EFFECT OF BIOCHAR AND CHITIN ON PLANT DEFENSE AND RHIZOSPHERE MICROBIOME OF STRAWBERRY

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Preface

This master thesis would not have been possible without the help of some people and therefore I want to thank them.

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List of abbreviations

ASV	Amplicon sequencing variant
BC	Biochar
CAGE	Cap analysis gene expression
CCI	Chlorophyll concentration index
cDNA	Complementary deoxyribonucleic acid
CEBiP	Chitin elicitor binding protein
COI1	Coronatine insensitive1
Cq	Quantification cycle
CTAB	cetrimonium bromide
DEPC	diethylpyrocarbonate
dNTP	Deoxynucleoside triphosphate
dUTP	deoxy-uridine triphosphate
EDTA	Diaminoethane tetraacetic acid
EST	Expressed sequence tag
ETI	Effector triggered immunity
HR	Hypersensitive response
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
JAZ	Jasmonate zim
MAMP	Microbe associated molecular pattern
MAP	Mitogen activated protein
MDS	Multidimensional scaling
MMP	Maximal mappable prefix
mRNA	Messenger ribonucleic acid
NB	Negative binomial
NPR1	Non expressor of pathogenesis related genes1
PAMP	Pathogen associated molecular pattern
PCA	Principal components analysis
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PGPF	Plant growth promoting fungi
PGPR	Plant growth promoting rhizobacteria
Poly-A	Polyadenylated
PR	Pathogenesis related
PRR	Pattern recognition receptor
PS	Peat substrate
PTI	Pathogen associated molecular pattern triggered immunity
qCLM	Quantile-adjusted conditional maximum likelihood
R protein	Resistance protein
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA seq	ribonucleic acid sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAR	Systemic acquired resistance
SBS	Sequencing by synthesis
TAE	Tris-Acetate-diaminoethane tetraacetic acid
TMM	Trimmed mean of M-values
16S rRNA	Small subunit ribosomal ribonucleic acid

Abstract

In this master's thesis the effect of biochar and chitin amendment on the strawberry plant bacterial microbiome was studied. Furthermore, the effect on the strawberry defense response against *Botrytis cinerea* was investigated.

Biochar is a by-product of biomass combustion. Chitin is a natural polysaccharide found in the arthropod exoskeleton and cell wall of fungi.

To study the strawberry plant bacterial microbiome amplicon sequencing was done. The rhizosphere microbiome, as well as the bulk soil microbiome was analyzed. The rhizosphere is the narrow zone surrounding the roots. The bulk soil is the zone of peat substrate not influenced by the plant. From the amplicon sequencing analysis it was concluded that both biochar and chitin have an effect on the composition of the bacterial microbiome. However, it seems that chitin has a larger effect. For all treatments it was seen that the composition of the microbiome changes over time. Also, the rhizosphere microbiome is different from the bulk soil microbiome, especially during the first weeks of plant growth. However, at the end of plant growth both microbiomes are more alike.

The strawberry defense response against *B. cinerea* was studied with gene expression analysis by RNA sequencing and RT-qPCR. Biochar and chitin do not have an effect on the basal strawberry leaf gene expression. Only when the plants are infected there is an effect of biochar and chitin visible. From the RT-qPCR it was also concluded that biochar is not able to enhance the strawberry defense response. The defense response of plants grown in biochar amended peat substrate was not very different from the defense response of plants grown in unamended peat substrate. Chitin does enhance the defense response. Already 1 week after infection, the expression of the tested defense related genes was enhanced. It could even be said that chitin has a priming effect. The expression of some defense related genes is already enhanced upon chitin amendment. However, the enhancement is small in non-infected plants. Only when plants are infected, the expression is enhanced strongly.

Dutch abstract

In deze thesis werd het effect van biochar en chitine op het bacterieel microbioom van de aardbeiplant onderzocht. Daarnaast werd ook het effect op de afweerrespons van aardbei tegen *Botrytis cinerea* bestudeerd.

Biochar is een nevenproduct van de verbranding van biomassa. Chitine is een natuurlijk polysacharide dat teruggevonden wordt in het exoskelet van Arthropoda en in de celwand van schimmels.

Om het bacterieel microbioom van de aardbeiplant te bestuderen werd 'amplicon sequencing' uitgevoerd. Het rhizosfeer microbioom alsook het microbioom van de 'bulk soil' werd onderzocht. De rhizosfeer is de nauwe zone die de wortel omgeeft. De 'bulk soil' is het deel van het substraat dat niet beïnvloed wordt door de plant. Uit de 'amplicon sequencing' analyse werd geconcludeerd dat zowel biochar als chitine een effect hebben op de samenstelling van het bacterieel microbioom. Het effect van chitine lijkt wel groter dan het effect van biochar. De samenstelling van het microbioom verandert ook doorheen de tijd. Dit tijdseffect werd in alle behandelingen gezien. Verder is het rhizosfeer microbioom verschillend van het microbioom in de 'bulk soil', vooral tijdens de eerste weken van de plantengroei. Beide microbiomen convergeren naar elkaar naarmate de planten verder ontwikkelen.

De afweerrespons tegen *B. cinerea* werd onderzocht via genexpressieanalyse met 'RNA sequencing' en RT-qPCR. Biochar en chitine hebben geen effect op de basale genexpressie van het blad. Enkel wanneer de planten geïnfecteerd zijn, is er een effect van biochar en chitine op de genexpressie zichtbaar. Uit de RT-qPCR analyse werd geconcludeerd dat biochar niet de afweerrespons van de aardbeiplant verhoogt. De afweerrespons van planten gegroeid in substraat met biochar toegevoegd was niet erg verschillend van de afweerrespons van planten gegroeid in substraat zonder toevoegingen. Chitine stimuleert wel de afweerrespons. De expressie van de onderzochte genen gerelateerd met defensie was gestimuleerd, zelfs al 1 week na infectie. Chitine heeft zelfs een 'priming' effect. Enkele genen werden al opgereguleerd na toedienen van chitine. Echter, deze opregulatie is beperkt in niet-geïnfecteerde planten. Enkel als de planten geïnfecteerd zijn, wordt de expressie van genen gerelateerd met defensie sterk gestimuleerd.

Objectives

Despite the popularity of strawberry as fruit, the production of the fruit is not easy for farmers. Strawberry plants are sensitive to a variety of pathogens, of which *Botrytis cinerea* can cause large fruit losses. To control this pathogen, multiple fungicides are used, leading to a variety of residues on strawberry fruits (Vervoort, Melis, Stoffels, & Van Delm, 2017). These residues can have a negative impact on the environment and on our health. Decreasing the amount of residues on food is thus an important challenge.

Biological control can be used instead of chemical control. Beneficial bacteria or fungi can control some pathogens that affect strawberry plants. For example, the bacterium *Bacillus subtilis* can be used as a biological control agent against *B. cinerea* (Williamson, Tudzynski, Tudzynski, & Van Kan, 2007). Also, cultural practices can be used to control diseases. These include changing humidity, temperature or light in greenhouses (Williamson et al., 2007). Biochar and chitin have been reported to have a positive effect on plant growth and resistance. Biochar is a by-product of biomass pyrolysis (Elad et al., 2010; Graber et al., 2010). Chitin is the second most abundant biopolymer in nature and can be found as a component of the arthropod exoskeleton and the cell wall of fungi (Malerba & Cerana, 2016; Sharp, 2013). It has been shown that biochar amendment to peat can lead to a higher fresh and dry plant weight, a lower susceptibility against *B. cinerea* and changes in the rhizosphere microbiome (De Tender et al., 2016a). The rhizosphere is the narrow zone surrounding the root (Mendes, Garbeva, & Raaijmakers, 2013). Chitin addition to potting soil resulted in a higher fresh weight of lettuce plants. In addition, a change in the rhizosphere microbiome was reported, including an increase in the abundance of species involved in biocontrol and plant growth (Debode et al., 2016). However, the mechanisms underlying this positive effect of biochar and chitin are not yet completely understood.

In this master's dissertation, the effect of biochar and chitin on the strawberry microbiome and defense response against *B. cinerea* will be studied. Therefore we will study two things. First, the rhizosphere microbiome of strawberry plants will be investigated through amplicon sequencing. More specifically, we are interested in bacteria related to plant disease responses that could be influenced by the application of biochar and chitin. Second, the expression of different defense related genes will be studied by RNA sequencing and verified by RT-qPCR on plant defense genes. This study can help understanding the mechanisms of biochar and chitin on plant growth and disease resistance. Enhancement of plant resistance by biochar or chitin amendment can reduce the use of fungicides in the strawberry cultivation.

Literature research

1. Introduction

Research on reducing chemical residues on fruits and vegetables is becoming more important. The use of pesticides in agriculture is getting more restricted and alternative techniques are being promoted by the European Union (European Commission, n.d.). Some organic compounds, such as biochar and chitin, can enhance the plant defense mechanism when they are added to the (potting) soil. These compounds are known as defense elicitors. If the plant defense can be stimulated by adding defense elicitors to the soil or potting soil, these compounds can partly replace the use of chemicals to control diseases and pests. Thereby reducing the amount of residues found on food.

Two environmental-friendly compounds, biochar and chitin, show positive effects on plant growth and defense in horti- and agriculture. Previous research of De Tender et al. (2016a) has shown a positive effect of biochar amended potting soil to the growth and resistance of strawberry plants against *Botrytis cinerea* (De Tender et al., 2016a). Debode et al. (2016) have shown a positive effect of chitin amended peat to the growth of lettuce plants. They also reported a change in the rhizosphere microbiome (Debode et al., 2016). The mechanisms underlying these effects however are not yet completely known.

In this master's dissertation, the effect of biochar amended and chitin amended peat on strawberry plant defense response will be studied. First, the rhizosphere microbiome will be investigated for potential bacteria that can enhance the plant defense response. For this study amplicon sequencing will be used. This is a method to study the entire microbial diversity present in a sample by studying the microbial DNA in the environment (Van Dijk, Auger, Jaszczyszyn, & Thermes, 2014). Second, the effect on the expression of defense related genes will be studied. A possible hypothesis here is that biochar and chitin can activate defense related genes, hereby triggering the defense response. The expression of defense related genes will be studied using RNA sequencing and RT-qPCR for validation.

In this master's dissertation we will focus on strawberry since this is an economical important fruit but the cultivation is not that easy for the farmers. Strawberry plants are sensitive for different pathogens and farmers have to use a variety of chemicals to control pests and diseases in the plant. The use of defense elicitors to partly replace these chemicals can thus be particularly useful in the strawberry cultivation.

In this literature research, first some theoretical background about the strawberry cultivation in Belgium and about *B. cinerea* and the control against this pathogen is given. Second, an overview of the known effects of biochar and chitin on plant growth, plant disease resistance and rhizosphere microbiome are given. Finally, high-throughput sequencing methods for studying the rhizosphere microbiome and expression of defense related genes are being discussed.

2. Strawberry

2.1. Economic importance

Worldwide, strawberry is an important fruit with a production of over 9 million tons in 2016. The main producer is China, with a production of almost 4 million tons in 2017. The current Belgian production amounts 48 000 tons per year, but is still increasing yearly ("FAOSTAT," n.d.). The price of fresh strawberries shows a yearly pattern (Figure 1). When the production is high, the price per kilogram is in the range of 2,5 to 4 euro. When the production is low, the price rises to 7-9 euros (De Samber, 2019).
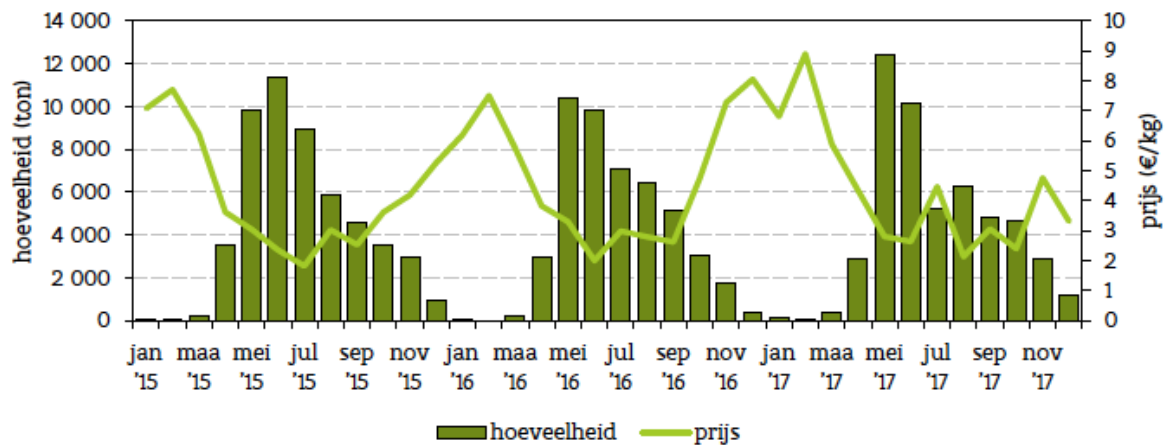
Figure 1: Monthly amount (ton) and price (euro/kg) of fresh strawberries delivered to Belgian producer organizations. Data from 2015 to 2017 (De Samber, 2019).

The common strawberry, *Fragaria x ananassa*, belongs to the family of the *Rosaceae* and the genus *Fragaria* (Hummer & Hancock, 2009). The dominant cultivar grown in Belgium is Elsanta, which accounts for 76% of the strawberry production (Van Delm et al., 2016).

The main diseases that occur on strawberry are powdery mildew (*Podosphaera aphanis*), grey mold (*Botrytis cinerea*), and diseases caused by soilborne pathogens such as *Phytophthora* spp. Chemical control used to be the main pest and disease management technique, but nowadays biocides and beneficials are used too (Van Delm et al., 2016).

2.2. Strawberry cultivation methods in Belgium

In Belgium, different cultivation systems are used to provide a year-round strawberry production. Plants are either grown in soil or in soilless substrate culture (Van Delm et al., 2016). Substrate culture in greenhouses is the most common cultivation system in Belgium (De Samber, 2019).

In the traditional soil cultivation, fresh plants are planted in August and harvested in June the following year. Another possibility is to use cold stored plants in soilless substrate culture. Fresh plants are planted in August until dormancy is achieved. Then, the plants are put in cold storage until planting (April to June). Hereby the harvest is delayed from mid-June until mid-September (Van Delm et al., 2016).
In the soilless substrate culture, three harvests can be achieved per year by planting cold stored plants at the end of December, end of May and in August. The harvest periods are from March until May, July to August and October until December, respectively (Lieten, 2013).

Modern substrates used in substrate culture are composed of peat moss or a mix of peat moss and coir. In this cultivation system there is no need for soil fumigants and the use of fungicides is less as compared to soil cultivation (Lieten, 2013).

Peat is extracted from peatlands. The first step in the extraction procedure is almost always drainage of the peatland. This is done to dry the peat and to make it easier to process and transport the peat. Next, the peat is removed from the peatland. This can be done by cutting the peat in blocks, sausage cutting or peat milling. Peat cutting can be done by hand or mechanically. Sausage cutting is the removal of peat below the surface. Peat milling is the most frequently used method for removal of peat. Here the peatland is drained and leveled by digging drains 15 meter apart. The drain depth is sequentially increased.

The extraction of peat causes irreversible damage to the natural ecosystem. The peatland will be restored, but this can take 50 to 500 years, depending on the severity of the extraction of the land (Lindsay, Birnie, & Clough, 2016). In addition, the process causes the release of particulate carbon, dissolved organic carbon and heavy metals that can end up in waterways. The combustion and decomposition of peat during and after production leads to the emission of carbon dioxide. Because of this, the use of peat is being questioned ("How is Peat Extracted? - Manitoba Peatlands," n.d.).

3. Botrytis cinerea
3.1. Introduction

Botrytis cinerea is an airborne plant pathogen that causes grey mold disease on different plants. *B. cinerea* is a necrotroph: the pathogen kills the cells of its host and feeds on dead material. Over 200 plant species can be infected by this fungus. Mature or senescent tissues are the most susceptible, but the pathogen usually enters the plant tissue at an earlier stage in crop development. It can remain quiescent for a long period before rotting tissues when the environment is beneficial and the host physiology changes. Favorable conditions for *B. cinerea* are high humidity, reduced light and moderated temperature (Williamson et al., 2007).

The pathogen has different modes of attack, diverse hosts and can survive as mycelia or as sclerotia in crop debris for a longer time, which makes the control of *B. cinerea* difficult (Williamson et al., 2007). Sclerotia are persistent structures that help fungi survive in hard conditions such as freezing temperatures or long-term absence of a host (Smith, Henkel, & Rollins, 2015). Sclerotia start growing in early spring to produce conidiophores and conidia. The conidia serve as a primary source of inoculum within a crop (Williamson et al., 2007).

3.2. Infection cycle

The infection cycle of *B. cinerea* begins with a conidium landing on the host plant. The conidia can be transported by wind over long distances. After attachment, the conidium germinates on the plant tissue under moist conditions and penetrates the plant tissue. When the plant defense barriers are broken, the fungus starts to outgrow vigorously. The most important barrier that the fungus needs to break is the plant cell wall. For this, *B. cinerea* excretes enzymes that rupture the cell wall. Eventually the fungus sporulates to produce new conidia for the next infection. An infection cycle may be completed within 3 to 4 days under optimal conditions (Kan, 2005).

The infection cycle in strawberry is shown in Figure 2. *B. cinerea* can infect flowers, fruits and leaves. This infection can be caused by overwintering sclerotia or conidia from infected neighbouring plants. The conidia germinate and spreads over the fruits and leaves. The fungus then starts to sporulate and forms new conidia or survives in crop debris. Fruit infections appear as soft rots on the fruits. Eventually the fruit becomes covered with grey masses of conidia (Figure 3 and Figure 4) (Petrasch, Knapp, van Kan, & Blanco-Ulate, 2019; Williamson et al., 2007).
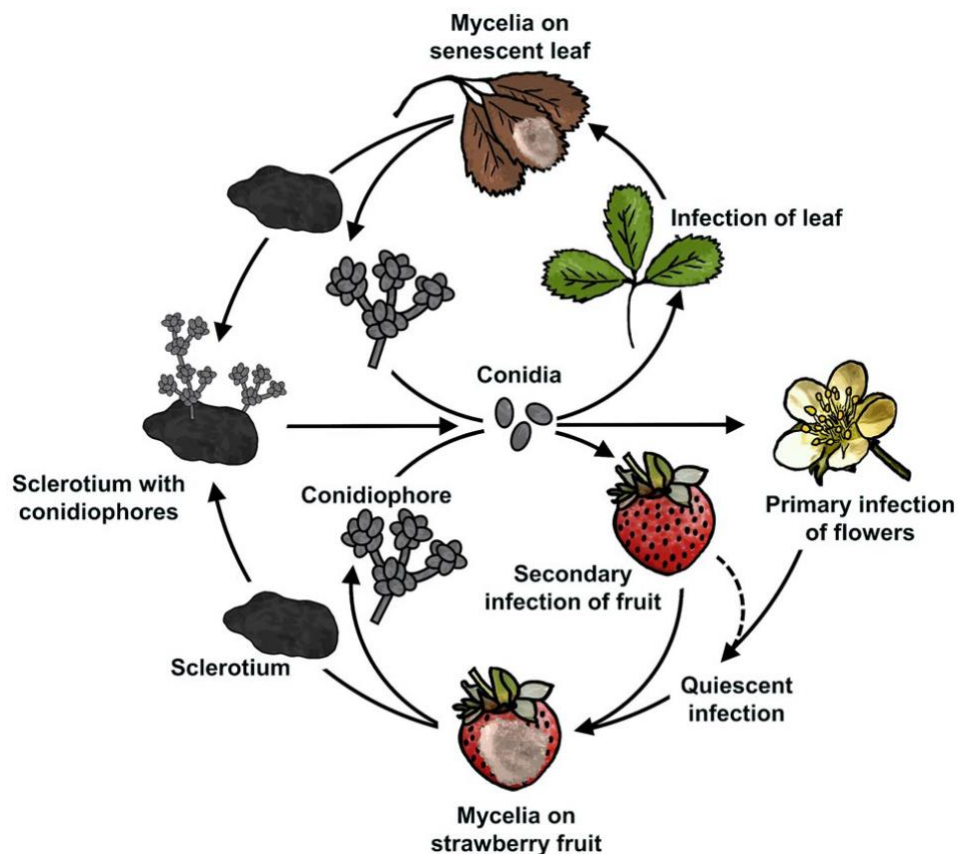
Figure 2: Infection cycle of B. cinerea in strawberry (Petrasch et al., 2019).

Figure 3: B. cinerea *infection on fruits (Williamson et al., 2007).*

Figure 4: B. cinerea *infection on leaves (Petrasch et al., 2019).*

3.3. Disease control

Grey mold can cause important strawberry fruit losses, up to 25% for untreated plants, worldwide. Control of *B. cinerea* can be achieved by frequent fungicide application. During flowering, strawberries are treated every week (Van Liefferinge, 2015). To prevent resistance, a variety of chemicals are used in the treatment against *Botrytis*. But this also results in multiple active ingredients left on the fruits. On 500 gram of strawberry, up to 15 fungicide residues can be found (Veiling Haspengouw, 2011). *Botrytis* control agents are responsible for 43% of active ingredients left on strawberry fruits (Vervoort et al., 2017). Compared to other fruits, there are more fungicide residues found on strawberry. Strawberry is very sensitive to diseases and pests and thus needs to be treated more often. The remark needs to be made however that the concentration of residues found on strawberry still remains below the legal standard (European Food Safety Authority, 2011).

Biological control of *B. cinerea* is also possible. This may include the application of filamentous fungi, such as *Trichoderma harzianum* or bacteria, such as *Bacillus subtilis* (Williamson et al., 2007). Also cultural practices may help in the control of grey mold. The disease is stimulated under high humidity, reduced light and moderated temperature. Hence it can be useful to provide adequate air movement and good light interception (Williamson et al., 2007).

4. Biochar
4.1.   Introduction

Biochar is a by-product of biomass pyrolysis: the thermal decomposition of biomass in the absence of oxygen. This is an exothermic process, which means that it produces more energy than invested in the heating process. The products of pyrolysis are a solid fraction, liquid bio-oil and gas biofuels (Figure 5). Bio-oil and biofuels are used as energy source or for the production of chemicals. The solid fraction is named biochar. Biochar can be applied to the soil, where its turnover is so slow that it leads to a net removal of carbon from the atmosphere. Pyrolysis of biomass and soil amendment by biochar is thus a carbon-negative process (Elad et al., 2010; Graber et al., 2010).
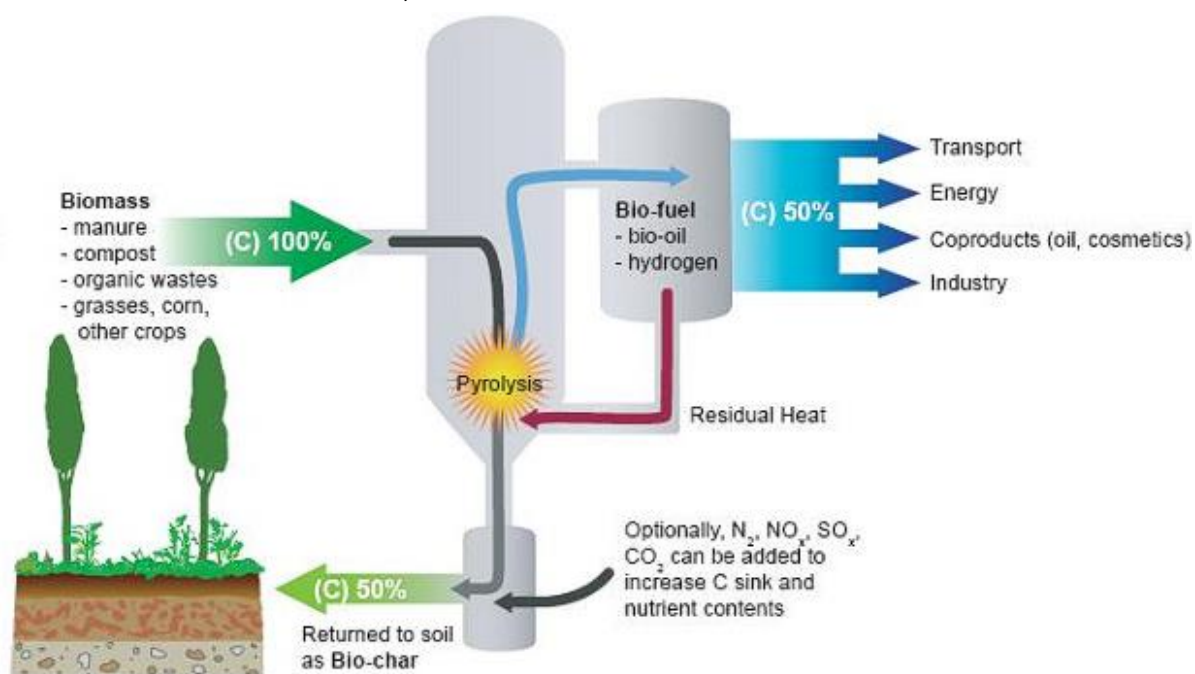
Figure 5: Pyrolysis of biomass. The products of this process are biochar, bio-oil and biofuels. Biochar is returned to the soil, bio-oil and biofuels are used for the production of energy or other coproducts ("Biochar as the new black gold | Grist," n.d.).

Next to the net carbon removal of the atmosphere, the application of biochar to the soil can be beneficial for crop cultivation. Biochar can for example supply nutrients, increase nutrient retention, improve the soil pH, neutralize phytotoxic compounds in the soil, improve soil physical properties and modify soil microbial populations and functions. Many of these effects are interrelated and can enhance each other (Elad, Cytryn, Meller Harel, Lew, & Graber, 2011). Different studies report an increase in soil pH (Rondon, Lehmann, Ramírez, & Hurtado, 2007; Steiner et al., 2007). Steiner et al. reported an increase in pH to almost neutral levels (Steiner et al., 2007). Chan et al. show that biochar amendment can increase the electrical conductivity, total nitrogen, total carbon and exchangeable cations content of the soil (Chan et al. 2008).

Novak et al. also show an increase in nutrient content of the soil, but they did not see an improvement in soil nitrogen status. Most of the micronutrients concentrations were not influenced by the addition of biochar. In addition, the cationic exchange capacity of the soil was not increased (Novak et al. 2009).

4.2.   Effects on plant growth promotion

Biochar amendment to soil can have a positive effect on plant growth. For example, it has been shown that biochar amendment results in an increase in maize yield (single application of 20 t/ha biochar), biomass production in bean (single application of 90 g/kg biochar) and grain yield in rice (Elad et al., 2011). Positive effects of biochar amendment to potting soil have also been reported. In the study of Graber et al., plant growth was stimulated in pepper after biochar addition (1-5% by weight) to a coconut fiber/tuff growing mix. The pepper plants had a larger leaf area, dry weight, number of nodes and yields of buds, flowers and fruits. In tomato, biochar also had a positive effect on plant height and leaf size. It did however, have no effect on flower and fruit yield (Graber et al., 2010). A positive effect of biochar addition to potting soil was also shown by Carter et al. The total biomass, root biomass, plant height and number of leaves of lettuce and Chinese cabbage were increased after biochar addition. Biochar was added to the potting soil in concentrations of 25, 50 or 150 g/kg (Carter, Shackley, Sohi, Suy, & Haefele, 2013).

It has been shown that biochar amendment to peat can increase the development of lateral roots in strawberry and the number and weight of fruits. However, so far, no effect on leaf and petiole fresh and dry weight is noticed. Following hypotheses for the effects on lateral root formation and on fruits are made (De Tender et al., 2016b):
- Biochar can function as a fertilizer. In a previous study it was shown that biochar addition only had a clear effect on strawberry growth when peat was not fertilized. In nutrient rich conditions, peat already has a high concentration of nutrients. In nutrient limiting conditions, the increase in nutrient content of the peat will supply necessary nutrients for the plant (De Tender et al., 2016a).
- Biochar can have an indirect effect through a change in the rhizosphere microbiome. An increase in plant growth promoting rhizobacteria (PGPR) could be responsible for the higher production of fruits and roots (De Tender et al., 2016b).
- Biochar can have a direct or indirect effect on the auxin pathway of the plant. Biochar application has been shown to induce auxin related genes (Viger, Hancock, Miglietta, & Taylor, 2015). Auxin has an important role in the formation of lateral roots. The root system of a plant regulates the capacity of the plant to take up nutrients and water. Also, the root system is important for sensing environmental conditions (Overvoorde, Fukaki, & Beeckman, 2010). Also, rhizosphere associated bacteria can produce auxin and interfere with the auxin pathway of the plant (Spaepen & Vanderleyden, 2011). Auxin is involved in almost all aspects of plant growth and development (Overvoorde et al., 2010).

However, neutral or even negative effects of biochar addition on plant growth have also been reported by some studies. Gravel, Dorais, & Ménard showed that the effect of biochar amendment to potting soil is plant species dependent when biochar is added in large concentrations. In their experiment they added biochar in a 1:1 ratio based on volume (Gravel et al., 2013). Nelissen et al. did not see an improvement in the yield of spring barley as a result of biochar addition (20 t/ha) to the soil. They also did not see an effect on soil chemical, physical and biological properties (Nelissen et al., 2015).

4.3.   Effects on plant resistance to diseases

Various publications reported a positive effect of biochar soil or potting soil amendment on disease resistance of different plant species. The mechanisms underlying this increase in disease resistance are not yet fully understood, but are probably linked with an activation of the plant's defense mechanisms.

Biochar can reduce the disease incidence of *Rhizoctonia solani* in cucumber. Biochar added to potting soil in low concentrations (0.5% on weight basis) reduced the disease incidence and severity. High concentrations of biochar (3% on weight basis) had a neutral or even negative effect (Jaiswal, Elad, Graber, & Frenkel, 2014). It has also been shown that biochar can reduce

the percentage of root lesions caused by *Fusarium oxysporum.* In this experiment, 1.5% and 3% biochar on weight basis was added to potting soil (Elmer & Pignatello, 2011).

Elad et al. showed that pepper and tomato were less susceptible to foliar pathogens after biochar addition to soil and potting soil medium. Biochar was added to the soil or potting medium in a ratio of 7:3 based on volume. But the site of infection of foliar pathogens is the leaves. Since the biochar location was different from the site of infection, this suggests that there was no direct toxicity toward the pathogens. The reduction of susceptibility is most likely caused by induced disease resistance. The observed induced systemic resistance could not be explained by effects on plant nutrition or improvements in soil water retention (Elad et al., 2010). Graber et al. suggest two hypotheses for the induced resistance observed upon biochar amendment. According to the first hypothesis, biochar addition causes shifts in microbial populations towards beneficial PGPR and plant growth promoting fungi (PGPF). It is possible that these shifts are stimulated by the residual organic tars in the biochar. In addition, the porous structure of biochar may provide physical refuge for beneficial microorganisms. The second hypothesis suggests that the systemic resistance is induced directly by the low levels of chemicals in biochar (Graber et al., 2010).

The first plant defense response that is activated upon infection by a pathogen is pathogen associated molecular pattern (PAMP) triggered immunity (PTI). PAMPs are conserved structure components of pathogens, such as flagellin and peptidoglycan. These components can also be present in nonpathogenic microorganisms. Therefore, these molecules are alternatively termed microbe associated molecular patterns (MAMPs). These PAMPs and MAMPs are recognized by pattern recognition receptors (PRRs) present on the plant cell surface. This recognition leads to the activation of different defense responses, including expression of pathogen responsive genes, production of reactive oxygen species (ROS) and mitogen activated protein (MAP) kinase signaling. Some pathogens can suppress PTI by secretion of effectors into the plant cell cytosol. Plants respond to these effectors through the development of resistance proteins (R proteins). These R proteins recognize effectors and activate effector triggered immunity (ETI). This defense response is associated with hypersensitive response (HR) (Chisholm, Coaker, Day, & Staskawicz, 2006; Thomma, Nürnberger, & Joosten, 2011).

Plant defense can also be stimulated before infection by a pathogen. Induced resistance is a physiological state in which the plant's defense mechanism is enhanced by specific stimuli. There are two forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is triggered by infection by a pathogen and leads to the accumulation of pathogenesis related (PR) proteins and salicylic acid (SA). ISR is triggered by colonization of plant roots by PGPR and PGPF. ISR is associated with signaling responses mediated by jasmonic acid (JA) and ethylene (Choudhary, Prakash, & Johri, 2007). In addition, plants can be primed for a more efficient activation of defense responses. The primed state of the plant is the physiological state in which a plant can activate its defense responses more rapid and/or stronger. This primed state can be induced by pathogens, beneficial microbes or natural or synthetic compounds. However, the fitness costs of priming are lower than those of directly induced defense (Conrath et al., 2006).

Biochar amendment in potting soil can also influence the expression of defense related genes. Meller Harel et al. reported that biochar amendment (1% or 3% on weight basis) induced the expression of defense related genes in non-infected strawberry plants. The plants are thus better prepared for infection. The defense related genes that were studied here are pathogenesis related protein 1 (*FaPR1*), osmotin like protein (*Faolp2),* Fraa3 allergen (*Fraa3*), lipoxygenase (*Falox*), and WRKY DNA binding protein (*FaWRKY1*). *FaPR1*, *Faolp2* and *Fra a3* encode PR proteins. *Falox* encodes a lipoxygenase enzyme and *FaWRKY1* encodes a transcription factor from the WRKY family (Meller Harel et al., 2012).

However, neutral or negative effects of biochar amendment are also reported by some studies. For example, biochar addition (0.3% or 1% on volume basis) to soil had no effect on the viability or reproduction of potato cyst nematodes. Biochar even reduced the positive effect of some other soil amendments (Ebrahimi et al., 2016).

4.4. Effects on rhizosphere microbiome

Biochar addition in soil or potting soil causes changes in microbial community composition and diversity in the rhizosphere. The rhizosphere is the narrow zone surrounding the plant roots. This zone contains a lot of different organisms, including bacteria, fungi, oomycetes, nematodes, protozoa, algae, viruses, archaea and arthropods (Mendes et al., 2013).

Biochar addition to soil or soilless growing media can cause changes in microbial community composition and diversity in the rhizosphere. Biochar addition (1 to 5% by weight) to potting medium increased the abundance of *Trichoderma* spp., *Pseudomonas* spp., *Bacillus* spp. and filamentous fungi in the rhizosphere of tomato and pepper plants (Graber et al., 2010). Kolton et al. also showed that biochar addition (3% by weight) to soil changes the composition of the rhizosphere of pepper plants. They reported an increase in the relative abundance of *Bacteroidetes* and a decrease of *Proteobacteria* as a result of biochar addition (Kolton et al., 2011).

The mechanisms that drive these changes are currently unknown. However, a few hypotheses have been suggested (De Tender et al., 2016a):
- Biochar can provide an additional habitat for bacteria and fungi and may provide a refuge place for fungal grazers (Thies & Rillig, 2009).
- Biochar may interfere with microbial intercellular signaling (Masiello et al., 2013).
- The effect on microbial composition can be due to the chemicals biochar contains. These chemicals may suppress some species and stimulate others (Kolton et al., 2011).
- Biochar can change the physicochemical characteristics of the soil. This may have an effect on the microbial communities. Biochar addition can increase the size of the soil water reservoir and increase the amount of plant available water. This changes the water holding capacity of the soil and this can potentially favor some bacterial species and disadvantage other species (Graber, Tsechansky, Lew, & Cohen, 2014).
- The effect on microbial composition can be due to the effect of biochar on nutrient composition of soil. Biochar can provide an additional nutrient source for the microbial community (De Tender et al., 2016a).
- Biochar may serve as a microbial source (De Tender et al., 2016a).

The relative abundance of the phyla *Planctomycetes* and *Actinobacteria* was increased in biochar amended potting soil (1 or 3% biochar by weight) (De Tender et al., 2016a). Some of the genera belonging to the *Planctomycetes* are known to oxidize ammonium (Buckley, Huangyutitham, Nelson, Rumberger, & Thies, 2006). *Actinobacteria* help in the degradation of complex polymers, such as lignin, chitin and cellulose. They also produce antimicrobial agents which help in the biocontrol of other bacteria and fungi (Sowani, Kulkarni, Zinjarde, & Javdekar, 2017). The relative abundance of *Proteobacteria* and *Acidobacteria* was decreased (De Tender et al., 2016a).

5. Chitin
5.1. Introduction

Chitin is the second most abundant natural polysaccharide, after cellulose. It is the major constituent of the exoskeleton of arthropods, the cell walls of fungi and the shells of crustacean and nematode eggs. At its pure state, chitin is a white, partially crystalline, odorless and tasteless solid. It is a long-chained linear, neutrally charged polymeric polysaccharide. Chitin can be deacetylated to chitosan (Figure 6). The commercial production of chitosan continues by exposing crustacean exoskeletons to high temperatures and alkali conditions. Chitosan has some favorable biological characteristics, such as biodegradability, biocompatibility, non-allergenicity and low toxicity to humans (Malerba & Cerana, 2016; Sharp, 2013).
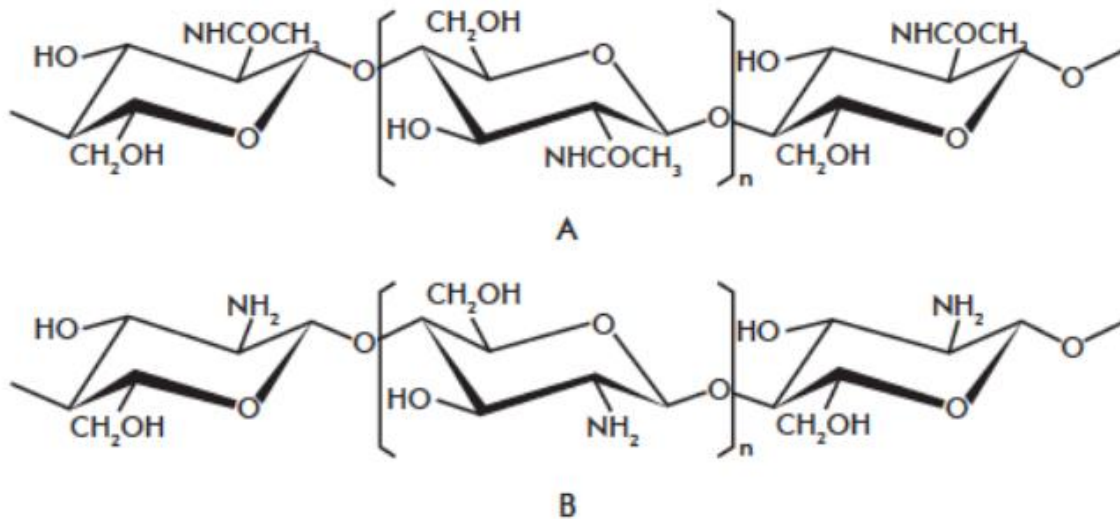
Figure 6: Structure of (A) chitin and (B) chitosan (Ramírez et al., 2010).

Chitin degrading enzymes are found in many different organisms, including bacteria, fungi, archaea, plants and even animals. Bacteria secrete chitinases mainly to obtain carbon, nitrogen and energy. Fungal chitinases have autolytic, nutritional and morphogenetic functions. Plants produce chitinases as a defense response to microbial infection. In vertebrate animals chitinases are found in the digestive tract.

Bacteria are thought to be the most important mediators of chitin degradation in nature. Chitin is degraded by two main pathways in bacteria (Figure 7). In the first pathway, the chitinolytic mechanism, the $(1\rightarrow4)$-β-glycoside bond is hydrolyzed. This hydrolysis is mediated by chitinases and leads to the formation of N-acetylglucosamine by N-acetylglucosaminidases. In the second pathway, chitin is deacetylated to chitosan. Chitosan can be further deaminated to glucosamine. This pathways involves the enzymes chitin deacetylase, chitosanase and glucosaminidase (Beier & Bertilsson, 2013; Frandberg, 1997).
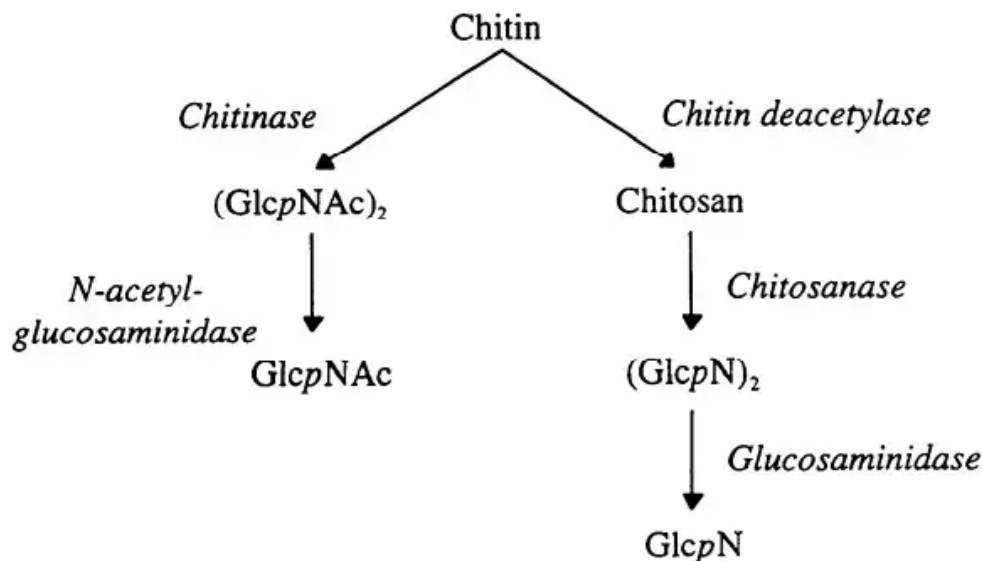
Figure 7: Main pathways of chitin degradation in bacteria (Frandberg, 1997).

The majority of chitin and chitosan used in agriculture as a soil amendment comes from the exoskeletons of crustaceans farmed for human consumption. Crustacean exoskeletons contain very high chitin content. Additionally, the use of these exoskeletons in agriculture provides a way of utilizing a major source of waste in the shrimp farming industry (Sharp, 2013). Chitin and chitosan can be applied in agriculture for four reasons (Ramírez, Rodríguez, Alfonso, & Peniche, 2010):

1. Regulating plant growth and development.

2. Protection of plants against pests and diseases before and after harvest.
3. Increasing the beneficial symbiotic plant-microorganism interaction.
4. Stimulating the action of antagonist microorganisms and biological control agents.

5.2.   Effects on plant growth promotion

It has been shown that chitin addition to potting soil has positive effects on plant growth. Debode et al. reported that the addition of chitin (2% by weight) increased the fresh weight and root development of lettuce. They also saw an increase in the relative abundance of PGPR and PGPF. This is possibly related to the positive effect of chitin on the lettuce growth (Debode et al., 2016).

Muymas et al. also reported a positive effect of chitin amendment to potting soil on growth and yield of lettuce. The chitin addition also improved the soil structure and plant nutrient supply. The degradation of chitin could provide useful nutrients and plant growth stimulators. In addition, the nitrogen uptake from the soil improved as a result of the degradation of chitin (Muymas et al. 2015).

5.3.   Effects on plant resistance to diseases

Various studies have reported that chitosan has potential antimicrobial activity. Hua et al. reported that gray mold disease incidence and disease severity in kiwifruit were reduced after chitosan treatment of the fruits (Hua et al. 2019). Chitosan also reduces the decay incidence of gray mold and blue mold in tomato fruit (Liu, Tian, Meng, & Xu, 2007). Chitin addition (2% by weight) to potting soil also has a negative effect on the survival of human pathogens, such as *Salmonella enterica* and *Escherichia coli* O157:H7, on lettuce leaves (Debode et al., 2016). Chitosan soil amendment has been shown to help in the control of both fungal and bacterial pathogens (Sharp, 2013). The antimicrobial effect of chitosan can be due to a direct effect, affecting growth and development of the pathogen, or due to the activation of the defensive mechanisms in the plant (Ramírez et al., 2010).
Chitin itself does not show a meaningful antimicrobial activity, possibly due to the insolubility in water and uncharged nature of chitin (Sharp, 2013).

Chitin and chitosan were found to be strong plant defense elicitors. Elicitors are compounds applied to plants in low concentrations. These compounds can activate biochemical, genetic and physical defense mechanisms in the plant. Chitin is a structural component of different pathogens and is not produced by the plant itself. Because of this, it is classified as a MAMP. Plants have developed PRRs for chitin perception. In rice, the chitin receptor is the receptor-like protein (RLP) chitin elicitor binding protein (CEBiP). This receptor associates with the receptor-like kinase (RLK) OsCERK1 upon binding to chitin. The activation of OsCERK1 by chitin perception triggers immune responses in rice. These immune responses include the activation of MAPK cascades and production of ROS (Kawasaki, Yamada, Yoshimura, & Yamaguchi, 2017). It has been reported that MAPK is involved in SA and JA signaling (Jagodzik, Tajdel-Zielinska, Ciesla, Marczak, & Ludwikow, 2018).

Various plant hormones can trigger the plant immune signaling network. SA and JA are important defense hormones. The SA response pathway is mostly activated when the plant is infected by a biotrophic pathogen. SA biosynthesis is stimulated upon recognition of PAMPs or effectors of pathogens. SA then activates the regulatory protein NON EXPRESSOR OF PR GENES1 (NPR1). Upon activation, NPR1 functions as a transcriptional coactivator of many different defense related genes. Among these genes, several encode proteins with antimicrobial activity. JA biosynthesis is activated when the plant is infected by a necrotrophic pathogen. After biosynthesis, JA can easily be metabolized to methyl jasmonate or conjugated to amino acids. These conjugates can bind to the CORONATINE INSENSITIVE1 (COI1) protein. This binding leads to the ubiquitinylation and proteasome mediated degradation of JASMONATE ZIM (JAZ) repressor proteins. Hereby, different JA responsive genes are activated (Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012).

A link between chitin treatment and jasmonate activation of plant defense has been shown. Exogenous methyl jasmonate application activates many of the same systemic responses and genes as chitin treatment. After the jasmonate signaling of the defense response, different downstream responses are activated. These include the production, release or activation of phenolics, terpenes and ROS. Cellular changes, such as membrane depolarization have also been detected. In addition, the formation of physical barriers, such as the deposition of callose and lignin, is induced, as well as the induction of programmed cell death (Sharp, 2013).

Since *B. cinerea* is a necrotrophic pathogen, the defense response of plants infected with the pathogen is most importantly mediated by a JA dependent signaling transduction pathway. It has been shown that JA appears to be involved in defense responses against *B. cinerea* in strawberry. Application of methyl jasmonate on strawberry fruits leads to a reduced *B. cinerea* incidence (Saavedra, Sanfuentes, Figueroa, & Figueroa, 2017).

5.4. Effects on rhizosphere microbiome

Chitin amendment to potting soil alters the environmental conditions in the rhizosphere to shift the microbial community towards beneficial organisms. Chitin and its derivatives can thus stimulate the action of antagonist microorganisms and biological control organisms (Debode et al., 2016). Debode et al., 2016 reported that the relative abundance of the phyla *Proteobacteria*, *Actinobacteria* and *Ascomycota* were increased as a result of chitin amendment (2% by weight). These phyla are known to promote plant growth. Chitin has also been reported to enhance the biological activity of *Cryptococcus laurentii* against blue mold rot in pear (Yu, Wang, Yin, Wang, & Zheng, 2008). Cretoiu, Korthals, Visser, & van Elsas reported higher microbial densities in soil amended with chitin. Especially the bacterial density increased after chitin addition. The relative abundance of the *Actinobacteria* and *Oxalobacteraceae* were increased in chitin amended soil. Species of these groups of bacteria are known to degrade chitin (Cretoiu et al., 2013). Cretoiu, Kielak, Schluter, & van Elsas also saw a change in the soil microbiome after chitin addition to soil. They reported an increase in the phyla *Proteobacteria, Bacteriodetes, Firmicutes, Actinobacteria* and *Acidobaceria* (Cretoiu et al., 2014).

Antagonist microorganisms work against pathogens via a number of methods, including the production of chitinases. Therefore, adding chitin to the soil may help these antagonist microorganisms by stimulating the production of chitinases. Chitin can also function as a nitrogen rich polysaccharide food source for the antagonist microorganisms, thus stimulating their growth and survival (Sharp, 2013).

6. High-throughput sequencing
6.1. Introduction

Sanger sequencing is limited by a low throughput. Next generation sequencing technologies made it possible to sequence multiple DNA molecules in parallel. This made it possible to identify new mutations underlying genetic diseases, identify epigenetic changes or analyze the transcriptome, all the transcripts present in a cell (Churko, Mantalas, Snyder, & Wu, 2013).

The cost per Megabase of DNA sequence is graphically represented in Figure 8. The data from 2001 through 2008 represent the costs of sequencing based on Sanger sequencing. The data from 2008 onwards represents the costs of sequencing using next generation sequencing platforms. The graph also shows Moore's law, which predicts a doubling of computer power every two years. The sequencing cost follows Moore's law from 2001 until 2008. Afterwards, the sequencing cost out-paces Moore's law. This coincides with the transition from Sanger sequencing to next generation sequencing. The costs thus dropped faster than would be expected by Moore's law (Wetterstrand, n.d.).
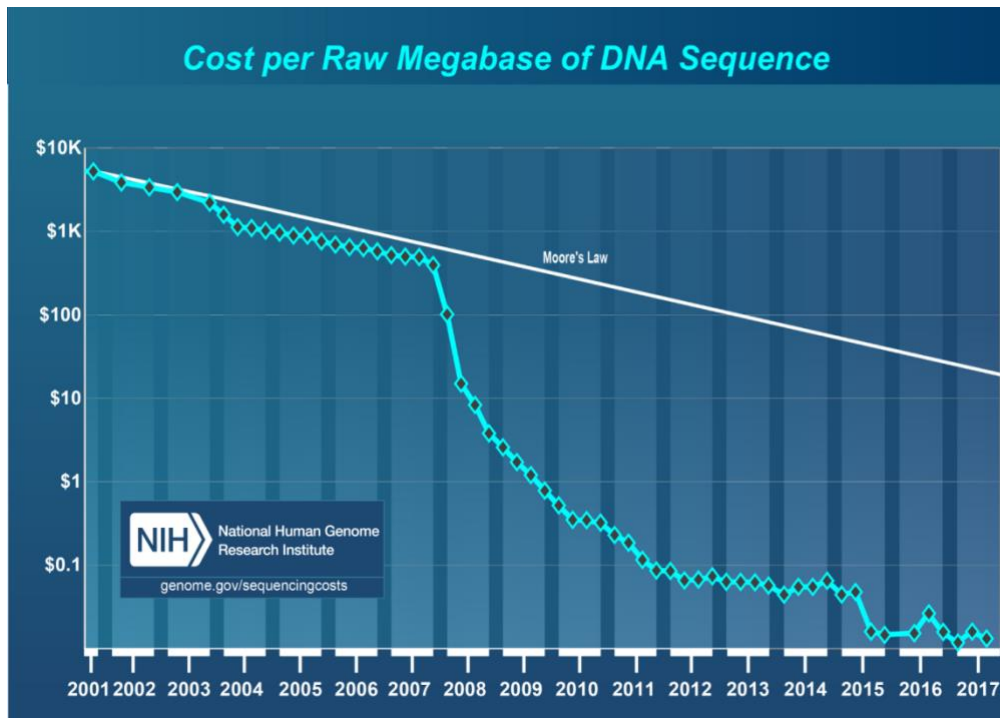
Figure 8: Cost per Megabase of DNA sequence. The cost of determining one megabase of DNA sequence of a specified quality (Wetterstrand, n.d.).

6.2.   Illumina sequencing

Illumina sequencing uses clonal amplification and sequencing by synthesis (SBS) chemistry to allow for rapid and accurate sequencing. The first step in the Illumina sequencing workflow after library preparation is cluster generation. The DNA templates are immobilized on a flow cell by hybridization via the adaptor sequences. Next, a polymerase is added to synthesize the complementary strands and the original templates are washed away. The strands are then clonally amplified by bridge amplification (Figure 9). The strands fold over and the adaptor regions hybridize to the flow cell. Polymerases are added to synthesize the complementary strands and forming a double stranded bridge. The bridge is then denatured to obtain two single stranded copies. This process is repeated multiple times. In this way, ca. thousand identical copies of each template are created. After the bridge amplification, the reverse strands are cleaved and washed off ("Sequencing Technology | Sequencing by synthesis," n.d.).

The second step is sequencing by synthesis. In this step, fluorescently labeled nucleotides, called reversible terminator nucleotides, are used. During each sequencing cycle, primers, polymerases and labeled deoxynucleoside triphosphates (dNTPs) are added. The label, which is added at the side of the base, functions as a polymerization terminator, so one nucleotide is incorporated at a time. After a nucleotide is added, the clusters are excited and a fluorescent light signal is emitted. This fluorescent signal is characteristic for the nucleotide that is added. The fluorescent label is then cleaved off to allow incorporation of the next nucleotide. This process is repeated until the desired read length is obtained. If the forward strands are sequenced, the 3' end of the strands are deprotected using a chemical group so they can fold over and bind with the adaptor sequence on the flow cell. Next, the complementary strand is synthesized, forming a double stranded bridge. The bridge is then linearized and the 3' ends are blocked. The forward strands are cleaved and washed off. The reverse strand is then sequenced in the same manner as the forward strand ("Sequencing Technology | Sequencing by synthesis," n.d.).

Bridge amplification

Fragments become double stranded
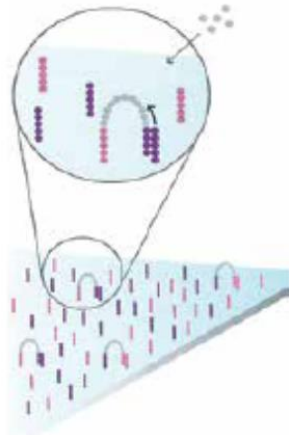
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.
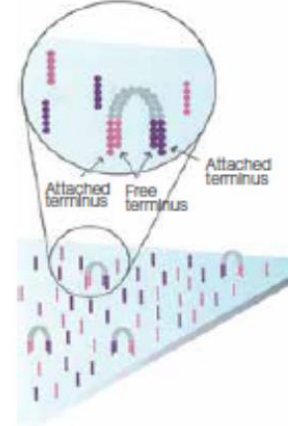
The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.

Denature the double stranded molecules

Complete amplification
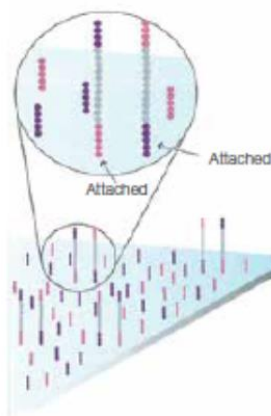
Denaturation leaves single stranded templates anchored to the substrate
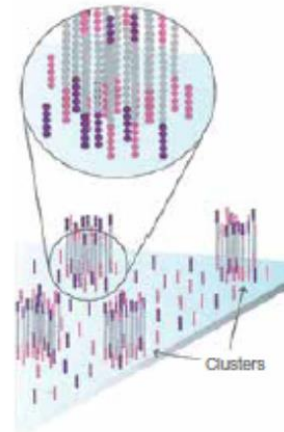
Several million dense clusters of double stranded DNA are generated in each channel of the flow cell

Figure 9: Bridge amplification. First the strands fold over and bind to the adaptor sequences. Then, the complementary strand is synthesized. The bridges are denatured to obtain single stranded templates. These steps are repeated multiple times to obtain clusters from each template present in the sample (Illumina, 2010).

Illumina has different sequencing platforms, among which the MiSeq and HiSeq platforms are frequently used. A comparison between both platforms is made in Table 1. The MiSeq platform is mostly used for amplicon sequencing, because of the longer read lengths that are obtained. The HiSeq platform is mostly used for shotgun metagenomics, sequencing all genomes present in a sample. This because of the higher sequencing depth (Dark, 2013; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013).

Table 1: Comparison of Miseq and Hiseq platforms of Illumina (Dark, 2013; Kozich et al., 2013; "MiSeq Specifications | Key performance parameters," n.d.).

Feature	MiSeq	HiSeq 2500 high output
Number of reads per run	20-25 million	3 billion
Read length	2x300 basepairs (bp)	2x100 bp
Run time	Up to 56 hours	11 days

6.3. Amplicon sequencing

Amplicon sequencing is a commonly used method to characterize the microbial diversity. It has been applied to evaluate bacterial diversity in different environments. Amplicon sequencing allows to study microbiomes from samples that are otherwise difficult to study (Van Dijk et al., 2014).

In amplicon sequencing, a community is sampled and DNA is extracted from it. Next, a taxonomically informative marker is targeted and amplified by PCR. This marker is present in all organisms of interest. However, the exact sequence can vary, hence the marker can be used for identification. The amplicons generated by PCR are then sequenced. To determine which species are present in the sample and their relative abundance, bioinformatics is used (Sharpton, 2014). In general, the reads are first filtered to remove bad quality reads. Next, the reads are assembled into clusters or aligned to reference sequences. Afterwards, function analysis of the clusters can be done, the number of reads per cluster can be calculated or statistical comparison between samples or treatments can be made (University of Oregon, n.d.).

6.3.1. Genetic markers

With amplicon sequencing, a specific genomic locus is targeted for amplification. This genomic locus, or marker, should be present in all organisms of interest in the sample. However, the marker should also have sufficient variation to be taxonomically informative. In other words, the marker must have a high interspecific variation but low intraspecific variation (Lindahl et al., 2013; Sharpton, 2014).

The small subunit ribosomal RNA (16S rRNA) locus is the most commonly used marker in bacterial diversity studies. This is both a taxonomically and phylogenetically informative marker (Sharpton, 2014). The 16S rRNA gene is a suitable marker for several reasons. The gene is universally distributed, so it is possible to target a wide variety of bacterial phyla. It is assumed to be only weakly influenced by horizontal gene transfer, since it is an essential part of the core gene set of bacteria. However, the 16S rRNA gene has some variable regions, meaning it allows for classification. The conserved regions allow for the design of PCR primers for various taxa at different taxonomic levels (Větrovský & Baldrian, 2013). The locus is graphically represented in Figure 10. The conserved regions are represented by the green boxes, the variable regions by the grey boxes.
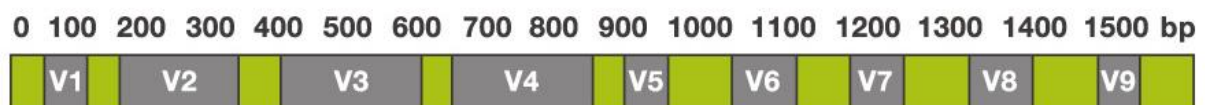
Figure 10: The small subunit ribosomal RNA (16S rRNA) locus in bacteria. The conserved regions are shown in green. The variable regions, named V1 to V9, are represented in grey (Ruturaj, n.d.).

For fungal diversity studies, the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) locus is mostly used as marker. The locus is graphically shown in Figure 11. The ITS regions consists of two highly variable spacers, named ITS1 and ITS2. The

Figure 11: The nuclear ribosomal RNA locus in eukaryotes. IGS = intergenic spacers, in most species composed of one or more internally repeated sequences (grey arrowheads). ETS = external transcribed spacer. ITS = internal transcribed spacer (Eickbush & Eickbush, 2007).

ITS region is frequently used as marker for the following reasons. (1) It is universally distributed. (2) The rRNA locus has some highly conserved regions across different groups of organisms. This allows for designing PCR primers for a wide range of taxa. (3) The ITS region has a high interspecific variation, but a low intraspecific variation. This region allows thus for classification (Lindahl et al., 2013).

6.3.2. Advantages and limitations

Amplicon sequencing makes it possible to efficiently discover, validate and screen genetic variants. High coverage is possible by multiplexing of thousands of amplicons per reaction. Furthermore, amplicon sequencing reduces costs and time in comparison with whole genome sequencing. In addition, it is possible to sequence multiple samples simultaneously. This can be done by adding an additional index/barcode to the sequences by a second PCR reaction ("Amplicon Sequencing Solutions," n.d.).

Amplicon sequencing also has some limitations. Since a PCR step is needed for amplification of the target gene, this introduces some biases. Because of these biases some part of the diversity in the sample can be missed. In addition, different estimates of diversity can be obtained. The obtained diversity estimate is dependent on the genomic locus that has been targeted. Different genomic loci have a differential power for resolving taxa. Sequencing errors and incorrectly assembled amplicons, for example chimeras, can produce artificial sequences. This results in a different diversity estimate (Sharpton, 2014).

The copy number per genome of the 16S rRNA varies from 1 up to 15 or more copies. This means that the same species can be counted multiple times in the data analysis, leading to an overestimation of the diversity. Since the read length is 2x300 bp, this means the maximum read length is 600 bp. Therefore, the taxonomical depth is limited (Větrovský & Baldrian, 2013). Amplicon sequencing only gives information about the taxonomical composition of a sample. The biological functions of the taxa cannot be determined with amplicon sequencing. Another limitation is that only taxa for which taxonomically informative genetic markers are available can be analyzed with amplicon sequencing (Sharpton, 2014).

6.4. RNA sequencing
6.4.1. Introduction

RNA sequencing (RNA seq) profiles the transcriptome by using high throughput sequencing methods. The transcriptome is the collection of all transcripts and their quantity present in a cell in a specific developmental stage or physiological condition. The analysis of the transcriptome allows determining all different forms of a transcript and the transcriptional structure of genes. In addition, it allows to quantify the changes in the expression level of a transcript during development and under different conditions (Gerstein, Snyder, & Wang, 2009; Kukurba & Montgomery, 2015).

Transcriptomics studies were initially done using hybridization based microarray technologies. These methods are high-throughput and have a relatively low cost, but also have some limitations. They require a priori knowledge about the sequences that are looked at. Cross-hybridization artifacts, formed by the analysis of highly similar sequences, complicate further analysis. Finally, microarrays are limited in their ability to accurately quantify very low and high expressed genes. Sequence based methods analyze the transcriptome by directly determining the sequence of the transcript. The first sequence based approaches for transcriptomics were based on expressed sequence tags (EST) libraries. This method is however low in throughput and not well suited for quantification of transcripts. To overcome these limitations, tag based methods were developed. Examples of these methods are serial analysis of gene expression (SAGE) and cap analysis gene expression (CAGE). But these methods cannot measure expression levels of splice isoforms. Also, novel gene discovery is not possible with these methods. RNA seq made it possible to analyze RNA through sequencing of the complementary

DNA (cDNA). Generally, a RNA seq experiment consists of the following steps. First, RNA is collected and converted to cDNA. Next, adaptors are attached to one or both ends of the cDNA (Kukurba & Montgomery, 2015). The adaptors are specific for the sequencing platform that is used. In addition, the adaptors make it possible to sequence multiple samples in the same run (University of Oregon, n.d.). Finally, the cDNA is sequenced on a next generation sequencing platform (Kukurba & Montgomery, 2015).

6.4.2. Advantages and limitations

RNA seq can be used to discover novel transcripts, since it is not limited to transcripts of existing genomic sequence. It can also be used to determine the exact location of transcription boundaries. In addition, sequence variations can be detected. RNA seq has very low background signal because sequences are precisely mapped to unique regions of the genome. It has a large dynamic range of expression levels, since it does not have an upper limit for quantification (Gerstein et al., 2009).

However, RNA seq also has some challenges, mostly concerning the bioinformatics analyses. Improvements in sequencing technologies make it possible to produce longer reads. New mapping methods are needed to precisely and efficiently align these long reads. As it becomes possible to sequence very low quantities of RNA, complex statistical approaches will be necessary to differentiate between technical noise and meaningful biological variation (Kukurba & Montgomery, 2015).

Material and methods

1. Plant material and growth conditions

The strawberry experiment, sampling and measurements were done from January 2018 until May 2018 and therefore finished before the start of this master thesis. Samples were stored under adequate conditions until further analysis. Samples taken for bacterial 16S rRNA gene amplicon sequencing, plant RNA sequencing and Rt-qPCR were prepared and analyzed in this thesis. In addition, the data collected on physiological parameters, such as plant fresh weight, plant dry weight, root weight and infection rate of fruits and leaves were also analyzed in this master's dissertation.

1.1. Strawberry growth conditions

Strawberry plants were grown either in peat substrate, peat substrate amended with 3% biochar or with 3% chitin. The peat substrate used in this experiment was NOVOBALT white peat 100% (AVEVE Lammens,Wetteren, Belgium). Biochar was prepared from the pyrolysis of holm oak at 650°C for 12-18 hours (Proininso S.A., Malaga, Spain). Chitin flakes were purified from crab shells (BioLog Hepp Gmbh (lot: 90200705)).
Additionally, fertilizer (1,9 g/L potting soil) (PGMix, Peltracom, Ghent, Belgium) and lime (7,7 g/L potting soil) (Dolokal extra, Ankerpoort NV, Maastricht, The Netherlands) were added to the mixtures.
For each treatment, 84 plants were grown. The plants were grown in the greenhouse at 20°C for 11 weeks. They were placed in the greenhouse according to a semi- randomized complete blocking design, in which each condition was put within a block and randomized within this block. In addition, 84 pots were filled with either potting soil, potting soil amended with 3% biochar or with 3% chitin for bulk soil microbiome analysis. In these pots no plants were grown.

1.2. Sampling
1.2.1.Overview

Starting from the first week of plant growth, strawberry plants were sampled weekly. The following samples were taken and parameters were measured: (1) Plant fresh weight was measured by removing the above ground plant parts and measuring the weight. (2) Plant dry weight was determined by drying the above ground plant parts and measuring the weight. (3) Root weight was determined by removing loose soil from the roots and measuring the weight of the root. (4) Chlorophyll content was measured using a CCM-200 chlorophyll content meter (Opti-Sciences Inc., Hudson, NH, USA). (5) The rhizosphere and soil was sampled for microbial analysis. (6) The leaves were sampled for gene expression analysis.

1.2.2.Rhizosphere & soil sampling for microbial analysis

At the start of the experiment all mixtures (peat substrate, peat substrate + 3%biochar, peat substrate + 3% chitin) were sampled for analysis. In addition, the strawberry rhizosphere was sampled from six plants before the start of the experiment.

From the first week of plant growth onwards, the rhizosphere was sampled from strawberry roots weekly in a completely randomized way. Six biological replicates were sampled for each growing medium at each timepoint. Rhizosphere sampling was done according to Lundberg et al. (2012). The aboveground plant parts were removed. Loose soil was removed from the roots by kneading and shaking. The roots were then placed in a 50 mL tube containing 25 mL phosphate buffer. The tubes were vortexed and the resulting turbid solution was filtered through a 100 µm nylon mesh cell strainer. The turbid filtrate was centrifuged for 15 minutes at 3200g. The resulting pellets were used for DNA extraction with the PowerSoil DNA isolation

kit (MoBio, Carlsbad, USA), according to the manufacturer's instructions. DNA was stored at -20°C until further use.

Simultaneously, six replicates per treatment were sampled weekly from the bulk soil samples in a completely randomized way. In total, a falcon tube of 50 mL was filled with potting soil. DNA extraction was done with the PowerSoil DNA isolation kit (MoBio, Carlsbad, USA), according to the manufacturer's instructions. DNA was stored at -20°C until further use.

1.2.3. Plant leaf sampling for gene expression analysis

The leaves of strawberry plants were sampled weekly in a completely randomized way. For sampling the leaves, 3 leaf punches, 11 mm diameter, were made per leaf. Per plant, 2 leaves were sampled. In total, 6 leaf punches per plant were made, randomly distributed over the plant. The material was collected in a 1.5 mL tube and immediately flash frozen in liquid nitrogen and stored at -80°C until further processing.

2. *Botrytis cinerea* bio-assay

Strawberry leaves were inoculated with *B. cinerea* after 8 weeks of plant growth. Leaves were inoculated using the method described by Meller Harel et al. (2012). *B. cinerea* was grown on potato dextrose agar and incubated at 20°C for 4 days. Agar disks with mycelium were cut out from the edge of the colony. These agar disks were placed, with the mycelium side down, on the surface of 3 young fully expanded strawberry leaflets. Per leaf, 3-6 disks were placed. As negative control, half of the plants were inoculated with agar disks without mycelium, thus only containing agar. The plants were sprayed with water and each pot was covered with a plastic box for 1 week to create a high humidity level to stimulate the development of *B. cinerea.* One week after inoculation, the resulting lesions on the leaves were recorded using a 0-4 disease scale. In this scale a score of 0 corresponds with 0% of the leaf area infected, 1 with less than 25% of the leaf area infected, 2 with 25-50% of the leaf area infected, 3 with 51-75% of the leaf area infected and 4 with more than 75% of the leaf area infected. After scoring the infection, the inoculated leaves were removed from the plants.

Strawberry fruits were inoculated from week 6 of plant growth onwards, this was the moment the first fruits appeared. Fruits were inoculated according to the method of Bhaskara, Belkacemi, Corcuff, Castaigne, & Arul (2000). *B. cinerea* was grown on potato dextrose agar for 2 weeks. Conidia were recovered by flooding the cultures with sterile water containing 0.1% (v/v) Tween 80. The mycelial suspension was filtered through 3 layers of sterile cheese cloth and the concentration was adjusted to 2×10^5 conidia/mL. Strawberry fruits were transferred to plastic boxes and were inoculated with 20 µL conidial suspension per fruit. The fruits were evaluated daily when the first symptoms appeared. Spoiled fruits were removed to avoid secondary infections.

3. Amplicon sequencing
3.1. DNA extraction

After sample collection, DNA was extracted using the PowerSoil DNA isolation kit and stored at -20°C until further analysis. This step was already done before the start of this master's dissertation.

3.2. Amplicon Polymerase chain reaction (PCR)

The bacterial V3-V4 region was amplified using PCR with region of interest specific primers with overhang adapters attached. The primers used to amplify the bacterial V3-V4 region were the primers suggested in the Illumina protocol (Illumina, 2013) and were selected from the Klindworth et al. publication (Klindworth et al., 2013).

16S Amplicon PCR Forward Primer:
5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer:
5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
The sequences indicated in red are the region specific nucleotide sequences. In black are the Illumina overhang adapter sequences.

The mastermix used for amplicon PCR consisted of the following components: 12.5 µL 2x Kapa HiFi Hotstart ReadyMix (Kapabiosystems, Wilmington, MA, USA), 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer and 9 µL MilliQ water. To this mastermix, 2.5 µL DNA (concentration: 5 ng/µL) was added. The PCR program followed the scheme listed in Table 2. All PCR reactions were done using a T100 Thermal Cycler (Bio-Rad).

Table 2: Amplicon PCR program.

Temperature	Time	
95°C	3 min	
95°C	30 sec	
55°C	30 sec	25/30x
72°C	30 sec	
72°C	5 min	

Gel electrophoresis was done to verify the amplification. For this 1.5% agarose (in 0.5 Tris-Acetate-EDTA (diaminoethane tetraacetic acid) (TAE) buffer) gel was used. The sample (5 µL) was mixed with dye (Midori green direct, NIPPON genetics, Germany) and loaded on the gel. The gel electrophoresis was done at 100 V for 20-25 minutes.

3.3. Amplicon PCR clean-up

The amplicons were purified from unincorporated primers and primer dimers. For this purification, the HighPrep PCR beads (MAGBIO, Gaithersburg, MD) were used.
HighPrep PCR Reagent (45 µL) was added to the PCR product and mixed thoroughly by pipetting up and down 8 times. The mixture was then incubated for 5 minutes at room temperature. The sample plate was placed on a magnetic separation device for 3 minutes and supernatant was removed. 70% ethanol (200 µL) was added and incubated for 1 minute at room temperature. The supernatant was removed. This step was repeated one time. The beads were dried by incubating the samples for 15 minutes at room temperature. The sample plate was removed from the magnetic separation device and 40 µL elution buffer (1x TE buffer, Sigma-Aldrich) was added. The buffer contains 10 mL 1M Tris pH 8 (MP Biomedicals Europe) and 2 mL 0.5M EDTA (Sigma Aldrich), the volume was adjusted to 1 L by adding milliQ water. The sample and TE buffer were mixed by pipetting up and down 20 times. The mixture was then incubated at room temperature for 3 minutes. The sample plate was placed back on the magnetic separation device and incubated for 1 minute. The cleared supernatant (eluate) was transferred to a new plate.

3.4. Index PCR

The adaptor sequence for Illumina sequencing and an index specific for each sample was ligated to the selected strain using PCR. The primers used in this PCR are Illumina Nextera XT index 1 primers (N7XX) and Illumina Nextera XT index 2 primers (SSXX) (Nextera XT-index kit). In total, 5 µL from each purified amplicon was used in the PCR reaction.

The mastermix used for amplicon PCR consisted of the following components: 25 µL 2x Kapa HiFi Hotstart ReadyMix (Kapabiosystems, Wilmington, MA, USA), 5 µL Nextera XT index primer 1 (N7xx) (Illumina), 5 µL Nextera XT index primer 2 (S5xx) (Illumina) and 10 µL

Molecular grade water. To this mastermix, 5 µL DNA was added. The PCR program followed the scheme listed in Table 3.

Table 3: Index PCR program

Temperature	Time
95°C	3 min
95°C	30 sec
55°C	30 sec
72°C	30 sec
72°C	5 min

(rows 2–4: 8x)

The PCR products were quality controlled using the Qiaxcel Advanced (QIAGEN, Germantown, MD, USA).

3.5. Index PCR clean-up

The PCR product was again purified using the same protocol as described in 3.3 Amplicon PCR clean-up.

3.6. Library quantification, normalization and pooling

The concentration of each sample was measured with the Quantus Fluorimeter (Promega, Madison, WI, USA). For this, the following mix was made: 187.5 µL milliQ water, 10 µL TE buffer, 1µL DNA and 0,5 µL quantifluor dye.
The concentration in nM was calculated using following formula:

$$\text{Concentration in nM} = \frac{\text{concentration in ng/µL}}{650 \frac{\text{g}}{\text{mol}} * \text{average library size}} * 10^6$$

The average library size was 600 bp.

The final library was diluted using 1x TE buffer[1] to 10 nM. Aliquots of 5 µL DNA were made from each library. The aliquots were mixed for pooling the libraries.

3.7. Sequencing

Sequencing of the libraries was done using the Illumina MiSeq v3 platform (2x 300bp) by Admera (South Plainfield, NJ, USA). Illumina uses a bridge amplification followed by sequencing by synthesis. These technologies were already described in 6.2 Illumina sequencing.

3.8. Bioinformatics analysis
3.8.1. DADA2 workflow

Samples were demultiplexed by the sequencing provider.

The raw data was first preprocessed by removing the primer sequences making use of Trimmomatic version 0.38 (Bolger, Lohse, & Usadel, 2014). Additional preprocessing, such as filtering, trimming and merging of forward and reverse reads, was done with DADA2 (Callahan et al., 2016). Reads were truncated at the positions where the quality dropped. For forward reads, this was at base 263. Reverse reads were truncated at base 240. Sequences with

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[1] This is the same buffer used in the PCR clean-up and consists of 1M Tris pH 8 and 0.5M EDTA

unassigned bases were removed. Forward reads with more than 3 expected errors and reverse reads with more than 5 expected errors were removed. The number of expected errors were calculated based on the quality score. Reads were truncated when the quality score was less than 2. Reads that matched against the phiX genome were removed. The phiX library is added as a quality control for cluster generation, sequencing and alignment. The library originates from the well characterized bacteriophage phiX genome.

Before the filtering and trimming step in the DADA2 pipeline, the quality of the samples was evaluated by the function plotQualityProfile (DADA2 package). In addition, the quality can be evaluated using FastQC (Andrews, 2010).
After the preprocessing, inference of amplicon sequencing variants (ASV) was done with DADA2. Therefore, an error model was created for every run. The error model was created from the data by alternating estimation of the error rates and interference of sample composition until they converge on a mutually consistent solution. The error model makes it possible to infer ASVs. All identical reads were combined into unique sequences with a corresponding abundance. Based on this information, a sequence table of all samples of a run was created. Afterwards, the sequence tables of each run were merged into one sequence table. In a next step, chimeras (a sequence that actually originates from two different sequences) were removed. In a last step, taxonomy is assigned to each ASV. The resultant count table with taxonomy information is used for further analysis.

3.8.2. Data exploration

The data exploration was done in R version 3.4.3.

Differences in richness between the different treatments and between bulk soil and rhizosphere were visualized with richness plots. To create these plots, the Shannon diversity index was used. This index uses both richness and evenness of the community. It is calculated using the following formula:

$$H = - \sum_{i=1}^{s} p_i * \ln(p_i)$$

With   H: Shannon diversity index
        s: Number of species
        p_i: Proportion of individuals found in species i
The differences in richness over the growth period of the strawberry plants were also visualized in a line graph. Statistical differences in richness between the treatments were studied for the rhizosphere samples. Therefore, generalized linear models were fitted for every timepoint.

The composition of the bacterial community was visualized using bar charts at the taxonomical level of phylum and genus.

3.8.3. Statistical analysis

The statistical analysis was done in R version 3.4.3.

An additional filtering step was done to remove samples with a low number of reads and low abundant ASVs. Only samples with a total number of reads above 8000 and ASVs with a count of 2 in at least 6 samples were retained for further analysis.

First, a PERMANOVA test was done to look if there were significant effects of the treatments, sample type (rhizosphere or bulk soil) and time on the composition of the bacterial community. PERMANOVA is similar to a classical ANOVA test, but PERMANOVA is more widely applicable. It is a geometric partitioning of multivariate variation in the space of a dissimilarity measure. This dissimilarity measure can be chosen. PERMANOVA uses a pseudo F statistic for testing the null hypothesis of no differences between groups in the space of the chosen dissimilarity measure. For Euclidean distances, this pseudo F statistic is the same as the F

statistic used in ANOVA. Permutations are used to obtain p values. This means that there is no assumption of multivariate normality. PERMANOVA allows to perform a meaningful analysis of high dimensional data with non-normal variables (Anderson, 2017).

Since PERMANOVA requires a dissimilarity measure, dissimilarity matrices were calculated. These matrices were calculated using the Bray-Curtis dissimilarity coefficient, calculated according to the following formula (Goslee, 2010):

$$ BC = \frac{\sum_{i=1}^{n}|p_i - q_i|}{\sum_{i=1}^{n}(p_i + q_i)} $$

With   BC: Bray-Curtis dissimilarity matrix

        p_i: i^{th} element of data vector p

        q_i: i^{th} element of data vector q

        n: total number of elements in data vector p and q

Before calculating the dissimilarity matrices, the data was split in 2 groups. The first group only contained the samples of the potting soil and biochar treatments. The second group only contained the samples of the potting soil and chitin treatments. For both groups, a dissimilarity matrix was calculated from the ASV count table. Homogeneity of variances was tested on both dissimilarity matrices using the betadisper function (R package vegan version 2.5-4, (J. Oksanen et al., 2019)). PERMANOVA was done for both groups using the respective dissimilarity matrix as input.

Principal coordinates analysis (PCoA) plots were made to look at the relation between the samples. This is similar to principal components analysis (PCA) plots, but PCoA is better fitted for non-Euclidean data. PCA tries to represent as much of the variance in the data as possible in the first axes. It does this by rotating the data. PCoA is similar to PCA, but uses dissimilarities (Jari Oksanen, 2017). PCoA plots were made using the cmdscale function with the dissimilarity matrices as input.

To look deeper at the effects of the treatments and time, a statistical analysis based on the EdgeR workflow was done (R package edgeR version 3.20.9, Robinson, McCarthy, & Smyth, 2010). For this analysis, only the data from rhizosphere samples were used. The count table was clustered at genus level before analysis. First, the data was normalized to account for technical variation between the samples. The default normalization method used in EdgeR is trimmed mean of M-values (TMM) normalization. This method takes the sequencing depth into account and corrects for the presence of highly abundant ASVs (Robinson & Oshlack, 2010). A negative binomial (NB) model with main effects for time and treatment, as well as treatment × time interaction, was used to model the counts. Overdispersion parameters of the NB model were estimated using Empirical Bayes estimation adopted by the quantile-adjusted conditional maximum likelihood (qCLM) method. Therefore, the overdispersion parameter of a single ASV was shrunk toward the common dispersion across all ASVs. Likelihood ratio tests were done on the appropriate contrasts of the model parameters to asses differences in bacterial microbiome composition. Benjamini-Hochberg False Discovery Rate procedure was used to correct for multiple testing (Robinson et al., 2010).

4. RNA sequencing

4.1. RNA extraction

RNA extraction was based on the method of Luypaert et al. (2017), with some minor modifications. For RNA seq analysis, samples from weeks 8 and 9 of plant growth were analyzed. For every treatment and timepoint, 3 biological replicates were used. In addition, samples from infected plants after 9 weeks of plant growth were analyzed. Also here, 3 biological replicates were used for every treatment.

4.1.1. Preparation

Retsch Tissuelyser II (QIAGEN, Germantown, MD, USA) sample blocks were treated with RNase away and stored at -80°C overnight. Zirconium beads were washed with soap, treated in 10% bleach for 30 minutes and baked for 8 hours in a weck jar at 180°C. The beads were then cooled at -80°C overnight. Spatulas, tweezers, bottles, caps, aluminum weigh shells and measuring cylinders were wrapped in aluminum foil and baked for 8 hours at 180°C. The working place, racks and equipment was cleaned with 10% fresh made bleach solution.

4.1.2. Preparation of solutions and extraction buffer

Sodium chloride (NaCl) with a concentration of 2.5 M was made by dissolving 3,653 g sodium chloride (Sigma-aldrich) in 20 mL UltraPure DNase/RNase free distilled water (Invitrogen) and heating to 60°C. The volume was adjusted to 25 mL and stored at room temperature.
Lithium chloride (LiCl) with a concentration of 4 M was made by dissolving 8.478 g lithium chloride (Sigma-aldrich) in 45 mL UltraPure DNase/RNase free distilled water and heating to 80°C. The volume was adjusted to 50 mL and stored at room temperature.
The extraction buffer was consisted of the following components: 0.3 g cetrimonium bromide (CTAB) (Sigma-aldrich), 0.5 g polyvinylpyrrolidone (molar mass 40000 g/mol) (Sigma-aldrich), 1 mL 1 M Tris-HCl pH 8 (Invitrogen), 5.6 mL 2.5 M NaCl, 0.4 mL 0.5 M Na_2EDTA (Invitrogen), 0.1 mL beta-mercapto-ethanol and 3.9 mL UltraPure DNase/RNase free distilled water. First, CTAB and polyvinylpyrrolidone were weighted. Next, Tris-HCl, NaCl, Na_2EDTA and UltraPure DNase/RNase free distilled water were added. Beta-mercapto-ethanol was added just before the extraction buffer was added to the samples. The extraction buffer was heated to 60°C in a warm water bath.

4.1.3. Extraction procedure

The tubes containing the leaf punches (which were stored at -80 °C) were distributed in the cold Retsch sample blocks in a symmetrical way and 3 cold zirconium beads were added to each tube with baked tweezers. During this step the sample blocks were placed in a box filled with liquid nitrogen. Leaf punches were grounded with the TissueLyser II for 2 times 90 seconds at 30 Hz. The sample blocks were inverted between the 2 grinding steps. Beta-mercapto-ethanol was added to the preheated (60°C) extraction buffer and the extraction buffer (700 µL) was added to each sample. The samples were vortexed vigorously for 1 minute and incubated for 10 minutes at 65°C and 850 rpm in the thermomixer. The samples were then centrifuged for 5 minutes at 15,800 g at room temperature. Supernatant (650 µL) was transferred to a new tube and an equal volume of chloroform was added. The samples were vortexed for 1 minute and mixed on a rotator for 5 minutes. Afterwards, the samples were centrifuged for 10 minutes at 15,800 g at room temperature. The upper aqueous phase was transferred to a new tube without disturbing the interface. The samples were then re-centrifuged at the same conditions to enhance purity of the extracted RNA. The supernatant (450 µL) was transferred to a new tube and an equal volume of 4 M LiCl was added. RNA was allowed to precipitate overnight at 4°C. The steps following the overnight incubation were done on ice. After the overnight incubation, the samples were centrifuged for 15 minutes at 15,800 g at 4°C and the supernatant was removed. The pellets were washed twice with 800 µL 75% ethanol and centrifuged for 15 minutes at 15,800 g at 4°C. The ethanol was removed and the remaining ethanol drops were removed during a short spin. The pellet was air dried for 20 minutes and re-suspended in 50 µL nuclease free water. The samples were kept for 2 hours on ice and mixed regularly. Afterwards, the concentration of the samples was measured with the nanodrop to select the type of DNase treatment. RNase free water was used as blank and the program was adjusted at RNA:40. Routine DNase treatment was done for samples with a concentration below 200 ng/µL. Heavy DNase treatment was done for samples with a concentration below 500 ng/µL. Samples with a concentration above 500 ng/µL were diluted to a concentration of 500 ng/µL. The DNase treatment was done using the DNA-free kit (Ambion, Thermo-Fisher Scientific). The DNase treatment was done on ice.

Routine DNase treatment

10x DNaseI buffer (5 µL) and DNaseI (2U) (1 µL) was added to the samples. The solutions were mixed by shaking the tubes and incubated for 30 minutes at 37°C in the thermomixer. DNase Inactivation Reagent (0.1V) (5 µL) was added, the inactivation reagent was vortexed prior to use. The mixtures were vortexed and incubated for 2 minutes at room temperature. During this incubation the tubes were constantly shaken. The samples were centrifuged for 1.5 minutes at 10000g at 4°C and supernatant was transferred to a new tube.

Heavy DNase treatment

RNase free water was added to the samples until a total volume of 100 µL. 10x DNaseI buffer (10 µL) and DNaseI (2U) (1 µL) was added to the samples. The solutions were mixed and incubated for 30 minutes at 37°C in the thermomixer. DNaseI (2U) (1 µL) was added to the samples and they were again incubated for 30 minutes at 37°C. DNase Inactivation Reagent (0.2V) (20 µL) was added, the inactivation reagent was vortexed prior to use. The mixtures were vortexed and incubated for 2 minutes at room temperature. During this incubation the tubes were constantly shaken. The samples were centrifuged for 1.5 minutes at 10000g at 4°C and supernatant was transferred to a new tube.

After the DNase treatment, the concentration of the samples was measured with the nanodrop. The RNA was stored at -80°C until further analysis.

4.2. Library preparation

This step was done by nxtgnt (Ghent, Belgium).

Before the library construction, the samples were enriched in messenger RNA (mRNA). This was done by selecting for polyadenylated (poly-A) RNAs. The 3' poly-A tail of mRNA was targeted using poly-T oligos attached to a substrate, such as magnetic beads.
After the enrichment of the samples for mRNA, the RNA was converted into cDNA. To preserve strand information, a chemical label, such as deoxy-UTP (dUTP) was integrated during the synthesis of the second-strand cDNA. Therefore, the second-strand cDNA can easily be distinguished from the first strand during library preparation.
To analyze multiple samples in a single sequencing lane, the samples were multiplexed. Unique indices, barcodes, were added to each RNA-seq library (Kukurba & Montgomery, 2015).

4.3. Sequencing

Sequencing of the libraries was done using the Illumina HiSeq 3000 platform. Illumina sequencing was already described in 6.2 Illumina sequencing.

4.4. Bioinformatics analysis
4.4.1. Data preprocessing

First, the quality of the raw reads was evaluated with FastQC version 0.11.8 (Andrews, 2010). The raw reads were trimmed with Trimmomatic version 0.38 (Bolger et al., 2014). The adapter sequences used for Illumina sequencing were removed using the ILLUMINACLIP option of Trimmomatic. Reads were also truncated if the mean quality score was fewer than 20 in a window of 5 bases using the SLIDINGWINDOW option of Trimmomatic. In addition, reads with a length of fewer than 20 bases were removed.

4.4.2. Alignment to reference genome

The reads were mapped to the *Fragaria ananassa* reference genome (Hirakawa et al., 2014) with STAR version 2.6.1d (Dobin et al., 2013). STAR sequentially searches for a maximal

mappable prefix (MMP) for every read. A MMP is the longest sequence in a read that exactly matches one or more sequences of the reference genome. Alignments of the entire read are built by stitching together all seeds that were aligned to the reference genome. A seed is a part of the read that is mapped. The seeds are first clustered together by proximity to a selected set of anchor seeds. All seeds that map within a predefined genomic window around the anchors are stitched together. A local alignment scoring scheme is used to score each stitched combination. The combination with the highest score is selected as the best alignment of a read (Dobin et al., 2013). The STAR algorithm is graphically shown in Figure 12 ("Alignment with STAR | Introduction to RNA-Seq using high-performance computing").
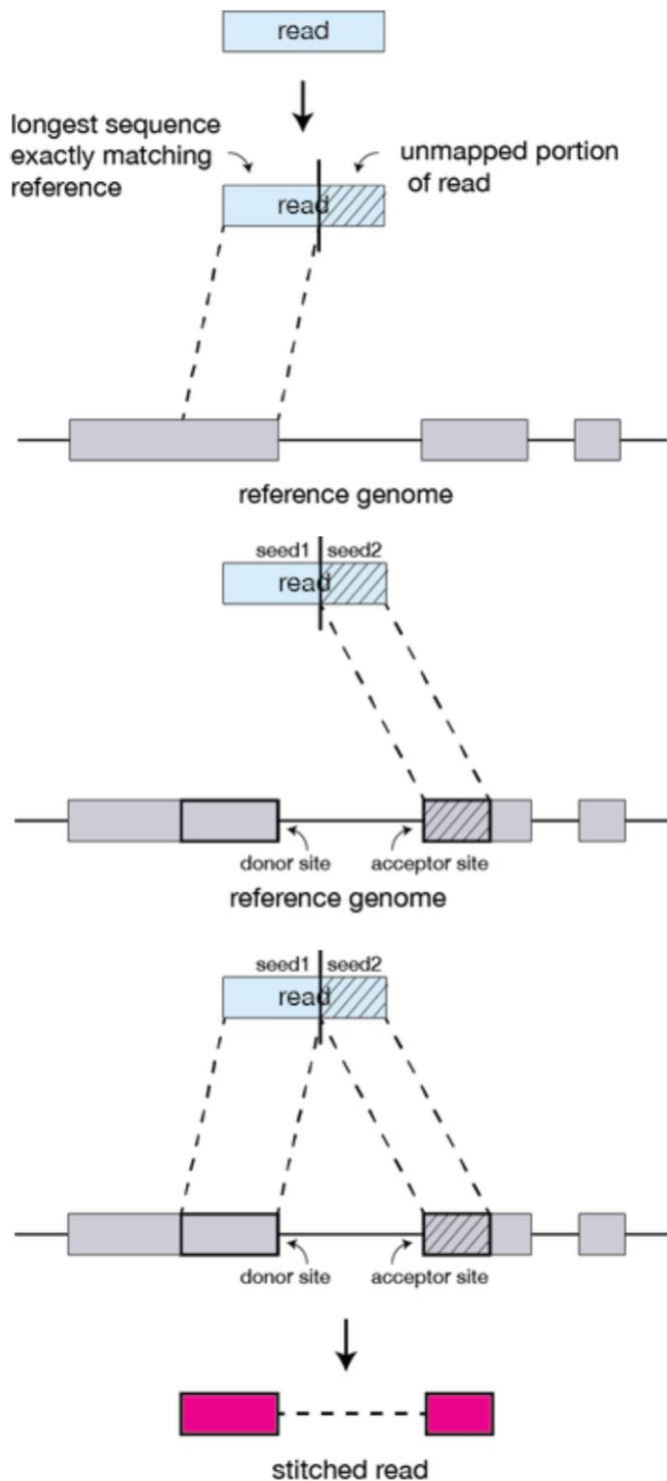

Figure 12: Star alignment algorithm ("Alignment with STAR | Introduction to RNA-Seq using high-performance computing," n.d.).

4.4.3. Building a count table

Using the alignments obtained by STAR and a file containing descriptions of the genes present in the reference genome, a count table was built. This was done in R version 3.4.3 using the function summarizeOverlaps (R package GenomicAlignments, Lawrence et al., 2013). Only reads that overlap any part of exactly one gene are counted. Reads that overlap multiple genes are removed. The resulting count table is then used for statistical analysis of differentially expressed genes.

4.4.4. Exploratory analysis

To look at the relation between the samples, a PCA plot was made using the plotPCA function (R package DESeq2, Love, Huber, & Anders, 2014). Before this plot was made, the data was first log transformed using the rlog function. This was done to stabilize the variance present in the data so that the variance is not dependent on the mean (Klaus & Huber, 2016).
In addition, a multidimensional scaling (MDS) plot was made using the plotMDS function (R package Limma, Ritchie et al., 2015). This plot is similar to a PCA plot, but it is based on dissimilarity matrices. Distances in the MDS plot represent leading fold changes, the root-mean-square average of the log fold changes for the genes that best differentiate each pair of samples (Ritchie et al., 2015).

4.4.5. Statistical analysis

The statistical analysis was done in R version 3.4.3 using the EdgeR workflow. Before the statistical analysis, the data was split in 2 groups. The first group only contained samples from non-infected plants, but from both weeks 8 and 9 of plant growth. The second group contained all samples from week 9 of plant growth, both from infected and non-infected plants. The statistical analysis was done for both groups separately.
The data was first normalized using TMM normalization to account for differences in sequencing depth (Robinson & Oshlack, 2010). For the first group a negative binomial (NB) model with main effects for treatment and time, as well as treatment $\times$ time interaction, was used. For the second group a NB model with main effects for treatment and infection, as well as treatment $\times$ infection interaction was used. Overdispersion parameters of the NB model were estimated using Empirical Bayes estimation adopted by the quantile-adjusted conditional maximum likelihood (qCLM) method. Therefore, the overdispersion parameter of a single gene was shrunken toward the common dispersion across all genes. Likelihood ratio tests were done on the appropriate contrasts of the model parameters to asses differences in bacterial microbiome composition. Benjamini-Hochberg False Discovery Rate procedure was used to correct for multiple testing (Robinson et al., 2010).
Gene annotation of the significantly differentially expressed genes was already done by Hirakawa et al. (2014). Similarity searches in the Interpro database and NCBI's non-redundant protein sequence database were done by InterProScan and BLASTX, respectively. BLAST searches were done for the sequences in the *Fragaria ananassa* reference genome against the *Fragaria vesca* (v1.1) genome (Hirakawa et al., 2014).

5. Real time quantitative polymerase chain reaction (RT-qPCR)
5.1. RNA extraction

RNA extraction was done based on the method of Luypaert et al. (2017) with some minor modifications. This protocol was already described in 4.1 RNA extraction. Samples from weeks 9 and 10 were analyzed using RT-qPCR. Samples from both infected and non-infected plants were used. Per treatment and timepoint 3 biological replicates were used.

5.2. cDNA synthesis

For first strand cDNA synthesis, the Tetro cDNA Synthesis kit (Bioline) was used. First, all RNA samples were diluted to obtain an equalized mass of 1.5 µg in a total volume of 12 µL.
The mastermix for cDNA synthesis was made using the following components: 12 µL Total RNA (mass 1.5 µg), 1 µL Oligo (dT) primer 10 µM, 1 µL 10 mM dNTP mix, 4 µL 5x RT buffer, 1 µL RiboSafe RNase Inhibitor and 1 µL Tetro Reverse Transciptase (200U/µL). This mix was prepared on ice in a RNase free tube.

The samples were then incubated for 2 hours at 45°C. The reaction was terminated by incubating for 5 minutes on 85°C and the samples were cooled on ice. DEPC treated water (80 µL) was added to the samples to adjust the volume of the cDNA to 100 µL. The quality of the cDNA was checked by reverse transcription PCR (RT-PCR), using one of the reference genes. The mastermix for RT-PCR consisted of the following components: 1 µL cDNA, 1 µL dNTPs (5 mM, Invitrogen), 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 2 µL 10x KEY buffer + 15 mM $MgCl_2$ (VWR), 0.4 µL 25 mM $MgCl_2$ (VWR), 0.2 µL Taq polymerase (5 U/µL, VWR) and 13.4 µL DEPC treated water. The program for RT-PCR followed the scheme listed in Table 4. RT-PCR was done using a T100 Thermal Cycler (Bio-Rad).

Table 4: RT-PCR program.

Temperature	Time	
95°C	5 min	
95°C	45 sec	
55°C	45 sec	35x
72°C	30 sec	
72°C	5 min	

The amplification was checked by gel electrophoresis. For this, 1.5% agarose (in 0.5 TAE buffer) gel was used. The sample (10 µL) was mixed with dye and loaded on the gel. The gel electrophoresis was done at 130 V for 20 minutes. The gel was placed in an ethidium bromide solution for 20 minutes. cDNA was visualized by placing the gel under UV-light (Gel Doc XR+ System).

5.3. RT-qPCR

In total, 10 genes were analyzed with RT-qPCR. The used reference genes are listed in Table 5. The target genes are listed in Table 6 and were previously reported as defense related genes in strawberry. The β-1,3-glucanase gene (FaBglu) has been grouped in the PR2 family of PR proteins. Calcium-dependent protein kinase (FaCDPK) has been reported to be involved in immune and stress signaling (Landi, Feliziani, & Romanazzi, 2014). The class II chitinase genes FaChi2-1 and FaChi2-2 have been grouped in the PR3 family of PR proteins (Khan & Shih, 2004). The phenylalanine ammonia lyase gene (FaPAL) is involved in the phenylpropanoid pathway. The PR1 gene (FaPR1) encodes for a PR protein of the PR1 family (Pombo, Rosli, Martínez, & Civello, 2011). The WRKY75 like transcription factor gene (FaWRKY1) has been reported to act as a positive regulator of the plant defense response (Amil-Ruiz et al., 2013).

Table 5: Reference genes used in RT-qPCR.

Gene	Gene description	Primer sequence (Forward (F) /Reverse (R))	Literature reference
FaRIB413	RNA interspacer (16S-23S) region	**F** ACCGTTGATTCGCA CAATTGGTCATCG **R** TACTGCGGGTCGGCAATCGGACG	(Amil-Ruiz et al., 2013)

FaACTIN	Actin	**F** GGGCCAGAAAGATGCTTATGTCGG **R** GGGCAACACGAAGCTCATTGTAGAAG	(Amil-Ruiz et al., 2013)
FaEF1a	Elongation factor 1-alpha	**F** TGGATTTGAGGGTGACAACATGA **R** GTATACATCCTGAAGTGGTAGACGGAGG	(Amil-Ruiz et al., 2013)

Table 6: Target genes used in RT-qPCR.

Gene	Gene description	Primer sequence (Forward (F) /Reverse (R))	Literature reference
FaBglu	β-1,3-Glucanase	**F** TATGGACGAAACGGTGACAA **R** AGGGTTGCACATTTTTCTGG	(Landi et al., 2014)
FaCDPK	Calcium-dependent protein kinase	**F** TCCGTTTTGAAGAACCCAAC **R** CCGTCCTCAGTTTCTGCTTC	(Landi et al., 2014)
FAChi2-1	Chitinase	**F** TCGGCACCACCGGAAGT **R** TGGGAGATCTGAGCAAGAAATG	(Khan & Shih, 2004)
FaChi2-2	Chitinase	**F** GGTCAAACCTCTCACGAAACCA **R** ATCCCCAAGCATAAGGACCAT	(Khan & Shih, 2004)
FaPAL	Phenylalanine ammonia lyase	**F** GATTTGAGGCATTTGGAGGA **R** CTTGCCTTAGCCTTTGCATC	(Landi et al., 2014)
FaPR1	Pathogenesis-related protein 1	**F** ACATGGATGCCAATCTAGC **R** CCACAGGTTCACAGCAGATG	(Pombo et al., 2011)
FaWRKY1	WRKY75 like transcription factor	**F** ACAGCATAAGATTAGGGATGAAGAAGGGAG **R** GCTTCTTCACATTGCAACCCTGATGCGTG	(Amil-Ruiz et al., 2013)

For setting up a RT-qPCR run, a mastermix and a samplemix were made for each combination of sample and gene. The mastermix consisted of the following components: 10 µL 2x sensimix SYBR No-ROX kit (Bioline), 1 µL 10 µM forward primer and 1 µL 10 µM reverse primer; The samplemix consisted of the following components: 1 µL cDNA and 7 µL water. The mastermix and samplemix were mixed together in 96 well plates. Two technical replicates were used for every sample. The program for RT-qPCR followed the scheme listed in Table 7. RT-qPCR was done using CFX Connect (Bio-Rad).

Table 7: RT-qPCR program.

Temperature	Time	
95°C	10 min	
95°C	25 sec	
58°C	25 sec	45x
72°C	20 sec	

For the melt curve analysis the temperature was raised from 65°C to 95°C in steps of 0.5°C. The fluorescence intensity was measured at every temperature. The average quantification cycle (Cq) value and melt curve analysis was determined with CFX Manager 3.1 (Bio-Rad). The Cq values were used for further analysis. The melt curves were evaluated per gene to check if the same fragment was amplified in every sample.

5.4. Statistical analysis

To visualize the relative gene expression of the seven defense genes a bar plot was made. Therefore, the relative gene expression was calculated using the following formula (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007):

$$\text{Relative gene expression} = \frac{\left(E_{\text{target}}\right)^{\Delta Cq\ \text{target}}}{\text{GeoMean}\left[\left(E_{\text{ref}}\right)^{\Delta Cq\ \text{ref}}\right]}$$

With E_{target}: Amplification efficiency for the amplification of the target gene

ΔCq target: Calibrator Cq – target Cq

Calibrator Cq: Average Cq value of control samples (in our case: only peat substrate)

Target Cq: Cq value of treated sample

GeoMean: Geometrical mean

E_{ref}: amplification efficiency for the amplification of the reference gene

ΔCq ref: Calibrator Cq – ref Cq

Ref Cq: Cq value of reference gene

For amplification efficiencies the theoretical value of 2 was assumed.

The statistical analysis was done with REST version 2009 (M. W. Pfaffl, Horgan, & Dempfle, 2002). The Cq for every combination of sample and gene was analyzed with REST. This software uses a mathematical model to analyze the gene expression data. The model uses the expression of reference genes to normalize expression levels of target genes. The geometric mean of all reference gene concentrations is calculated. The concentration of the target gene is then divided by this geometric mean to obtain the relative expression of that target gene. An average expression value indicating gene regulation is calculated for every target gene. However, REST 2009 also reports 95% confidence intervals for the expression levels. Bootstrapping techniques are used to calculate 95% confidence intervals. A pair wise fixed reallocation randomization test calculates a p-value, which is a measure of whether the result is statistically significant. This test uses the following randomization scenario: If any observed difference between samples and controls is due only to chance, then values could be randomly switched between the 2 groups and no greater difference than the initial observed difference would be seen (M. Pfaffl & QIAGEN, 2009).

Results

1. Introduction

First, some plant physiological parameters will be discussed. Second, we will focus on changes in the bacterial community of the strawberry plant rhizosphere and bulk soil. Third, the strawberry plant gene expression is studied by RNA sequencing and RT-qPCR. In this part, we mainly focus on plant defense genes.

For each part, we will study the following: (1) Is there an effect of the treatment (biochar or chitin amendment); (2) Is there an effect of time. For the bacterial community analysis, we will also study: (3) Are there differences between the rhizosphere and bulk soil.

2. Preliminary data

These data were obtained before the start of this thesis, but were analyzed in this thesis. Plant fresh and dry weight, root weight, fruit yield and infection rate of the leaves were analyzed with generalized linear models for every timepoint. The data of these physiological parameters is shown in Appendix. Plant fresh weight of non-infected and infected plants is shown in Figure 22 (non-infected plants) and Figure 23 (infected plants), respectively. Plant dry weight of non-infected and infected plants is shown in Figure 24 (non-infected plants) and Figure 25 (infected plants), respectively. It seems that plants grown in biochar amended peat substrate have a smaller fresh and dry weight. Plants grown in chitin amended peat substrate seem to have a bigger fresh and dry weight. However, none of these differences are statistically significant. When comparing non-infected plants with infected plants, it seems that both the fresh and dry weight is slightly reduced in the biochar treatment by the infection. In the control and chitin treatments, the fresh and dry weight does not seem to change much upon infection. Root weight of non-infected and infected plants is shown in Figure 26 (non-infected plants) and Figure 27 (infected plants), respectively. The root weight seems to be higher in the biochar treatment compared to the control treatment (peat substrate, PS), but no significant differences are found. Chitin lowers the root weight. From week 9 of plant growth onwards, the difference is even significant for both non-infected and infected plants. The root weight is not changed much in the control and chitin treatments by the infection. However, in the biochar treatment the root weight seems to be reduced upon infection. The fruit yield of non-infected and infected plants is shown in Figure 28 (non-infected plants) and Figure 29 (infected plants), respectively. Biochar and chitin reduce the number of fruits, but non-significant. The fruit yield is not changed upon infection in the control and chitin treatments. In the biochar treatment, the fruit yield seems to be increased slightly. The infection rate of the leaves is shown in Figure 30. The infection rate in the biochar treatment is similar to the infection rate in the control treatment. However, the infection rate is reduced, although not significantly, upon chitin amendment.

3. Effect of biochar and chitin on the bacterial community
3.1. Community complexity

To study the effect of biochar and chitin amendment on the bacterial microbiome of strawberry, amplicon sequencing of the 16S rRNA gene was done for both rhizosphere and bulk soil samples. To study differences in community richness, the richness was plotted over time (Figure 13). From this figure it can be seen that at week 1 the richness in the rhizosphere is higher in the biochar and chitin treatments compared to the control treatment. The difference between biochar and control is even significant. After week 1, the richness decreases in the biochar and chitin treatments. However, from week 7 of plant growth the richness is higher in the biochar and chitin treatments compared to the control treatment. For biochar, the difference in richness is significant. At the end of plant growth the richness is significantly higher in both the biochar and chitin treatments compared to the control treatment. It can also be concluded that the richness changes over time. In the control treatment the richness increases until week

5. After week 5 the richness decreases and from week 7 onwards increases again slightly. In the biochar and chitin treatments the richness decreases first. In the biochar treatment the richness increases from week 4 onwards. In the chitin treatment the richness increases from week 7 onwards.
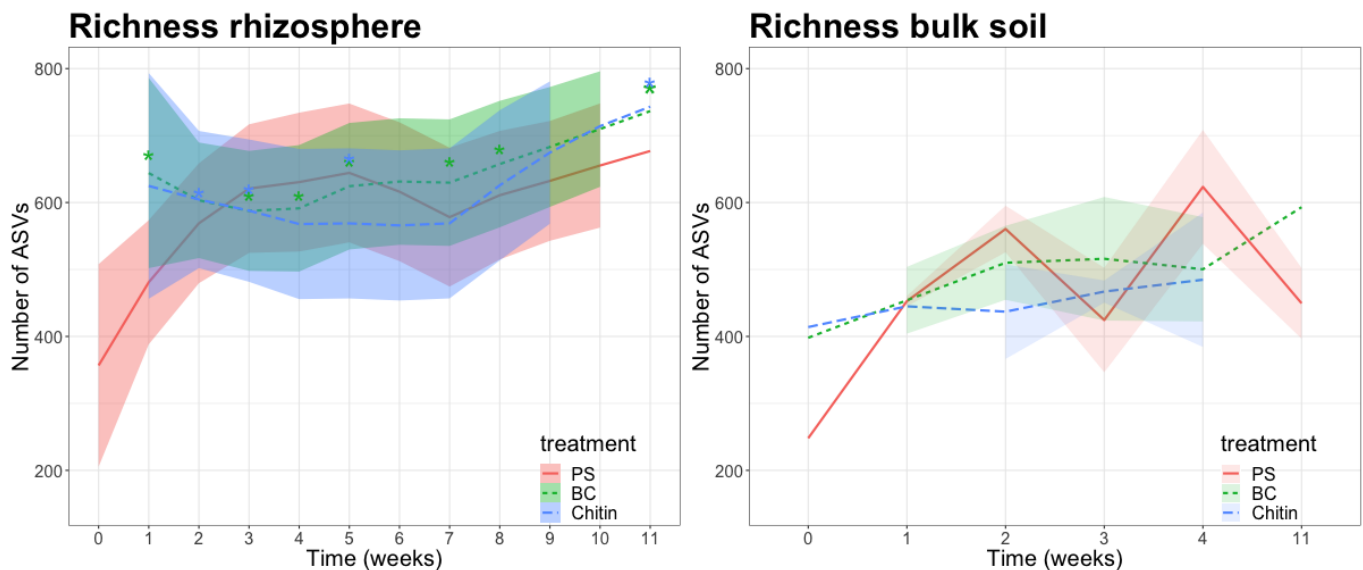

Figure 13: Line graph of the bacterial richness in strawberry plants rhizosphere (left) and bulk soil (right). 95% confidence intervals are shown. Statistical significant differences compared to the control treatment (PS) are indicated with an asterisk. PS = peat substrate, BC = biochar, ASV = amplicon sequence variant.

3.2. Community composition

To look if there is an effect of (1) treatment; (2) time and (3) compartment (bulk soil versus rhizosphere) on the bacterial microbiome composition, a PERMANOVA test was done. From this test it can be concluded that both biochar and chitin change the plant bacterial microbiome composition significantly ($p < 0.05$). In addition, the composition changes over time, there is thus also an effect of time ($p = 0.001$). The bacterial rhizosphere microbiome is also different from the microbiome found in the bulk soil ($p = 0.001$).

These community differences were visualized using PCoA plots (Figure 14). The significant effect of **biochar amendment** on the bacterial communities present in the bulk soil and rhizosphere of the strawberry plants is not clear within the plot. There is only a minor distinct clustering of the bacterial communities of the samples in the biochar treatment (Figure 14A). From Figure 14C it can be seen that there is a clear time effect on the rhizosphere microbiome. The bacterial communities change over time, both in the control and biochar treatments. The separate clustering between the bacterial communities present in the rhizosphere and those in the bulk soil is especially noticed in the first timepoints, followed by a convergence of the communities in the later timepoints (Figure 14A). The effect of **chitin amendment** on the bacterial communities is more visible. The bacterial communities of the samples in the chitin treatment can be separated from those in the control treatment (Figure 14B). There is again a time effect visible on the bacterial rhizosphere communities (Figure 14D). Also here differences between the bacterial communities present in the rhizosphere and those in the bulk soil are especially noticed within the first timepoints (Figure 14B).
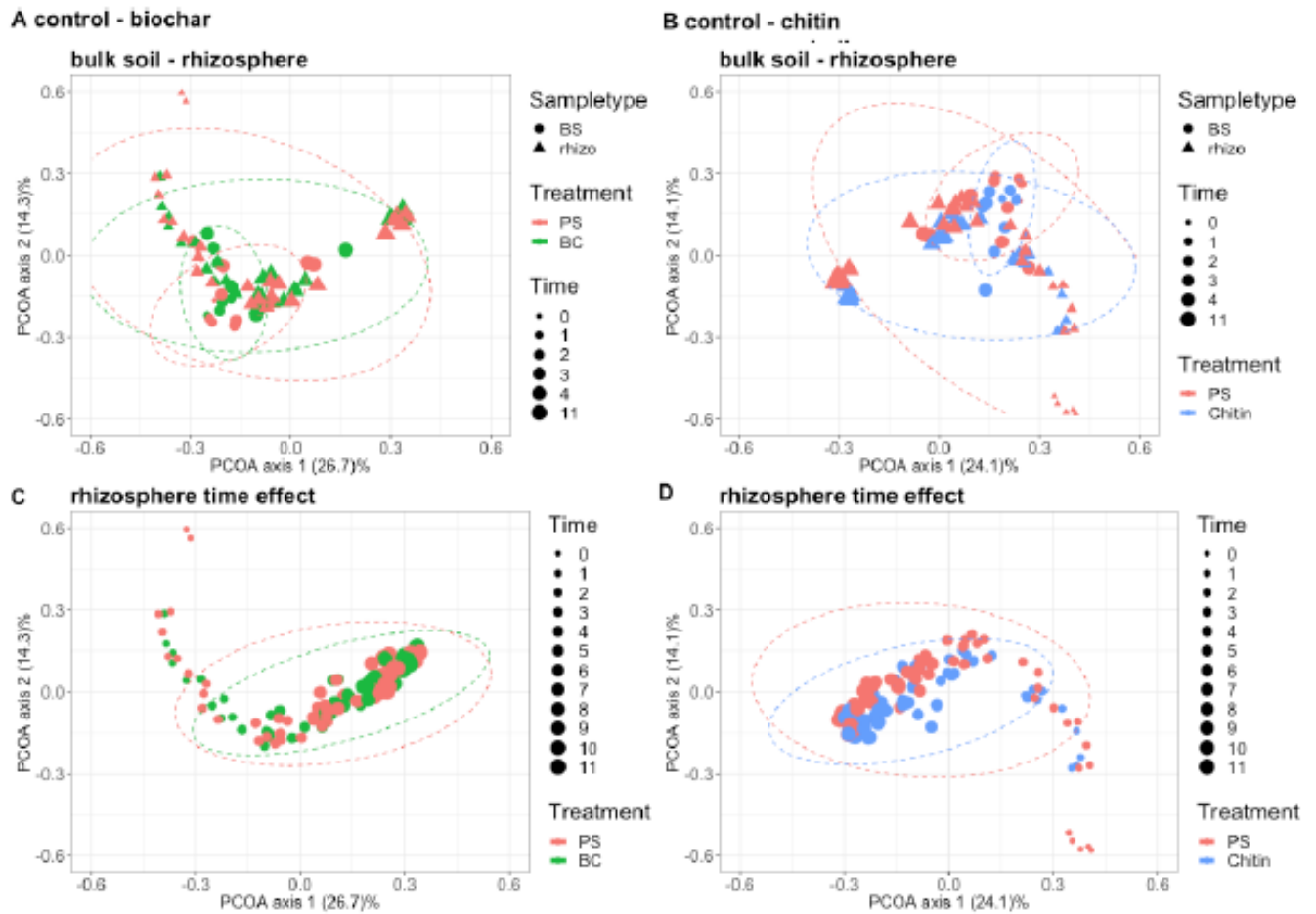
Figure 14: PCoA plots visualizing the bacterial communities present in the strawberry plant rhizosphere and bulk soil. Figures A and C show the bacterial communities present in the control (peat substrate, PS) and biochar (BC) treatments. Figures B and D show the bacterial communities present in the control and chitin treatments. 95% confidence intervals are shown as ellipses. BS = bulk soil, rhizo = rhizosphere.

To indicate which bacterial groups are responsible for effects of treatments, shifts in time and bulk soil/rhizosphere differences, the bacterial communities were first illustrated by bar plots. Average relative abundances were calculated for every treatment, timepoint and taxonomical group and the data was either grouped by phyla (Figure 15), or families (Figure 16 and Figure 17). The starting point (time 0) is actually the same for all treatments, as this represents the plant's rhizosphere before planting in the peat substrate growing media. The bulk soil samples of week 11 of the chitin treatment were removed from the analysis, because the total number of reads in these samples was too low. For both the rhizosphere and the bulk soil, there is no distinct treatment effect. However, there is a time effect visible, as was also seen in Figure 14. The first 4 weeks of plant growth, the bacterial communities of the rhizosphere are more different from those in the bulk soil. But at the end of plant growth, at week 11, both communities are more alike to each other, which was also illustrated by the PCoA plot.
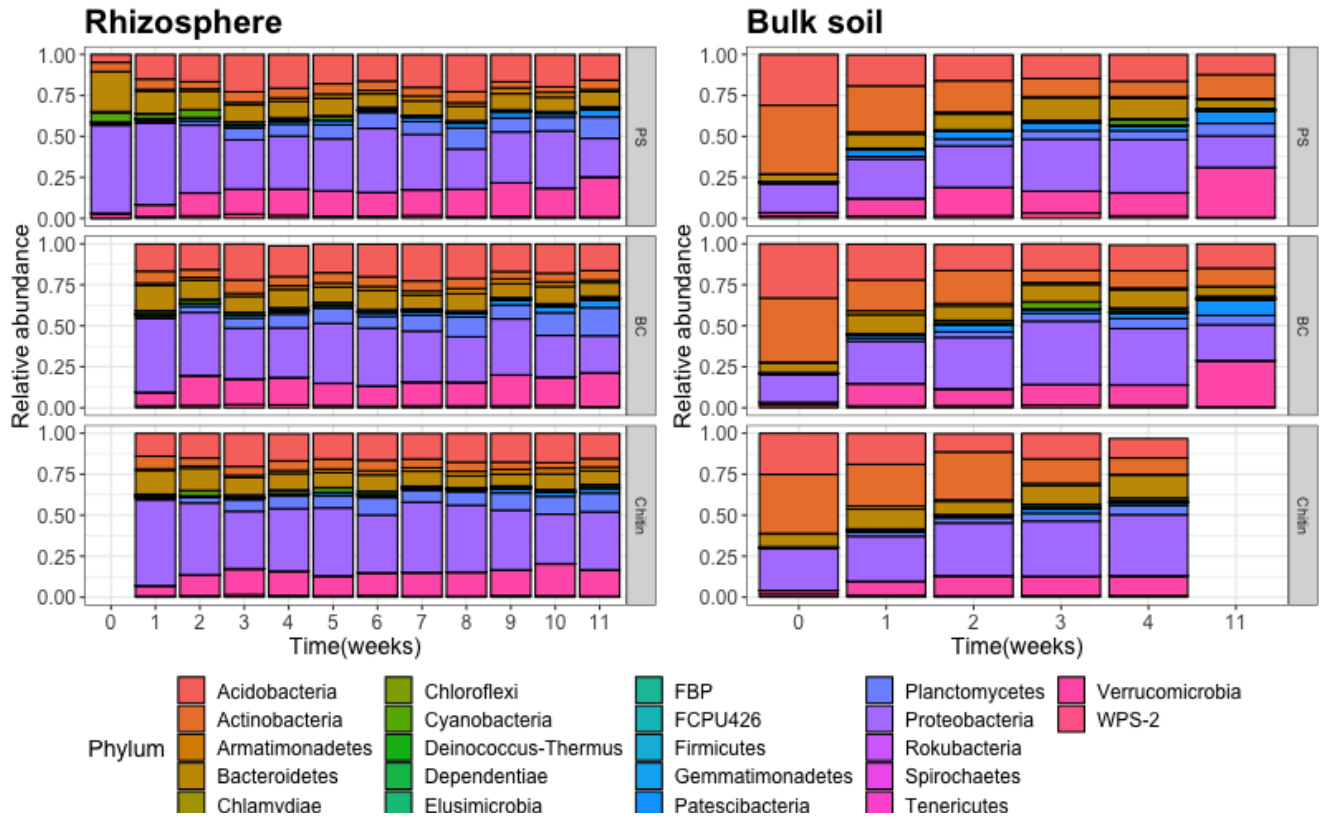
Figure 15: Bar plot of the bacterial phyla present in the rhizosphere (left) and bulk soil (right) of strawberry plants. The starting point (time 0) is the same for all treatments. This timepoint represents the rhizosphere of the plant before planting in the peat substrate growing media. Timepoint 11 of the chitin treatment is removed because the total number of reads for this timepoint was too low.
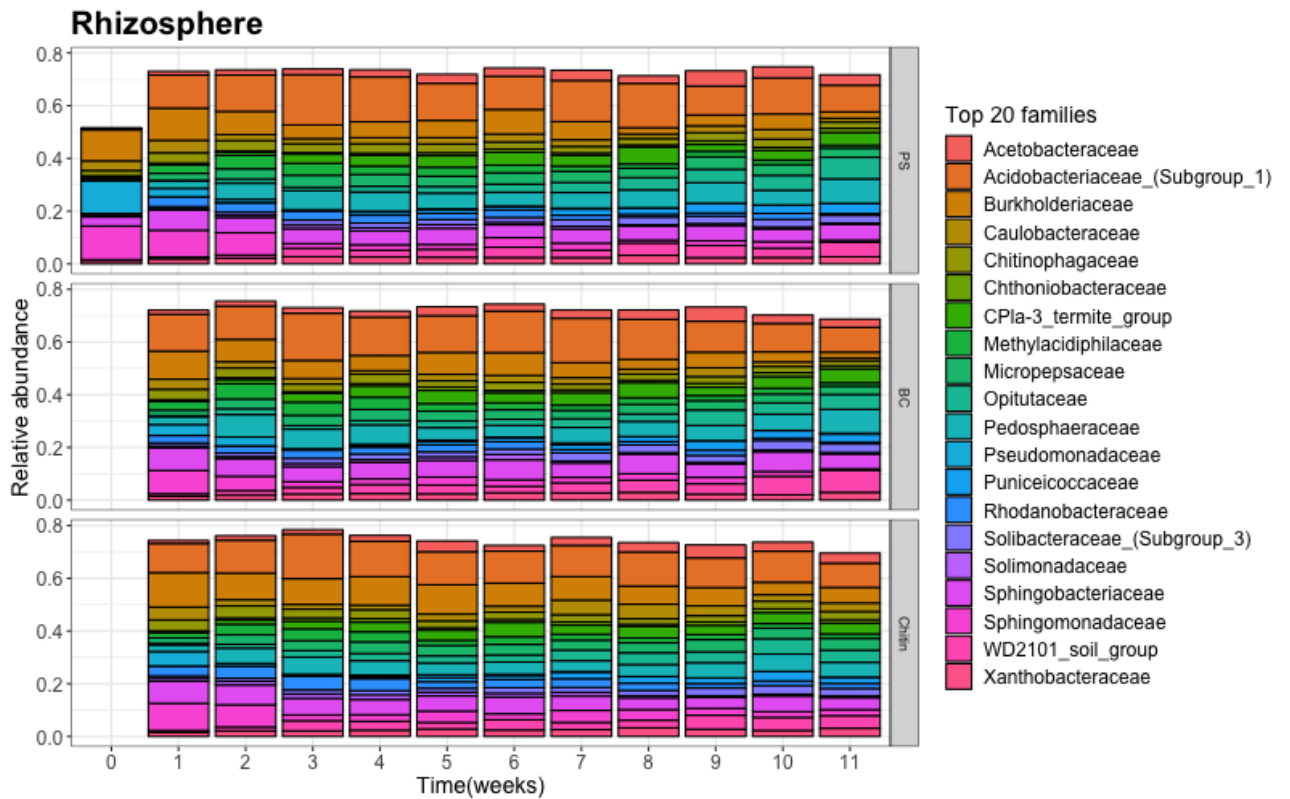

Figure 16: Bar plot of the 20 most abundant bacterial families present in the rhizosphere of strawberry plants. The starting point (time 0) is the same for all treatments. This timepoint represents the rhizosphere of the plant before planting in the peat substrate growing media.
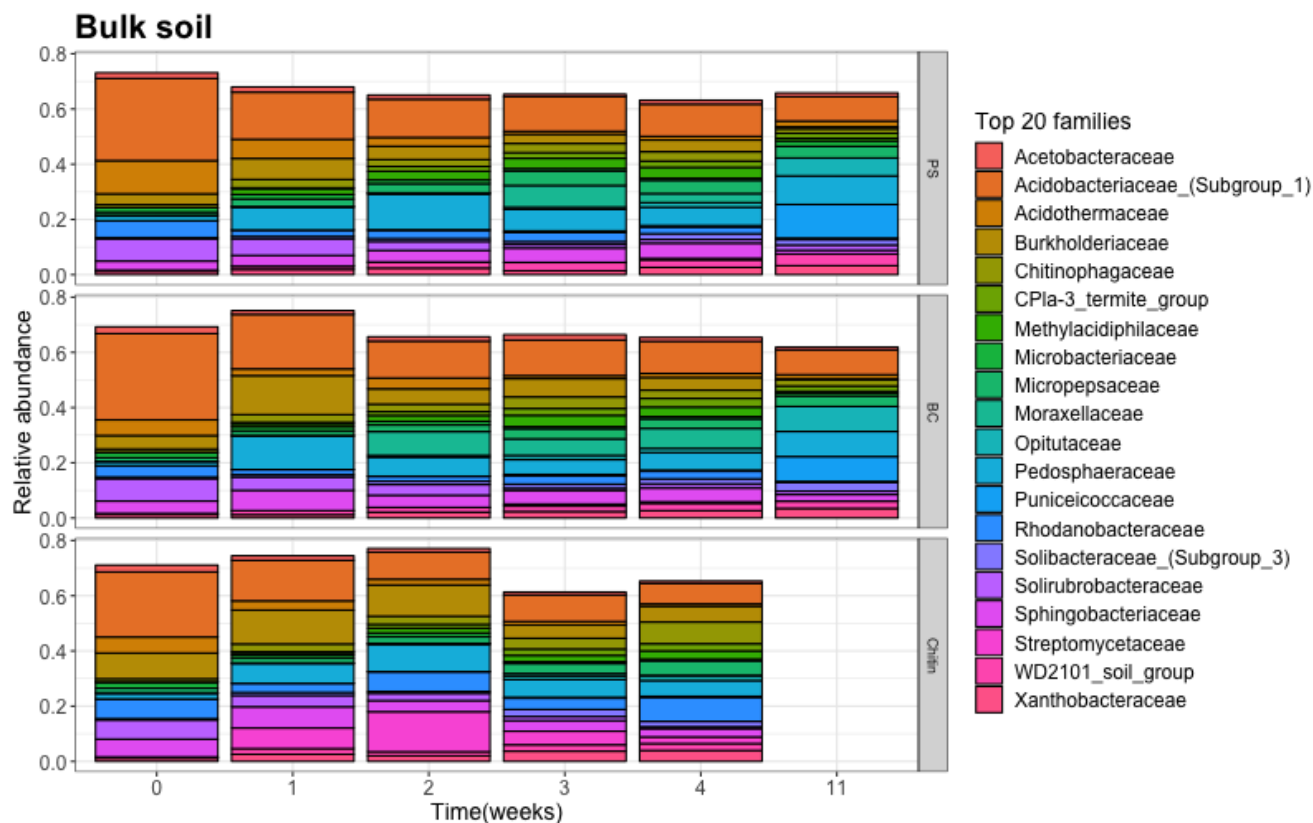
Figure 17: Bar plot of the 20 most abundant bacterial families present in the bulk soil of strawberry plants. Timepoint 11 of the chitin treatment is removed because the total number of reads for this timepoint was too low.

To look if there are significant differences in the bacterial communities between the different treatments, a statistical analysis was done using the edgeR pipeline. We therefore focused on the rhizosphere community by which the data was grouped by genus. Only the data from week 8 to week 11 was tested for statistically significant differences. It has already been shown for biochar amendment that most changes in bacterial community composition occur later in plant growth, from week 6 onwards (De Tender et al., 2016). From Figure 13 it can also be seen that only from week 7 onwards the bacterial richness is higher in the biochar and chitin treatments compared to the control treatment. From the statistical analysis it can be concluded that **biochar amendment** has few statistically significant effects on the bacterial community composition. Only at week 11 there were statistically significant differences between the genera found in the control and biochar treatment. Three genera, *Taibaiella* (BC: 4.8 ± 2.9, PS: 2.0 ± 1.3), *Pseudomonas* (BC: 172.6 ± 167.9, PS: 10.0 ± 4.5) and *Micropepsis* (BC: 8.0 ± 5.0, PS: 4.4 ± 4.4), were more abundant in the biochar treatment compared to the control treatment. **Chitin amendment** has a larger effect on the bacterial community composition. Here, significant differences were found at week 10 and 11. The genera with a statistically significant difference in abundance between the control and chitin treatment are given in Table 8.

Table 8: Genera differentially abundant between control and chitin treatment. Statistically significant differences are shown in bold.

Genus	WEEK 10		WEEK 11	
	PS	**Chitin**	**PS**	**Chitin**
Streptomyces	**80.4 ± 34.1**	**142.0 ± 77.0**	**0.0 ± 0.0**	**143.6 ± 49.3**
Phenylobacterium	**384.8 ± 100.0**	**326.8 ± 95.7**	**44.6 ± 12.0**	**362.2 ± 68.2**
Streptacidiphilus	**175.8 ± 73.6**	**214.8 ± 97.2**	**0.0 ± 0.0**	**299.2 ± 46.7**
Leptothrix	**375.6 ± 110.8**	**349.2 ± 142.9**	**24.2 ± 8.7**	**371.4 ± 25.4**
Actinospica	**44.0 ± 18.3**	**57.6 ± 23.0**	**2.0 ± 1.2**	**68.4 ± 8.0**
Acidibacter	**211.4 ± 32.2**	**227.2 ± 60.3**	51.8 ± 7.4	205.6 ± 22.2

Bradyrhizobium	**63.6 ± 31.7**	**114.8 ± 65.7**	17.4 ± 7.3	150.2 ± 40.6
Prosthecobacter	**90.6 ± 39.0**	**88.6 ± 26.3**	33.8 ± 7.8	7.2 ± 2.2
Methylovirgula	**84.0 ± 25.2**	**73.8 ± 8.8**	85.6 ± 12.6	77.6 ± 18.0
Burkholderia-Caballeronia-Paraburkholderia	1987.8 ± 482.5	1579.4 ± 295.2	582.0 ± 181.2	**1645.2 ± 343.8**
Aliidongia	102.4 ± 32.1	116.2 ± 49.2	4.2 ± 4.2	**93.6 ± 23.6**
Rhodanobacter	1158.2 ± 163.3	947.8 ± 202.2	194.6 ± 29.1	**678.2 ± 70.8**
Pseudomonas	29.4 ± 8.5	37.6 ± 31.3	10.0 ± 4.5	**9.6 ± 8.4**
Telmatospirillum	323.6 ± 96.5	243.6 ± 106	55.0 ± 25.5	**160.4 ± 16.1**
Micropepsis	54.6 ± 13.0	48.8 ± 13.6	4.4 ± 4.4	**49.8 ± 20.0**
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	60.0 ±14.0	53.6 ± 22.8	7.6 ± 4.0	**56.2 ± 15.8**
Caulobacter	45.0 ± 9.1	32.4 ± 9.8	3.6 ± 2.2	**18.0 ± 2.2**
Dyella	250.6 ± 47.5	194.4 ± 52.3	29.2 ± 10.4	**126.8 ± 16.2**
Hyphomicrobium	15.6 ± 6.1	3.0 ± 3.0	0.0 ± 0.0	**21.4 ± 4.3**
Novosphingobium	1291.2 ± 208.9	1036.2 ± 193.6	221.4 ± 66.1	**768.6 ± 139.6**
Occallatibacter	3859.8 ± 642.0	3421.0 ± 256.3	1951.0 ± 160.9	**1935.4 ± 155.2**
Galbitalea	171.6 ± 38.4	159.2 ± 9.4	135.8 ± 16.8	**118.2 ± 13.2**
Candidatus_Solibacter	1246.6 ± 157.1	1408.2 ± 164.8	773.0 ± 98.9	**719.4 ± 54.4**

4. Effect of biochar and chitin on the plant's gene expression
4.1. Overall gene expression by RNA sequencing

Samples from week 8 and 9 of plant growth, just before and one week after infection with *B. cinerea*, were used for RNA sequencing to study the effect of biochar and chitin on strawberry leaf gene expression. In addition, the effect of biochar and chitin on the strawberry defense response against *B. cinerea* was investigated. To study both effects, the data was split in 2 groups. To look at the effect of biochar and chitin on basal[2] gene expression, strawberry plants sampled at week 8 and 9 that were not infected were investigated (group 1). To look at the effect on the strawberry defense response against *B. cinerea* in combination with biochar or chitin addition, strawberry plants sampled at week 9 that were either infected or not infected were studied (group 2). The statistical analysis was done using the edgeR pipeline.

The results from the statistical analysis on the data from group 1 is given in Table 9. From this table it can be concluded that both biochar and chitin do not affect the basal gene expression of strawberry leaves. Only at week 9 there is 1 gene significantly upregulated, with a log2 fold change of 5, in the chitin treatment. This gene has been annotated as an auxin-induced protein (Hirakawa et al., 2014).

Table 9: Statistically significant differentially expressed genes between the control (PS) and biochar (BC) treatments and between the control and chitin treatments at week 8 (t8) and week 9 (t9).

upregulated/ downregulated	t8PS – t8BC	t8PS – t8Chitin	t9PS – t9BC	t9PS – t9Chitin
down	0	0	0	0
up	0	0	0	1

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[2] The term basal gene expression is used to indicate the gene expression in non-infected plants

The results from the statistical analysis on the data from group 2 is given in Table 10. Biochar amendment does not seem to have a big effect on the strawberry defense response against *B. cinerea*. Only a few genes are significantly differentially expressed. The effect of chitin amendment is stronger: in total 262 genes were upregulated and 219 genes downregulated upon chitin amendment and infection. Whereas in the control treatment, only 46 genes were upregulated and 86 genes downregulated upon infection. When comparing the gene expression in infected plants, 55 genes were upregulated and 7 genes downregulated upon chitin amendment. Also remarkably are the high log2 fold changes observed in all treatments. Given the high number of differentially expressed genes in the chitin treatment, only the gene annotation of the 20 most up- and downregulated genes was investigated in detail. The gene annotations of the differentially expressed genes are given in Table 12, Table 13, Table 14 and Table 15.

Table 10: Statistically significant differentially expressed genes between the control (PS) and biochar (BC) treatments and between the control and chitin treatments at week 9 (t9).

upregulated/ downregulated	t9PS- t9PS infection	t9PS – t9BC infection	t9PS – t9Chitin infection	t9PS infection – t9BC infection	t9PS infection – t9Chitin infection
down	86	1	219	0	7
up	46	14	262	2	55

Table 11: Gene annotation of significantly differentially expressed genes between control treatment and control with infection treatment at week 9 (t9PS-t9PS infection).

Gene ID	Gene annotation (Hirakawa et al., 2014)	Log2 fold change
FANhyb_rscf00005726.1.g00001.1	Alpha-amylase/subtilisin inhibitor-like	-7,27
FANhyb_rscf00002447.1.g00002.1	Hypothetical protein PRUPE_ppa018413mg	-4.45
FANhyb_icon00035972_a.1.g00001.1	Unknown protein	-4.32
FANhyb_rscf00000003.1.g00046.1	Probable E3 ubiquitin-protein ligase HERC3-like	-4.27
FANhyb_rscf00003859.1.g00001.1	Galactinol synthase 2-like	-4.22
FANhyb_icon00027359_a.1.g00001.1	Uncharacterized protein LOC101299163	-3.83
FANhyb_rscf00000569.1.g00007.1	GDSL esterase/lipase At1g33811-like	-3.69
FANhyb_rscf00000030.1.g00022.1	Cytochrome P450 94A1-like	-3.58
FANhyb_rscf00007376.1.g00001.1	Isoflavone 2'-hydroxylase-like	-3.57
FANhyb_icon20235835_s.1.g00001.1	Unknown protein	-3.54
FANhyb_rscf00000036.1.g00021.1	Universal stress protein A-like protein-like isoform 2	3.64
FANhyb_icon00008755_a.1.g00001.1	Uncharacterized protein LOC101310098	3.66
FANhyb_rscf00000012.1.g00021.1	Uncharacterized protein LOC101305153	3.77
FANhyb_rscf00000050.1.g00021.1	Sugar transporter ERD6-like 16-like	3.95
FANhyb_icon19730437_s.1.g00001.1	MATE efflux family protein 6-like	4.07
FANhyb_rscf00005149.1.g00001.1	Hypothetical protein VITISV_015109	4.65
FANhyb_rscf00000476.1.g00003.1	Anthranilate synthase component I-1, chloroplastic-like	4.94
FANhyb_rscf00000476.1.g00003.1	Anthranilate synthase component I-1, chloroplastic-like	4.94

| FANhyb_icon00015158_a.1.g00001.1 | (-)-germacrene D synthase-like | 5.86 |

Table 12: Gene annotation of significantly differentially expressed genes between control treatment and biochar with infection treatment at week 9 (t9PS-t9BC infection).

Gene ID	Gene annotation (Hirakawa et al., 2014)	Log2 fold change
FANhyb_rscf00000642.1.g00003.1	Polyphenol oxidase	-8.2
FANhyb_rscf00003700.1.g00001.1	Uncharacterized N-acetyltransferase p20-like	1.3
FANhyb_rscf00001060.1.g00002.1	Chaperone protein DnaJ-like	1.6
FANhyb_rscf00000005.1.g00035.1	Sugar isomerase (SIS) family protein	1.7
FANhyb_rscf00001389.1.g00001.1	Ribonucleoprotein A	1.7
FANhyb_rscf00002652.1.g00001.1	Stress response protein NhaX-like	1.8
FANhyb_rscf00000002.1.g00026.1	DNAJ heat shock N-terminal domain-containing protein	1.9
FANhyb_rscf00000700.1.g00003.1	Hypothetical protein PRUPE_ppa1027140mg	2.2
FANhyb_icon00002769_a.1.g00001.1	Zinc finger protein CONSTANS-LIKE 9-like	2.3
FANhyb_icon00003278_a.1.g00001.1	Hypothetical protein PRUPE_ppa013060mg	2.5
FANhyb_rscf00000382.1.g00011.1	Cold regulated gene 27	2.7
FANhyb_icon19730437_s.1.g00001.1	MATE efflux family protein 6-like	2.8
FANhyb_rscf00000752.1.g00004.1	Unknown protein	3.4
FANhyb_icon00015166_a.1.g00001.1	Auxin responsive SAUR protein	3.4
FANhyb_icon00027985_a.1.g00001.1	Unknown protein	4.1

Table 13: Gene annotation of significantly differentially expressed genes between control treatment and chitin with infection treatment at week 9 (t9PS-t9Chitin infection). Only the 20 genes with the highest log2 fold change are given.

Gene ID	Gene annotation (Hirakawa et al., 2014)	Log2 fold change
FANhyb_icon00038937_a.1.g00001.1	Cytochrome P450 94A1-like	-7.9
FANhyb_rscf00000446.1.g00001.1	Uncharacterized protein LOC101311157	-7.5
FANhyb_rscf00002447.1.g00002.1	Hypothetical protein PRUPE_ppa018413mg	-7.4
FANhyb_icon20590414_s.1.g00001.1	Unknown protein	-7.1
FANhyb_rscf00001142.1.g00001.1	Delta(8)-fatty-acid desaturase-like	6.3
FANhyb_rscf00000570.1.g00006.1	Unknown protein	6.5
FANhyb_rscf00003013.1.g00001.1	MLO protein 2	6.7
FANhyb_rscf00002956.1.g00002.1	Inorganic pyrophosphatase 1-like	6.7
FANhyb_icon00006059_a.1.g00001.1	Uncharacterized protein LOC101303288	6.8
FANhyb_rscf00004635.1.g00001.1	Laccase-14-like	6.8
FANhyb_icon00012271_a.1.g00001.1	Glycerophosphodiester phosphodiesterase gde1-like	6.9
FANhyb_rscf00003673.1.g00001.1	Purple acid phosphatase 22-like	7.2
FANhyb_rscf00004194.1.g00001.1	Glutamate receptor 2.8-like	7.9
FANhyb_rscf00000220.1.g00010.1	Putative E3 ubiquitin-protein ligase LIN-1-like	8.6

	Long-chain-fatty-acid--AMP ligase FadD26-like	8.7
FANhyb_icon00012354_a.1.g00001.1	Long-chain-fatty-acid--AMP ligase FadD26-like	8.7
FANhyb_icon00000987_a.1.g00001.1	SPX domain-containing protein 3-like	8.8
FANhyb_icon20322791_s.1.g00001.1	Unknown protein	8.9
FANhyb_rscf00003480.1.g00001.1	Uncharacterized protein LOC101296428	9.4
FANhyb_icon00012099_a.1.g00001.1	Unknown protein	10
FANhyb_rscf00002154.1.g00001.1	Probable purple acid phosphatase 20-like	11.5

Table 14: Gene annotation of significantly differentially expressed genes between control with infection treatment and biochar with infection treatment at week 9 (t9PS infection-t9BC infection).

Gene ID	Gene annotation (Hirakawa et al., 2014)	Log2 fold change
FANhyb_icon20235835_s.1.g00001.1	Unknown protein	2.5
FANhyb_rscf00000003.1.g00046.1	probable E3 ubiquitin-protein ligase HERC3-like	2.9

Table 15: Gene annotation of significantly differentially expressed genes between control with infection treatment and chitin with infection treatment at week 9 (t9PS infection-t9Chitin infection). Only the 20 genes with the highest log2 fold change are given.

Gene ID	Gene annotation (Hirakawa et al., 2014)	Log2 fold change
FANhyb_icon00015158_a.1.g00001.1	(-)-germacrene D synthase-like	-8
FANhyb_icon00042885_a.1.g00001.1	Phosphoenolpyruvate carboxykinase [ATP]-like	-5.8
FANhyb_rscf00004066.1.g00001.1	Uncharacterized protein LOC101305000	4.6
FANhyb_icon00015473_a.1.g00001.1	Unknown protein	4.9
FANhyb_rscf00001142.1.g00001.1	Delta(8)-fatty-acid desaturase-like	5.4
FANhyb_rscf00000290.1.g00005.1	Polyvinylalcohol dehydrogenase-like	5.7
FANhyb_rscf00002956.1.g00002.1	Inorganic pyrophosphatase 1-like	6.1
FANhyb_iscf00392735_s.1.g00001.1	Unknown protein	6.2
FANhyb_icon00006059_a.1.g00001.1	Uncharacterized protein LOC101303288	6.3
FANhyb_icon00009955_a.1.g00001.1	Glycerophosphodiester phosphodiesterase gde1-like	6.3
FANhyb_icon00012271_a.1.g00001.1	Glycerophosphodiester phosphodiesterase gde1-like	6.6
FANhyb_icon00012099_a.1.g00001.1	Unknown protein	7.3
FANhyb_rscf00002154.1.g00001.1	Probable purple acid phosphatase 20-like	7.7
FANhyb_rscf00003673.1.g00001.1	Purple acid phosphatase 22-like	8.2
FANhyb_rscf00000220.1.g00010.1	Putative E3 ubiquitin-protein ligase LIN-1-like	8.6
FANhyb_icon00012354_a.1.g00001.1	Long-chain-fatty-acid--AMP ligase FadD26-like	8.7
FANhyb_rscf00004635.1.g00001.1	Laccase-14-like	8.7

FANhyb_icon00000987_a.1.g00001.1	SPX domain-containing protein 3-like	8.8
FANhyb_icon00016707_a.1.g00001.1	Predicted protein gi|222835118|gb|EEE73553.1	8.8
FANhyb_rscf00003480.1.g00001.1	Uncharacterized protein LOC101296428	9.4

4.2. Defense gene expression by RNA sequencing

To further look at the effects of biochar and chitin on the strawberry defense response against *B. cinerea*, samples from week 9 (one week after infection) and week 10 (two weeks after infection) of plant growth were analyzed with RT-qPCR. Both samples from infected and non-infected plants were used. Non-infected plants were inoculated with agar containing no mycelium. For the samples of week 9, 7 genes previously reported as defense related genes in strawberry were tested. For the samples of week 10 only 6 genes were tested. This because for one gene, FaCDPK, there were too few samples with successful amplification for this gene. For the samples of week 10 in the peat substrate treatment with infection, only 2 biological replicates were used in the analysis. There was no successful amplification of any of the tested genes in the third biological replicate. Also, for the samples of week 10 in the biochar treatment with infection, only 2 biological replicates were used in the analysis of the gene FaChi2-1. There was no successful amplification in the third replicate for this gene.

Relative gene expression values for the samples of the biochar treatment from week 9 and week 10 are shown in Figure 18 (week 9) and Figure 19 (week 10), respectively. One week after infection, the defense related genes are downregulated in the biochar treatment. Although most differences in expression are not statistically significant. However, there is also a downregulation in the control treatment upon infection. It thus seems that biochar is not able to enhance the defense response. The gene Bglu is downregulated upon biochar amendment ($p = 0.03$), even without infection. CDPK is downregulated in the biochar treatment with infection. Since this downregulation is only statistically significant compared to the control treatment ($p = 0$) and the biochar treatment ($p = 0$) without infection, it seems that this is caused by the infection rather than the biochar amendment. WRKY1 is downregulated upon biochar amendment in infected plants ($p = 0$).

Two weeks after infection, the negative effect of biochar is disappeared. Now there is even a positive, but non-significant, effect of biochar amendment on the defense response. The defense related genes are now upregulated. Some genes, Bglu, Chi2-1 and WRKY1, are even more upregulated upon infection in the biochar treatment compared to the control treatment. These genes thus seem to be stimulated by biochar. PAL is significantly upregulated upon infection ($p = 0.03$). Possibly the positive effect of biochar on the defense response is too late. The pathogen has already infected the plant and has possibly spread to other plant parts or other plants.
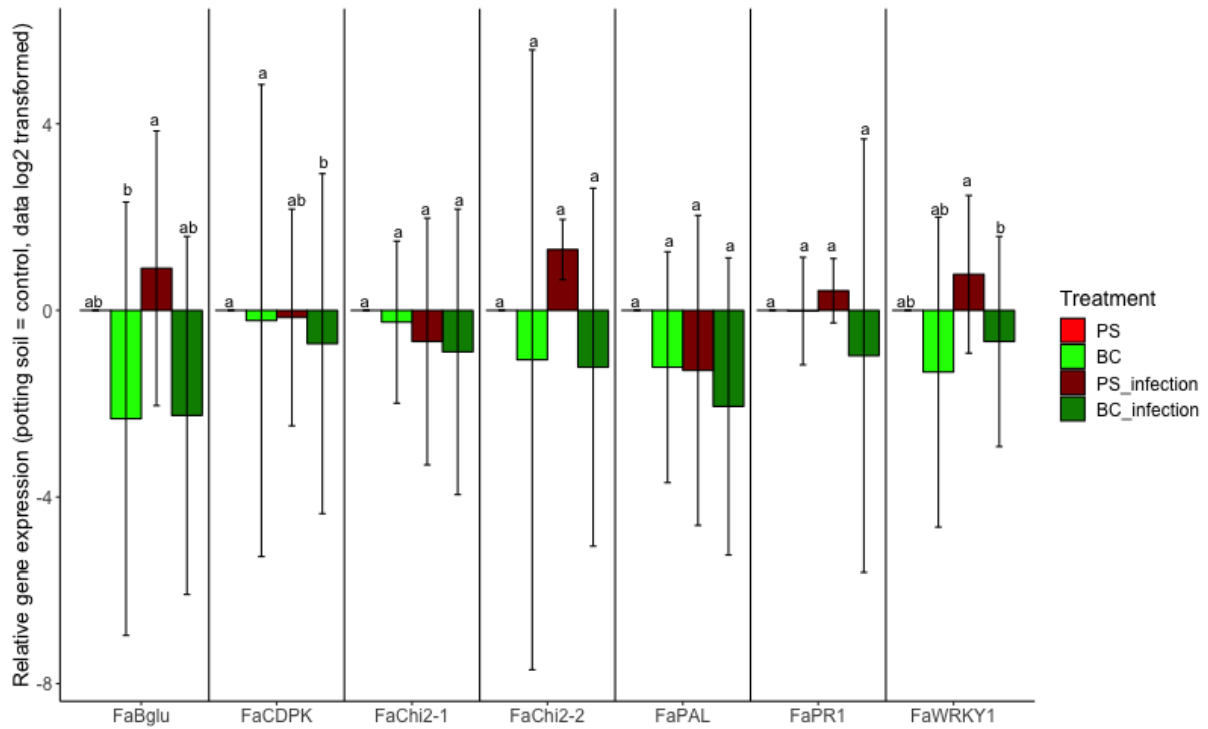
Figure 18: Relative gene expression values of seven defense genes in strawberry for peat substrate (PS) and biochar (BC) treatments. Data from week 9 of plant growth is shown. Expression values are expressed in log 2 fold changes. The PS treatment is used as control treatment, for this treatment the expression value is set to 0. Statistically significant differences are indicated with different letters.
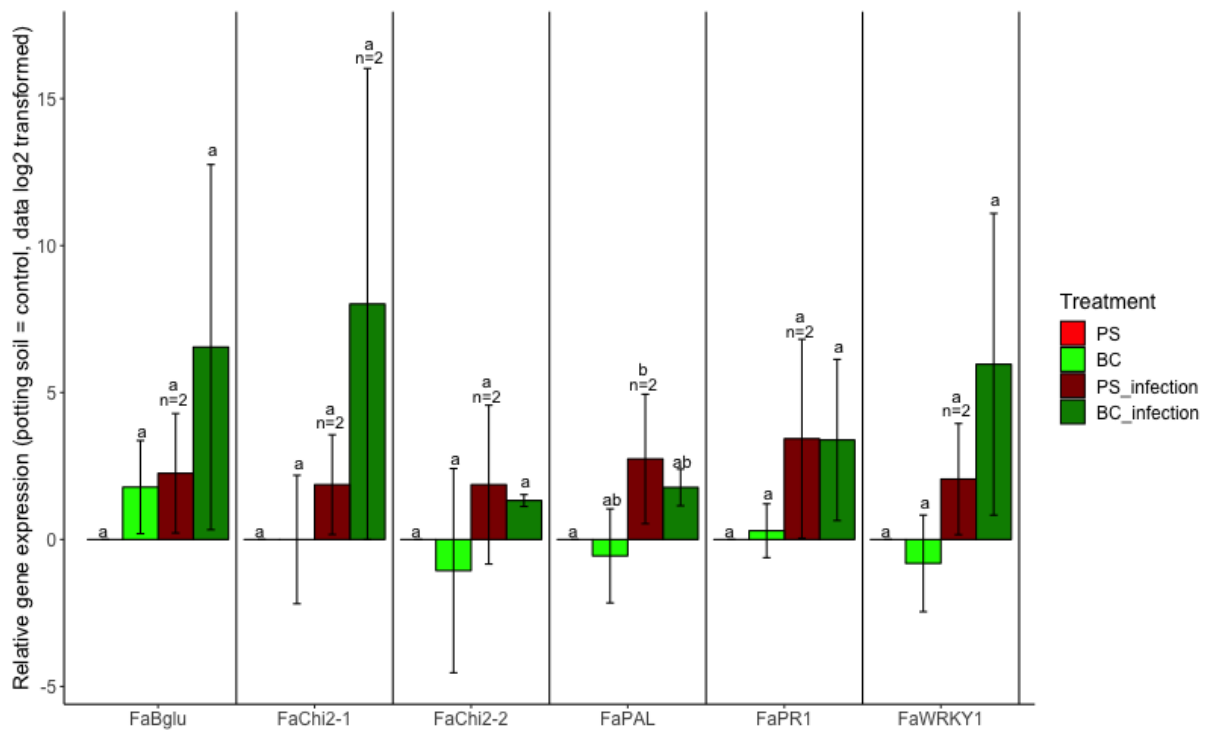

Figure 19: Relative gene expression values of six defense genes in strawberry for peat substrate (PS) and biochar (BC) treatments. Data from week 10 of plant growth is shown. Expression values are expressed in log 2 fold changes. The PS treatment is used as control treatment, for this treatment the expression value is set to 0. Statistically significant differences are indicated with different letters. For the PS treatment with infection, only 2 biological replicates were used because there was no successful amplification in the third replicate. Also for the gene Chi2-1 in the BC treatment with infection only 2 biological replicates were used. There was no successful amplification of this gene in the third replicate.

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Relative gene expression values for the samples of the chitin treatment from week 9 and week 10 are shown in Figure 20 and Figure 21, respectively. Chitin amendment does have a positive effect on the defense response. Already one week after infection there is an upregulation of the defense related genes. Chi2-1 is significantly upregulated in the chitin treatment compared to the control treatment with infection (p = 0.03). Chitin is added to the growth medium, it is thus logical that chitinase genes are upregulated in the plant to degrade this chitin. Chi2-2 is not significantly upregulated upon chitin addition. PR1 is significantly upregulated upon chitin addition compared to the control treatment without infection (p = 0.02) and with infection (p = 0). This gene is strongly stimulated by chitin amendment. Chitin is a PAMP and thus triggers PTI. The expression of different genes is stimulated upon activation of PTI. PR1 is one of the genes stimulated during PTI. The strong upregulation of PR1 upon chitin amendment is thus expected. WRKY1 is upregulated in the chitin treatment by the infection (p = 0.02). In the control treatment it is also somewhat upregulated, although not significant. In addition to stimulation of the defense genes, there also seems to be a priming effect of chitin for the genes Bglu, Chi2-1, Chi2-2 and WRKY1. These genes are strongly upregulated upon chitin amendment and infection but not upon chitin amendment only. The defense response is more efficient in plants grown in chitin amended peat substrate. These results agree with the results from the RNA seq analysis. Chitin itself does not have an effect on the strawberry plants. But when the plants are infected, there is a clear effect of chitin visible.

Two weeks after infection, chitin still has a positive effect on the defense response. All defense genes are upregulated. Bglu is significantly upregulated upon chitin amendment and infection (p = 0). PAL is significantly stimulated by the infection (p = 0.03). However, the gene is downregulated by chitin amendment compared to the control treatment with infection (p = 0). PR1 is again significantly upregulated upon chitin amendment without infection (p = 0.03) and with infection (p = 0.01). The priming effect is still visible two weeks after infection.
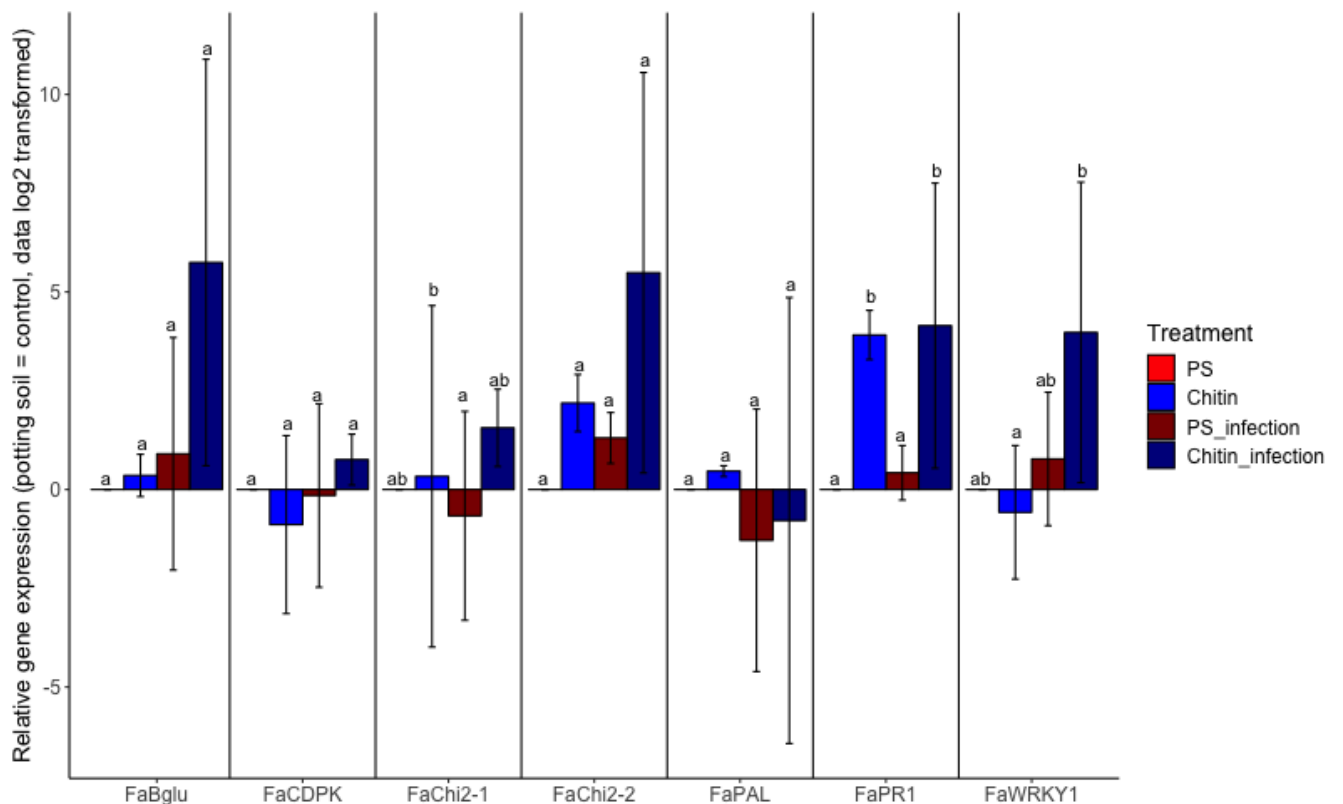

Figure 20: Relative gene expression values of seven defense genes in strawberry for peat substrate (PS) and chitin treatments. Data from week 9 of plant growth is shown. Expression values are expressed in log 2 fold changes. The PS treatment is used as control treatment, for this treatment the expression value is set to 0. Statistically significant differences are indicated with different letters.
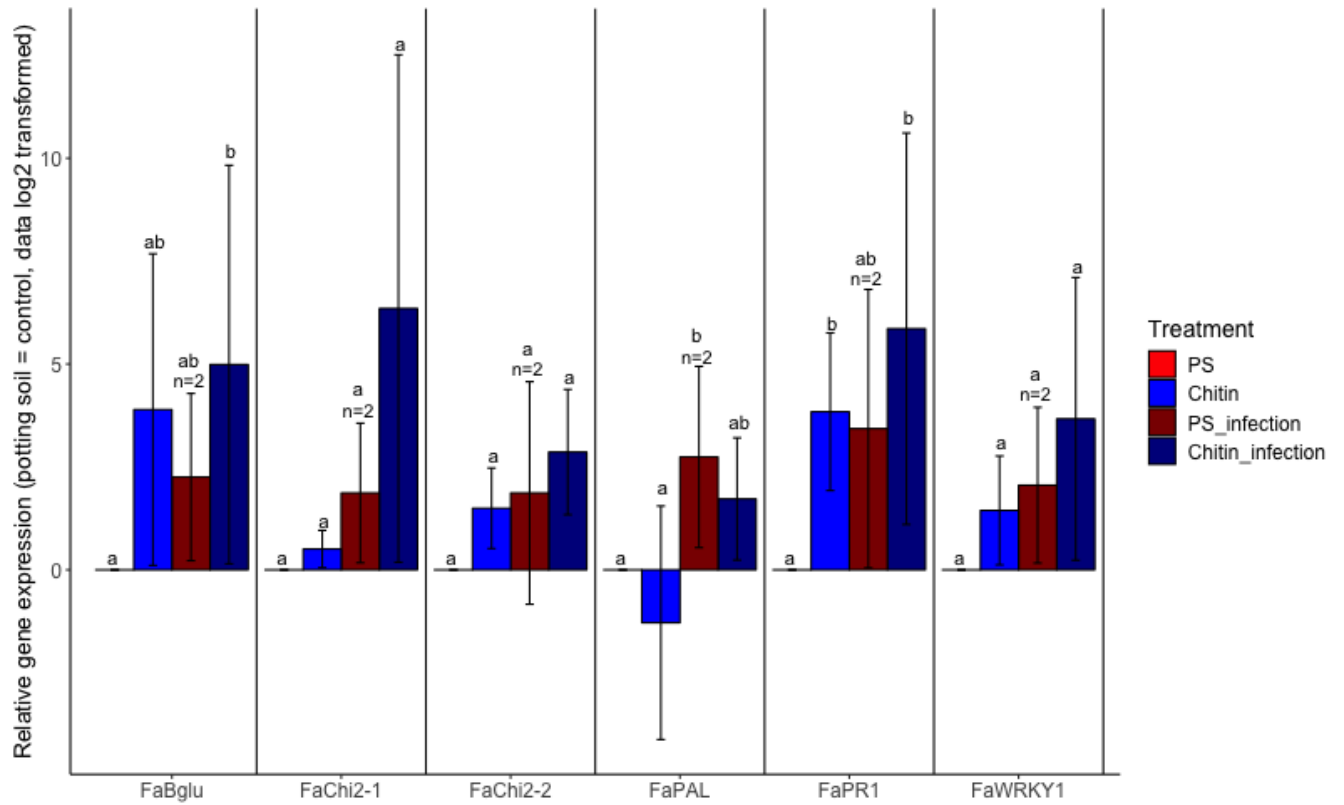
Figure 21: Relative gene expression values of six defense genes in strawberry for peat substrate (PS) and chitin treatments. Data from week 10 of plant growth is shown. Expression values are expressed in log 2 fold changes. The PS treatment is used as control treatment, for this treatment the expression value is set to 0. Statistically significant differences are indicated with different letters. For the PS treatment with infection, only 2 biological replicates were used because there was no successful amplification in the third replicate.

Discussion

1. Introduction

This thesis can be divided in two parts: (1) The effect of biochar and chitin on the bacterial microbiome of strawberry plants; (2) The effect of biochar and chitin on the defense response against *B. cinerea.* First, the effects on the bacterial community will be discussed. Second, the effects on the defense response will be studied.

2. Microbiome analysis

In this thesis, the effect of biochar and chitin on the bacterial microbiome of strawberry plants is investigated. To study the bacterial microbiome, amplicon sequencing of the 16S rRNA gene was done. From this analysis it can be concluded that both biochar and chitin amendment result in a higher bacterial richness from week 7 of plant growth onwards. For biochar amendment it has already been reported that the increase in richness is only seen at later stages of plant growth (De Tender et al., 2016b). It is possible that both biochar and chitin can serve as a nutrient source for some bacteria. These bacteria can then be attracted by the plant and cause the increase in richness seen here.
The richness was also compared between the rhizosphere and peat substrate not influenced by the plant (further referred to as bulk soil) for all treatments. From this comparison it can be seen that the richness is higher in the rhizosphere than in the bulk soil. This result is in contradiction with results previously reported in soil experiments (García-Salamanca et al., 2013; Weisskopf, Fromin, Tomasi, Aragno, & Martinoia, 2005). These studies report a lower richness in the rhizosphere compared to bulk soil, indicating a plant-derived selection for specific microorganisms which can for example help in plant growth and nutrient acquisition (Hartmann, Schmid, Van Tuinen, & Berg, 2009; Weisskopf et al., 2005). A possible explanation for these contradictory results is that part of the microbial community in the rhizosphere observed in peat substrate originates from the strawberry plants microbiome before planting in peat substrate. The strawberry plants used in the experiment were first grown in soil. It is possible that some bacteria present in this soil were still attached to the roots when the strawberry plants were transplanted to peat substrate and remained on these roots during the experiment. This could partly explain the higher richness seen here. However, at the beginning of the experiment the richness was about the same between rhizosphere and bulk soil. Probably there is thus also an attraction of bacteria from the bulk soil environment to the root of the plant. When looking at the community composition, it can be concluded that the rhizosphere and bulk soil microbiomes are different from each other during the first 4 weeks of plant growth. However, at the end of the experiment, both microbiomes are more alike. Possibly, the effect of the plant on the rhizosphere microbiome becomes smaller as the plant develops to the senescence stage.

In terms of community composition, biochar amendment increased the relative abundance of 3 bacterial genera: (1) *Taibaiella*, (2) *Pseudomonas* and (3) *Micropepsis*. These results are in line with previous experiments in which also an increase in abundance of *Taibaiella* and *Pseudomonas* upon biochar amendment to peat substrate was noted (De Tender et al., 2016b; Graber et al., 2010).
The genera *Taibaiella* and *Micropepsis* have been reported to be involved in the nitrogen cycle (Harbison, Price, Flythe, & Bräuer, 2017; Zhang et al., 2009). *Pseudomonas* spp. are known as biocontrol agents used in the control of phytopathogens (Walsh, Morrissey, & O'Gara, 2001). In addition, it has been reported that certain *Pseudomonas* spp. can have a plant growth promoting effect (Berg, 2009). However, there is no increase in plant fresh or dry weight of plants grown in biochar amended peat substrate compared to plants grown in peat substrate. The root weight seems to be higher in the biochar treatment, although there are no significant differences.

Chitin amendment resulted in a significant increase in relative abundance of 19 genera and a decrease in relative abundance of 4 genera at the end of the experiment. Similar as for the biochar addition, the abundance of the genera *Pseudomonas* and *Micropepsis* were increased. Amongst the more abundant genera are *Pseudomonas* and *Streptomyces*. Both genera are known for their plant growth promoting effect (Berg, 2009). The increased abundance of plant growth promoting bacteria can explain, at least in part, the increase, however not significant, in plant fresh and dry weight in plants grown in chitin amended peat substrate observed during the strawberry experiment. In contrast to the increase in plant fresh and dry weight, the root weight is significantly reduced for plants grown in chitin amended peat substrate, an effect which is significant from week 9 of plant growth onwards. The genera that decreased in abundance included *Methylovirgula* and *Candidatus solibacter*. Debode et al. (2016) also reported a decrease in the genus *Candidatus solibacter* by chitin amendment to peat substrate in which lettuce was grown (Debode et al., 2016).

Besides the effect of treatment on the bacterial microbiome composition, there is also a time effect visible. The microbiome composition changes over time. This time effect is seen in biochar and chitin amended peat substrate, as well as in potting soil without amendments. This result is in accordance with the results seen in De Tender et al. (2016b).

3. Defense response against *B. cinerea*

To study the effect of biochar and chitin on the strawberry defense gene expression, RNA sequencing and qPCR were used. Leaf samples of just before and one week after infection with *B. cinerea* were analyzed with RNA sequencing. Both the effect of biochar and chitin on the strawberry leaf gene expression, as well as the effect on the defense response against *B. cinerea* is investigated. For RT-qPCR 7 known defense related genes in strawberry were analyzed. Leaf samples of plants one week and two weeks after infection were analyzed with RT-qPCR.

Biochar and chitin do not have an effect on the strawberry leaf **basal gene expression**. This can be concluded based on the RNA sequencing analysis of non-infected samples. Only one gene is significantly upregulated in plants grown in chitin amended peat substrate. This gene is predicted to encode for an auxin-induced protein (Hirakawa et al., 2014). This can be related with the seemingly higher plant fresh and dry weight in the chitin treatment compared to the control treatment. Auxin plays an important role in plant growth and development. In the shoot, auxin accumulation stimulates growth (Vanneste & Friml, 2009).

The infection with *B. cinerea* already has a large effect on the gene expression of plants grown in unamended peat substrate. In total, 86 genes are downregulated and 46 genes are upregulated **upon infection**. The gene with the strongest downregulation encodes an alpha-amylase/subtilisin inhibitor. These proteins are involved in plant defense. In barley, alpha-amylase/subtilisin inhibitors have a specificity for microbial proteases. This suggest that they are involved in the plant defense against microorganisms (Nielsen, Bønsager, Fukuda, & Svensson, 2004). Also a gene encoding a PRUPE protein is downregulated upon infection. PRUPE proteins are still uncharacterized ("PRUPE_ppa1027140mg - Uncharacterized protein - Prunus persica (Peach) - PRUPE_ppa1027140mg gene & protein," n.d.). Two genes encoding anthranilate synthases are upregulated. Anthranilate synthase is involved in the synthesis of tryptophan. It has also been reported to contribute to penetration resistance to the causal agent of powdery mildew in barley (Hu, Meng, & Wise, 2009).

When the plants are infected there is an effect of both biochar and chitin. Although the effect of biochar on the gene expression is smaller than the effect of chitin. Only 1 gene, encoding for a polyphenol oxidase, is significantly downregulated in plants grown in biochar amended peat substrate upon infection. This is also the gene with the highest log fold change. Polyphenol oxidase proteins are known to play a positive role in plant defense against pathogens (Li & Steffens, 2002; Raj, Sarosh, & Shetty, 2006; Thipyapong, Hunt, & Steffens, 2004). This gene is strongly downregulated, suggesting that biochar does not seem to be able

to enhance the strawberry defense response against *B. cinerea*. This can also be seen from the leaf infection rates of plants grown in peat substrate and plants grown in biochar amended peat substrate. The infection rates seem to be alike in both treatments. Several genes encoding PRUPE proteins are upregulated upon infection in biochar amended plants. Different DnaJ proteins are also upregulated by biochar. These chaperone proteins are involved in different processes, such as protein homeostasis under environmental stress (Rajan & D'Silva, 2009).

Chitin amendment seems to have a bigger effect on the gene expression in infected plants. In the chitin treatment, 482 genes are significant differentially expressed upon infection. While in the control treatment only 132 genes are significant differentially expressed upon infection. Chitin strongly enhances the expression of purple acid phosphatase genes. Purple acid phosphatases are involved in phosphate acquisition in plants. It has also been reported that these proteins can play a role in peroxidation and maintaining basal defense against some pathogens (del Pozo et al., 1999; Ravichandran, Stone, Benkel, & Prithiviraj, 2013). Glycerophosphodiester phosphodiesterase is also upregulated by chitin. These proteins catalyze the hydrolysis of different glycerophosphodiesters (Van Der Rest et al., 2004). They have also been reported to be involved in maintaining cellular phosphate homeostasis under phosphate starvation (Cheng et al., 2011). Chitin stimulates the expression of a MLO protein. It has been reported that loss of function of MLO genes reduces the susceptibility of various plant species to powdery mildew (Pessina et al., 2016). In barley for example, the MLO protein is necessary for successful penetration of the host cell wall by the fungus. However, it is possible that MLO proteins are not involved in the pathogenesis of *B. cinerea* (Panstruga, 2005). Similar as in the control treatment, a gene encoding a PRUPE protein is downregulated by chitin. Since these PRUPE proteins are differentially expressed in all treatments, a functional analysis of these genes could explain why these genes are differentially expressed in all treatments. Possibly, PRUPE proteins could play a role in plant defense. Further research is needed to prove this statement.

The strawberry defense response against *B. cinerea* was also studied with RT-qPCR, both 1 and 2 weeks after infection. One week after infection, biochar is not able to enhance the gene expression of the analyzed defense related genes. Most genes are even downregulated upon biochar amendment. However, 2 weeks after infection there is an upregulation of the defense related genes. For both timepoints, the gene expression profile seen in the biochar + infection treatment is similar to the profile seen in the control treatment after infection. It thus seems that the defense response in plants grown in biochar amended peat substrate is not that different from the profile of plants grown in peat substrate. This can also be seen from the analysis of the infection rates of the leaves. From this figure it can be seen that the infection rate in the biochar treatment is similar to the infection rate in the control treatment. The results from the RT-qPCR analysis are in agreement with the results from the RNA sequencing analysis. From the RNA seq analysis can be seen that biochar only has a small effect on the leaf gene expression. It can thus be concluded from both analyses that biochar has a small effect on the defense response of strawberry against *B. cinerea*.
Meller Harel et al. also studied the defense response of strawberry plants against *B. cinerea* using RT-qPCR on strawberry leaf samples. In their experiment, they also looked at the expression of FaPR1 and FaWRKY1. However, they reported an upregulation of FaPR1 and FaWRKY1 upon infection in combination with biochar amendment to a coconut fiber:tuff mix. This upregulation was already seen 1 week after infection (Meller Harel et al., 2012). Whereas in this thesis, there is a downregulation seen of both these genes. It is possible that these different results can be explained by the differences in plant growth conditions and growth medium used in both experiments.

In contrast to biochar amendment, chitin amendment has a positive effect on the defense response of strawberry plants against *B. cinerea*. Already 1 week after infection, there is an upregulation of all genes analyzed with RT-qPCR upon chitin amendment in combination with infection. However, there is not always a significant upregulation. In the control treatment the defense response upon infection does not seem to be induced that much. The pathogen *B. cinerea* will try to suppress the plant defense response. This can explain why the defense

response is not induced much. In the chitin treatment, the defense response is already strongly induced. Also in the non-infected plants chitin induces the expression of some defense related genes. Two weeks after infection, the upregulation is even higher for most genes. The stronger defense response upon chitin amendment can also be linked to a lower infection rate of leaves in the chitin treatment. The infection rate is much, but not significant, lower in the chitin treatment compared to the control treatment.

Since the effect of chitin becomes stronger when the plants are infected, it can be said that chitin act as a priming agent. A priming agent is defined as a component that triggers a state of enhanced capability to induce elicitor-induced cellular defense responses (Paré et al., 2005). When the plants are not infected, the effect of the chitin amendment on the expression of defense related genes is low. Only PR1 responds strongly to chitin. Since chitin is a PAMP it is logical that PR1 is stimulated by chitin amendment. Chitin will be recognized and stimulate PTI. PR1 is upregulated upon activation of PTI (Ali et al., 2018; Kawasaki et al., 2017). However, when the plants become infected the defense response is strongly enhanced in plants grown in chitin amended peat substrate. Whereas the defense response in plants grown in peat substrate is much lower, especially one week after infection. This priming effect of chitin can also be seen in the results from the RNA sequencing analysis. There, it was concluded that chitin does not alter gene expression in non-infected plants. Only when the plants were infected there was a clear effect of chitin on the leaf gene expression. Chitosan, a derivative of chitin, has previously been reported to induce resistance against different pathogens (Faoro, Maffi, Cantu, & Iriti, 2008; Iriti et al., 2010). Faoro et al. showed that chitosan can induce SAR in barley plants (Faoro et al., 2008). Iriti et al. hypothesized that chitosan can have a priming effect when applied at low concentrations, while it can directly stimulate defense mechanisms when applied at high concentrations (Iriti et al., 2010).

In this thesis it has been shown that biochar and chitin amendment do have an effect on the bacterial rhizosphere and bulk soil microbiome composition of strawberry plants. Although this effect is rather small. Both the rhizosphere and bulk soil microbiome composition change over time. This time effect seems to be more important than the effect of biochar and chitin. The change over time in the rhizosphere microbiome is probably caused by the different plant developmental stages. At the first 4 weeks of plant growth, the rhizosphere microbiome is different from the bulk soil microbiome. However, at the end of plant growth both microbiomes are more alike in composition.

The strawberry plant defense response - against *B. cinerea* - of plants grown in biochar amended peat substrate is not very different from the defense response of plants grown in peat substrate. However, chitin amendment strongly stimulates the plant defense response. This enhancement is already seen 1 week after infection. Furthermore, it can be said that chitin primes the strawberry defense response against *B. cinerea*. Functional analysis of some of the differentially expressed genes could provide more insight in the effect of biochar and chitin on the plant defense.

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Appendix

1. Plant fresh weight
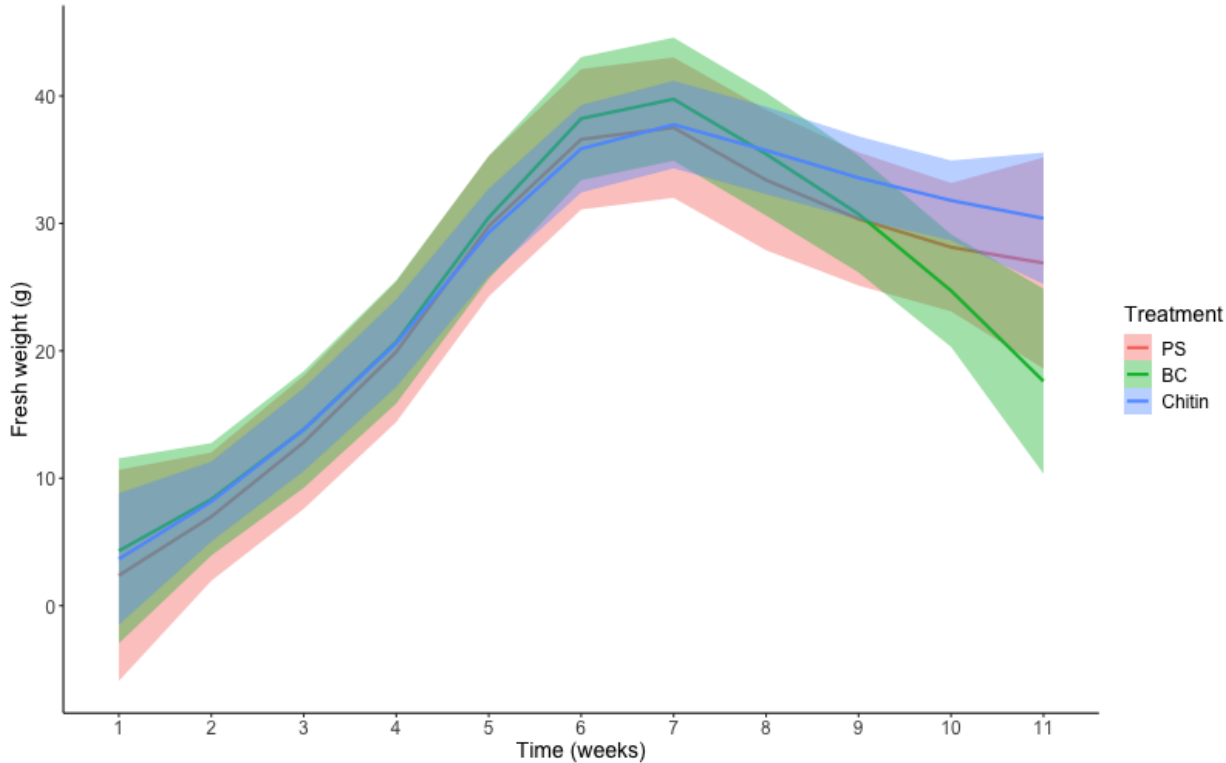
Figure 22: Line graph of plant fresh weight of non-infected plants. 95% confidence intervals are shown. PS = peat substrate, BC = biochar.
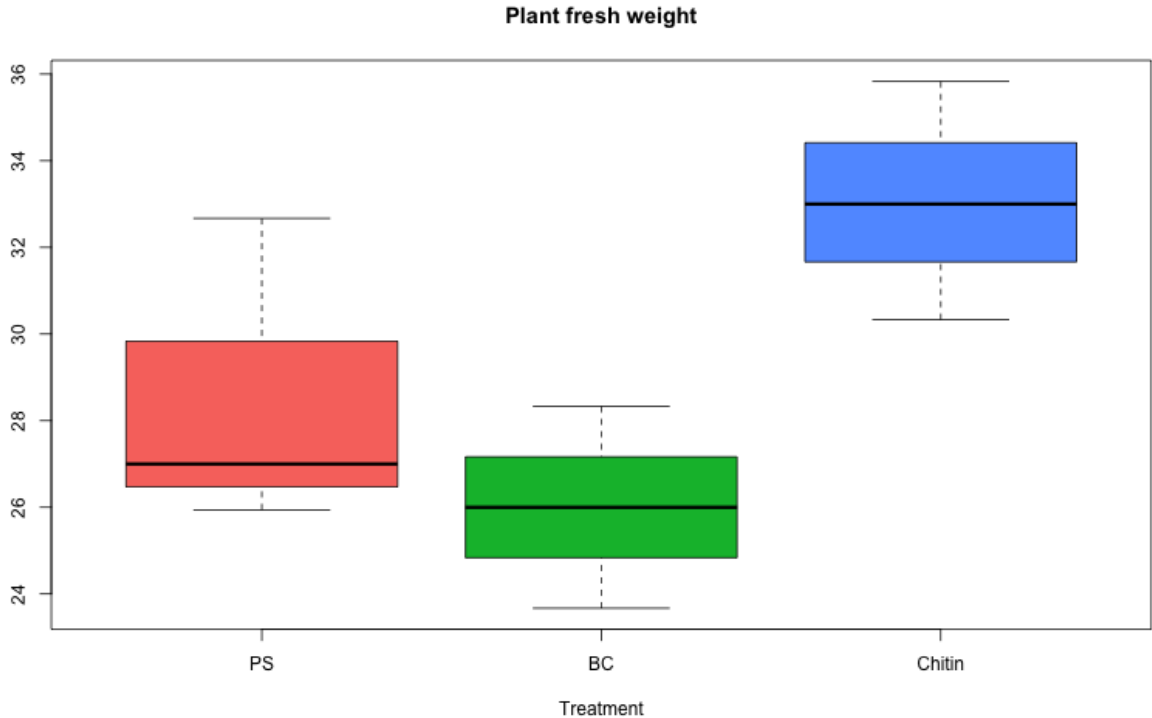

Figure 23: Boxplots of plant fresh weight of infected plants.
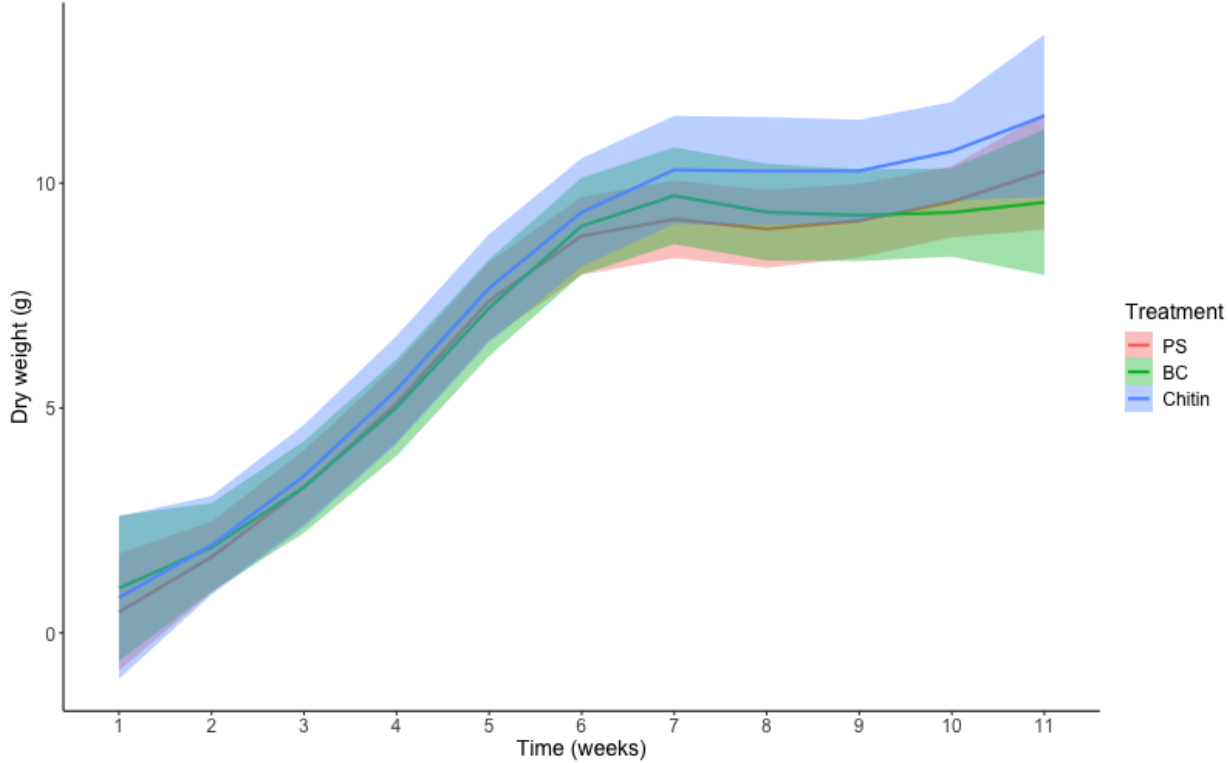
2. Plant dry weight

Figure 24: Line graph of plant dry weight of non-infected plants. 95% confidence intervals are shown.
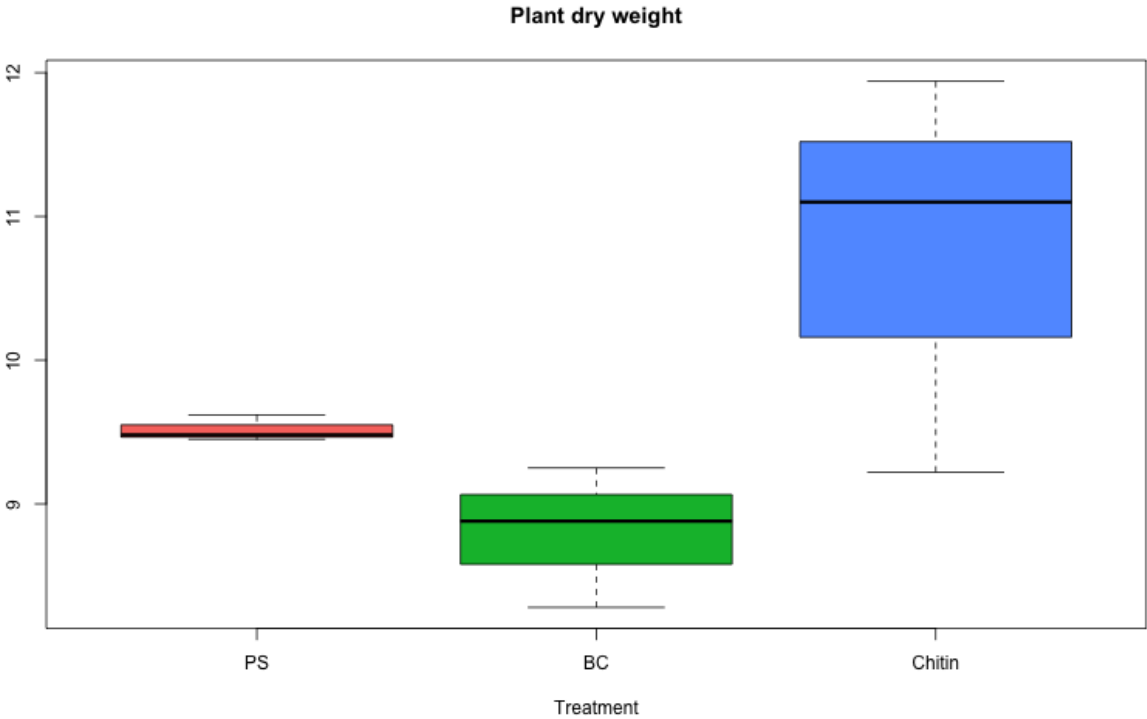

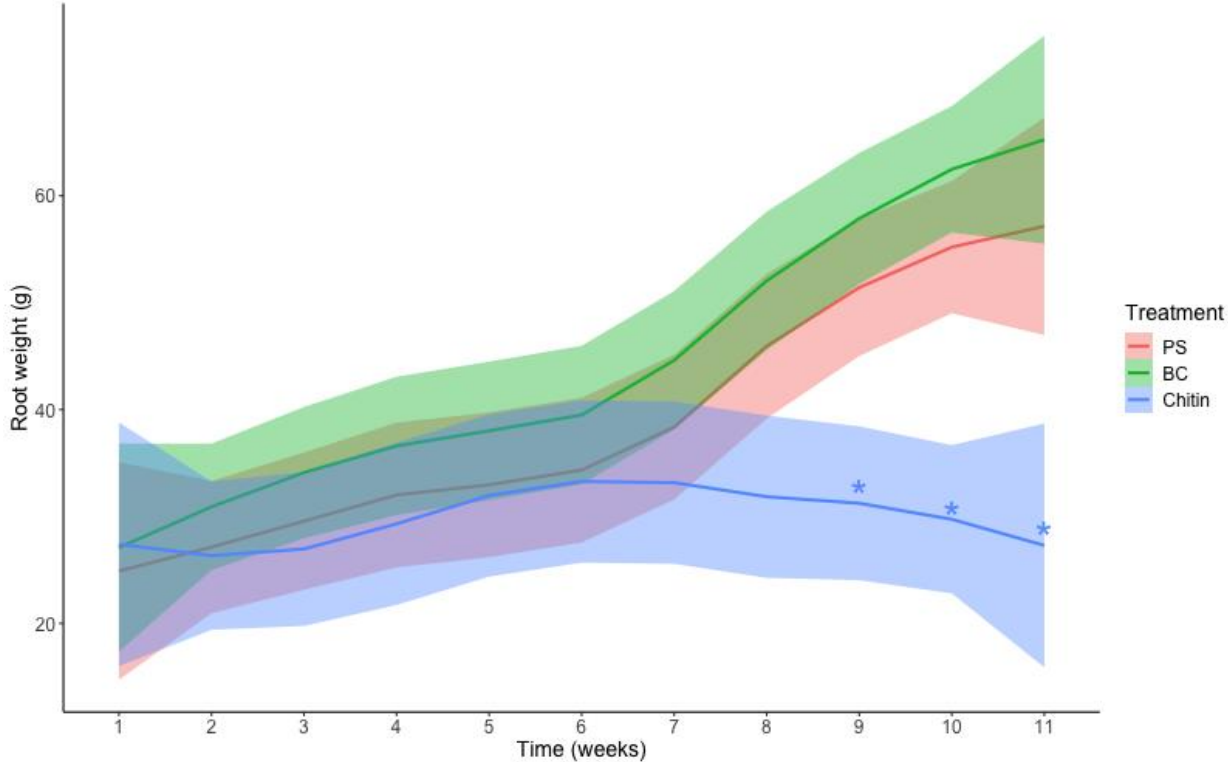*Figure 25: Boxplots of plant dry weight of infected plants.*

3. Root weight

Figure 26: Line graph of root weight of non-infected plants. 95% confidence intervals are shown. Statistically significant differences compared to control treatment (PS) are indicated with an asterisk.
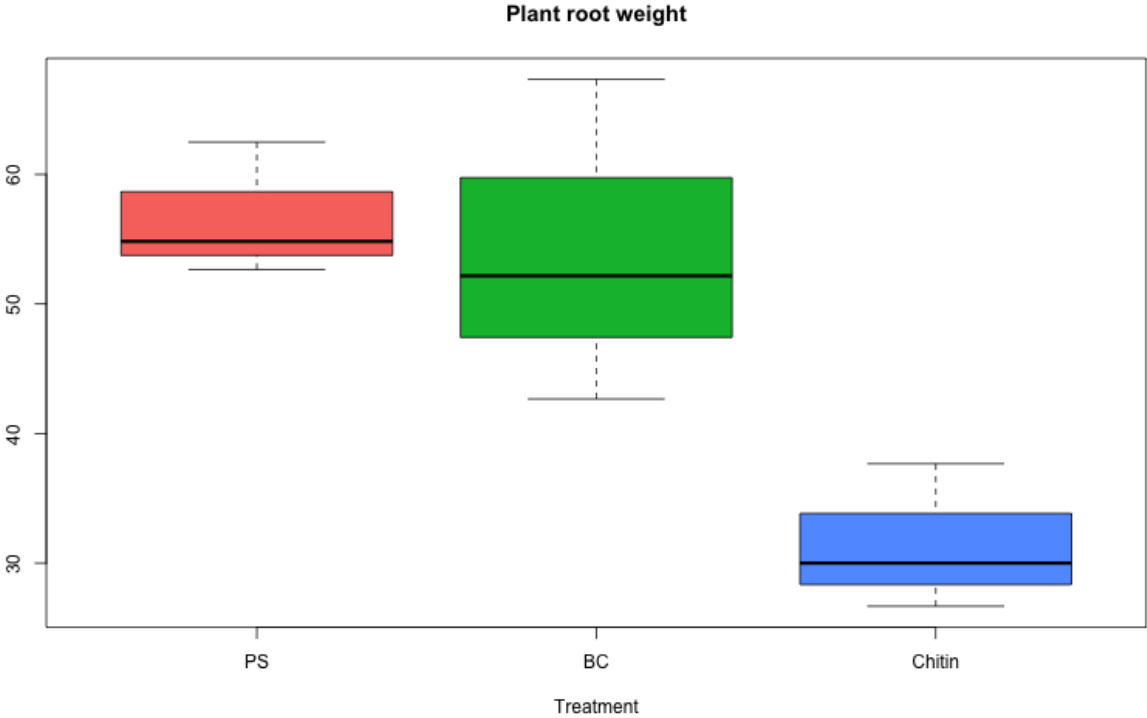

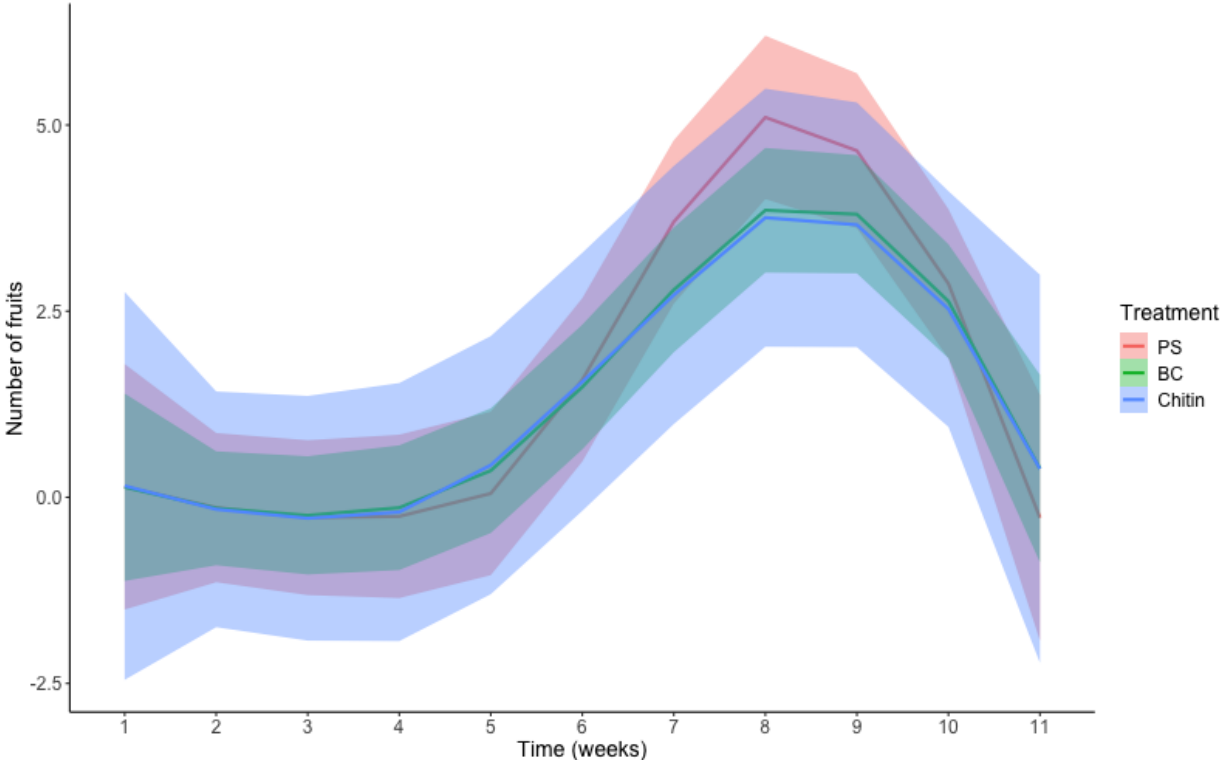*Figure 27: Boxplots of root weight of infected plants.*

4. Fruit yield

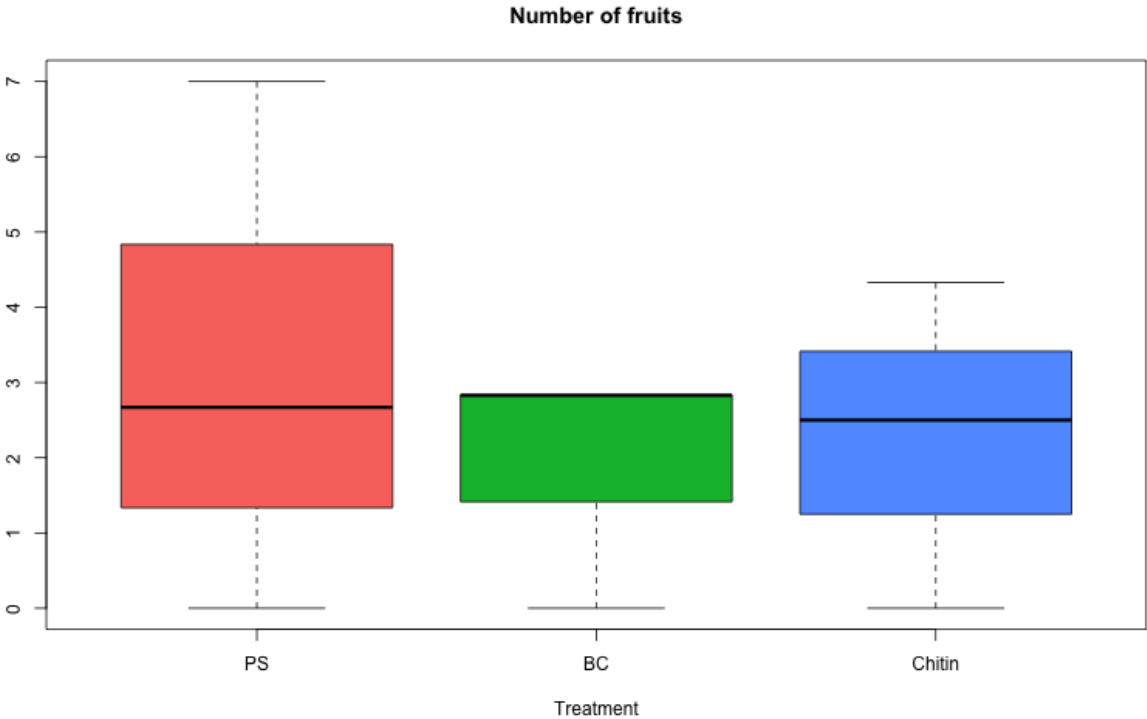*Figure 28: Line graph of fruit yield of non-infected plants. 95% confidence intervals are shown.*

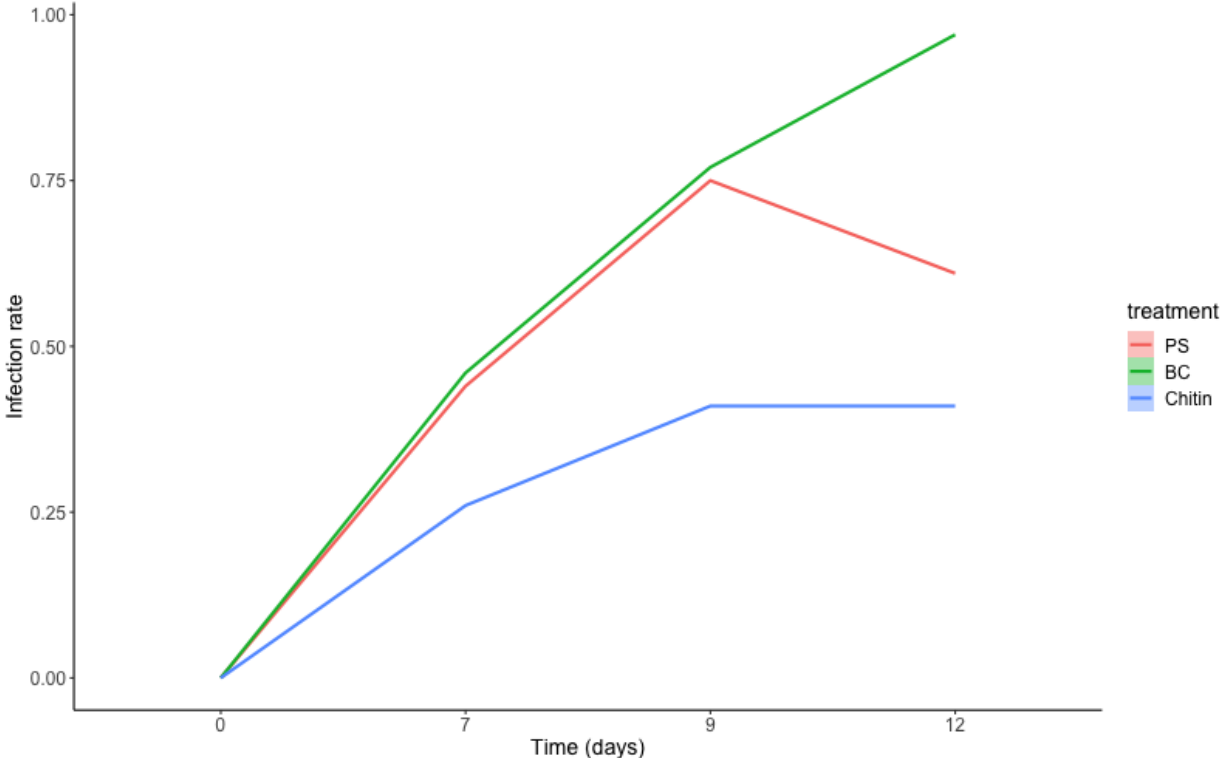*Figure 29: Boxplots of fruit yield of infected plants.*

5. Infection rate leaves

Figure 30: Line graph of the infection rate of leaves.