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Induction of Plant Defence and Secretion of Volatile Compounds by *Colletotrichum acutatum* in Leaf Infection Assay of Woodland Strawberry

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Induktion av växtförsvar och utsöndring av lättflyktiga ämnen av svamp-patogenen *Colletotrichum acutatum* i ett bladinfektionsförsök med smultron

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Abstract

Consumers demand healthy food with less residues of pesticides, in addition to the global awareness of the negative impact such chemicals have caused to the environment during the last decades. Farmers and other agricultural participants are far away from completely end up the usage of pesticides but currently used practice could stepwise be improved in pest management. One major step in this progress is to improve tolerance in our existing cultivars and/or breed new cultivars based on improvements in research in such way that includes plant-pest interactions on the genomic, transcriptomic, and metabolomic level. Plant tolerance (in stronger sense *resistance*) against pests is a great area of research aiming for reduction in usage of pesticide. One possibility to improve our knowledge of tolerance/resistance, which partially is based on plant produced antimicrobial compounds, is to bioassay such compounds (or secondary metabolites) with plants collected from nature. Woodland strawberry (*Fragaria vesca*) is a close relative to garden strawberry (*Fragaria x ananassa*). *Fragaria vesca* co-existis with important pathogens to *F. x ananassa* and this wild relative is used as the model plant for research in pathogen/pest-interactions of *F. x ananassa.* In this work, different fungal pathogens were bio assayed to determine tolerance traits of one *F. vesca* genotype whose detached leaves has (in previous infection assay) showed to be resistant against the fungal pathogen *Colletotrichum acutatum,* causing anthracnose disease of *F. x ananassa*. An inhibition assay was performed with *F. vesca* leaf extract, resulting in inhibition of the fungal pathogen *Botrytis cinerea* (causing grey mould on *F. x ananassa* fruit). This experiment was practicing the well-established theory of pathogen associated molecular pattern (PAMP)-Triggered Immunity (PTI), with responses like *de novo* production of antimicrobial compounds. Moreover, *C. acutatum* has been shown to secret β-caryophyllene (a volatile sesquiterpenoid) in resent infection assay with strawberry fruits. Interestingly, β-caryophyllene is also produced by a large number of different plant species. Some plant-secreted volatiles repeals and some attract different lifeforms (e.g. insects) to plants and this phenomenon indicates that volatiles could be essential for the pathogen lifestyle. However, *C. acutatum* was used for infection assay of *F. vesca* leaves to determine if β-caryophyllene is secreted during this particular (leaf infection) interaction. Gas Chromatography Mass Spectrometry (GC-MS) analysis was performed for detection of volatiles, resulting in higher abundancy of β-caryophyllene and other volatile sesquiterpenoids in infected leaf samples, in comparison with uninfected leaves (control). RNA extraction of the same leaf samples which were used for the GC-MS analysis was also performed to reveal that the increased abundancy of β-caryophyllene in infected samples, was due to the pathogen secretion and not by the leaf itself. The enzyme which catalyzes the biosynthesis of this product (β-caryophyllene) is coded by the *C. acutatum* Sesquiterpenoid synthase gene (*CaTPS*), the gene of interest in this study. *CaTPS* RNA was not detected during PCR step, most likely due to fail in cDNA synthesis which relies on the purity level of the RNA sample taken from the infected *F. vesca* leaf tissue.

1 Disposition

2 Introduction

2.1 Plant defense towards fungal pathogens

Interactions between plants and associated pathogens are complex. The fungal phyto pathogens have to overcome a plethora of host defenses which include physical and chemical barriers. The latter can be divided into constitutive (always present in plant) or induced production of secondary metabolites (SM) which are chemicals that are not needed for general growth or reproduction with antimicrobial properties against various pests and pathogens (Agrawal, A.A., 1999; Wittstock & Gershenzon, 2002; Freeman et al., 2008). These bioactive compounds can be phytoalexins (biosynthesized *de novo* in response to plant receptor stimulus) and they accumulate rapidly during pathogen infection (explained in detail later in this section). The constitutive defenses and its related compounds increases under various stresses (such as pathogen attack) and these are called phytoanticipins. In addition to chemical defenses, plants also have physical barriers like different plant structures such as the cuticle (a layer of wax coating the surface of different plant tissues) and the cell wall (mainly a complex network of polysaccharides surrounding the plant cell membrane) (Malinovsky et al., 2014). Fungal pathogens need to overcome these physical barriers and bioactive compounds in order to penetrate the plant cell and facilitate successful infection.

The inducible defense is an evolutionary result of the trade-off between growth/reproduction and defense. In other words, some plant defense systems are only induced in presence of pathogens, in purpose of saving resources for other metabolic processes (i.e. growth and reproduction). Plants have evolved two lines of strategies to recognize pathogens. The first line of defense perceives molecules essential to pathogens (such as fungal cell wall components) which can be of polysaccharide (*e.g.* beta-1,3 glucan) or chitin origins (Yoshimi, Miyazawa & Abe, 2017). These are often referred to pathogen-associated molecular patterns (PAMPs) and they are recognized trough stimulation of pattern recognition receptors (PRRs) which has a transmembrane position (Erwig et al., 2017). The fungal cell wall can be degraded by extracellular pathogenicity related (PR) proteins (in this context enzymes like chitinases and 1,3 glucanases). The degradation products (e.g. chitin fragments and oligosaccharides), degraded by the PR proteins are then being recognized by PRRs as foreign molecules, Figure 1. This particular stimulation activates the PAMP-trigged immunity (PTI) (Qi et al., 2011).

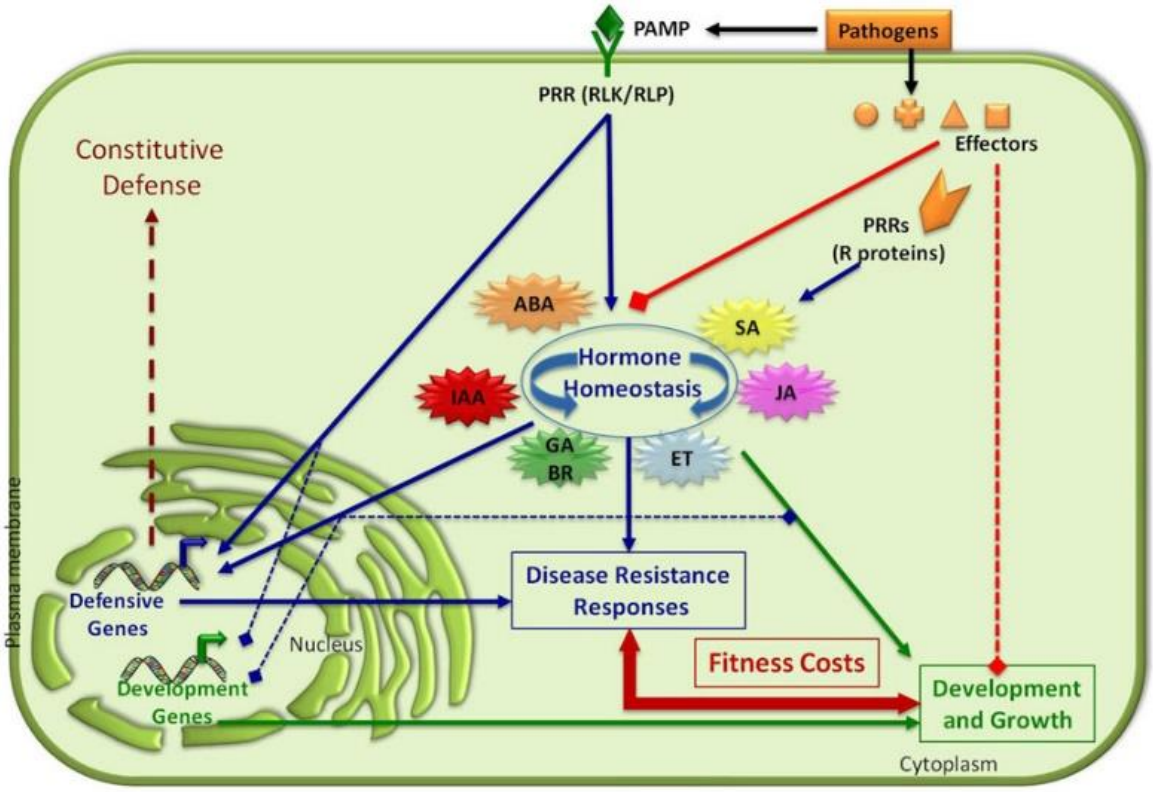

Figure 1, PAMPs are detected by transmembrane PRRs (attached to the cell membrane) (Erwig et al., 2017). Effectors released by the pathogen interferes with defense pathways such as hormone signaling pathways. PRRs (e.g. NBS-LRRs) can detect these effectors. Successful recognition of PAMPs and effectors induces defense responses. Activation of defense responses is a fitness cost for the host (Denancé et al., 2013).

The second line of defense is the gene-for-gene type interaction between fungal avirulence proteins and plant resistant proteins (R-proteins), the gene products of resistant genes (R-genes) (Van de Wouw, Elliott & Howlett, 2014). Both avirulence and virulence proteins are together defined as *effectors* because they are proteins that aim to manipulate the host cell to benefit the pathogen (Irieda, Ogawa & Takano, 2016). Effector-trigged immunity (ETI) is activated via R-protein recognition of fungal effectors (Qi et al., 2011). This is done by intracellularly located nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs) (Nishimura & Dangl, 2014) and/or nucleotide binding, leucine-rich repeats (NBS-LRR) plant resistance receptors (both are examples of PRRs) (Mchale et al., 2006). It is worth to mention that gene-for-gene interaction between plant tissue (such as leaf) and fungal effectors could lead to hypersensitive response (HR) or programmed cell death (PCD) (Cuming, 2009). The HR leads to dead plant tissue in and around the infection cite, which in its turn, isolates the pathogen inside dead tissue.

The first and second line of defense are accompanied by a set of induced responses which can prevent the attack of fungal pathogens. There are complex pathways in each and every defense response as seen in Fig. 1. Different phyto-hormones, which are chemicals that regulate plant growth, are also involved in different first/second- line defense pathways (e.g. jasmonic acid (JA), salicylic acid (SA) and ethylene (ET)). SA signaling is relevant to mention due to its importance in the systemic acquired resistance (SAR). SAR induces long lasting immunity towards various pathogens (Vidhyasekaran, 2015). This means that plants produce bioactive compounds towards different pathogens, as a result of pre-induced resistance activated by a single pathogen.

2.2 Lifestyles of fungal pathogens in relation to plant defense

Some pathogenic fungi depend on living plant tissue like biotrophic fungi which are dependent on host metabolism, while other kills the plant tissue (via secretions of cell wall degrading enzymes or toxins) and uses its dead cell components as substrate for their own metabolism (called necrotrophic fungi). In addition, there are pathogens which can shift from biotrophic to necrotrophic lifestyle depending of the stage of infection and defense responses called hemi-biotrophic fungi. Necrotrophic pathogens benefits from HR and related responses because the plant does the same as the pathogen - causing necrotic tissue. *Botrytis cinerea* is an important agricultural necrotrophic pathogen which has a huge host range. This pathogen was not able to infect plant tissue in an experiment with *Arabidopsis thaliana* mutant strain which could not produce HR (Govrin & Levine, 2000). This plant-fungus interaction study indicates that other defense responses (in absence of the HR) contributes to the suppression of necrotrophic pathogen infections. It is plausible that the previously mentioned SAR and the induced production of phytoalexins are the key factors of this defense. Therefore, necrotrophic fungi are possibly behind the induction of the essential components needed for suppression of the pathogens. Pétriac et al. (2016) showed that the spore suspension of a necrotrophic pathogen *Plectosphaerella cucumerina* (fungal model organism – co-exist with *A. thaliana* in nature) induces different defense pathways depending on the spore suspension concentration (or density). High density of spores induces JA dependent defenses and low spore density induces SA dependent defenses (Pétriac, Stassen & Ton, 2016). A most recent study by Amil ruiz et al., (2016) showed that genes associated with JA and SA pathways are activated during the infection of *Colletotrichum acutatum*. A part of this BSc study is based on

induction of phytoalexins based on the SAR theory by using a fungal spore suspension with lower concentration than the one used for infection inoculation of *C. acutatum.*

2.3 *Colletotrichum spp*. & Woodland strawberry (*F. vesca*)

So far, focus of this introduction has been on describing important plant defense responses against fungal pathogens. The fungal pathogen of interest in this study is *Colletotrichum acutatum.* This pathogen is important because it has been reported to cause devastating crop losses to various plant species. *Colletotrichum acutatum* belongs to the division of Ascomycota and causes anthracnose disease of garden strawberry (*Fragaria* x *ananassa*) cultivars with great yield losses and affecting commercial plantation worldwide (Perez et al., 2005). It is highly specialized to the tissue it infects, which varies depending on the host; for strawberries, infection sites vary from leaves, flowers, preharvest fruits (in some case postharvest symptoms) and roots (Peres el al., 2005). *Colletotrichum spp.* belongs to top 10 most important phyto pathogens in the world (Dean et al., 2012). *Colletotrichum acutatum* uses different lifestyles depending on the host it infects and the stage of infection. In strawberries, it primarily uses the necrotrophic lifestyle (Perez et al., 2005). *C. gloeosporioides* is a close relative to *C. acutatum* causing huge crop losses in strawberry production. Transcriptome (gene expression) analysis of *C. gloeosporioides* has been performed through an infection assay with woodland strawberry *F. vesca* and data suggests that genes coding for the previously mentioned NB-LRR proteins (R-protein/PRR) are activated (Li et al., 2013). Moreover, transcriptomes of genes coding for β-1,3-glucanases (PR-protein) was detected after inoculation of *C. acutatum* in garden strawberry *Fragaria x ananassa* (Zamora et al., 2012).

To date, only a few studies have been performed on *C. acutatum* interactions with *F. vesca* (the model plant used in research of the Rosaceae family). *F. vesca* is less complicated to study at the genetical level – it is a diploid species – in comparison too garden strawberries (*Fragaria x ananassa*) which is an octoploid species (Amil-Ruiz et al., 2016). In addition, resistance in garden strawberries seems to be quantitatively inherited, which makes resistance breeding even harder (Amil-Ruiz et al., 2016). The whole genome of *F. vesca* is sequenced (Shulaev et al., 2010) and this species has co-existed together with various pathogens and evolved defense related compounds against them. One way of using this wild relative to garden strawberry, is to investigate the resistance levels among different

genotypes. The resistance could be determined through inoculation assays as a first step, followed by selection of genotypes to be used for transcriptome analysis. Fungal transcriptomes are also interesting to analyze to determine presence of fungal secondary metabolites during the infection.

2.4 Role of fungal secondary metabolites and effector secretion

Plants produce many molecules in interaction with fungal pathogens. In response, fungal pathogens also produce different molecules that could be used either to suppress the plant defenses (Embengue et al., 2016) or in competition against other microbes. For example, *Sclerotinia sclerotiorum* secretion of oxalic acid interferes with several plant defense responses (e.g. PCD), on the other hand, oxalic acid contributes to the induction of PCD (Kim et al., 2008). Interestingly, Irieda et al. (2016) showed that hemi-biotrophs secrete effectors from the biotrophic state part of the hyphae that allows the pathogen to regulate PCD responses. The garden strawberry cv. Houkouwase accumulates triterpenoids (cyclic C30 bioactive molecules with multiple combinations of functional groups) in the unripe fruits as a response to infection with *Colletotrichum musae* (Hirai et al., 2000). Three of these triterpenoids where isolated and used in inhibition assay, where two of them inhibited growth of *C. musae*. Another class of terpenoids is sesquiterpenoid (STs, C15) which often are volatile bioactive molecules. Both triterpenoids and STs are bioactive compounds known as important antimicrobials (*e.g.* phytoalexins and phytoanticipins) to ward off plant pathogens in many plants. The fungal genes and their terpenoids produced during plant infections are from several studies shown to have important roles in mycotoxin (toxic to mammals) and phytotoxin (toxic to plants causing disease symptoms) biosynthesis (Cardoza et al., 2011; Hohn & Desjardins, 1992; Proctor et al., 1995; Rynkiewicz et al., 2001; Trapp et al., 1998). This make terpenoids important both on the plant as well on the pathogen side, but information is lacking or remain poorly understood during the interaction between *Colletotrichum* spp. and strawberry plants. STs have been detected during *in vitro* growth of *C. acutatum* on strawberry fruit medium, indicating these volatiles could be essential for the infection process (Amby et al., 2016). Among the detected compounds, β-caryophyllene, a product of *C. acutatum* sesquiterpenoid synthase (CaTPS). In the same study, gene expression of *CaTPS*, coding for this essential β-caryophyllene producing enzyme, confirmed that the enzyme was active both *in vitro* and during fruit infection. Interestingly, Amby et al. also showed that a higher amount

of novel (unidentified) STs was detected during *in vitro* growth on strawberry fruit medium, compared to potato dextrose broth (PDB) medium. In total, 24 ST related compounds were detected from the both media. Characterization of STs and their roles as possible virulence related molecules could contribute to a deeper understanding of virulence-strategies of *C. acutatum.*

To date, no study has been made on terpenoid secretion by *C. acutatum* during leaf infections in *Fragaria* spp. or any other plant species. Since the whole genome is sequenced for *F. vesca*, gene expressions in response to volatiles secreted by *C. acutatum* can be determined. In addition, fungal transcriptome analysis or qRT-PCR (with primers specific for CaTPS) could be performed to determine if the gene of interest is expressed (i.e. *CaTPS*) during leaf infection.

2.5 Objects of this study

This experimental study and volatile analysis was performed to determine if *C. acutatum* secrets terpenoid volatiles during infection of *F. vesca* leaves, as seen for the pathogen during the *in vitro* growth on medium and garden strawberry fruits (Amby et al., 2016). This observation is important because volatiles and especially β-caryophyllene could be of great importance to understand the lifestyle and interaction ecology of *C. acutatum* and strawberry plants. This type of knowledge, in its turn, could contribute to better management of anthracnose disease and the role of volatiles in general for other fungal pathogens. In addition, PAMP-triggered immunity (PTI) of one pathogen could possibly induce defense against other fungal pathogens. It is possible that genotypes with high tolerance of *F. vesca* also has a broader spectrum of tolerance against different pathogens, with eventually more diversity among their secondary metabolites. The cell is like a fabric producing a lot of molecular products, whereof production of antimicrobial compounds could wary depending on environmental stimulus and on the genetical level. It could be possible to induce defense pathways and phytoalexins in *F. vesca* leaves, by inoculation with relative low concentration of *C. acutatum* spore suspension, just to stimulate the PRRs without having the leaves eventuate in infection. The observations will be done with focus on inhibition to determine tolerance of *F. vesca* with (a sort of primed or induced) leaf extract against various pathogens to receive a first indication of broad spectrum tolerance, or any tolerance at all.

2.6 Hypotheses

1) I hypothesize that *CaTPS* (*C. acutatum* gene coding for sesquiterpenoid synthase) is activated and fungal sesquiterpenoids are secreted by *C. acutatum* during leaf infection of woodland strawberry.

2) I also hypothesize that spore inoculation in the center woodland strawberry leaflet (out of three leaflets on a compound leaf) will induce defense responses (e.g. production of phytoalexins) in the other two (non-infected) neighboring leaflets.

3 Material and methods

3.1 Sesquiterpenoid detection and CaTPS *gene expression analysis*

3.1.1 Leaf samples and surface sterilization

Leaf sterilization was performed before inoculation of *F. vesca* genotype 5A (susceptible or less tolerant to *C. acutatum* infection, identified in previous experiments by Amby). A susceptible genotype was used for this experiment, aiming for high infection and growth of fungal biomass to receive more volatile compounds which makes the comparison to the background (leaves without inoculation) more reliable. 30 compound leaves of 5A were collected and surface sterilized, by first washing them in a bowl filled with tap water, thereafter dipping them for 30 secs in 70 % ethanol, followed by 60 secs in 250 ml/l sodiumhypochlorite at 0,376 M (mol/l). As a last step, leaves were washed with tap water again (by soaking them for approx. two min) to rinse of the disinfection agents. The sterilized leaves were stored in sealed zip lock bags in two days at 4 ℃, before they were used for inoculation. Surface sterilization is important because there are endophytes living on the leaf tissue which could interfere with the infection of *C. acutatum*.

3.1.2 Spore suspension preparation and quantification

Colletotrichum acutatum spores were collected and counted to prepare a spore suspension (spores diluted in water) for inoculation of the collected (and surface sterilized) genotype 5A leaves. The fungal pathogen that was used for this experiment (*C. acutatum*, isolate SA 0-1) was previously isolated in Denmark and stored in 10% glycerol at -80℃ as a stock culture (Sundelin et. al., 2005). From this fungal stock, mycelium plugs were transferred to petri dishes (PDs) with potato dextrose agar (PDA, Sigma aldrich) and propagated for ca. 14-21 days (Amby et al., 2016). New mycelial plugs were transferred to new plates with fungal medium (Amby et al., 2016). All samples were incubated at room temperature for 7 days (Amby et al., 2016). Hereafter, for about one week, the plates with fungal mycelia were exposed daily to ca. 8 hr. of black light (near ultraviolet radiation) to promote fungal sporulation at 25-28 ℃ (Amby et al., 2016). At 14 days in total the spores where collected from the plates by pouring ca. 15 mL of sterile Milli-Q H_2O over the surface of the mycelia. Spores were collected by first scraping the whole surface of the media (with fungal mycelia) with a sterile scalpel in order to disperse the spores in the Milli-Q H_2O. Thereafter, spores were filtered through a 100 µm cell strainer

fitted on a 50 mL falcon tube. In this way, only fungal spores will pass through whereas mycelium are trapped in cell strainer. Spores were stored at 4℃ until use (latest a day after). The spores were counted under a microscope by using a Haemocytometer (http://home.cc.umanitoba.ca/~adam/lab/Haemocytometer), and the final concentration of the spore suspension was 9 x 10^5 spores/ml; Amby et al. (2016) used 1 x 10^6 as their final spore suspension concentration.

3.1.3 Inoculation and incubation of the leaves

The stored surface sterilized 5A leaves where used for the infection assays. Six transparent plastic boxes (PBs) with lids were used as inoculation chambers to achieve high humidity during the incubation step. All 5A leaves were wounded by using a pipet tip which was cut with a scissor (approximately 5 mm from the tip) first to get a rugged surface. The cut pipette tip was pressed (with the rugged surface) gently on each side of the mid vein on the upper surface (adaxial side) on every leaflet of the compound leaves to make entry points for the *C. acutatum* spores (Fig. 2). Five wounded leaves where placed in each PB. The prepared spore suspension was poured into a sterile PD. The leaves were then inoculated by submerging only the adaxial side in the spore suspension for ca. 5 sec. The inoculated leaves were transferred into their respective PBs with one plastic screen was placed in bottom of each PB. Five PBs (a total of 25 leaves) were inoculated with spore suspension and one PB (with five leaves) where mock-inoculated with sterile MQ H_2O (the control samples). Sterile paper tissue was placed below each leaf before they were placed in the incubator, and sterile water was added regularly for leaves to maintain a moisture environment in the PBs. All PBs were placed in an incubator at 30 ℃ day temperature, 90% RH (for 12h) respectively 25 ℃ night temperature, 90% RH (for 12h). The samples were incubated for 12 days (there are

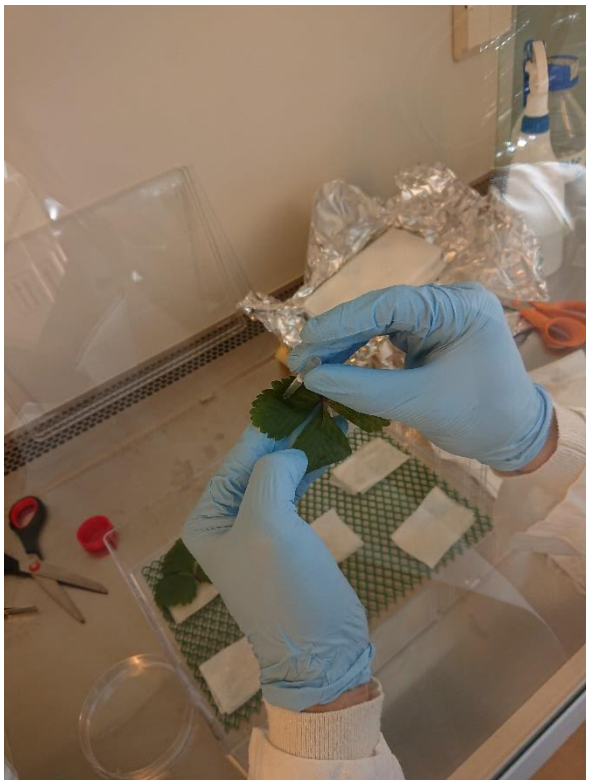

Figure 2, wounding the surface sterilized F. vesca *5A leaves to make entry points for* C. acutatum *spores.*

no straight rules of incubation conditions in infection assay as long as infection occur and leaves are not stressing to much). Condition levels were advised by Amby.

3.1.4 Terpenoids profiling of *Colletotrichum acutatum* during in vitro and in planta growth

- *In planta*

The incubated 5A leaves showed clear symptoms of infection twelve days past inoculation (12-dpi), Fig. 3. Five infected and five control leaves were placed inside ten cryogenic tubes (volume 5 mL) with one compound leaf in each tube sealed with screw caps for releasement and accumulation of volatile compounds (Fig. 4). The plastic tubes with samples (replicates) were placed in the incubator (at previous described conditions) for further two days in order to keep the same conditions during the incubation time. The first Gas Chromatography Mass Spectrometry (GC-MS) analyses were performed on two of the infected samples using SPME fiber (used for collecting volatiles), two days past incubation (2-dpic). Fiber (with collected volatiles) were injected into GC-MS instrument by Sebastian Larsson Herrera (PhD student at the Swedish University of Agriculture Science, Alnarp). The last three infected samples were tested three days later (*i.e.* in total 5-dpic in cryogenic tubes). The increased incubation time for the remaining three infected samples was simply to compare the accumulation of volatiles with the first two samples.

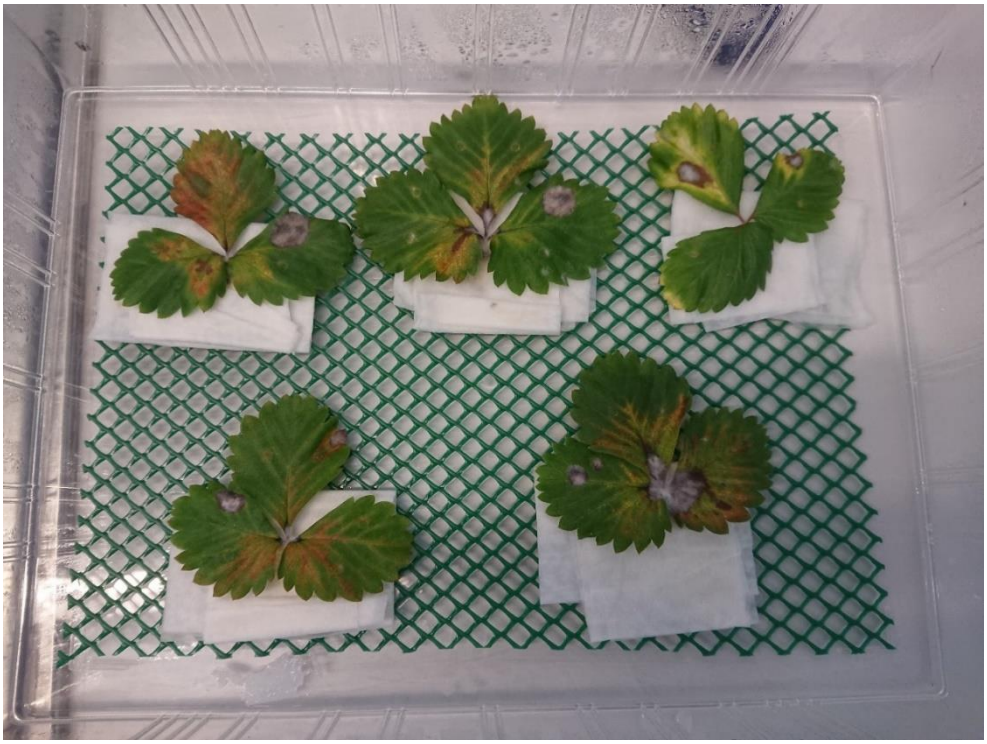

Figure 3, symptoms of C. acutatum *infection of* F. vesca *5A leaves, 12-dpi.*

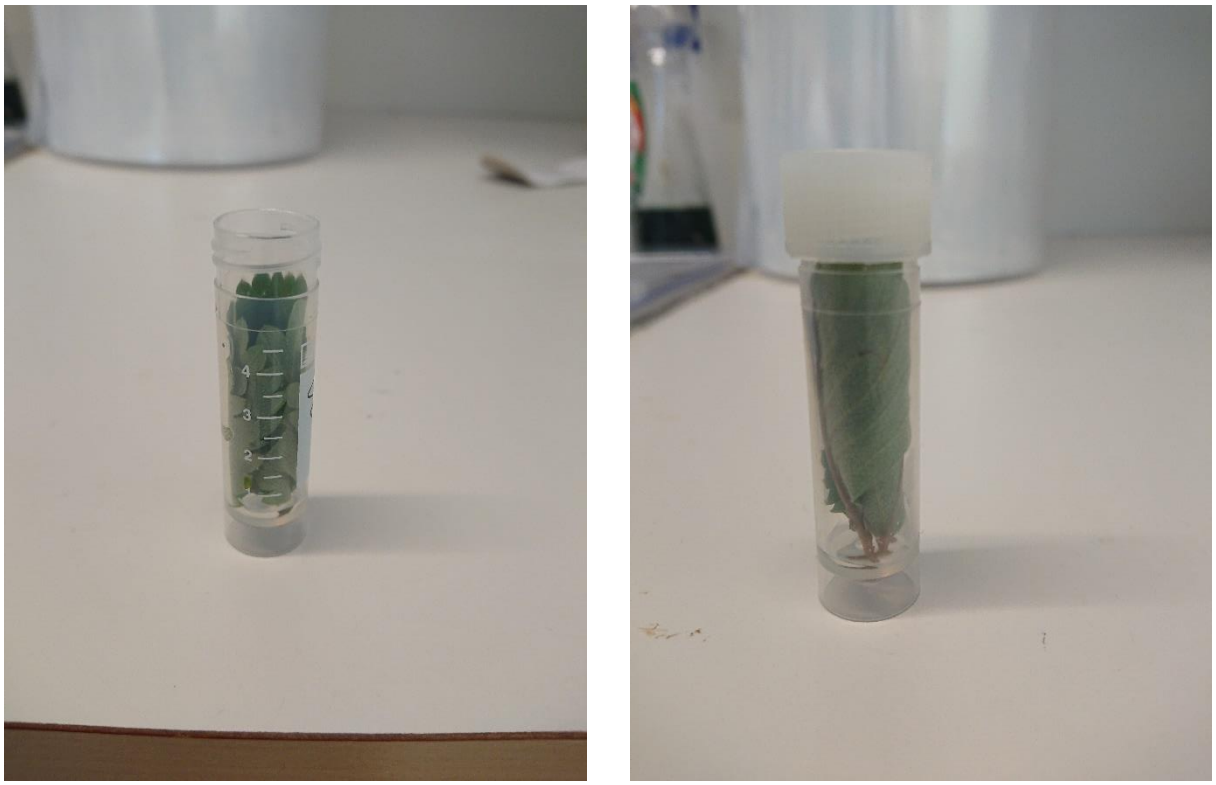

Figure 4, Infected F. vesca *5A compound leaf rolled and placed inside a cryogenic tube for incubation - accumulation of volatile compounds.*

- *In vitro*

Colletotrichum acutatum was grown *in vitro* on PDA plates for seven days at room temperature in darkness. Hereafter plates were exposed to short wave UV light six - eight hours per day, for five days to promote sporulation (Damm et al. (2012) used this method). PDA medium were transferred to 20 ml solid phase microextraction (SPME) headspace glass vials (see photo). The medium was inoculated with 1 mL of *C. acutatum* spore suspensions (10^5/mL) and incubated for 14 days in room temperature under normal light and dark regimes. Four biological replicates where used and divinylbenzene/carbon/polydimethylsiloxane coated SPME fiber was injected into the 10 ml vial to collect the volatile metabolite with 1 cm of the fiber exposed for 15 min at 25 °C. After the absorption step, samples were inserted into the injection port of a GC-MS for thermal desorption and volatile detection.

3.1.5 RNA extraction of infected *Fragaria vesca* leaves

RNA was extracted from the infected samples for gene expression analysis of *CaTPS* gene to verify that sesquiterpenoids (STs) are produced and secreted specifically by *C. acutatum* and not by the infected leaf itself. The last three samples (the infected leaves that were incubated for five days) were snap frozen in liquid nitrogen and stored in -80℃ freezer (until use) after

performing GC-MS analysis for use in gene expression studies; RNA is highly reactive in room temperature and could even get degraded in ordinary freezer temperatures at -20 ℃ when not diluted in to a special buffer that followed the RNeasy Plant mini kit (Qiagen). Whole infected leaves were ground in liquid nitrogen using a sterile mortar and pestle. Total RNA was extracted using the RNeasy extraction kit. This was performed following the manufacturer's instructions but with the following modifications: in order to lyse material samples, 50 mg plant material were used and transferred into a mixture of lysis solution/2-Mercaptoethonal supplemented with 5 % w/v of polyvinylpyrrolidone (PVP 40; Sigma-Aldrich) in 750 µl solution. Each of the washing step in the protocol were performed one extra time and samples were eluated using 50 µl RNase free water. The concentration of the total RNA sample was determined using a NanoDrop ND-1000. The DNA-free DNA removal kit (Thermo Fischer) were used for removal of contaminating DNA following the manufacturer's instructions.

3.1.6 cDNA preparation and gene expression of CaTPS during *Fragaria vesca* leaf infection

The prepared total RNA was used for generating first-strand cDNA using iScript cDNA synthesis (BioRad) from 1µg total RNA with random primers. The cDNA was needed for amplification during the PCR step and verification of the *CaTPS* gene expression. The gene expression analysis of CaTPS gene characterized by Amby et al. (2016) was performed using conventional PCR analysis with primers designed for the CaTPS full length clone using DreamTaq green DNA polymerase (Thermo Scientific) under recommended thermal cycle conditions following the manufacturer's instructions. Also, primers used for normal quantitative PCR (small size products of 50-200 base pairs) of CaTPS gene of interest as well as using the housekeeping β-tubulin gene of *C. acutatum* were used (Amby et al. 2016). The PCR amplification products of both genes were visualized and analyzed using gel electrophoresis. The band intensity of each PCR product represents the activity of gene.

3.1.7 Microscopy of infection

Microscopy was performed after 3-dpi and 5-dpi to keep trac of the infection and to make sure that nothing else was growing and interfering with *C. acutatum* infection on the *F. vesca* 5A leaves (Fig. 5; Fig. 6).

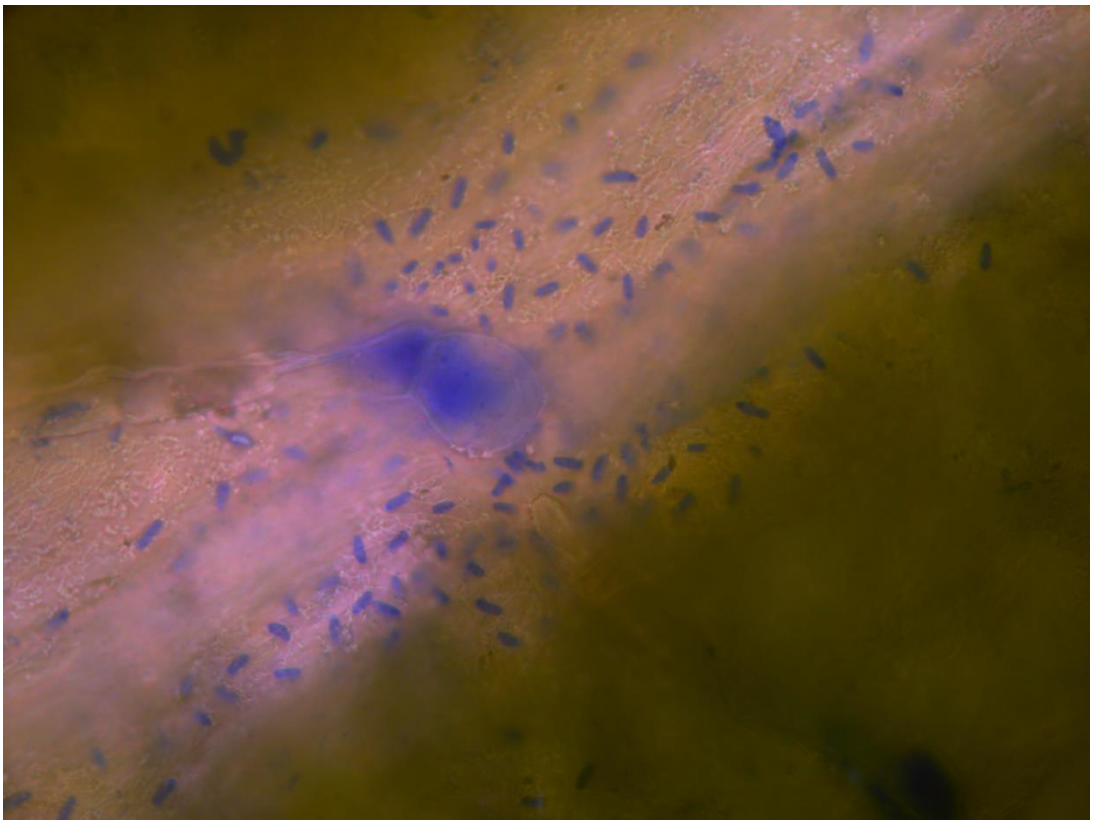

Figure 5, Colletotrichum acutatum *spores clustered in and around the wounded site of* F. vesca *5A leaf, 3-dpi.*

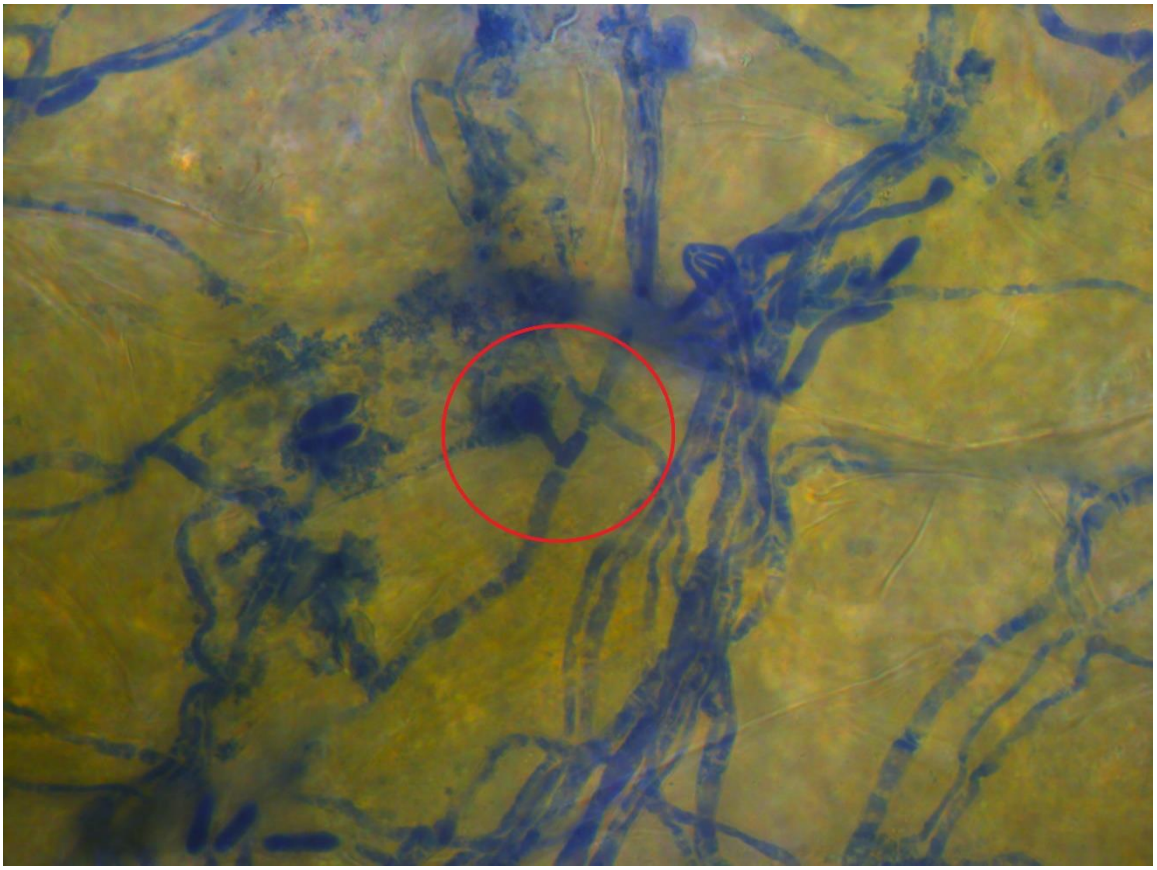

Figure 6, Colletotrichum acutatum *mycelium growth on* F. vesca *5A leaf, 5-dpi. Germ tube and appressorium formation is presented in the marked area – red circle.*

3.2 Leaf extract pathogen inhibition assay – LEPIA

Fragaria vesca 24A (genotype of interest for this particular analysis) has previously shown from detached leaf infection assay to be fairly resistant against *C. acutatum* infection with no visual anthracnose disease symptoms as showed for the susceptible genotype 5A, which was used for the previously described assay.

3.2.1 Leaf samples and surface sterilization

Twenty-four samples of *F. vesca* (genotype 24A) leaves were collected and sterilized according to previously described sterilization method with the same purpose – to make sure that epiphytes were killed. The sterilized leaves were stored in sealed zip lock bags in three days at 4℃, before they were inoculated.

3.2.2 Inoculation and incubation of leaves

This step was performed to induce defense responses and possible production of phytoalexins. Four plastic boxes (PBs) were used as inoculation chambers. Six leaves of 24A (less susceptible genotype) were placed on a plastic screen (which was placed in the bottom of each box). Twelve of these leaves were inoculated by pipetting a 25 µl drop of spore suspension (5 x 10^5/ml) on each adaxial side of the mid vein, on the center compound leave for all leaf samples (Fig 7). The twelve remaining ones where inoculated with sterile MQ H_2O, serving as the control samples. All PBs with treatments were placed in an incubator at 30℃ day temperature and 90 % RH (for 12h), respectively 25℃ night temperature and 90% RH (for 12h). Lower concentration of spore suspension (e.g. 5 x 10^5/ml) was used because the aim of LEPIA was to induce PTI in the leaf. The inoculation was therefore done without wounding the leaves.

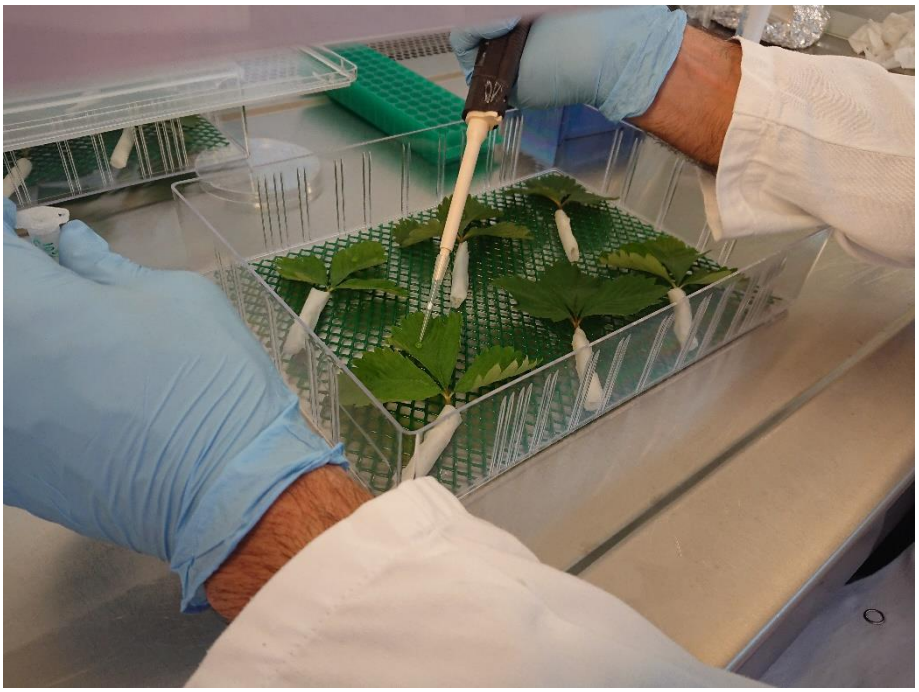

Figure 7, F. vesca *24A leaves inoculated with spore suspension without wounding the surface.*

3.2.3 Preparation of fungal medium

PDA was prepared as substrate for the LEPIA. 31.2 g PDA was dissolved (according to the instruction on the package 39 g/l with 800 ml MQ H_2O in a blue cap glass bottle. Three bottles where prepared in total (enough volume for preparing 60-PDs). The PDA solution was autoclaved and used for preparing 60PDs (100 mm x 15mm) with media which were UV treated for 10-min and stored in sealed plastic bags.

3.2.4 Preparation of fungal pathogen cultures

In total, five different pathogens were used for leaf extract inhibition observations: *Botrytis cinerea* (causing grey mould symptoms on strawberry fruits), *Alternaria solani* (early blight disease, tomato and potato isolates), *Verticilium dahliae* (wilt disease) was collected from various researchers at The Swedish University of Agriculture Science in Alnarp and transferred to the new PDA media in order to start healthy cultures for LEPIA: A small piece (0,5-1 cm^2) of pathogen containing medium was cut out from each of the collected cultures with a sterile scalpel and placed in the center of petri dishes containing the new PDA media. Five biological replicates were prepared for each of the pathogens. *C. acutatum* were also transferred to new PDA media.

3.2.5 Leaf Extract preparation

In order to make the control and induced leaf extract, the control and spore inoculated (induced) 24A leaves were homogenized using liquid nitrogen and sterilized pestle mortar; liquid nitrogen vas added in small portions during the whole pestle mortar process to prevent the leaf material from thawing. 80% MeOH was used as organic solvent for metabolite extraction. Approx. 3 g (wet weight) of homogenized leaf material were mixed with 200 ml 80% MeOH in blue cap bottles and placed on a shaking table (100-rpm) overnight in cold room (ca. 10℃) Next day the solvents were separated from grounded tissue by passing it through a sterile Whatman no. 1 (pore size 11 µM) filter paper. A rotary evaporator was used to evaporate the MeOH at 40℃ and 320mbar (Fig. 8). The evaporation was completed when the extract started to get thick (green glue-like consistency) and diluted again with 10ml 80% MeOH to get the end product. Same procedure was used for the control and the induced leaf extract. Both of the extracts where filter sterilized by passing it through a PTFE membrane with pore size of 0.22 µm to assure no contamination (*e.g.* bacteria, yeast etc. from surroundings) was carried over during the evaporation steps. The extract was stored in 4℃ until use.

Figure 8, A rotary evaporator was used to separate the organic solvent (MeOH) from the extract.

3.2.6 Filter paper disk preparation and LEPIA set up in 3 parts

First part of the inhibition experiment setup followed a standardized method for inhibition assay for human pathogenic bacteria and yeast (Bauer et al., 1959). The exceptions in this study was that one single concentration was used for each treatment and the experiment was

performed on phyto pathogens. A hole-puncher was used for preparing circular paper disks in equal sizes (approx. 5 mm diameter). The paper disks were autoclaved in tin foil and stored in an 80℃ oven to evaporate excess water/humidity carried over from the autoclave step.

3.2.6.1 Part 1

The sterilized paper disks were socked with the induced- and control-extract (+ and C), and the negative control (MeOH) until saturated, using 25 µl of fluid per paper disk. The treated paper disks were transferred onto PDA medium with fungal growth, using two paper disks per treatment. Each replicate (petri dish containing fungal species and PDA) included three treatments (the induced- and control-extract, and the negative control (MeOH)) with two technical replicates per treatment which was six paper disks per petri dish in total. Five replicates were produced for each pathogen.

3.2.6.2 Part 2

LEPIA was repeated with some adjustments because no inhibition appeared in *part one* setup. To improve LEPIA and eliminate the possibility of interference in-between the different treatments (and the risk of receiving nested dependent inhibition results), the nested trial - which means that all treatments were placed in the same PD - was discarded. The pathogens that were used (for part one) was also discarded (due to lack of time to trial all of them again). The paper disks were also discarded to avoid the compounds from adhere to the paper (the leaf extract diffuses from the filter paper into the gel).

The treatments (+, C and MeOH) was not added together in each replicate. Instead, each treatment (with the same volume used for part one) was added directly to the media (without filter paper disks) to four spots in each replicate. Each spot had 2cm distance from the petri dish center point where the *B. cinerea* mycelium cubes were placed. Five replicates were produced for each treatment in total, and sterilized MQ H_2O was added to the mock samples (a new step performed for comparison of fungal growth relative no treatment). The mock samples also had a major role, to serve as stop signal for the experiment: when one (of five replicates) of the mock samples reached its maximum areal growth (limited by the area of the petri dish).

3.2.6.3 Part 3

Repeating LEPIA for the third time, with some changes performed. The number of replicates was increased to ten per treatment because the data from part two trial was not normally distributed. The same treatments were used as previously described in part one and two trial, but the added volume was changed to 50 µl per spot. The volume was increased because the concentration of antimicrobial components is less in a leaf extracts, compared with the antibiotics used in traditional inhibition assays, where a single substance is added as treatment in several concentrations. This was not an option for the extracts – where all secondary metabolites were blended.

Each treatment was added to four spots with equal distance from the center point in each petri dish (like in part two) but they were not added to the media directly. Instead, four holes were made in media for each sample with a small metal pipe (diameter of 7mm). 50 µl of treatment was added to each hole (four holes in each replicate) where the leaf extracts diffused in minutes. *Botrytis cinerea* was used as pathogen of interest. The incubation was made at room temperature in darkness, for five days. Data were collected on day six with the same criteria used in part two – when the mock reaches maximum area.

The major change in part three: added volume for the negative control (80% MeOH) was reduced to the volume that corresponds the mass percentage of MeOH in the other treatments (+ and C), in order to ad equal amount of MeOH in each treatment. In this way, the fungal pathogen was not exposed to more MeOH in the negative control. The added volume of 80 % MeOH was therefore de creased to 28 µl (corresponding volume to the same mass % (weight) in the other samples).

4 Results

4.1 GC-MS

In total, 3 infected and 3 control *F. vesca* 5A samples were analyzed with GC-MS. The result shows that β-caryophyllene is secreted with higher abundancy during leaf infection, in comparison to the control samples (leaves without *C. acutatum*) (Fig. 9).

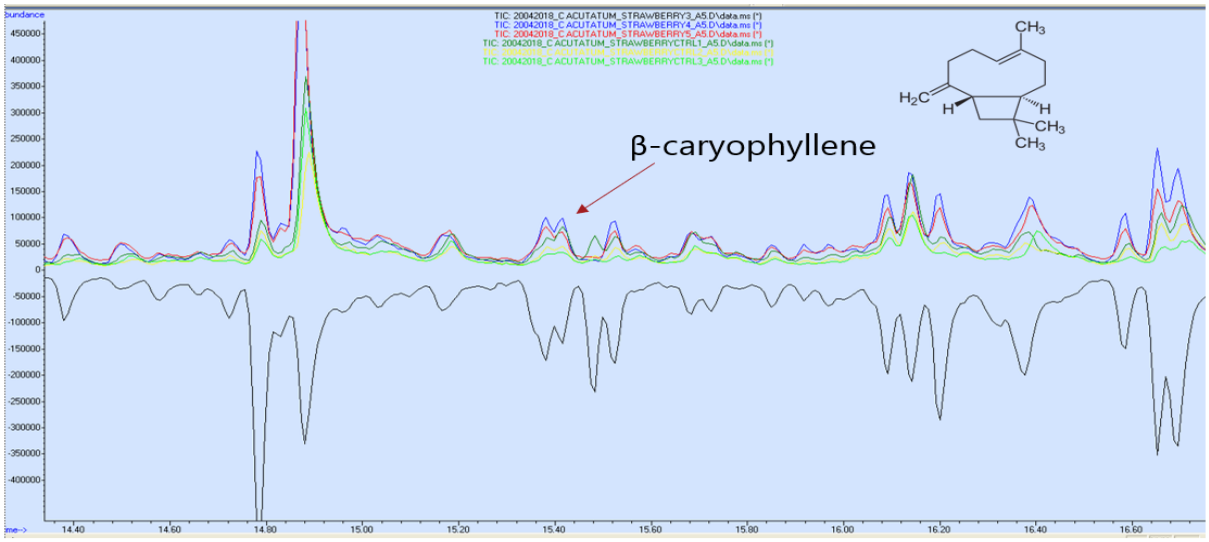

Figure 9, chromatogram showing the SPME-GC-MS result of volatiles from 3 infected F. vesca *5A leaf samples (black, blue & red graphs), and 3 control leaf samples (dark green, yellow & light green graphs), 5-dpi.*

Peaks of interest with typical terpenoid profiles were found by comparing the mass spectra of the in vitro growth (*C. acutatum* on PDA) with the NIST and Wiley libraries. Several terpenoids were found using these criteria for identification, but most importantly two peaks (*i.e.* sesquiterpenoid 2 and β-caryophyllene) biosynthesized by *C. acutatum* and CaTPS were found (Fig. 10), as previously characterized by Amby et al. (2016).

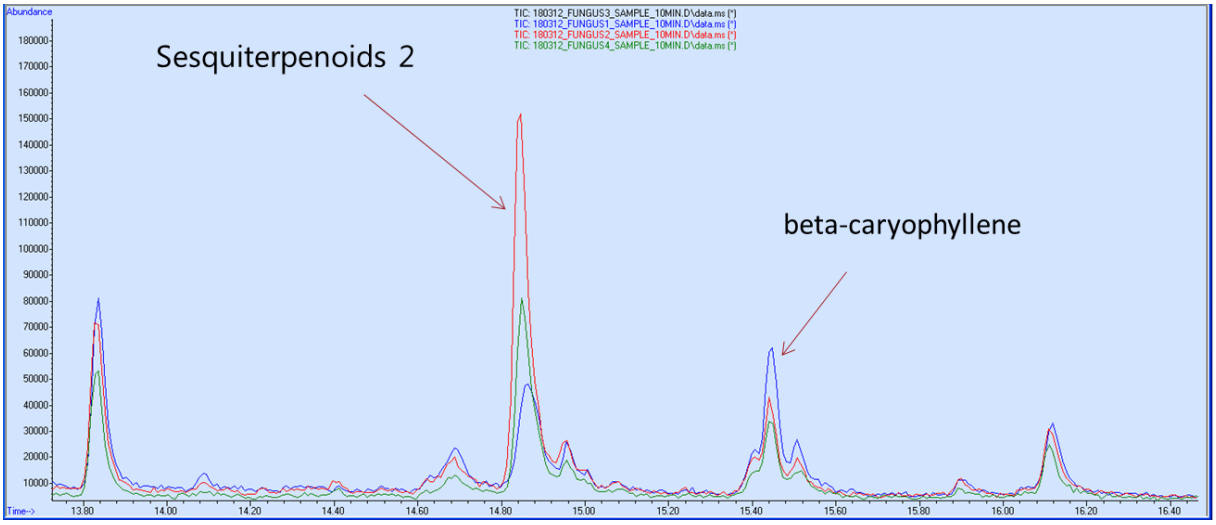

*Figure 10, chromatogram showing the SPME-GC-MS result of the background (*C. acutatum *grown on PDA), after 14 days of in vitro growth. Several peaks were identified having similarity to mass spectra profiles of sesquiterpenes (NIST and Wiley libraries).*

4.2 Gene expression analysis

RNA quality and quantity of the infected 5A leaf samples (snap freeze right after performing GCMS) were identified using Nano drop, resulting in concentration levels from 70-100 ng/µl, and purity level (260/280 ratio) of 1.3 – 1.4 (accepted purity level should be at least 1.8 for cDNA synthesis).

cDNA result was not measured with Nano drop. The cDNA was used to make amplification (PCR) for the verification of *CaTPS* (enzyme-gene expression). The gel electrophoresis (separation of PCR products by size) showed no abundancy (bands on gel) of amplified products corresponding to *CaTPS* cDNA at all, not even for the housekeeping gene β-tubulin (pictures of gel not shown).

4.3 LEPIA part 1, 2 & 3

4.3.1 Part 1

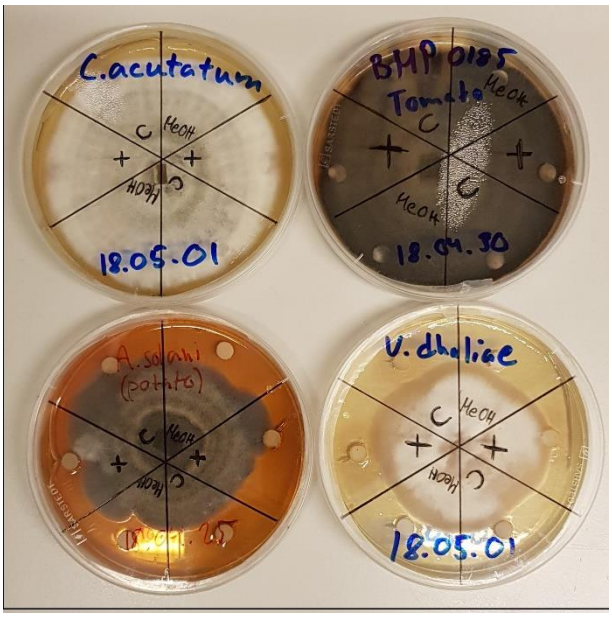

Figure 12, fungal pathogens exposed to different treatments: C (control, extract without any induction of defense), + (extract of leaves with added C. acutatum *spore suspension) and MeOH (80% methanol).*

The leaf extract pathogen inhibition assay (LEPIA) was performed 3 times – presented as 3 parts. The results were not measured for part 1 assay. There was no pattern of inhibition around the filter paper disks which were submerged in the induced extract (+) in any of the samples (i.e. *C. acutatum, A. solani* (tomato isolate), *A. solani* (potato isolate) and *V. dahlia* (Fig 12), all replicates not shown. Data was not collected.

4.3.2 Part 2

ANOVA analysis was made to compare area growth between the treatments. *Botrytis cinerea* had some differences growth and coloration between the treatments, (Fig. 13). The induced extract (+) samples show more coloration in the center part of media, compared to the other treatments and the mock (growth without treatment). There were also changes in the visual mycelia density between the samples (not distinguishable in the figure), with slightly lower density of mycelia in parts of the samples with the induced- and control-extract.

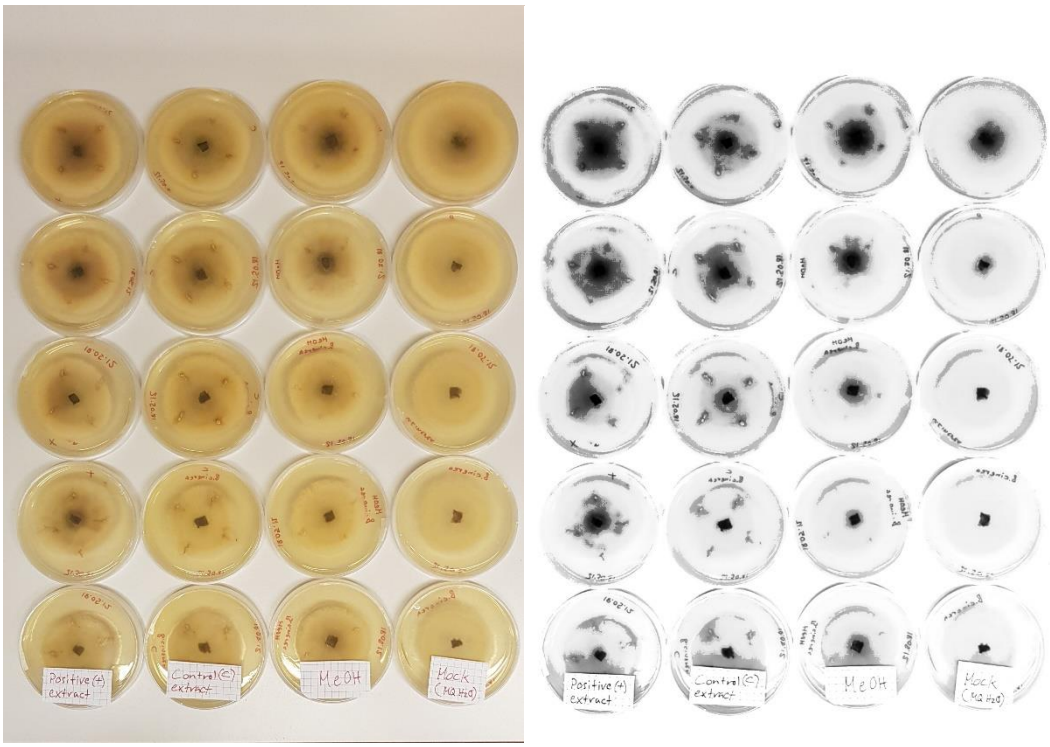

Figure 13, B. cinerea *grown in different treatments as shown in the figure (+, C, MeOH and Mock) (left). Black and white filter is used to exhibit different colorations between the treatments (right).*

A boxplot was made using data of area growth from all samples, (Fig. 14). The box for the induced extract (positive (+)) shows that the spread is lower within these replicates and this treatment also showed the lowest standard deviation (data not shown). The majority of the MeOH observations is clustered on the lower side of the mean value (pointer) while (+) shows the oposit. Overall, data are not normally distributed in the different treatments, with the (+) treatment closest to normal distribution. There were no sigificant difference between the treatments (data not shown). Comparisons of treatment mean values are presented in Fig. 15.

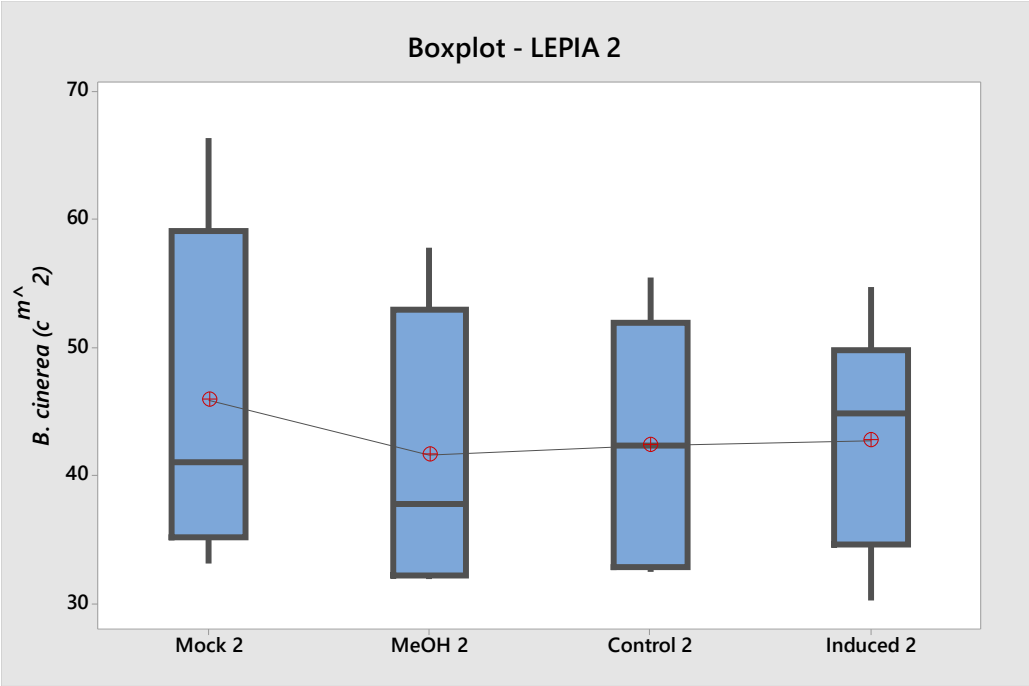

Figure 14, boxplot of the different treatments (Induced, Control and MeOH) and Mock (fungal growth without treatment) for second part LEPIA, after five days of B. cinerea *growth. Red pointers represent the mean value of each treatment and black bars represents the median value.*

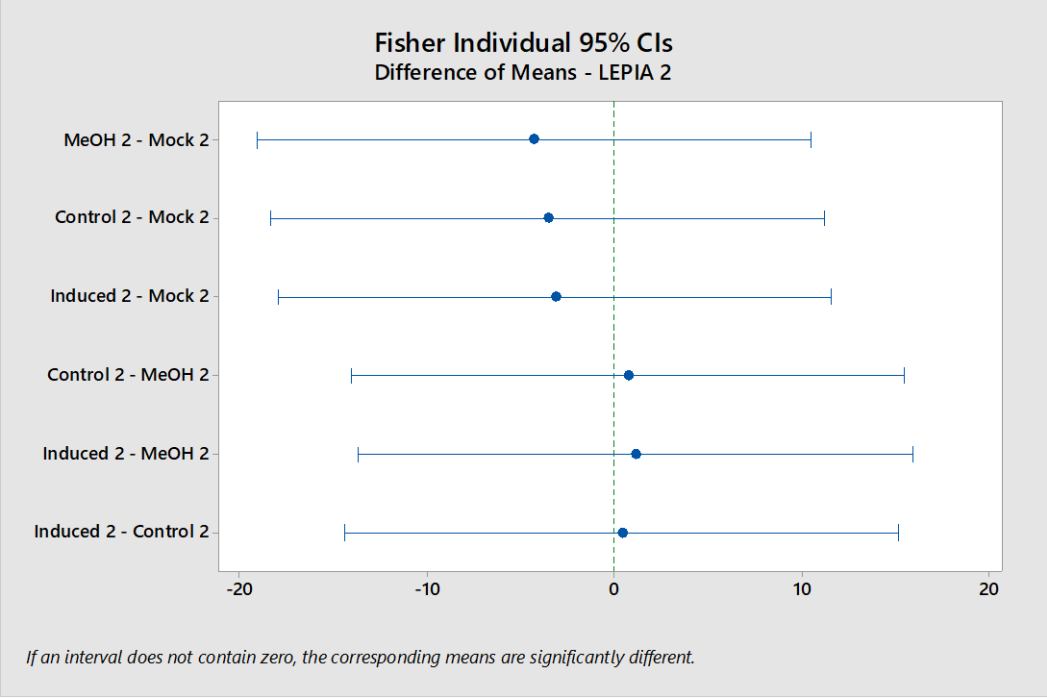

Figure 15, Fisher´s individual, comparison between mean values of treatments in LEPIA 2.

4.3.3 Part 3

Area growth of *Botrytis cinerea* for LEPIA 3 is presented in Fig. 16. The spread is lower in part 3 samples (with ten replicates for each treatment) in comparison with part 2 samples (with five replicates for each treatment). Both mean and median values are decreasing gradually from mock in direction to the induced extract (Fig. 16). Comparisons of treatment mean values are presented in Fig. 17.

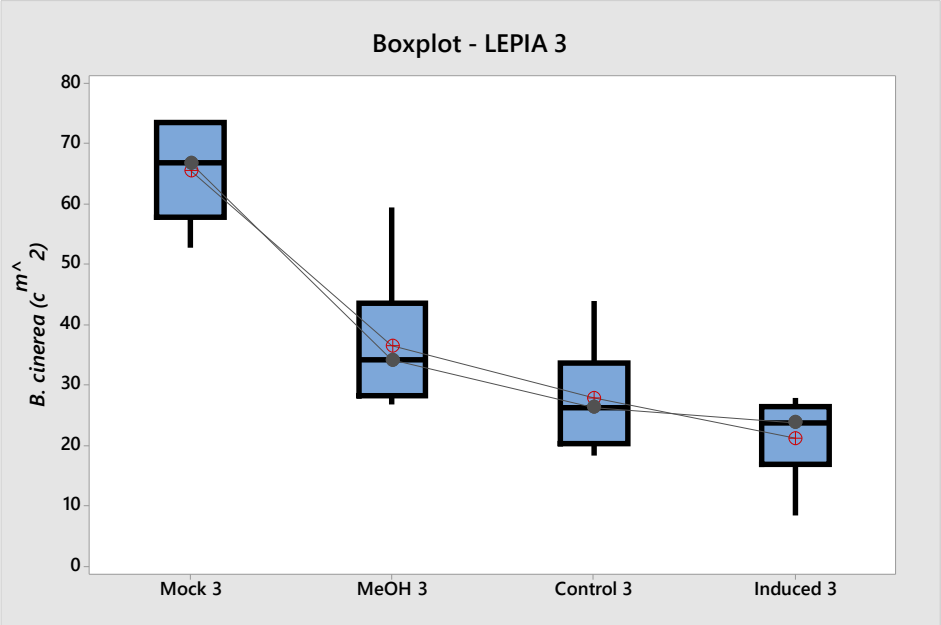

Figure 16, boxplot of the different treatments (Induced, Control and MeOH) and the mock (fungal growth without treatment) for LEPIA 3, after five days of B. cinerea *growth. Red pointers represent the mean value of each treatment and bars with black dots represents the median value.*

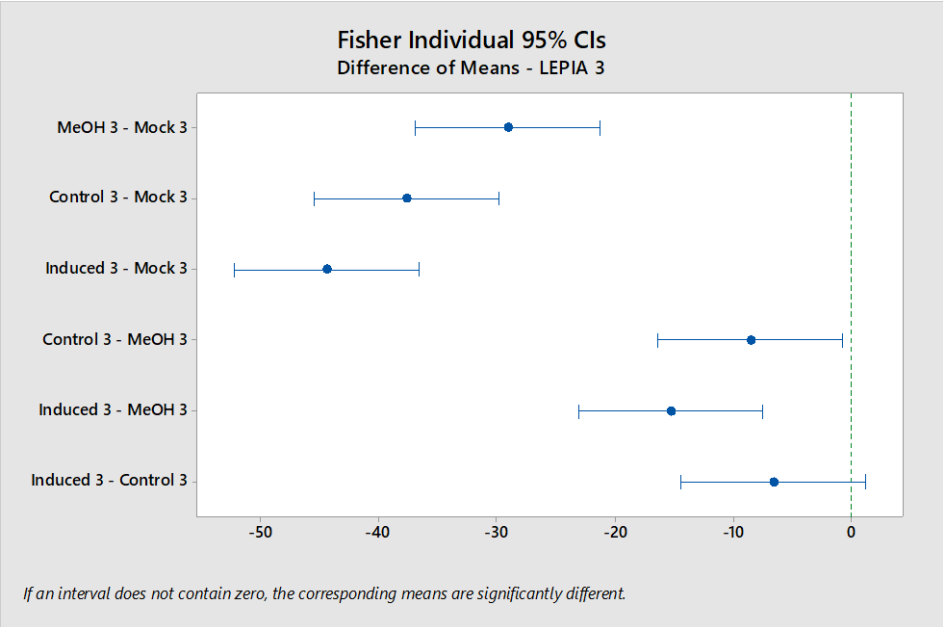

Figure 17, Fisher´s individual, comparison between mean values of treatments in LEPIA 3.

In MeOH treatment, most sample areas are positioned under the treatment mean value; there is a few MeOH samples that reaches higher area (Fig. 18). The same pattern follows for control samples but median value is slightly closer to the mean, in contrast to the induced samples which show the opposite pattern, with some samples being more suppressed than the majority. Another way to visualize this pattern is by stacking the growth area of all replicates within each treatment (Fig. 19). Growth development is showing a decreasing pattern for induced extract samples (+).

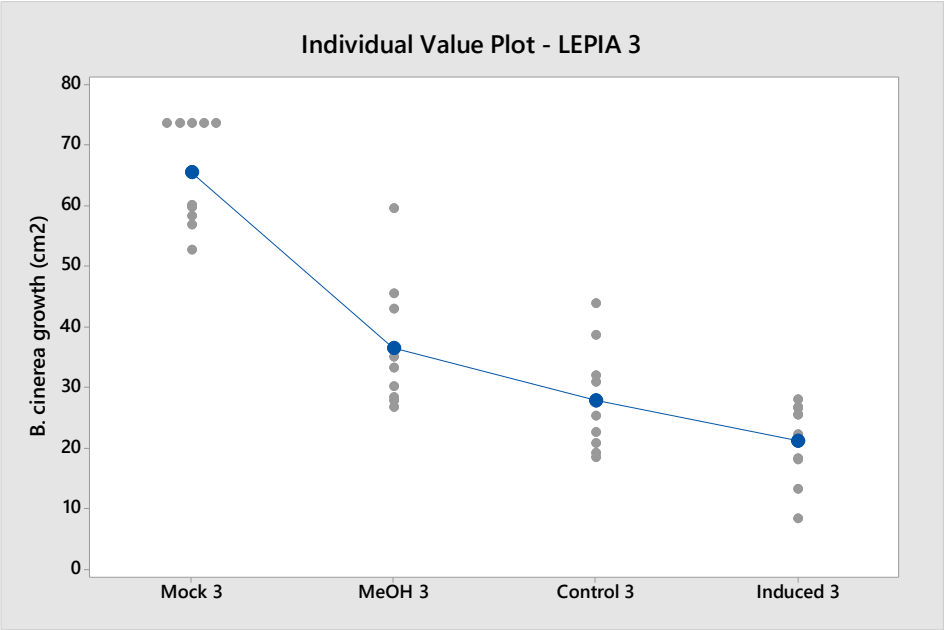

Figure 18, all individual replicates plotted against their respectively treatment.

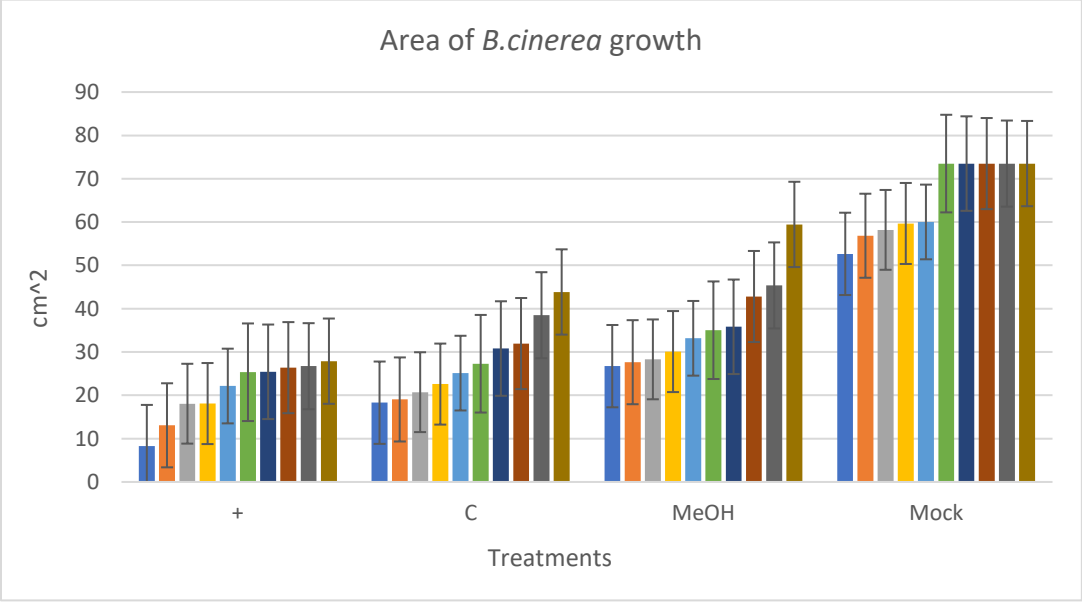

Figure 19, all samples of each treatment for LEPIA, 3 in order of magnitude within the treatments.

5 Discussion

5.1 Resubmit on hypotheses

None of the stated hypotheses could be fully proved with significance on the statistical level (for LEPIA) neither by quantification (of abundancy) nor in the transcriptome level (of secreted volatiles), so far. However, there is a significant difference between the negative control (MeOH) and control sample in LEPIA part 3. Induced extract sample mean is close to being significantly different from control mean (Fig. 17).

5.2 *Volatile*

Characterization of STs and their role as possible virulence related molecules could contribute to a deeper understanding of the infection strategies in use by *C. acutatum.* Volatiles in general has many fundamental roles as communication molecules between different species (e.g. plants and insects) (Kramer, R. & Abraham, W.-R., 2012). Even though many plants produce volatiles to attract different natural enemies against pests (e.g. herbivores), particularly β-caryophyllene could be phytotoxic (or give disadvantageous) effects to plants (Garcia-Pajon & Collado, 2003). For example, a Maize line whit no ability to produce β-caryophyllene has a higher tolerance against *Colletotrichum graminicola* in comparison with the mutant Maize strain which produces the compound during infection of *C. graminicola* (Fantaye et al., 2015). On the other hand, β-caryophyllene secretion in the root zone attracts nematodes which prey on root eating insect larvae. By stating this background, *C. acutatum* could potentially secrete β-caryophyllene in purpose of attracting herbivores to the plant. Pathogens benefits from entry points (caused by herbivores) on the plant tissue. Further, spores of *C. acutatum* could possibly be distributed over the plant surface and even between plants with insects serving as vectors.

The present results show that β-caryophyllene has higher peaks in the infected samples as compared with control samples (Fig. 9). The hypothesis in this part of the experiment is that *C. acutatum* produces this compound during infection of *F. vesca* leaves. The higher peaks shown for the infected leaves (Fig. 9) could of course be due to upregulated production of β-caryophyllene by the leaf itself, in response to the infection. The question is why the pathogen should not be producing the compound during the leaf infection, when our mock sample (background) *does*. Fig. 11 shows a chromatogram of volatiles when *CaTPS* (gene

coding for *C. acutatum* sesquiterpenoid synthase) was incorporated in yeast, resulting in large peaks of β-caryophyllene and other STs.

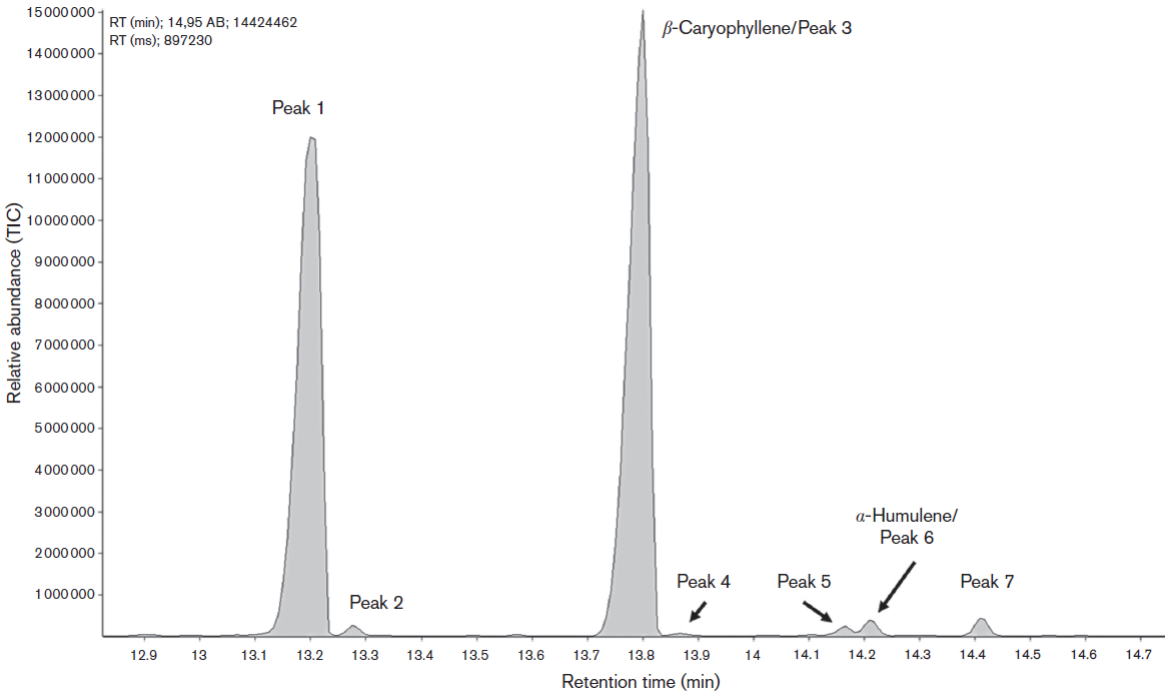

Figure 11, chromatogram (Amby et al., 2016) *of volatile sesquiterpenoids produced by transgenic yeast with the* CaTPS gene inserted. Y-axis showing abundancy and x-axis showing the retention time – when fragments of molecules are detected.

So, when the CaTPS (enzyme) is expressed, it produces the compound of interest. Further, to strengthen the hypothesis and the fungal secretion of STs during the infection, transcriptome analysis of *C. acutatum* mycelia (infecting *F. vesca* 5A leaves) was performed in order to reveal gene expression of *CaTPS*. Unfortunately, the RNA extraction had too low purity level. There was likely to much impurities interfering in the cDNA synthesis, resulting in no amplification during the PCR step. There is a very high chance the low purity is due to phenolics and anthocyanins which is often high in strawberry fruits and leaves. In the end of this project I realized that there are special RNA extraction-kits suited for the strawberry leaf. At least, I improved my knowledge about difficulties in RNA extraction on this particular species - *F. vesca*.

5.2.1 *CaTPS* transcriptome and RNA extraction improvements

Possible difficulties which could be time consuming and interfere with other steps in the experiment time schedule should be investigated before starting such work for the next time. If there are any signs of them, start to investigate the possible issues before even starting the

project; Investigate extraction protocols of strawberry leaves and compare them with each other.

5.3 LEPIA

In purpose of studying tolerance and/or resistance traits, researchers have collected different genotypes of *F. vesca* from different geographical areas in Sweden. It is worth to mention why resistance in *F. vesca* is relevant to study when questing for phytoalexins, because in general, the selection pressure of pathogens in nature only allow the fittest and most adapted individuals to survive and reproduce. *F. vesca* is a close relative to garden strawberries (*Fragaria x ananassa*), and similarities could be found in their genomes. This means that *Fragaria x ananassa* cv. could be engineered (e.g. CRISPR CAS9), based on gene expressions related to resistance in their wild relatives (e.g. *F. vesca*). Unfortunately, the amount of leaf extract and the overall setup procedure used for LEPIA part 1-2 did not cause the inhibition grade I was aiming for, with no significant differences between the treatments. On the other hand, LEPIA part 3 show that the induced *F. vesca* 24A extract (+) could potentially inhibit (or suppress) the growth of *B. cinerea* with significance by increasing the volume extract added to the spots of each petri dish. In addition, a higher number of replicates seems to give more reliable data.

5.3.1 Improvements for LEPIA

Many steps that could be modified in the leaf extraction methods. For example, pressure settings of the rotary evaporator could be set to the value that corresponds to evaporation of MeOH at room temperature – instead of using 40 ℃ as evaporation temperature. By stating this information, proteins and other compounds (with antimicrobial properties) could be denaturated (loose its specific 3-dimentional structure) respectively degraded by heat or by oxidation. Another improvement could be to inoculate intact plants and use neighboring compound leaves for making the extract. The inoculated leaves used for LEPIA suffered slightly from heat related stress, even though we incubated them at high humidity and lowered the night temperature. Finally, I have to admit that the adjustment of MeOH for part 3 assay was mistaken, because the percentage of MeOH in both C and + extract is more or less corresponding to 80 %. In other words, no adjustments of the negative control (MeOH) treatment, to make sure that the experiment is done correctly for the next time.

5.4 Summary

There are many pieces left to get a broader perspective of *C. acutatum* and its behavior during infection of *F. vesca*. More individuals of the collected *F. vesca* genotypes have to be included in further bio assays to compare tolerance among the individuals. Ultimately, to apply interaction assays with other pests (e.g. the strawberry leaf beetle) to find out if one *C. acutatum* resistant or tolerant *F. vesca* genotype has traits suited to tolerate or repeal the insect as well. This type of research in improvements of understanding interactions and behavior in agricultural production systems contributes to reduction in usage of pesticides by improvements in strawberry resistance breeding together with practical production steps that could be improved. Chemicals that are used in pest management do not only affect the target pathogen but also other microorganisms in the environment, in and around the production system; those that are beneficial for plants with suppressing/competing properties against pathogens.

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