuing stress), adaptation process.
3. End phase: exhaustion (long-term stress), stress intensity is too high.
4. Regeneration phase: partial or full regeneration of the physiological functions.

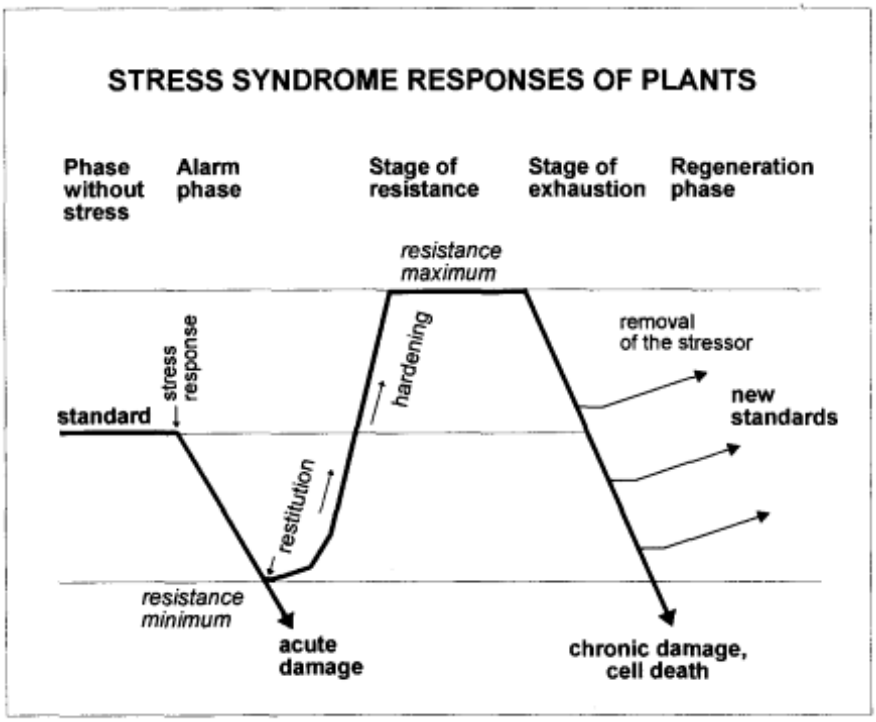

Figure 1: Phase sequences and responses induced in plants by stress exposure (Lichtenthaler, 1998).

All these responses are intervened by plant growth regulators (phytohormones) which play an important role in the adaptation of plant to environmental changes suggesting the existence of a network between the different hormone signalling pathways (Peleg and Blumwald, 2011).

2.1.1 Plant Growth promoting rhizobacteria / fungi.

In 1904, Lorenz Hiltner was the first scientist who described the term „rhizosphere". The rhizosphere is a layer of the soil surrounding the roots of plants. It is a dynamic complex of plant roots with soil microorganisms, particularly bacteria or fungi, for which the rhizosphere form a very beneficial habitat (Gnanamanickam, 2006; Calvo *et al.*, 2014). Plant roots secrete many organic compounds, for example organic acids or monosaccharides, which are very attractive for soil microorganisms and can also act as their nutrients (de Weert and Bloemberg, 2007; Podile and Kishore, 2007).

Over 80% of plants worldwide are colonized with this divers microflora with which they create a symbiotic relationship and subsequently may lower the effects of biotic and abiotic stresses on plants (van Loon and Bakker, 2007). Root-microbe interactions or the interactions between microorganisms are the main types of rhizosphere interactions that can be either neutral, beneficial or harmful.

The beneficial microbes positively affecting plants were firstly defined by Joseph Kloepper and Milton Schroth. They named them plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1981). PGPR enhance plant growth by increasing plant biomass, yield, root proliferation and by production or even degradation of plant hormones (Ryu *et al.*, 2003a). Beneficial rhizosphere fungi promoting plant growth are called plant growth-promoting fungi (PGPF) (Le Mire *et al.*, 2016). In addition to this, PGPF have also the ability to protect plant against the deleterious microorganisms. They have also demonstrated the positive effects on seed germination, plant growth and stress tolerance by hormone synthesis, activation of induced systematic resistance (ISR), systemic acquired resistance (SAR) or inhibition of growth of other pathogens (Kanchiswamy *et al.*, 2015; Li *et al.*, 2016). ISR and SAR are two types of a plant resistance formed as a result to plant defence against non-pathogenic (ISR) or pathogenic (SAR) organisms (Ryu *et al.*, 2004). Recent studies have reported that certain PGPF strains promote plant growth through the production of plant growth-promoting compounds such as phytohormones and volatiles (Hossain *et al.*, 2017).

2.1.2 Volatile compounds (VCs)

Currently, plant growth promotion by VCs produced by diverse microorganisms is the most recently identified mechanism.

Volatiles emitted by phylogenetically diverse fungi or bacteria are small molecules with masses not more than 300 Da, with low polarity and a high vapour pressure, e.g. aldehydes, ketones or alcohols. These substances are essential for many plant-microbe interactions and so can promote the growth and flowering of plants by facilitating nutrient uptake, photosynthesis and defence responses (Sánchez-López *et al.*, 2016a; Ryu *et al.*, 2003b). However, some mixtures of VCs can have an opposite effect and so may inhibit the plant growth.

Plant growth promotion of VCs is not limited to beneficial microorganisms but include also many different phytopathogens. In 2010, Ezquer *et al*. described how VCs from different microbes, including Gram-positive and Gram-negative bacteria and fungi, affect the starch metabolism. After 2 days of exposure of potato leaves (*Solanum tuberosum* L.) to VCs, the measurement of starch and soluble sugars in plant leaves cultured in presence or absence of microbial cultures showed that all tested fungi and bacteria emitted VCs which promoted a high accumulation of starch in potato leaves combined with the increased plant growth even without physical contact between plants and microbes (Li *et al.*, 2011). According to this, the term MIVOISAP (MIcrobial VOlatiles Induced Starch Accumulation Process) was established. For further demonstration of MIVOISAP, time-course analyses of starch accumulation in leaves of *Arabidopsis* exposed to continuous presence or absence of AA VCs was performed. The obtained results exhibit that MIVOISAP in *Arabidopsis* plants enhanced the starch biosynthesis and caused changes in the expression of genes involved in multiple processes (Li *et al.*, 2011). Based on the results obtained in previous studies, after exposure of *Arabidopsis* plants to phytopathogenic fungi AA, promotion of plant growth was associated with enhanced photosynthesis, accumulation of starch and cytokinin production (Sánchez-López *et al.*, 2016a).

Findings about how microbial VCs may positively affect plant growth and other physiological processes indicate that the interactions between plants and pathogenic microorganisms are very diverse and complex. All these information arise questions about potential application and ecological significance of VCs in the near future.

2.2 Plant biostimulants

In the last decades, the population has risen dramatically and it is predicted to increase by 34% in 2050, being necessary to satisfy the growing demand in agriculture (FAO, 2009). With the resource depletion and ecosystem degradation, the main challenge for agriculture is to increase the crop yield. With the demand of biostimulants continuously increasing in the marketplace and the need to satisfy the requirements of crops, companies are introducing many innovative products composed of different ingredients (Sharma *et al.*, 2016). The problem becomes with the unregulation in the legislation, because of the unspecific framework in the lack of a formal definition and acceptance of the concept. The use of biostimulants contributes to make agriculture more sustainable and environmentally friendly. On top of that, biostimulants offer an alternative to synthetic protectants. They provide a possible new approximation for the regulation of physiological processes in plants to stimulate growth, improve plant tolerance to environmental disturbances, to alleviate stress-induced limitations and to increase the yield. (Yakhin *et al.*, 2017).

2.2.1 Evolution of the definition of biostimulants

During the years, there have been several attempts to define plant biostimulants. Initially, the term "biogenic stimulant" was used in 1933 by Prof. V.P. Filatov in the USSR. He defined it as a biological material derived from various organisms, including plants, that after exposure to various stressors could affect metabolic processes in animals and plants (Yakhin *et al.*, 2017). After this idea, there were further developments of the biostimulants definition including the applications of those compounds on plants. Real plant biostimulant approach was firstly used in 1994 by Herve, who defined biostimulants as "products that, in small quantities, have reproducible benefits in plant growth and is ecologically benign" (Herve, 1994). Since that moment, biostimulants were considered as a subgroup of growth regulators. Along the years, several studies have greatly contributed to clarify and specify the conceptual and methodological development of biostimulants. However, despite the efforts, the term "biostimulant" is still not well defined due to diversity of contributions with a high breadth of concept.

The evidence of that fact was shown by two industries, one in Europe and one in North America. Each of these has adopted their own definitions describing the effects of biostimulants on plants. The European Biostimulant Industry Council (EBIC) in 2012

adopted definition of biostimulnts as “containing substances or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/ benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality”. On the other hand, the “Biostimulant Coalition” of North America described the term as “substances, including microorganisms, that are applied to plant, seed, soil or other growing media that may enhance the plants ability to assimilate applied nutrients, or provide benefits to plant development” (Povero *et al.*, 2016; Calvo *et al*., 2014).

Considering that biostimulants will take an important role in the agriculture in the future, it is important to establish a legal framework for marketing and regulation of these products to solve the problematic exposed at the beginning of this section.

2.2.2 Evolution of biostimulants classification

In the same manner as the evolution of the definition, the classification of biostimulants has changed. One of the first classifications of them was conducted by Filatov who classified 4 main groups of “biogenic stimulants”, including carboxylic acids and hydroxy acids, unsaturated acids and phenolic aromatic acids (Yakhin *et al.*, 2017). In 2007, Kaufman *et al.* summarized the definition of biostimulants as substances with a variety of formulations classified into three major groups depending on their original source and content: humic substances (HS), hormone containing products (HCP), and amino acid containing products (AACP). A year later, Basak (2008) suggested the classification of biostimulants based on their mechanism of action and also on their origin. But it was not until 2012 when du Jardin (du Jardin, 2012), provided in-depth classification. In contrast, Bulgari *et al.* (2015) proposed that biostimulants should be defined only by their physiological effect on plants rather than the composition. Based on the bibliographic analyses of reviews focused on plant growth promotion and biotic stress, du Jardin (du Jardin, 2012, 2015) firstly classified eight main groups of compounds reported as biostimulants. These groups involve: humic and fluvic acids, protein hydrolysates and other N-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, beneficial bacteria and beneficial fungi.

Humic substances (HS) are the most common organic molecules that may vary in their origin. They are end products of microbial decomposition and chemical degradation of dead biota in soils. There have been many positive effects of HS on plant growth

identified including nutrient uptake, change in root morphology, improvement of soil structure and solubility of phosphate and of the micronutrients (Halpern *et al.*, 2015).

Protein based-products are divided in two groups: amino acids and peptides mixtures obtained by enzymatic protein hydrolysis from plant sources. There are 20 structural amino acids involved in protein synthesis. By the application of some amino acids, e.g. histidine, proline, glycine or arginine, the effects of stress factors on plants can lower. The main purpose of the use of amino acids as a biostimulant is the protection against abiotic and biotic stress, enrichment of soil microbial activity and subsequently biomass production. The accumulation of other N-containing substances, including polyamines and betaines, have also been reported to better stress tolerance in plants (Calvo *et al.*, 2014; Halpern *et al.*, 2015).

There are five main inorganic beneficial elements including aluminium (Al), selenium (Se), silicon (Si), cobalt (Co) and sodium (Na). All these elements present in the soil positively affect plant growth, increase the quality of plant products and enhance plant stress responses (du Jardin, 2015). Inorganic salts from essential chemical elements, such as phosphates and phosphites, silicates, chlorides or sulphates act as a protection against soil microorganisms. They either directly suppress fungicidal effects or indirectly sustain plant defence reactions among others (du Jardin, 2012a).

To enhance plant productivity and soil fertility, seaweed extracts have been widely used. By liquefying of seaweeds, these extracts can be easily applied on plants in order to improve mineral uptake and plant growth, flowering and yield. Seaweed extracts are basically mixtures of plant hormones, micronutrients and saccharides which also help to lower abiotic and biotic stresses (Calvo *et al.*, 2014; Halpern *et al.*, 2015).

Chitosan is a form of chitin with a different signalling pathways resulting in a fungal pathogen protection, tolerance to various environmental stresses and improvement of trait quality (du Jardin, 2015).

In the case of beneficial bacteria, it is worth mentioning their classification (1) based on the way of interaction with plants (from soil to the cells, via the seeds, etc.) and (2) dependent on their agricultural use as a biostimulants considering the taxonomical, functional and ecological diversity (endosymbionts and rhizosperics (PGPR)). Influence in nutrition and plant growth, development and stress tolerance, among others are the principal effects promoted by these kind of biostimulants (du Jardin, 2015).

Fungal-based products are mostly non-pathogenic fungi with a physical interaction with plant roots. This interaction is either mutualistic symbiosis or parasitism. As

described previously, rhizosphere fungi which have many beneficial effects in plants are called plant growth-promoting fungi (PGPF). Furthermore, there are several studies describing the beneficial effects of VCs emitted by various microorganisms including fungi. Up to now, only 69 fungi have been identified as volatile emitters. Taking in consideration that at least a million of microbial species is expected to exist on the Earth, up to date only around 10,000 of them have been identified (Lemfack *et al.*, 2014). However, other studies have been performed proving the evidence that not only non-pathogenic but also pathogenic fungi without physical contact are beneficial for plants (Sánchez-López *et al.*, 2016a).

2.2.3 Mode / Mechanism of action

Agriculture marketplace has the crucial obligation to perform the registration of the products used to ensure their practical, safe and legitimate application. Due to the lack of a clear definition of biostimulants, the registration and classification in the system may vary depending on the regions. Moreover, if we consider that biostimulants have a complex multicomponent composition, it is difficult to discover their modes/ mechanisms of action. Knowing that many jurisdictions regulatory practices require an identification of each compound present in the product make the regulation of biostimulants almost impossible. Therefore, there is a real need to ensure that all these products have clear benefits on crop productivity but with still unknown mode of action.

Mode of action may be defined as a specific effect on a discrete biochemical or regulatory process. In contrast, mechanism of action would have an impact on general biochemical, molecular pathways or physiological processes (Yakhin *et al.*, 2017). A summary of main mechanisms targeted by biostimulants are shown in **Figure 2**.

For a high number of biostimulants, neither a specified mode of action nor a mechanism of action has been identified. Moreover, multicomponent biostimulants contain biologically irrelevant concentrations of essential elements. Even though that the modes of action of these elements are well known they are not useful in low doses. In a specific case of VCs, the identification of all compounds present is sometimes untenable. For these reasons, given the difficulty in determining the mode of action or the recognition of all the compounds, the main objective of this bachelor thesis is focused on the biostimulant research and validation performing a bioassay. Using this protocol, it could

be possible to determine the mechanism of action without any requirements for the determination of the compound/s induced plant response.

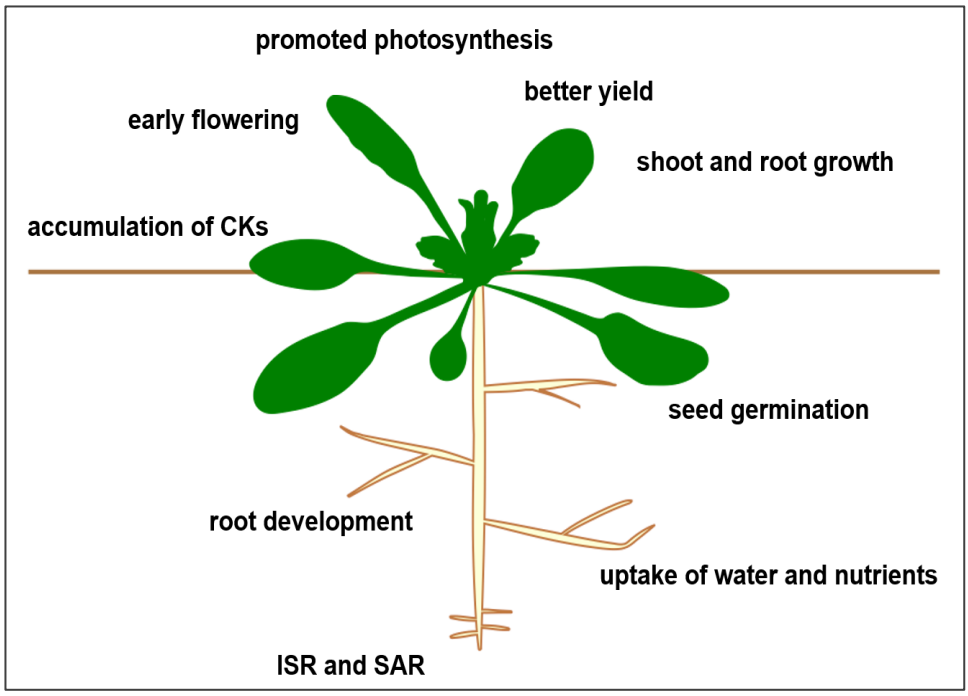

Figure 2: Summary of main mechanisms targeted by biostimulants on plant physiological processes.

2.3 High-throughput phenotyping platforms (HTPP)

Besides the increasing world population, there is a general interest and high demand on a plant research community to forward the progress in breeding programmes, with the need to accurately measure increasing number of plants and number of parameters. The release of crop varieties characterized by improved water and nutrient use efficient and tolerant to environmental stresses is a primary target of a biotechnology industry. In order to ensure higher plant productivity and better adaptation to various climatic modifications, it is necessary to understand the interaction between phenotype and genotype of a plant (Tester and Langridge, 2010; Yang *et al.*, 2017). Since 1909, when Johannson firstly presented concept of the genotype-phenotype, phenotype was described as a total constitution of a plant comprising of many different characteristics that can be determined by different analytical methodologies (Junker *et al.*, 2015). During last few years, gene sequencing has undergone a rapid evolution. However, because of the absence of sufficient knowledge about plant phenotypes, the use of the information obtained from gene sequencing is not suitable for the identification of appropriate plant traits (Yang *et*

al., 2017). According to this, genotypic and phenotypic characterization are equally important for generating the relationship between genes and traits (Rahaman *et al.*, 2015). Therefore, the higher is the resolution of the phenotype analysis it is more likely that new genes and complex interactions will be revealed.

In general, phenotyping is defined as a set of various methodologies and protocols to asses plant parameters and characteristics that can be expressed quantitatively or qualitatively (Fiorani and Schurr, 2013; Krajewski *et al.*, 2015). Throughout the history of plant breeding, hundreds or thousands of measurements have been necessary to select specific individuals or identify particular regions in the genome. Traditionally, whole shoot phenotyping involves techniques such as visual assessment of plants, manual measurements of biomass and leaf dimensions, making it a time-consuming and labour-intensive process (Berger *et al.*, 2012). Since plant phenotyping became a part of science, the evolution in the technique and the increase of the accuracy has been required. This technique has a potential to reveal physiological and morphological traits related to plant responses.

A non-destructive 2D high-throughput phenotyping (HTP) was firstly described in 1999, presenting the applicability of a projected leaf area as a substitute for a classical biomass determination (Arvidsson *et al.*, 2011). Recently, HTPP have been widely installed in a greenhouses or growth chambers using robotically driven arms with imaging technologies for obtaining information about plant growth and developmental processes. These approaches provide a chance to combine various methods of automated, simultaneous, non-destructive analyses. Thanks to a number of measurements performed during plant life cycle, HTPP also help to evaluate several plant traits in one experimental setup (Humplík *et al.*, 2015).

2.3.1 High-throughput phenotyping (HTP) imaging methods

To obtain HTP data about phenotypes for subsequent quantitative analyses of plant traits (e.g. size, colour or growth of a plant), HTPP use a variety of imaging sensors (Chen *et al.*, 2014; Awlia *et al.*, 2016).

One of the most widely used imaging technique for the evaluation of a plant and rosette growth is a visible red-green-blue (RGB) imaging. This technique operates with the wavelengths ranging from 400 to 700 nm of the electromagnetic spectrum providing 2D images. (Fiorani and Schurr, 2013; Humplík *et al.*, 2015; Rahaman *et al.*, 2015).

Biomass formation is a dominant trait of a plant development. This characteristic may be defined as the weight of a whole plant or a part of a plant (mostly the shoots) in a particular point of its lifespan. This trait can be easily evaluated by weighing of the fresh (FW) and dry (DW) weights. Unfortunately, manual measurements of the plant leaf dimensions and subsequent plant growth analyses are very tedious and so cannot be used for large-scale experiments. Another problem is that the classical biomass determination also includes the destruction of a plant and so allows only the end-point analyses. This is the reason why the use of digital cameras is preferred for the evaluation of the growth rate on time followed by software image analysis (Humplík *et al.*, 2015; De Diego *et al.*, 2017). In addition, RGB imaging can also provide the quantification of greenness parameters for the analysis of growth, leaf geometry and photosynthetic pigments.

The main disadvantage of the use of RGB imaging method is an inability to applicate a top-view analysis on crop plants with vertical growth. Moreover, in case of the leaf overlap of older plants, the analysis of projected area would not be so precise. For these reasons, new assessments and improvements of RGB imaging should be performed.

Chlorophyll fluorescence imaging (CFIM) being another type of the methodologies used for high-throughput phenotyping provide the information about plant photosynthetic capacity. Besides the fact that this method is inexpensive and non-destructive, it also offers the option to measure the rate of electron transport, effectivity of photosystem II or the range of nonphotochemical quenching.

CFIM is based on the primary adaptation of plants on darkness followed by the illumination and measurement of excited chlorophyll fluorescence signal using a weak flashes (Humplík *et al.*, 2015). The capture of light energy by chlorophyll molecules has three options in the cells. The first one is the photosynthesis by photochemistry. The second option is a dissipation of energy as heat or energy re-emission as fluorescence. All processes are co-existing and values of most of the parameters cannot be compared. The only possible way to do it is with relative changes of the parameters. The quantum yields responsible for the use of the absorbed light energy are the best option (Humplík *et al.*, 2015; Awlia *et al.*, 2016).

Thermoimaging is another HTP imaging technique, which uses cameras in order to measure spatial heterogeneity of heat emissions from leaves. The heat is basically an electromagnetic radiation in the infrared region (8-13 μm). It also evaluates the leaf and canopy temperature by monitoring the transpiration of stomata and the final water status (Fiorani and Schurr, 2013a; Humplík *et al.*, 2015).

2.4 Phytohormones

Phytohormones, also referred as plant growth regulators, are small, organic molecules, usually produced in a very low concentrations (from nano- to micromolar), which play a very important role in different phases of plant development, such as germination, growth, reproduction and also defence responses to various environmental stresses (Wani *et al.*, 2016). Plant hormones are divided into several main classes including auxins, abscisic acid (ABA), cytokinins (CKs), gibberellins (GA), salicylic acid (SA) and jasmonic acid (JA), ethylene (ET), brassinosteroids (BRs) and strigolactones (SL) (**Figure 3**) (Pan *et al.*, 2008; Fahad *et al.*, 2015; Wani *et al.*, 2016). Thanks to various sectors of science, e.g. genetics, biochemistry or molecular biology, it is possible to concrete a roadmap from the biosynthesis to perception and action of many of these phytohormones.

In this bachelor thesis, three groups of phytohormones (CKs, auxins and ABA) have been selected in order to understand the biosynthetic mechanisms and signal transduction pathways in response to fungal VCs.

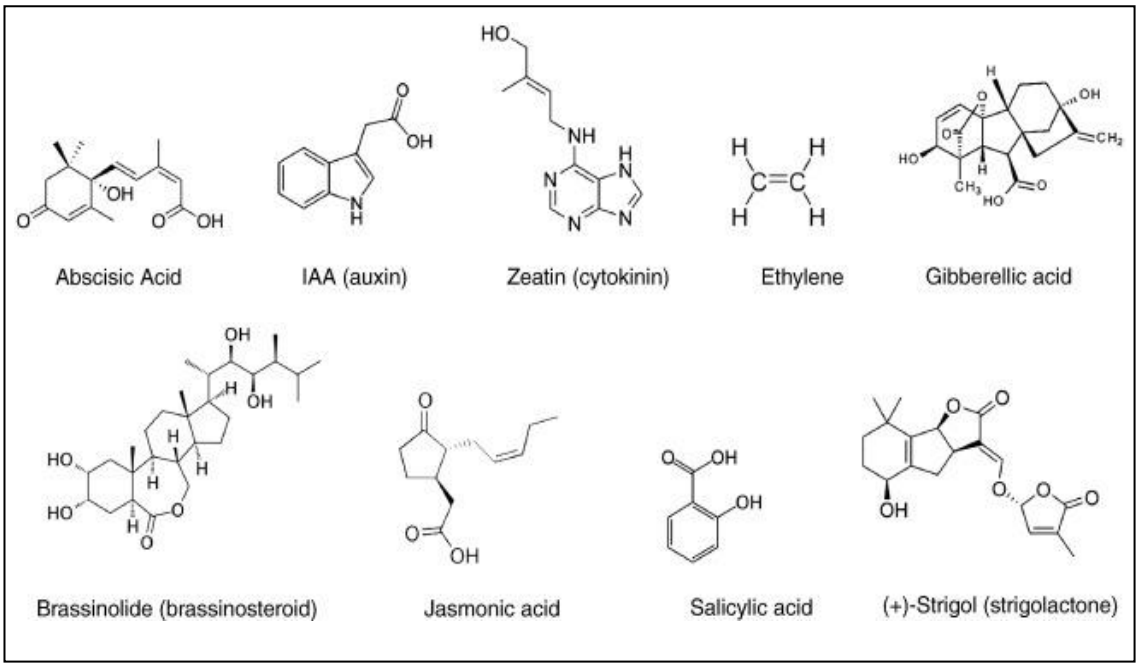

Figure 3: The main representatives of each group of phytohormones (Wani *et al.*, 2016)

2.4.1 Auxins

Auxins are chemically diverse substances that mainly regulate plant development and growth stages. They also play an important role in many physiological processes such as activation and control of plant meristem, cell expansion, root initiation, apical dominance and bud formation (Pan and Wang, 2009; Davies, 2010).

Most of auxins are made out of aromatic system which can be composed of indole, phenyl or naphthalene with carboxyl group as a side chain (Bajguz and Hayat, 2009). The most common form of plant auxin is indole-3-acetic acid (IAA) but at the same time, many other naturally active forms of auxins have been identified (Shigenaga and Argueso, 2016)

The first plant hormone named as auxin was discovered in 1926 (Crozier, 1987). Despite the fact that this group of plant hormones have been identified for almost 100 years, the biosynthesis and signalling pathways are still not fully understood. Nevertheless, few biosynthetic pathways have so far been introduced (Wani *et al.*, 2016).

In higher plants, biosynthesis of IAA can be performed in leaves, cotyledons and roots (Ljung *et al.*, 2001)**.** As shown in **Figure 4**, the plants use two basic pathways for biosynthesis of IAA which originate from essential amino acid tryptophan (Trp). One of them is tryptophan-dependent and the other pathway is tryptophan-independent (Woodward and Bartel, 2005; Ehlert *et al.*, 2008; Shigenaga and Argueso, 2016).

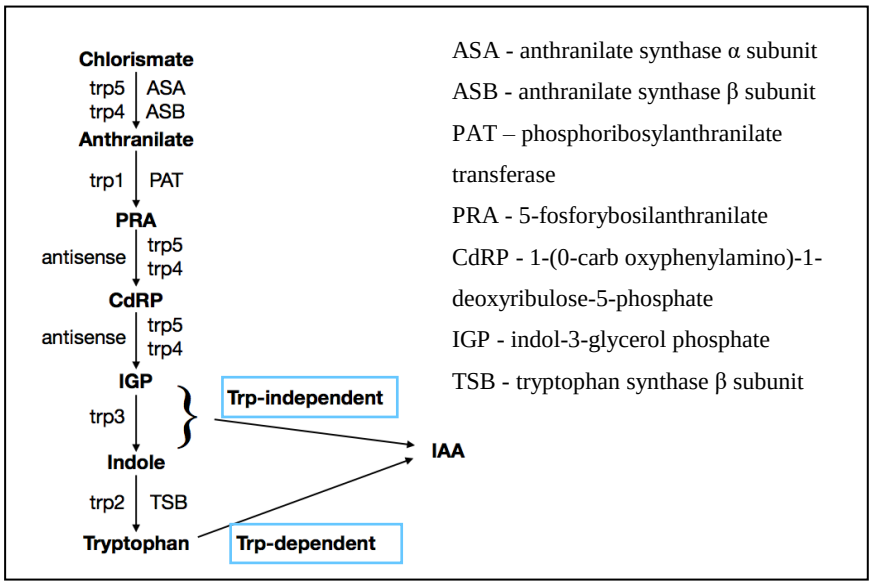

Figure 4: Biosynthesis of auxins in *Arabidopsis thaliana*. Synthesis of IAA through the Trp-independent or Trp-dependent pathway.

2.4.2 Abcisic acid

Abscisic acid (ABA), firstly mentioned in 1960s, is a plant growth regulator playing an important role in plant development, particularly cell differentiation, seed dormancy and germination, senescence and stress responses (Finkelstein, 2013). ABA also regulates plant embryogenesis and physiological adaptation to different environmental stimuli such as drought, salinity, osmotic stress, low / high temperatures, pathogens or lack of water, by change of its concentration (Davies, 2010).

Since the discovery, many analogues of ABA and their metabolites were synthetized but only S-(+)-ABA is naturally synthetized in plant (**Figure 5**) (Zaharia *et al.*, 2005).

There are two different ways of ABA synthesis: direct and indirect. In direct pathway, two genera of phytopathogenic fungi, *Cercospora* and *Botrytis*, synthetize ABA from isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) which were firstly synthetized from mevalonate. From two IPP and one DMAPP, *trans*-farnesyl pyrophosphate is created (Hirai *et al.*, 2000).

In plants, the biosynthesis of ABA is more complex. Carotenoids are synthetized from IPP and converted into geranylgeranyl pyrophosphate (GGPP). The first step of carotenoid synthesis is a conversion of GGPP into phytoene, which is followed, by conversion to xanthoin through the synthesis of *β*-carotene and xanthophyll. Lastly, xanthoin is converted into ABA. This pathway is known as indirect or also as a non-mevalonate pathway of ABA synthesis (Seo and Koshiba, 2002).

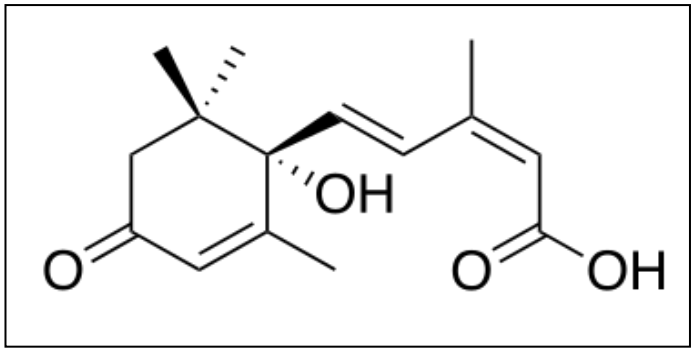

Figure 5: Structure of naturally synthetized *S*-(+)-abscisic acid.

2.4.3 Cytokinins

In 1955, the first cytokinin, 6-furfurylaminopurine, was identified by Miller and Skoog. They named it kinetin (Spíchal, 2012). This group of plant hormones regulates many aspects of growth and development, including cell division, seed dormancy and germination, senescence, plant organ development and response to biotic or abiotic stresses (Werner and Schmülling, 2009; Davies, 2010). CKs are not only restricted to plants. They can also be found in number of different organisms ranging from bacteria to humans. Commonly occurring CKs are relatively simple derivates of adenine with N^6-side chains that differ in the structure of the N^6 substituent (Romanov, 2009). The majority of biologically active CKs found in *Arabidopsis* plants have a saturated or unsaturated aliphatic side chains with isoprenoid origin. There are also phytohormones which do not have a modified isopentenyl side chain like isopentenyl adenine (iP). (Kieber and Schaller, 2014; Fahad *et al.*, 2015).

Almost 20 years later since discovery of kinetin, D. Letham identified the first free CK isolated from maize (*Zea mays* L.) endosperm and named it zeatin (Romanov, 2009; Kieber and Schaller, 2014). A scheme of CK biosynthesis, interconversions and degradation in plants is shown in **Figure 6**.

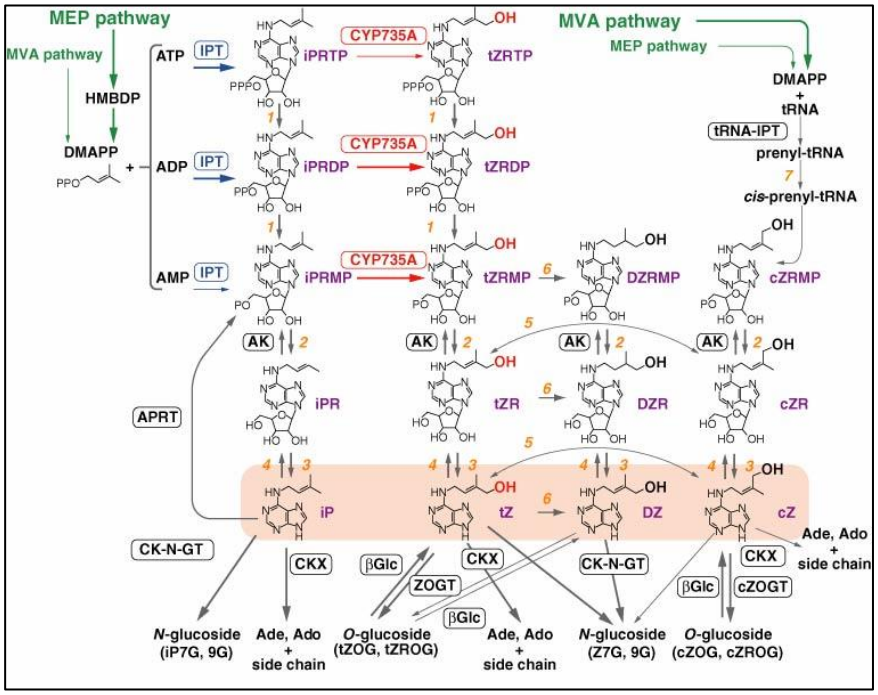

Figure 6: Scheme of cytokinin biosynthesis, interconversions and degradation. Synthesis of N^6 side chains of iP (isopentenyl adenine) and *t*Z (*trans*-zeatin) from methylerythriol phosphate (MEP pathway). Derivation of *c*Z (*cis*-zeatin) side chain from the mevalonate (MVA pathway) (Sakakibara, 2006).

2.4.4 Crosstalk between phytohormones

Regulation of a plant growth is one of the major functions of phytohormones. They are also critical factors which play an essential role in improvement of plant metabolism and physiology under environmental changes. Plants have developed a complex defence response by modulating the gene expression or directly affecting the physiology of the cell (Pandey, 2017). These signal pathways have been investigated indepently trying to create a model of a linear pathway. Nevertheless, these transduction mechanisms must interconnect to allow plants to coordinate and reorganize their reactions using limited sources for survival (Yoshioka and Shinozaki, 2009). One of the techniques used for the validation of crosstalk between different groups of hormones is the use of different plant mutants. This method provides a means of dissecting the signal transduction pathways controlling physiological processes and elucidating molecular mechanisms.

It is worth to mention several studies about the interactions between different phytohormones in many plant species and in different environmental conditions. In general, ABA is considered to be a regulator of growth and developmental processes in response to stress. The interaction of this hormone with auxins has been described in plant-pathogen interactions observing changes in the levels of auxins after the exposure of *Arabidopsis* plants to different bacterial strains. Induction of auxins were shown as well as upregulation of ABA genes (Yoshioka and Shinozaki, 2009). The increased levels of ABA produced seeds with accumulated high level of auxin. Auxin action in seed dormancy requires ABA signalling pathway (and vice versa), indicating that the roles of auxin and ABA in seed dormancy are interdependent (Chen *et al.*, 2014).

Another example is the analysis of abiotic stresses in tobacco roots where ABA and CKs were elevated acting as antagonists of auxins (Havlová *et al.*, 2008). ABA and CKs inhibit lateral root development resulting in a decrease of the total amount of auxins. This suggested that ABA and CKs act synergistically in some biological processes under concrete growth conditions (Ha *et al.*, 2012). In contrast, CKs appear to act as antagonists to many physiological processes mediated by ABA. For example, ABA-induced stomatal closure, leaf senescence, and leaf and fruit abscission are reversed by exogenous application of CK while CK-mediated release of seed dormancy contrasts with ABA inhibition of germination (Cowan *et al.*, 1999). This antagonism may be the result of metabolic interaction of CKs when they share, at least in part, a common biosynthetic origin with ABA.

3. List of chemicals and devices

3.1 Biological material

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

3.2 Chemicals

- ammonia solution – NH_3 (25%), Suprapur
- deuterium labeled internal standards:

$^{13}C_6$-IAA, $^{13}C_6$-oxIAA, $^{13}C_6$-IAAsp, 13C_6-IAGlu, D_6-ABA

$^{13}C_5$-tZ, D_5-tZR, D_5-tZ7G, D_5-tZ9G, D_5-tZOG, D_5-tZROG, D_5-tZRMP, $^{13}C_5$-cZ, D_3-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
- distilled water
- ethanol (EtOH) – H_5C_2OH (96%), Lach-Ner s.r.o.
- formic acid – HCOOH (98%), Honeywell Fluka
- chloric acid – HCl (35%), Lach-Ner s.r.o.
- liquid nitrogen – N_2
- 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 – HNO_3 (65%), Lach-Ner s.r.o.
- phytagel, Sigma Aldrich
- potassium hydroxide – KOH (1M), Lach-Ner s.r.o.
- sucrose, Merck spol. s.r.o.
- triton X-100, Sigma Aldrich

3.3 Solutions and media

- Sterilization solution:

  70% EtOH with 0.01% Triton X-100

- Bieleski buffer (BS):

  75% MeOH, 5% HCOOH, Milli-Q water

- Phosphate Buffer Solution (PBS), pH 7.00:

  0,05M PBS, 0.02% DEDTCA

- 1x MS medium (plants):

  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 (plants):

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

- 1x MS medium (fungi):

  MS (4.4 $g.l^{-1}$), sucrose (30$g.l^{-1}$), KOH (1M) – pH 5.7, phytagel (6 $g.l^{-1}$)

- Ammonium hydroxide NH_4OH (0,35M):

  6.25 ml 25% NH_3, 243.75 ml Milli-Q water

- Ammonium hydroxide (NH_4OH 0,35M) in 60% MeOH:

  6.25 ml 25% NH_3, 93.75 ml Milli-Q, 150 ml MeOH

- Nitric acid – HNO_3 (65%):

  76.9 ml 65% HNO_3, 23.1 ml Milli-Q

- 1M HCOOH:

  9.625 ml 98% HCOOH, 240.375 ml Milli-Q

- 1% FAc:

  2 ml 98% HCOOH, 198ml Milli-Q

- 10% MeOH in 1% FAc:

  10 ml 98% MeOH, 90 ml 1% FAc

- 70% MeOH in 1% FAc:

  70 ml 98% MeOH, 30 ml 1% FAc

3.4 Equipment

- analytical balance XA110/2X, RADWAG
- autoclave STERIVAP HP IL, BMT Medical technology s.r.o.
- automatic pipette, Eppendorf Research Plus
- Benchtop Vaccum Concentrators, Labconco
- 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
- incubator shaker Innova 42, New BrunswickScientific
- laminar flow cabinet, MERCI s.r.o.
- magnetic mixer, LAVAT
- mixer mill Retsch MM400, Retsch GmbH
- pH meter ORION STAR A111, ThermoScientific
- phenotyping platform Olophen (FYTOSCOPE FS-WI-XYZ), PlantScreen™ PSI
- protein concentrator (filter size 10 kDa), Sartorius steim biotech
- rotator Stuart SB3, Stuart-Equipment
- ultrasonic bath TRANSSONIC T310, Elma®
- Visiprep Spe Vacuum manifold SUPELCO, Sigma Aldrich
- vortex mixer WIZARD IR, VELP® SCIENTIFICA
- vortexer HEATHROW SCIENTIFIC, P-lab a.s.

3.5 Devices

- Multi-well plates (6- and 12 multi-well plates), Jetbiofil, Guangzhou, China
- SPE columns:
  C18 (octadecyl C18/18%, 100 mg/1 ml), Applied Separations, Inc
  C8 (500 mg /3 ml), Agilent technologies, Inc
  MCX (300 mg/1 ml), OASIS®, Waters Corporation
- ceramic beats (ZrO_2)
- nanosep MF centrifugal devices with Bio-Inert® membrane, Pall Corporation
- parafilm M, Sigma Aldrich
- sterile plastic boxes Square Bioassay Dishes 245 x 245 x 25 mm, ThermoFisher Scientific
- square Petri dish (12 x 12 cm), P-lab a.s.
- vials with inserts, Sigma Aldrich

4. Methods

General overview of established experimental protocol is schematized in **Figure 7**. This protocol was used for the optimization of the method, its validation, and biostimulant response and growth interaction studies. The protocol consists of several steps including seed sowing, cold stratification, transfer of seedlings into the multi-well plates, time-course RGB-imaging, data processing and analysis (based on De Diego *et al.*, 2017).

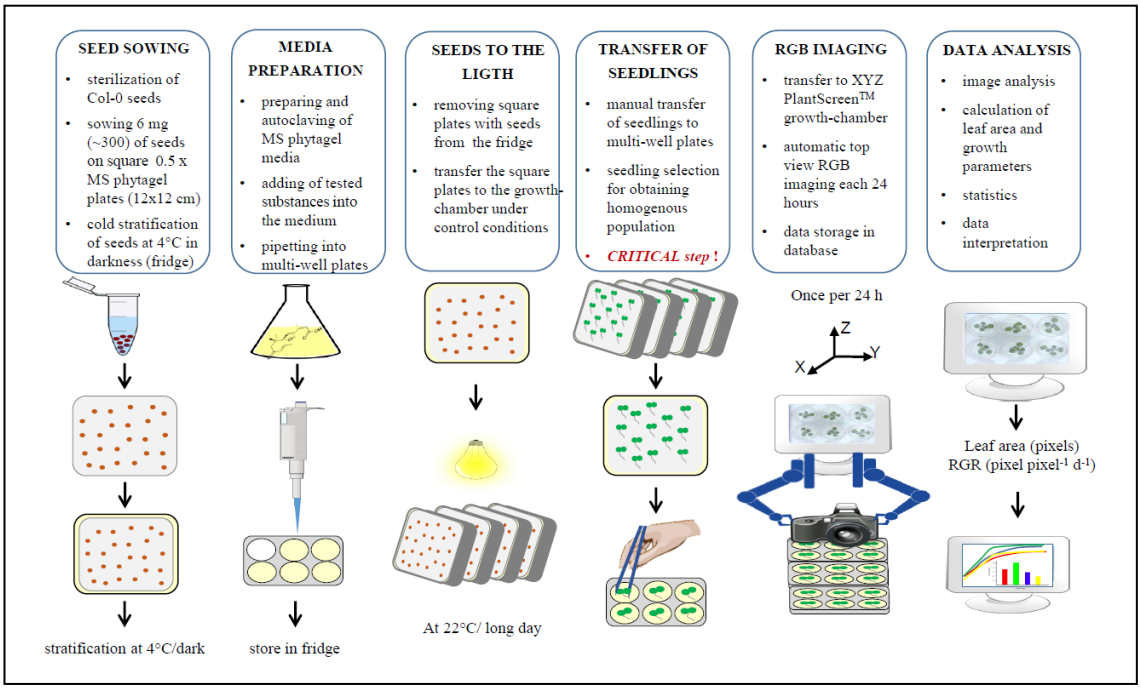

Figure 7: High-throughput plant phenotyping protocol for screening of *Arabidopsis* rosette growth in multi-well plates under controlled conditions in a XYZ PlantScreen™ growth chamber with RGB imaging and followed with data analysis.

4.1 Plant growth and culture conditions

Seeds of *A. thaliana* ecotype Col-0 were surface-sterilized using 200 µl of 70% ethanol enriched with 0,01% Triton X-100 during 10 min shaker and thereafter they were washed three times with the same solution. Afterwards, seeds were sown on a square Petri dish (12 x 12 cm) containing 0.5x Murashige-Skoog (MS) medium (2.2 g of MS, 0.5 g of MES to keep the pH and supplemented with 0.6% of gelling agent phytagel; pH 5.7 adjusted with KOH) and kept at 4°C in dark to synchronize the germination. After 4 days of stratification, plates were transferred into a growth-chamber with controlled conditions (22°C, 60% humidity, photoperiod 16/8h with photon irradiance of 150 µmol photons of PAR m^{-2} s^{-1}) for another 4 days and kept at vertical position.

4.2 *Alternaria alternata* culture conditions

Pathogenic fungi AA was cultured in Petri dishes (6 colonies per each one) containing 1x MS medium (4.4 g MS; pH 5.7, supplemented with 3% of sucrose and gelling agent 0.6% of phytagel). In order to carry out the growth of the colonies, they were maintained in an incubator during one week at 26ºC in dark.

4.3 Preparation of *Alternaria alternata* exudates

After the performance of the protocol cited above, for the preparation of exudates, colonies of AA were collected and transferred into Erlenmeyer flask containing 125 ml of sterile distilled water. Afterwards, flask was enclosed with a sterile cotton lid and placed into incubator shaker for 24 hours at 26°C and 160 g. Aqueous solution with dissolved exudates was then filtrated using vacuum pump and the filtrate was subsequently diluted four times to a total volume of 500 ml. Protein concentrator with a pore size filter of 10 kDa was used for a second filtration of exudates. The exudates were stored at -20ºC to maintain the stability of the solution (**Figure 8A, 8B**).

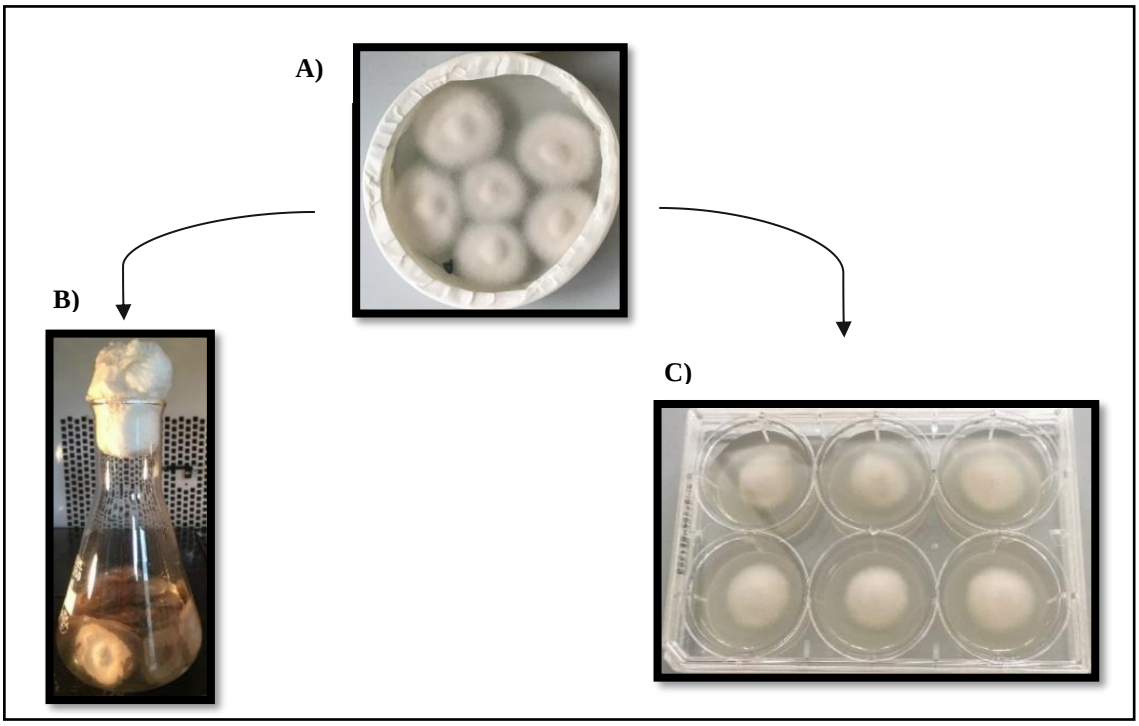

Figure 8: A) Colonies of AA in Petri dish with 1x MS medium supplemented with sucrose; B) preparation of AA exudates; C) colonies transferred into 6 multi-well plates for the experiment with VCs.

4.4 Establishment of plant stress conditions

4.4.1 Exposition to exudates

Three days after germination, *Arabidopsis* seedlings of a similar size were transferred under sterile conditions into multi-well plates (6- and 12 multi-well plates) (one seedling per well) containing 6.35 ml and 2.7 ml respectively of 1x MS medium (4.4 g MS, 0.5 g MES, 0.6% gelling agent phytagel; pH 5.7) supplemented with an amount of AA exudates corresponding to 0.1 µl ml^{-1} and 0.01 µl ml^{-1}. Plates were afterwards sealed with a perforated transparent foil avoiding condensation and allowing gas and water exchange.

4.4.2 Exposition to volatiles

Fungi were cultured on 6 multi-well plates containing 6.35 ml of 1x MS medium enriched with sucrose in an incubator at 26°C and 55% humidity for one week (**Figure 8A, 8C**).

To investigate the effect of volatiles on *Arabidopsis* plants, seedlings were cultured as in the previous experiment. Both cultures (plants and fungi) were placed without lids and without physical contact into sterile plastic boxes (Square Bioassay Dishes 245 x 245 x 25 mm, ThermoFisher Scientific) and properly sealed with parafilm as illustrated in **Figure 9**. As negative controls treatment, multi-well plates containing sterile culture media without fungi were enclosed in the boxes adjacent to the plants.

Figure 9: Sterile plastic boxes (Square Bioassay Dishes) used for the demonstration of the effect of AA VCs on growth of *Arabidopsis thaliana*, A) multi-well plates with MS medium and with *Arabidopsis* seedlings as a control, B) multi-well plates containing *Arabidopsis* seedlings exposed to VCs emitted by AA colonies.

4.5 Biometrical parameters

4.5.1 High-throughput phenotyping analysis

The multi-well plates with the transferred *Arabidopsis* seedlings were placed onto the OloPhen platform with PlantScreen^{TM} XYZ system composed of a robotically driven arm with RGB camera in a growth chamber with a controlled environment and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czech Republic) (**Figure 10**) (De Diego *et al.* 2017). The conditions were set to stimulate a long day with a regime of at 22ºC/20ºC in a 16/8 h light/dark cycle, an irradiance of 120 µmol photons of PAR $m^{-2}s^{-1}$ and a relative humidity of 60%.

In the experiment performed with exudates, measurements were carried out once per day during seven days while for the experiment with volatiles, there were two measurements, the first one just before the application of volatiles and secondly seven days after treatment.

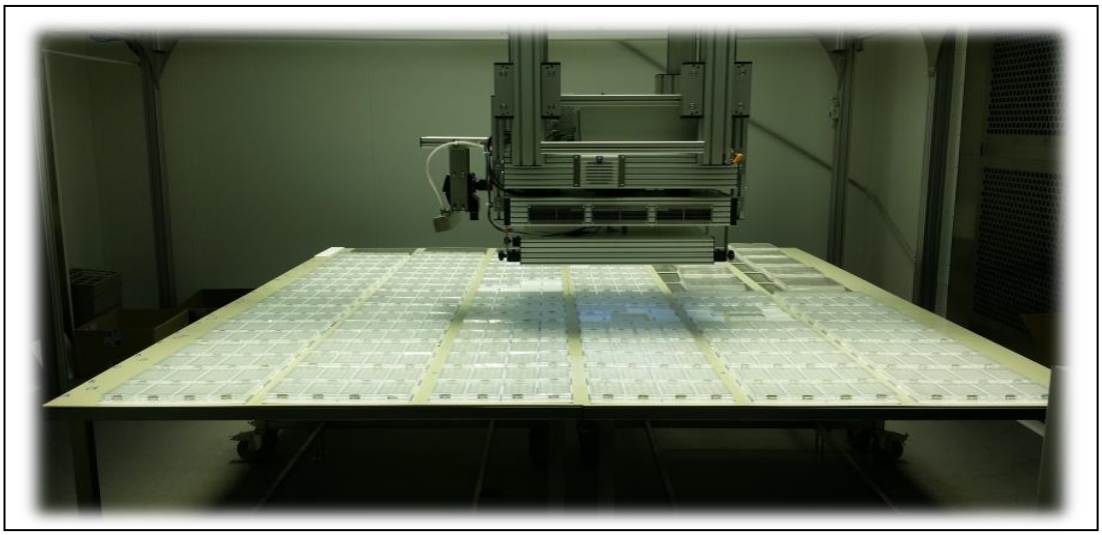

Figure 10: High-throughput phenotyping platform OloPhen with robotically driven arm with RGB camera over the multi-well plates with *Arabidopsis* plants installed in a XYZ PlantScreen^{TM} growth chamber with controlled growth conditions.

4.5.2 Biomass (Fresh weight)

To perform the traditional biometric methods for the analysis of *Arabidopsis* rosette growth, the rosettes of individual plants grown *in vitro* in both experiments were collected at the last day of measurements and the fresh-weight (FW) of individual plant rosettes was determined. Subsequently, they were freeze-clamped and stored in Eppendorf tubes at -80°C.

4.6 Analysis of phytohormones

In order to perform the analysis of different groups of phytohormones, protocols established by the department of Plant Growth Regulators (LRR) of Palacký University were followed as described above.

4.6.1 Preparation of samples for quantification of cytokinins

Samples were prepared by grinding and homogenizing approximately 20 mg (FW) of a rosette material. Four and five plates for 6 and 12 multi-well plates respectively were used as biological replicates. To each sample, 1 ml of extraction agent (Bieleski buffer (BF)), 30 μl of a mixture of deuterium labelled-internal standards (IS) of CKs and three ZrO_2 beads with a diameter of 2 mm were added. Thereafter, the Eppendorf tubes were covered and their content was homogenized for 3 min using a MM 301 ball mill at a frequency of 27 Hz. Homogenized samples prepared by this procedure were further sonicated for 5 minutes in ultrasonic bath to increase the extraction efficiency of studied substances from plant tissues. The samples were then left 30 min on a laboratory rotator operating at 4°C. Tissue extracts were centrifuged at 15,000 g at 4°C for 5 min. After this procedure, a re-extraction was performed in the same way as described above. Supernatants were again collected and placed into the same Eppendorf tubes having a final volume around 2 ml.

The procedure of CK purification consists of two steps. Firstly, supernatants were removed from each Eppendorf tube using an automatic pipette and purified by C18 SPE columns (100 mg sorbent / 1 ml cartridge volume) as follows:

1. Activation of sorbent: 2 ml of 100% MeOH
2. Equilibration of the sorbent: 2 ml of extraction solution (BF)
3. Application of supernatant: 2 ml sample
4. Elution: 3 ml of BF (into a clean borosilicate tube)

The extracts purified by the above method were evaporated to 0.5 ml by a vacuum concentrator removing the water phase. Consecutively, the second part of the purification of the tissue extracts by MCX OASIS® SPE columns (30 mg sorbent / 1 ml cartridge volume) was performed as follows:

1. Activation of sorbent: 2 ml of 100% MeOH
2. Equilibration of the sorbent: 2 ml of Milli-Q H_2O
   2 ml 50% HNO_3

	2 ml of Milli-Q H_2O
	1 ml of 1 M HCOOH
3. Application of supernatant:	2 ml of 1 M HCOOH + 2 ml sample
4. Elution:	1ml of 0.35 M NH_4OH in water
	1 ml of NH_4OH in 60% MeOH (into a clean borosilicate tube)

Purified extracts were then evaporated to dryness by a vacuum concentrator. The resulting sample residue in each tube was reconstructed in a volume of 50 µl of 10% MeOH, filtered through a centrifuge filter, and pipetted into a conical vial from where it was injected onto the ultra-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS / MS) system for the quantitative analysis of CKs.

4.6.2 Preparation of samples for quantification of auxins and ABA

In the same way as the protocol of cytokinins, 20 mg (FW) of *Arabidopsis* samples were prepared. 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 with a diameter of 2 mm were added. Samples were homogenized for 10 min using a MM 301 ball mill at a frequency of 27 Hz.

The homogenized samples were then left 10 min on a laboratory rotator operating at 4°C. Tissue extracts were afterwards centrifuged at 15,000 g and 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 33 µl of 1 M HCl.

The process of purification of auxins and ABA was performed by C8 SPE columns (500 mg of sorbent / 3 ml cartridge volume) as follows:

1. Activation of sorbent:	2 ml of 100% MeOH
2. Equilibration of the sorbent:	2 ml of 1% FAc
3. Application of supernatant:	1 ml sample
4. Wash:	2 ml 10% MeOH in 1% FAc
5. Elution:	2 ml of 70% MeOH in 1% FAc (into a clean borosilicate tube)

Purified tissue extracts were evaporated to dryness by means of a vacuum concentrator, and the resulting residue was reconstructed in each tube analogously to the preparation of samples for quantification of CKs.

4.7 Statistical analysis and data representation

In high-throughput phenotyping system, RGB images (resolution 2500 x 2000 pixels) of a single plate with a file size of approximately 10 MB in the PNG compression format were stored in a database on a server, using a filename containing information about the acquisition time and the (x, y) coordinates of the camera. The data were automatically stored in PlantScreen XYZ database, exported by PlantScreen Data Analyser software and analysed using an in-house software routine implemented in MatLab R2015.

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

To test the reliability of the method, correlations between the green area and FW were calculated using Pearson's coefficient and the significance determined after ANOVA.

5. Results

5.1 Optimization and validation of HTP method

The assay designed for high-throughput phenotyping analysis described previously was used for the analysis of *Arabidopsis* rosette growth *in vitro* using 6 and 12 multi-well plates. To optimize and validate HTP method used for the experiments included in this bachelor thesis, the traditional biometric methods, gravimetric determination of plant fresh weight (FW) and the measurement of the green area estimated by RGB imaging were compared. After seven days of growing in the platform, *Arabidopsis* rosettes from both experiments (exudates and VCs) were harvested and the FW of individual rosettes was determined. Afterwards, the correlations between these two parameters were calculated by regression using Pearson's coefficient and the significance was determined using ANOVA.

As indicated in **Figure 11**, highly significant positive correlations were obtained in both cases with *R*-square correlation coefficients of 0.84 and 0.71 for the experiment with AA exudates and VCs respectively.

5.2 Application of biostimulants

5.2.1 Plant responses to fungal exudates isolated from *Alternaria alternate.*

Arabidopsis plants were cultured on sucrose-free solid 1x MS medium supplemented with specific concentrations of exudates (0.1 or 0.01 µl ml^{-1}) isolated from phytopathogenic fungi AA. After one week of exposure to different concentrations of AA exudates, the growth of *Arabidopsis* plants was compared based on the data obtained from high-throughput phenotyping.

In both cases (6- and 12 multi-well plates), there was no increase of the plant size during the first 2 to 3 days. After one week of exposure to AA exudates, the growth of *Arabidopsis* plants increased up to approximately 1.3-fold and 1.2-fold compared to controls in 6 and 12 multi-well plates respectively. In 6 multi-well plates, the highest growth was observed after 7 days of exposure to AA exudates of 0.01 µl ml^{-1} (**Figure 12A**). On the other hand, the best result in 12 multi-well plates was obtained using the concentration of 0.1 µl ml^{-1} with 1.3-fold increase in the rosette size compared to control (**Figure 12B**).

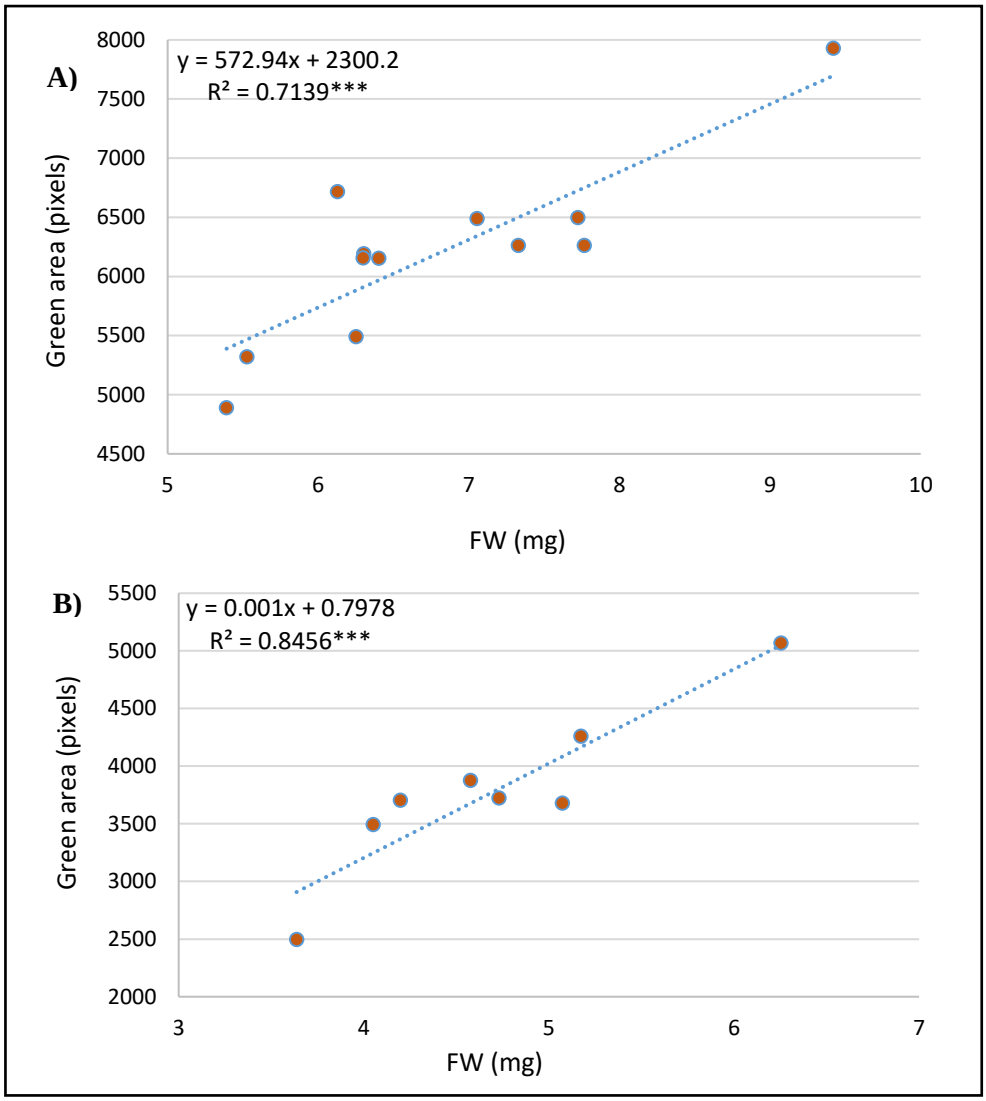

Figure 11: Validation between high-throughput phenotyping and biomass analysis for the study of *Arabidopsis* rosette growth in multi-well plates exposed to: A) AA exudates and B) volatile compounds. Correlation between the projected green area (pixels) and fresh weight (mg) of *Arabidopsis* seedlings grown in 6 and 12 multi-well plates with 1x MS for 7 days. The equation of the curve and the Pearson's correlation coefficient with significance according to ANOVA were calculated. ***$p < 0.001$.

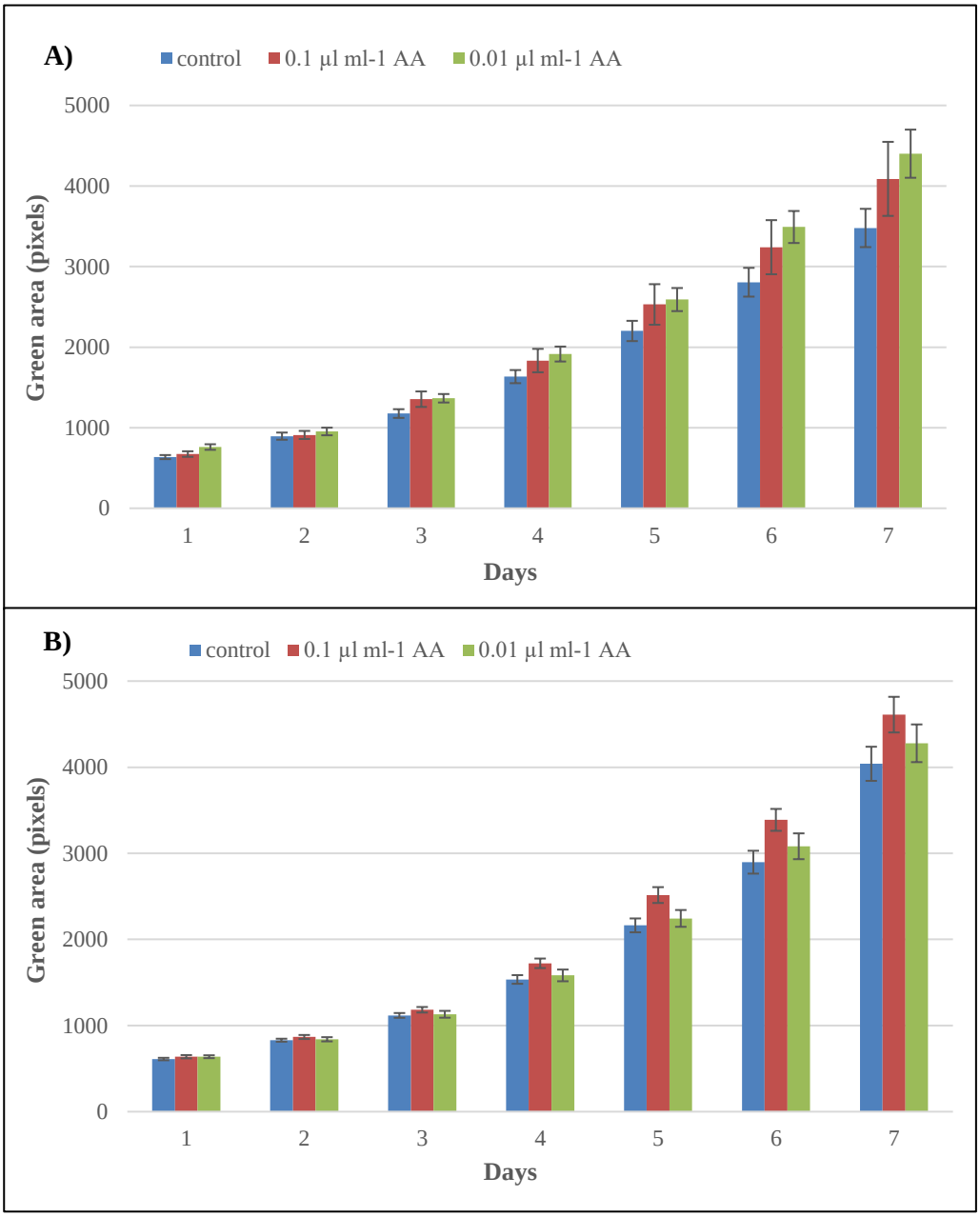

Figure 12: *Arabidopsis* plants cultured in A) 6 and B) 12 multi-well plates in absence or presence of specific concentrations of exudates (0.1 and 0.01 µl ml^{-1}) isolated from phytopathogenic fungi AA for one week.

5.2.2 Plant responses to volatile compounds emitted by *Alternaria alternata*

Arabidopsis seedlings were cultured on 1x MS medium in absence or continuous presence of adjacent cultures of phytopathogenic fungi AA in a closed environment. The experiment was performed in sterile plastic boxes (Square Bioassay Dishes 245 x 245 x25mm) with no physical contact between the plants and the microbial cultures (**Figure 9**).

After 7 days of exposition, the growth of treated and non-treated *Arabidopsis* plants was compared. In case of plants grown in 6 multi-well plates, the growth of treated *Arabidopsis* plants was lower than the growth of control plants (**Figure 13A**).

Conversely, in 12 multi-well plates, the growth of *Arabidopsis* plants after 7 days of exposure to VCs increased only 1.03-fold relative to controls (**Figure 13B**). It is worth mentioning a problem arose during the performance of the experiment specifically with 6 multi-well plates. Even though that this problem also occured in plants grown in 12 multi-well plates it had not that significant impact on plants. At the end of the experiment, it was possible to observe that there was a bacterial contamination of plants. It can be assumed that this could have happened during the manipulation of plants in the transfer step of the bioassay due a cross-contamination with the lab material used for that purpose.

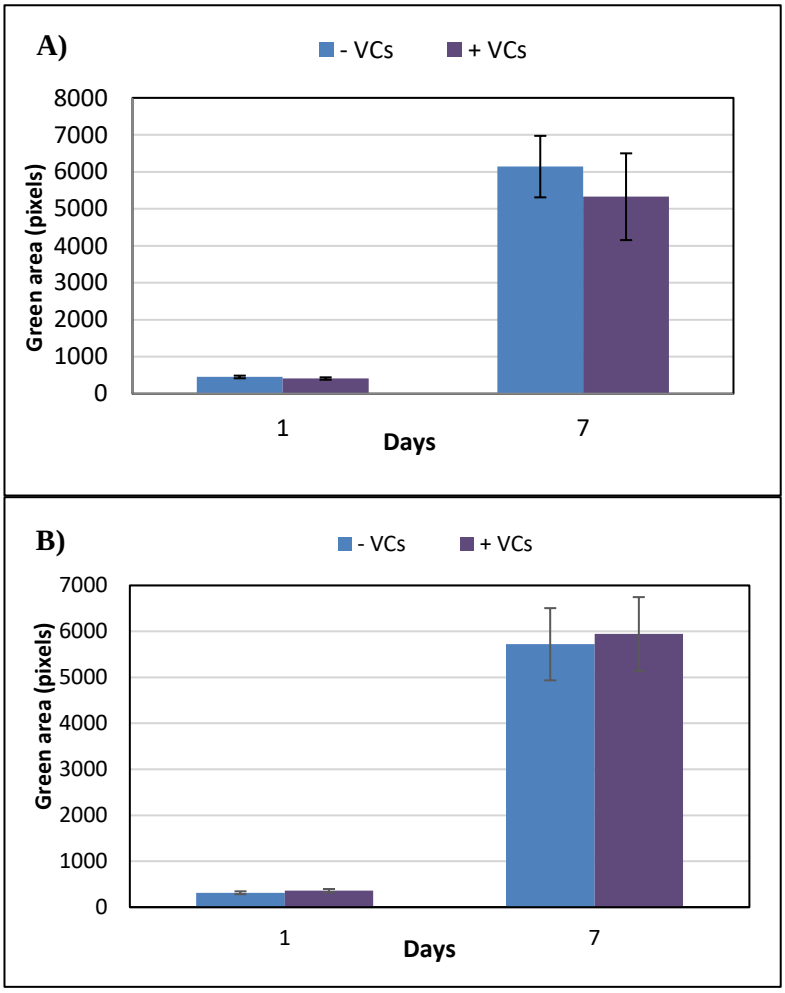

Figure 13: *Arabidopsis* plants after 7 days of cultivation on 1x MS medium in A) 6 and B) 12 multi-well plates in absence or presence of fungal volatile compounds (VCs).

5.3 Phytohormones

After the extraction and purification of phytohormones from different replicates of plant samples for each treatment and condition, the quantification of levels of auxins, ABA and CKs was assessed.

5.3.1 Auxins and ABA

As illustrated in **Table 1**, different fractions of auxins were observed in two different experiments, in *Arabidopsis* seedlings exposed to AA exudates and to VCs.

In the experiment with AA exudates, fractions of indol-3-acetic acid (IAA) and 2-oxoindol-3-ylacetic acid (oxIAA) were identified in both, 6 and 12 multi-well plates (**Table 1A**). While in plants with 6 multi-well plates the levels of IAA increased 1.4-fold, in 12 multi-well plates the levels of IAA decreased about the same value relative to controls.

With the concentration of AA exudates 0.01 µl ml^{-1}, the levels of oxIAA in 6 multi-well plates decreased (1.3-fold) whereas with the concentration of 0.1 µl ml^{-1} it slightly increased. Differently, the levels of oxIAA in 12 multi-well plates slightly increased compared to controls (**Table 1B**). Regarding ABA, a significant increase occurred in plants of both multi-well plates treated with 0.1 µl ml^{-1} AA exudates, reaching 1.5-fold of the control values. However, after the statistical Kruskal Wallis analysis, significant differences between the results were not observed.

In experiment with AA VCs, new auxin related metabolites appeared in the *Arabidopsis* leaves, which were not present in the previous experiment. Specifically, some amino acid conjugates of IAA, IAGlu and IAAsp, were observed in plants exposed to AA VCs (**Table 1B**). It is known that IAA is the most biologically active auxin, but its conjugates help to maintain IAA homeostasis.

The levels of IAA in both, 6- and 12 multi-well plates, increased almost twofold more than in control plants. On the other hand, the concentrations of oxIAA and IAGlu decreased (1.2- to 1.5-fold). The reduction in the values of IAGlu was highly significant relative to controls. While in 6 multi-well plates, the levels of IAAsp were quite lower, in 12 multi-well plates the levels increased relative to controls. Conversely, the levels of ABA doubled in 6 multi-well plates and in 12 multi-well plates the levels halved.

Table 1: Auxins and ABA contents (pmol g^{-1} FW) in *Arabidopsis* plants cultured in solid MS medium (6 and 12 multi-well plates) in absence or presence of A) exudates or B) volatile compounds (VCs) emitted by AA. Significant differences to Kruskal Wallis test ($p < 0.05$) were calculated.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

A)	**6 multi-well plates**								
	Control			**0.1 µl ml^{-1} AA**			**0.01 µl ml^{-1} AA**		
IAA	273.13	±	11.35	380.03	±	68.17	353.59	±	43.93
ox - IAA	769.18	±	93.12	599.60	±	84.44	799.31	±	20.56
ABA	3.89	±	0.68	4.68	±	0.47	5.86	±	3.14

	12 multi-well plates								
	Control			**0.1** µl ml^{-1} **AA**			**0.01** µl ml^{-1} **AA**		
IAA	354.34	±	26.72	262.04	±	40.78	257.72	±	30.44
ox - IAA	700.62	±	25.77	757.35	±	76.99	764.33	±	88.31
ABA	4.50	±	0.35	4.39	±	1.19	4.55	±	1.36

B)	**6 multi-well plates**					
	- VCs			**+ VCs**		
IAA	581.28	±	38.85	1183.07	±	116.41
ox - IAA	3048.62	±	188.85	2543.72	±	185.88
IAGlu	47.21	±	3.52	40.56	±	1.98
IAAsp	19.60	±	1.61	18.08	±	4.68
ABA	3.08	±	0.13	6.34	±	1.26

	12 multi-well plates					
	- VCs			**+ VCs**		
IAA	483.07	±	23.46	797.62	±	128.55
ox - IAA	1849.47	±	179.81	1475.51	±	155.37
IAGlu	51.34	±	3.04	35.09 **	±	2.08
IAAsp	16.44	±	1.46	20.51	±	1.67
ABA	3.25	±	0.30	2.41	±	0.16

5.3.2 Cytokinins

CKs are important determinants of plant growth. For the assessment of a possible involvement of CKs in the responses of *Arabidopsis* plants to AA exudates and VCs, the concentrations of CKs in mature *Arabidopsis* leaves were measured.

The results obtained from plants cultured in 6 and 12 multi-well plates indicate that AA exudates caused a slight increase of the total 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway-derived CKs, up to 1.10-fold compared to controls (**Table 2A, 2B**).

In 6 multi-well plates, the highest accumulation of CK levels was observed in the precursors of iP (isopentenyl adenine) and *t*Z (*trans*-zeatin) ribosides (iPRMP and *t*ZRMP). These levels increased in plants exposed to both concentrations of exudates

(0.01 µl ml^{-1} and 0.1 µl ml^{-1}). On the other hand, in 12 multi-well plates these levels decreased (**Table 2A**).

Different results were observed with the ribosides of iP and *t*Z (iPR and *t*ZR). In 6 multi-well plates the content of iPR and *t*ZR in *Arabidopsis* seedlings decreased from 1.4-fold (0.1 µl ml^{-1}) to 1.6-fold (0.01 µl ml^{-1}) (**Table 2A**), while in 12 multi-well plates, the levels of these ribosides were very similar as in controls (**Table 2B**).

In plants grown in 6 multi-well plates, the content of free bases of iP and *t*Z in presence of both concentrations of AA exudates (0.1 µl ml^{-1}, 0.01 µl ml^{-1}) decreased. Similarly, the levels of DHZ (dihydroxy zeatin) and *c*Z (*cis*-zeatin) in all samples were lower (**Table 2A**). While the levels of iP, *t*Z and DHZ in 12 multi-well plates increased from 1.5- to 2.5-fold in plants treated with 0.1 µl ml^{-1} AA exudates, the levels of these bases decreased up to 1.35-fold with 0.01 µl ml^{-1} compared to controls (**Table 2B**). In case of cZ, the content was lower for 0.1 µl ml^{-1} than for 0.01 µl ml^{-1}.

The content of inactive *N*- and *O*-glycosylated forms of CKs increased in most plates but the levels of iP7G, iP9G, *t*ZROG, DHZROG (with 0.1 µl ml^{-1}), and the levels of iP9G, DHZ9G, DHZROG (with 0.01 µl ml^{-1}) in 6 multi-well plates slightly decreased. In all treated plants, the glycosylated forms of *c*Z were also lower compared to controls (**Table 2A**). Similarly, the levels of glycosylated forms in 12 multi-well plates mostly increased but using 0.01 µl ml^{-1} AA exudates, the content of iP9G, DHZ9G, *t*ZOG and *t*ZROG decreased relative to controls (**Table 2B**).

Similarly, as in the experiment with AA exudates, in 6 and also in 12 multi-well plates, the total content of 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway-derived CKs was higher than in controls, but not for the mevalonate (MVA) pathway.

The most significant results were observed in plants cultured in 12 multi-well plates where the levels of iP and *t*Z ribosides (iPR and *t*ZR) and their precursors (iPRMP and *t*ZRMP) increased 1.5-fold compared to controls. After statistical analysis, it was possible to check a high significant difference in the values of *t*ZR. While in 6 multi-well plates the increase of iPR and *t*ZR was only slight, the levels of iPRMP and *t*ZRMP slightly decreased. Differently, the content of *c*Z ribosides (*c*ZR) in treated plants grown using 6 multi-well plates was 1.4-fold higher than controls. However, in 12 multi-well plates the level of *c*ZR and *c*Z riboside precursor (*c*ZRMP) decreased 1.7-fold compared to control.

Regarding free bases, the content of iP and DHZ in plants from 6 and 12 multi-well plates increased compared to controls but the levels of *c*Z in 6 multi-well plates decreased.

It is necessary to mention that in this experiment the content of *t*Z in multi-well plates was not observed.

Regarding glycosylated forms of CKs in 6 multi-well plates, the levels of DHZ7G, DHZ9G, DHZOG and DHZROG slightly increased while the levels of other glycosylated forms decreased by an average 1.3-fold. The most significant change was observed in content of *t*Z glycosylated form (*t*ZOG), which was lower compared to control.

In comparison, the results in 12 multi-well plates achieved similar results except the levels of iPZG, iP9G and DHZROG which were lower than in control.

The levels of glycosylated forms of *c*Z in plants from 6 multi-well plates were slightly lower mainly due to the reduction of *c*Z7G and *c*ZOG concentration. Conversely, the level of *c*Z9G was higher compared to control. In 12 multi-well plates, the total content of glycosylated *c*Z halved. This result was caused by a statistically significant decrease in *c*ZOG (3-fold) and *c*Z7G (2.25-fold).

Table 2: The levels of CKs content (fmol g^{-1}) in leaves of *Arabidopsis* plants cultured in A) 6 multi-well plates and B) 12 multi-well plates on 1x MS medium supplemented with specific concentrations of exudates (0.01 µl ml^{-1} or 0.1 µl ml^{-1}) isolated from AA. MEP and MVA pathways of CK synthesis are shown separately. Significant differences to Kruskal Wallis test (p<0.05) were calculated. *p < 0.05; **p < 0.01; ***p < 0.001.

A) 6 multi-wells plates		MEP pathway (plastid) derived CKs									MVA pathway (cytosol) derived CKs									
		Control			0.1 µl ml^{-1} AA			0.01 µl ml^{-1} AA				Control			0.1 µl ml^{-1} AA			0.01 µl ml^{-1} AA		
Precursors	iPRMP	11182.99	±	2152.62	14923.00	±	1235.46	13847.92	±	1567.40			±			±			±	
	tZRMP	3220.83	±	618.22	3198.66	±	338.58	3103.28	±	486.26	cZRMP	4877.78	±	308.70	5408.50	±	208.72	4580.27	±	300.63
	DHZMP	103.85	±	26.64	96.84	±	7.00	78.75	±	7.56			±			±			±	
	∑ (%)	14507.67	±	2797.48	18218.50	±	1581.04	17029.95	±	2061.22		4877.78	±	308.70	5408.50	±	208.72	4580.27	±	300.63
Active forms	iPR	1594.94	±	101.68	1049.40	±	91.68	953.93	±	145.35			±			±			±	
	tZR	711.43	±	210.41	545.44	±	103.95	477.60	±	117.17	cZR	688.67	±	192.20	380.66	±	50.33	344.14	±	50.31
	DHZR	144.83	±	30.83	134.80	±	2.03	115.31	±	3.35			±			±			±	
	∑ (%)	2451.20	±	342.92	1729.65	±	197.67	1546.83	±	265.86		688.67	±	192.20	380.66	±	50.33	344.14	±	50.31
	iP	906.82	±	38.16	705.39	±	54.47	686.98	±	34.39			±			±			±	
	tZ	706.12	±	68.70	642.83	±	42.88	415.33	±	72.52	cZ	7005.89	±	567.09	5353.48	±	704.45	4802.45	±	409.44
	DHZ	144.83	±	30.83	134.80	±	2.03	115.31	±	3.35			±			±			±	
	∑ (%)	1757.76	±	137.68	1483.02	±	99.38	1217.62	±	110.25		7005.89	±	567.09	5353.48	±	704.45	4802.45	±	409.44
Glycosylated (inactive) forms	IP7G	13901.29	±	601.05	13874.89	±	520.44	15103.52	±	790.38			±			±			±	
	tZ7G	7018.79	±	773.75	7915.70	±	1640.39	8334.84	±	1310.22	cZ7G	4738.42	±	211.43	3421.38	±	341.47	3435.88	±	189.52
	DHZ7G	1312.34	±	158.02	1440.36	±	166.21	1464.68	±	168.79			±			±			±	
	IP9G	1099.03	±	124.66	1050.83	±	42.76	1082.33	±	37.35	cZ9G	-	±	-	-	±	-	-	±	-
	tZ9G	2073.68	±	216.14	2156.26	±	445.28	2481.29	±	745.76			±			±			±	
	DHZ9G	112.03	±	14.45	187.47	±	54.43	78.83	±	11.39			±			±			±	
	tZOG	1533.55	±	76.33	2396.16	±	557.47	2186.88	±	409.94	cZOG	780.67	±	100.82	627.83	±	68.53	645.95	±	77.29
	DHZOG	217.15	±	16.08	-	±	-	166.87	±	1.07			±			±			±	
	tZROG	422.86	±	49.12	363.83	±	34.65	472.35	±	33.70	cZROG	1029.10	±	42.24	991.30	±	118.57	963.11	±	198.03
	DHZROG	408.62	±	69.47	332.48	±	19.49	326.62	±	74.73			±			±			±	
	∑ (%)	28099.34	±	2099.07	29717.99	±	3481.12	31698.22	±	3583.33		6548.19	±	354.49	5040.50	±	528.57	5044.94	±	464.84
TOTAL	∑ (%)	46815.98	±	5377.16	51149.16	±	5359.20	51492.63	±	6020.66		19120.52	±	1422.49	16183.14	±	1492.07	14771.79	±	1225.22

Table 2: (continued)

B) 12 multi-wells plates		MEP pathway (plastid) derived CKs									MVA pathway (cytosol) derived CKs									
		Control			0.1 µl ml^{-1} AA			0.01 µl ml^{-1} AA				Control			0.1 µl ml^{-1} AA			0.01 µl ml^{-1} AA		
	iPRMP	12435.55	±	1197.13	12563.08	±	1012.79	10879.36	±	769.82			±			±			±	
	tZRMP	3051.67	±	973.97	-	±	-	2409.91	±	180.63	cZRMP	4901.11	±	414.83	5774.15	±	714.33	6320.46	±	328.08
	DHZMP	86.59	±	19.75	-	±	-	71.81	±	6.19			±			±			±	
Precursors	∑ (%)	15573.81	±	2171.10	12563.08	±	1012.79	13361.08	±	956.64		4901.11	±	414.83	5774.15	±	714.33	6320.46	±	328.08
	iPR	1506.98	±	269.03	2352.39	±	242.61	2079.07	±	423.59			±			±			±	
	tZR	690.89	±	40.37	-	±	-	922.30	±	190.69	cZR	747.58	±	117.08	911.50	±	99.46	1115.40	±	312.74
	DHZR	155.60	±	19.31	-	±	-	152.02	±	15.44			±			±			±	
	∑ (%)	2353.47	±	328.72	2352.39	±	242.61	3153.38	±	629.72		747.58	±	117.08	911.50	±	99.46	1115.40	±	312.74
	iP	868.81	±	111.71	872.00	±	96.31	711.25	±	37.53			±			±			±	
Active forms	tZ	501.16	±	123.43	764.09	±	216.72	375.47	±	49.42	cZ	5522.40	±	231.73	4605.85	±	378.70	3847.46	±	436.39
	DHZ	155.60	±	19.31	399.45	±	146.88	152.02	±	15.44			±			±			±	
	∑ (%)	1525.57	±	254.45	2035.53	±	459.91	1238.73	±	102.40		5522.40	±	231.73	4605.85	±	0.38	3847.46	±	436.39
	IP7G	13864.85	±	564.18	15730.94	±	796.52	16029.18	±	1093.11			±			±			±	
	tZ7G	5271.48	±	620.13	8397.14	±	1258.23	6526.65	±	668.96	cZ7G	3205.68	±	141.26	3572.63	±	287.11	3887.65	±	238.03
	DHZ7G	1488.45	±	420.20	-	±	-	1377.23	±	129.53			±			±			±	
	IP9G	1217.46	±	91.16	1272.11	±	168.95	1183.07	±	165.44	cZ9G	-	±	-	-	±	-	-	±	-
	tZ9G	2172.35	±	108.18	2990.35	±	382.80	2234.43	±	272.39			±			±			±	
Glycosylated (inactive) forms	DHZ9G	114.36	±	23.21	-	±	-	73.89	±	8.54			±			±			±	
	tZOG	1906.72	±	294.83	2419.33	±	458.95	1659.78	±	90.26	cZOG	644.65	±	98.85	678.61	±	67.97	694.85	±	51.19
	DHZOG	172.98	±	16.69	-	±	-	168.59	±	11.60			±			±			±	
	tZROG	554.18	±	170.37	-	±	-	459.75	±	30.84	cZROG	1147.36	±	149.81	1164.32	±	111.10	1116.71	±	104.70
	DHZROG	335.20	±	43.83	-	±	-	351.21	±	39.67			±			±			±	
	∑ (%)	27098.03	±	2352.78	30809.86	±	3065.45	30063.79	±	2510.32		4997.69	±	389.91	5415.57	±	466.18	5699.21	±	393.92
TOTAL	∑ (%)	46550.88	±	5107.05	47760.87	±	4780.76	47816.97	±	4199.08		16168.79	±	1153.55	16707.06	±	1280.34	16982.53	±	1471.13

Table 3: The levels of MEP and MVA pathway-derived CKs (fmol g^{-1}) in leaves of *Arabidopsis* plants cultured in A) 6 multi-well plates and B) 12 multi-well plates on 1x MS medium in enclosed plastic boxes in continuous presence or absence of VCs emitted by *Alternaria alternata*. Significant differences to Kruskal Wallis test ($p<0.05$) were calculated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

A) 6 multi-wells plates	**MEP pathway (plastid) derived CKs**							**MVA pathway (cytosol) derived CKs**						
		- VCs			**+ VCs**				**- VCs**			**+ VCs**		
	iPRMP	5947.47	±	724.70	6653.01	±	465.37			±			±	
	tZRMP	4065.71	±	183.30	3058.13	±	556.73	cZRMP	4960.68	±	523.18	3728.93	±	404.34
	DHZMP	-	±	-	-	±	-			±			±	
Precursors	∑ (%)	10013.18	±	908.00	9711.14	±	1022.09		4960.68	±	523.18	3728.93	±	404.34
	iPR	738.69	±	53.92	870.56	±	134.18			±			±	
	tZR	893.42	±	110.71	692.61	±	72.39	cZR	188.07	±	16.06	251.97	±	50.69
	DHZR	70.25	±	9.18	83.48	±	7.71			±			±	
	∑ (%)	1702.36	±	173.82	1646.64	±	214.28		188.07	±	16.06	251.97	±	50.69
	iP	14592.78	±	1043.93	12404.50	±	498.79			±			±	
Active forms	tZ	-	±	-	-	±	-	cZ	27085.18	±	6413.81	22568.05	±	1600.90
	DHZ	500.00	±	57.61	508.28	±	52.75			±			±	
	∑ (%)	15092.78	±	1101.54	12912.78	±	551.54		27085.18	±	6413.81	22568.05	±	1600.90
	IP7G	23751.07	±	619.16	18654.51	±	1215.61			±			±	
	tZ7G	12965.85	±	625.10	10956.45	±	667.26	cZ7G	4909.12	±	135.07	3826.49	±	312.63
	DHZ7G	1767.46	±	128.82	1888.00	±	124.96			±			±	
	IP9G	1066.07	±	108.44	728.88	±	45.65	cZ9G		±			±	
Glycosylated (inactive) forms	tZ9G	4592.81	±	777.13	3923.13	±	442.16			±			±	
	DHZ9G	76.71	±	11.28	78.62	±	11.01			±			±	
	tZOG	3861.97	±	318.61	1341.69	±	835.84	cZOG	308.21	±	62.76	291.49	±	43.09
	DHZOG	71.69	±	4.66	78.97	±	3.83			±			±	
	tZROG	384.03	±	56.70	285.39	±	14.31	cZROG	1034.89	±	81.52	1136.85	±	50.61
	∑ (%)	48537.66	±	2649.91	37935.63	±	3360.64		6252.22	±	279.35	5254.83	±	406.33
TOTAL	∑ (%)	75345.98	±	4833.27	62206.20	±	5148.55		38486.15	±	7232.40	31803.77	±	2462.26

Table 3: (continued)

B) 12 multi-wells plates		MEP pathway (plastid) derived CKs						MVA pathway (cytosol) derived CKs					
		- VCs			+ VCs				- VCs			+ VCs	
Precursors	iPRMP	3939.09	±	385.88	5547.83	±	311.51			±		±	
	tZRMP	2301.02	±	207.68	4183.25	±	519.64	cZRMP	5034.13	± 565.79	3185.84	±	292.33
	DHZMP	-	±	-	-	±	-			±		±	
	∑ (%)	6240.11	±	593.55	9731.08	±	831.16		5034.13	± 565.79	3185.84	±	292.33
Active forms	iPR	636.17	±	99.75	672.48	±	120.20			±		±	
	tZR	505.96	±	98.38	954.36***	±	138.36	cZR	234.83	± 32.77	141.12	±	24.56
	DHZR	75.32	±	7.89	93.30	±	7.28			±		±	
	∑ (%)	1217.45	±	206.02	765.77	±	265.84		234.83	± 32.77	141.12	±	24.56
	iP	30336.35	±	3860.68	20395.37	±	931.49			±		±	
	tZ	-	±	-	-	±	-	cZ	30180.24	± 5321.38	29922.02	±	3007.02
	DHZ	376.22	±	28.94	416.99	±	22.15			±		±	
	∑ (%)	30712.57	±	3889.63	20812.37	±	953.64		30180.24	± 5321.38	29922.02	±	3007.02
Glycosylated (inactive) forms	IP7G	31034.84	±	500.58	12492.89***	±	1445.33			±		±	
	tZ7G	13159.30	±	482.36	10815.90	±	1473.87	cZ7G	5103.42	± 396.20	2278.42**	±	341.14
	DHZ7G	1979.45	±	112.78	1940.06	±	212.15			±		±	
	IP9G	1249.23	±	148.06	519.78**	±	41.19	cZ9G		±		±	
	tZ9G	4578.33	±	698.18	4758.05	±	481.21			±		±	
	DHZ9G	61.60	±	10.66	66.89	±	7.59			±		±	
	tZOG	2467.36	±	610.15	2814.06	±	457.05	cZOG	262.55	± 54.05	77.23	±	13.15
	DHZOG	80.68	±	4.41	102.89	±	4.86			±		±	
	tZROG	387.09	±	47.19	365.33	±	60.12	cZROG	1617.71	± 247.03	1197.06	±	192.13
	∑ (%)	54997.89	±	2614.37	20863.20	±	4183.37		6983.68	± 697.28	1274.29	±	546.41
TOTAL	∑ (%)	93168.01	±	7303.58	52172.41	±	6234.01		42432.88	± 6617.21	34523.27	±	3870.32

6. Discussion

6.1 Validation of high-throughput phenotyping method for *Arabidopsis* rosette growth in presence of fungal exudates or volatile compounds

Recently, the use of high-throughput phenotyping platforms (HTPP) as a method for obtaining and subsequent analysis of specific traits of plant growth in different environmental conditions have been widely used (Arvidsson *et al.*, 2011).

In this bachelor thesis, the HTP method was used for the validation and optimization of a fast, robust and reproducible high-throughput *in vitro* bioassay for *Arabidopsis* plants focused on the identification of mechanisms of action of biostimulants. Positive effects of the interaction between plants and fungal exudates and VCs emitted by AA were examined. High-throughput phenotyping Olophen platform was used as a tool to give reliable and reproducible information about the effect of biostimulants.

As presented in **Figure 11A** and **11B**, the data obtained from HTPP and from manual measurements of FW proved high correlations between these two methods. Based on the results, it is possible to confirm that HTP method is suitable for obtaining reproducible data for analysis of *Arabidopsis* rosette growth *in vitro*, avoiding significant differences between replicates used in different treatments and conditions (De Diego *et al.*, 2017).

6.2 Exudates and volatiles emitted by phytopathogenic fungi *Alternaria alternata* promote growth of *Arabidopsis* rosettes

During the decades, plants have developed many response mechanisms to defend themselves against pathogenic fungi and bacteria. Conversely, many recent studies have provided the information that some phytopathogenic microorganisms may have also a positive effect on a plant growth and development (Ryu *et al.*, 2003; Ezquer *et al.*, 2010; Sánchez-López *et al*., 2016a; Sánchez-López *et al*., 2016b).

This bachelor thesis includes the experiments about the possible positive interaction between plants and microorganisms, involving the effects of AA exudates and VCs. From the results presented above it is clear that both, exudates and VCs promoted growth of *Arabidopsis thaliana* plants.

In the experiment with AA exudates, the biggest changes in *Arabidopsis* rosette growth were visible after 4 days of cultivation. After one week of exposure of *Arabidopsis* plants to AA exudates, the most significant increase in a rosette growth was observed in 6 multi-well plates with 0.01 µl ml^{-1} AA exudates and in 12 multi-well plates with the

concentration 0.1 µl ml^{-1} (**Figure 12A, 12B**). Based on these results, it can be assumed that the amount of nutrients contained in MS medium in 6 multi-well plates was sufficient for plant nutrition. On the contrary, in 12 multi-well plates, the plants exhausted all the nutrients way earlier than the once present in 6 multi-well plates. Consequently, the supplies of nutrients after 4 days started to be limited and the exudates became an alternative source of a plant nutrition observing better results with the higher concentration of applied exudates (Zhang and Forde, 2000)

In terms of results obtained in the experiment with VCs in which *Arabidopsis* plants were exposed to AA for one week, the highest growth rate was observed in 12 multi-well plates. In case of 6 multi-well plates, the growth of *Arabidopsis* rosettes was slightly inhibited due to the presence of contamination produced during the manipulation process of the bioassay. Despite the contamination, it is worth to mention that survival of plants was high enough (**Figure 13A, 13B**) (Wajahatullah *et al.*, 2009).

6.3 Presence of *Alternaria alternata* exudates and volatiles affect the content of auxins and ABA

It is generally known that plant hormones are important regulators of a plant growth. Specifically, ABA is the most important for plant responses to stress conditions.

In experiments included in this thesis, *Arabidopsis* plants were exposed to biotic stress in form of AA exudates or VCs. All measured data indicate that the changes in the levels of ABA were affected by the presence of these stress factors. The most significant results were obtained in *Arabidopsis thaliana* plants cultured in multi-well plates with addition of AA exudates (0.1 µl ml^{-1}) and in plants cultured in 6 multi-well plates treated with VCs. Regarding the 12 multi-well plates, the content of ABA was quite lower than in control plants probably because of the contamination which caused more stress on treated and also non-treated plants (**Table 1**).

Concerning auxins, the main difference between the effects of AA exudates and VCs was the presence of different auxin conjugates (**Table 1**). While AA VCs increased the levels of IAA, oxIAA, IAGlu and IAAsp, in the experiment with exudates, only IAA and oxIAA were detected. Recently, Sánchez-López *et al.* (2016) published a scientific research in which they analysed the content of free amino acids in leaves of *Arabidopsis* mutant *pgi1-2* exposed to continuous presence of VCs emitted by AA. This study revealed that the content of glutamic acid (Glu), aspartic acid (Asp) and other amino acids was significantly higher in leaves treated with fungal VCs. Based on this, it can be considered

that unlike exudates, VCs caused the presence of IAGlu and IAAsp in leaves of *Arabidopsis thaliana.* However, the levels of both, IAGlu and IAAsp in leaves treated with VCs decreased compared to controls, with statistical significant differences in case of IAGlu. These results suggest that plants preferred the free forms of amino acids to use them as compatible solute to respond against the stress induced by AA VCs (Forde and Lea, 2007).

6.4 *Arabidopsis* plants respond to fungal exudates and volatile compounds by changes in levels of MEP and MVA-derived CKs

As already mentioned, CKs have a key role in many plant developmental processes. In addition to this, CKs are very important in the interaction of a plant with many different environmental conditions, mainly biotic and abiotic stresses. Many recent studies have already proved that CKs promote high accumulation of starch in *Arabidopsis* leaves, enhance photosynthesis, flowering and growth (Ezquer *et al.*, 2010; Sánchez-López *et al.*, 2016b).

The results presented in **Table 2A** and **2B** show that the levels of MEP-derived CKs in leaves of *Arabidopsis* plants treated with AA exudates are slightly higher than in control leaves. These results indicate that exudates promoted changes in the levels of these CKs. On the other hand, the levels of MVA-derived CKs were lower than in non-treated plants. These differences were most likely caused by different synthetic pathways of CKs which AA exudates activate.

In addition, plants have an ability to detect VCs emitted by diverse microorganisms (Sánchez-López *et al.*, 2016b). From **Table 3A** and **3B** it is clear that the content of MEP and also MVA-derived CKs was reduced. Furthermore, the presence of the most biologically active hormone *t*Z was not detected because its level was under limit of detection (LOD). Compared to the experiment of Sánchez-López *et al.* (2016b) in which the levels of MEP and MVA-derived CKs were higher than in control plants, it is clear that due to the contamination of our experiment, the synthesis of these CKs was rather inhibited. Even though that the previously mentioned phytohormones and the growth of *Arabidopsis* rosettes was down-regulated, the presence of VCs supported the ability of plants to survive in these unfavourable conditions.

Considering all the results presented in this bachelor thesis, it is possible to say that the effects of exudates and VCs emitted by *Alternaria alternata* had a significant impact on a growth of *Arabidopsis* rosettes. Furthermore, it has been proved that the effect of

AA exudates and VCs activate different pathways of hormone synthesis, specifically auxins, ABA and cytokinins.

7. Conclusion

The main purpose of the bachelor thesis was the implementation of the study about possible positive interaction between plants and microbes using *Alternaria Alternata* as an example.

There were two types of biostimulants used in order to study the effects of biotic stress on plant growth: exudates and VCs emitted by the phytopathogenic fungi AA.

After one week of exposure to these already mentioned stress factors, exudates and VCs, the growth of *Arabidopsis* plants was determined using two different methodologies, classical biomass determination and HTP as a fast, easy and reproducible method.

The results obtained from the experiments proved that exudates and VCs have promoted the growth of *Arabidopsis* rosettes and also caused changes in levels of different fractions of phytohormones affecting various plant developmental processes.

In general, it can be assumed that the use of biostimulants on agriculturally important plants has a huge perspective in near future. On the bases of the results included in this thesis, even though that the VCs have a better impact on *Arabidopsis* growth, the exudates have more advantages in their general application. The use of HTPP provides an automated and non-destructive method with the possibility to perform simultaneous analyses of plant growth, morphology and photosystem efficiency in one run. Taking in account the advances in this integrative system, the application of exudates in contrast with VCs provide the possibility to analyse higher number of plants, the application of wide range of different concentrations and a faster and easier way of application.

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