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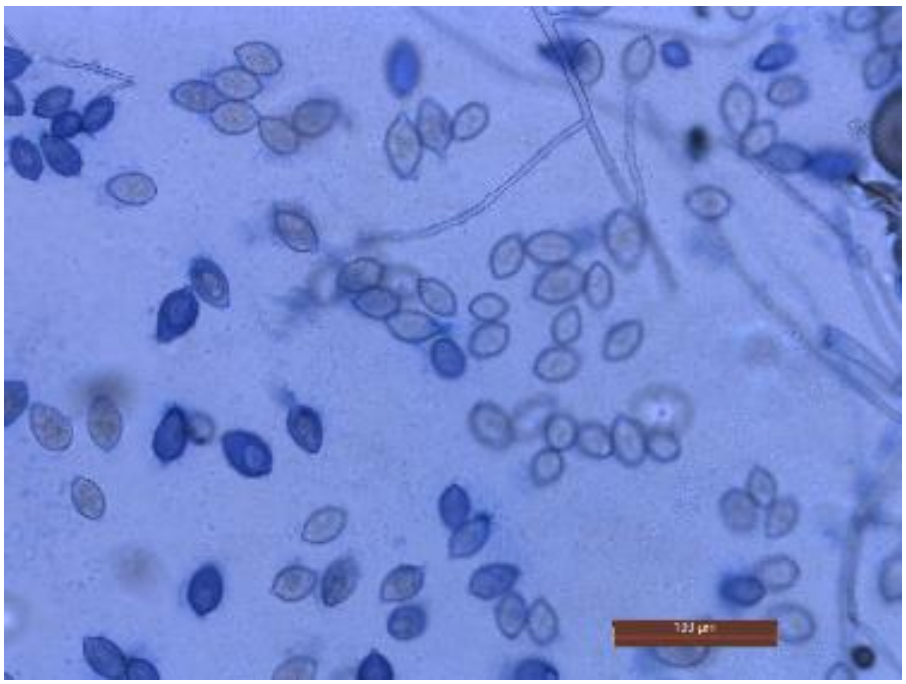

Potential of *Xenorhabdus bovienii,* symbiont of entomopathogenic nematodes, against plant pathogens of tomatoes

Einsatz von Bakterientoxine gegen Pflanzenschädliche und Krankheiten

Master's thesis

by

Manuela Anele

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Natural Resource Science

Research group biocontrol

Supervisors:

Dr. Esther Fischer, Zürcher Hochschule für Angewandte Wissenschaften, Grüental, 8820 Wädenswil

Prof. Dr. Jürg Grunder, Zürcher Hochschule für Angewandte Wissenschaften, Grüental, 8820 Wädenswil

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Phytophthora infestans seen under a light microscope (Photo: Manuela Anele, 2018)

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Address:

ZHAW Dept
Zürcher Hochschule für
Angewandte Wissenschaften
Life Sciences und Facility Management
Grüental, Postfach
CH-8820 Wädenswil

Author:

Anele Manuela
Zentralstr. 167
8003 Zurich

Abstract

The purpose of this following master's thesis is the examination of the potential of *Xenorhabdus bovienii*, a symbiont of entomopathogenic nematodes, against the plant pathogens *Phytophthora infestans* and *Colletotrichum coccodes*.
This document is based on a literature research on tomato as a cultivar, the plant pathogens *Phytophthora infestans* and *Colletotrichum coccodes,* the biology of the bacterium *Xenorhabdus bovienii* as well as its potential as a biocontrol organism against plant pathogens. Based on the insight gained in the literature research trails with two different bacterial strains of *Xenorhabdus bovienii* were elaborated. This is followed by the conduction of different experiments, including the establishment of a successful infection protocol for *Phytophthora infestans* on tomato, *in vitro* confrontation assays and various *in planta* pot trials with tomato cuttings. While the results showed no significant antagonistic effect against *Colletotrichum coccodes*, the bacterium was able to significantly inhibit the development of *Phytophthora infestans* both *in vitro and in planta.* Consulting the results obtained in the different trials and the evidences from the literature research, statements about the promising potential of *Xenorhabdus bovienii* as an antagonist against *Phytophthora infestans* are formulated.

Zusammenfassung

Die vorliegende Masterarbeit hat die Untersuchung der potentiellen Schutzwirkung des Bakterium *Xenorhabdus bovienii*, ein Symbiont entomopathogener Nematoden, gegen die Pflanzenpathogene *Phytophthora infestans* und *Colletotrichum coccodes* zum Ziel. Als Basis dieser Arbeit dient eine Literaturrecherche in den Bereichen Tomatenanbau, den Pflanzenpathogene *Phytophthora infestans* und *Colletotrichum coccodes,* die Biologie des Bakteriums *Xenorhabdus bovienii*, wie auch dessen Potential als Pflanzenschutzmittel. Basierend auf den Erkenntnissen aus der Literaturrecherche wurden verschiedene Experimente mit zwei unterschiedlichen *Xenorhabdus bovienii* Bakteriensträngen erarbeitet. Darauf folgte die Umsetzung der Experimente, wobei sowohl ein erfolgreiches Infektionsprotokoll für *Phytophthora infestans* auf Tomaten, wie auch *in vitro* confrontation assays und *in planta* Topfversuche mit Tomatenstecklinge durchgeführt wurden. Die Resultate konnten keinen signifikanten Schutz gegen *Colletotrichum coccodes* aufzeigen. Hingegen zeigten die Versuche eine signifikante Reduktion des Wachstums von *Phytophthora infestans* sowohl *in vitro* wie auch *in planta*. Unter Einbezug der Resultate aus den Versuchen und den Hinweisen aus der Literatur werden anschliessend Aussagen zum vielversprechenden Potential von *Xenorhabdus bovienii* als Antagonist gegen *Phytophthora infestans* aufgeführt.

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List of abbreviations

Phy=*Phytophthora infestans*

Col=*Colletotrichum coccodes*

Xen= *Xenorhabdus bovienii*

LBD=Late blight disease

DAI= Days after inoculation

1 Background

1.1 Tomato as a cultivar

Tomato (*solanum lycopersicum*) is a member of the large family of Solanaceae. Its origin can be traced back to the Anden region. However, until today it is not possible to determine the exact location of its provenance nor the time of domestication (Bai & Lindhout, 2007).
In the fifteenth century, the crop had already experienced a high degree of domestications. After being introduced to the European continent its domestication was intensified, leading to a large spectrum of varieties (Nowicki, Kozik, & Fooland, 2013). Even though the cultivar is considered a tropical plant, farmers are growing it almost all over the world. Depending on the environmental conditions, the crop is grown outdoors or indoors in green houses, in soil or hydroponically (Nelson, 2008). The demand for fresh and processed tomato varieties increased steadily over the last few years leading to a production of 177 million tons of tomatoes worldwide in 2016 (Nowicki, Kozik, & Fooland, 2013) (Horti Daily, 2018).
Nelson (2008) describes the tomato cultivar as one of the most widely grown vegetable food crop worldwide, that comes in second after potato (Nelson, 2008). According to VSGP (2015) the vegetable cultivar with the highest production volume in Switzerland (in kg) are tomatoes and they scored second place on the most consumed vegetables list in 2013. Due to the climatic conditions, the cultivation of tomatoes in Switzerland is almost exclusively done in green houses (Michel & Terrettaz, 2011) (Verband Schweizer Gemüseproduzenten VSGP, 2015).

1.2 Late blight disease of tomato

The biggest concerns in tomato production worldwide are caused by diseases, which lead to devastating economical losses (Nowicki, Kozik, & Fooland, 2013). More than 200 different pathogens affecting tomatoes have been described so far but according to Nowakowska et al. (2014) the most destructive is the late blight disease (LBD) caused by *Phytophthora infestans* (Nowakowaska et al., 2014). The disease is mostly famous for being the causing agent of the Irish potato famine in 1840 (Yoshida, et al., 2013).
After the discovery of the New World, potato as a crop was introduced to the European continent. Only after the passing of three centuries the pathogen *Phytophthora infestans* found its way to Europe. Even though it was able to spread to different parts of Europe, it was in Ireland where the epidemic reached a level of catastrophic magnitude. According to Bourk (1964, cited in Yoshida, et al., 2013) this was due to the population's high dependence on potato production. The Great Famine caused the death of approximately 1 milion people and around the same number had to flee the country (Turner,2005 cited in Yoshida et al., 2013).

Even though in present times *P. infestans* can be found worldwide (Nowicki, Kozik, & Fooland, 2013) Nelson (2008) states that, where the climatic conditions are mostly cool, moist, rainy and humid, the probability of its occurrence are higher.
Most important hosts of *P. infestans* are crops of the Solanacea family like tomato, nightshade and potato. (Nelson, 2008) The pathogen is able to infect all parts of the plant that grow above ground and is not limited to a certain development stage of the host (Nelson, 2008). According to Yoshida et al. (2013) the oomycetes success as a plant pathogen is mostly due to its high virulence adaptability. It shows a high level of diversity, both regarding the genome and phenotype. In addition, its asexual and sexual reproduction system prove to be highly efficient according to Nowicki et al. (2013).
If no counter measures are applied, the disease is able to destroy crops of potato or tomato in matters of days with the possibility of complete yield loss (Nowicki, Kozik, & Fooland, 2013). Economical losses can also be in form of lower fruit quality and shorter shelf life. Further, it can lead to higher costs for fungicide application, both monetary and environmental (Nowicki et al., 2012).
Phytophthora infestans is a fungus-like organism and is defined as an oomycete, which are included in a kingdom separate from the true fungi. A trait that separates the oomycetes from the true fungi is the absence of cell walls that contain chitin (Fry & Goodwin, 1997). Oomycetes are divided into two orders, the Saprolegniales and Peronosporales. The latter includes *Phytophthora* and other significant plant pathogens such as *Pythium spp.* (Nowicki, Kozik, & Fooland, 2013). Like Pythium spp., this pathogen shows a highly effective reproduction system, both asexual and sexual. *P. infestans* can produce thousands sporangia in its asexual form (Nowicki et al., 2012). According to Nelson (2008), the infection of a host usually happens by the transportation of sporangia or mycelial fragment to the host plant by either wind, splashing water or a combination of wind and rain.

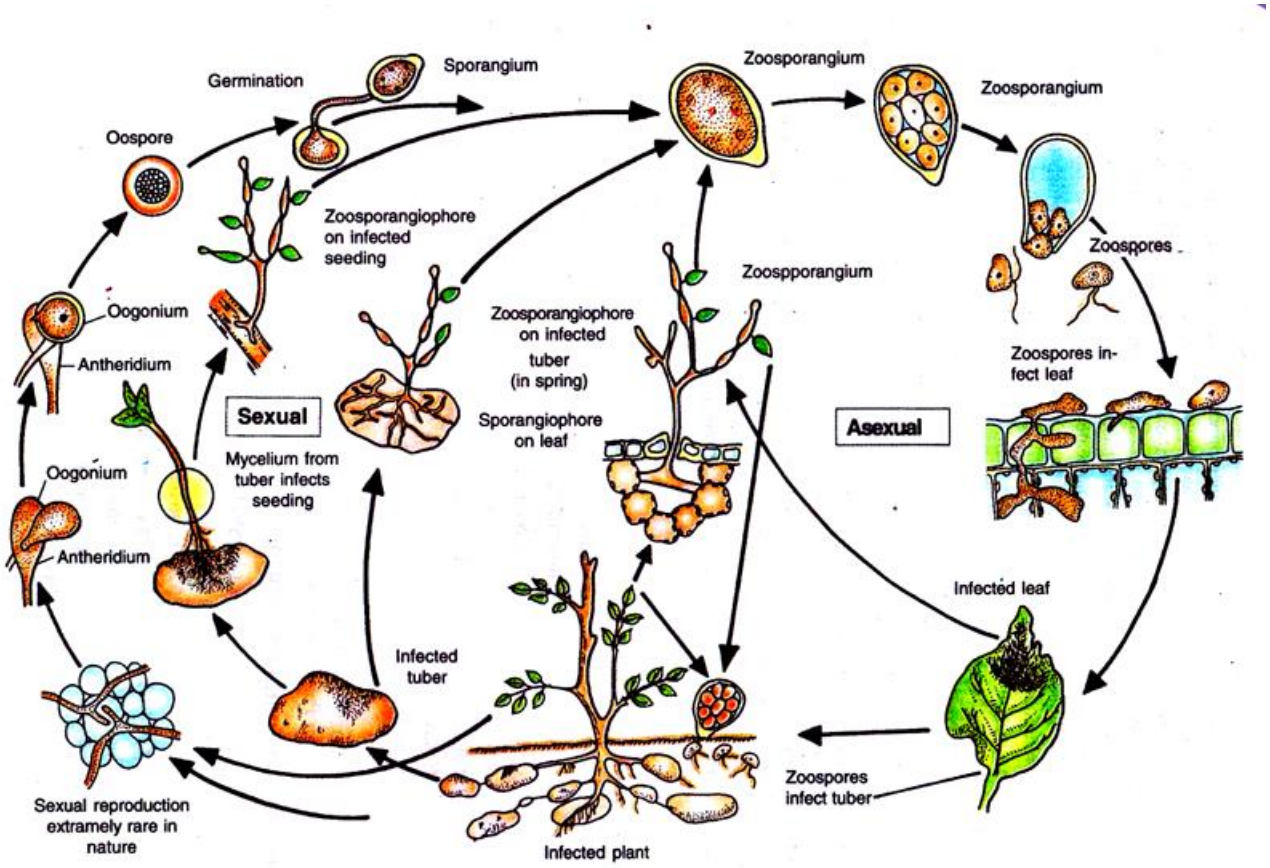

Fig. 1: Lifecycle of Late blight disease caused by of the pathogen P. infestans (Heller, 2004).

Once the host has been reached, the way of penetration of the plant depends on the environmental factors. For a successful infection, the plant tissue must be covered with a water film. Is this not the case, the motile germinated spores are not capable to reach the possible penetration site (Hardham and Blackman ,2010). Previous studies indicate that *Phytophthora infestans* sporulates mostly in dark periods and that the presence of light can have an inhibiting effect on the sporulation. (Nowicki et al., 2012)

The germination can occur in two different ways, depending on the weather condition. Temperatures higher den 21°C stimulate direct germination of the sporangium, which leads to the extension of germ tubes in a duration of 8- 48 hours. The optimum for this process lies at 25°C. If the temperature is below 21 °C, the optimum being around 12°C; zoosporogenesis occurs (Fig. 2 below). The sporangia releases around eight biflagellate zoospores (asexual spores) which are motile in the water film. They detach their flagella and after encysting, start the production of germ tubes. Penetration of the host is possible through the cuticle of the leaf or the stomata. The latter occurring less frequently. For the infection of the plant, the germ tubes convert into appressoria, which ultimately invade the host tissue. At the optimum temperature, this process needs approximately two hours (Nowicki, Kozik, & Fooland, 2013).

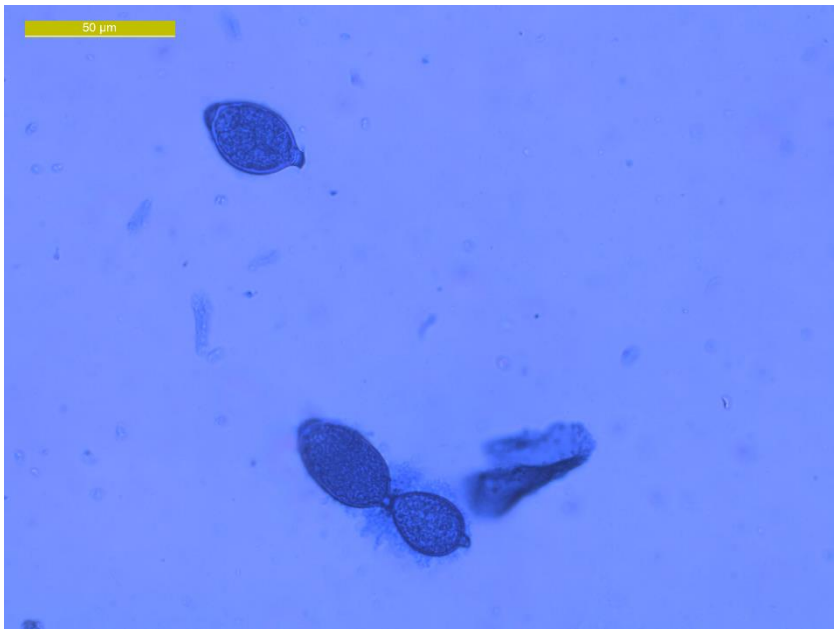

Fig. 2: Sporangia of *P. infestans* releasing zoospores due to low temperature.

The pathogen is heterothallic and seems to have two mating types A1 and A2. Sexual reproduction and therefore the formation of oospores is only possible, if the mycelia of the two types interact. Oospores, large and thick-walled, differ from asexual spores in their capability to survive for a long period of time in dead plant tissue or soil. According to Nowicki et al. (2012), the combination of the two mating types allows for sexual recombination and thus the formation of new and possibly more dangerous stains.

It takes generally up to five to ten days for the first symptoms of late blight disease to appear. As seen in figure 3 the symptoms of LBD can become visible on different parts of the plant. (USAblight, 2018). In general the first symptoms that can be spotted on the host, are water-soaked spots on leafs, petioles and stems. They can grow rapidly and cover up the whole abaxial surface of the leaf with lesions as described by Nelson (2008)

Under humid condition sporulation of the oomycete on the plant can be observed leading to shriveled and necrotic leafs, which in the end die (USAblight, 2018). In addition, the green tomato fruit can develop greasy spots and lesions (Nelson, 2008).

Fig. 3: Symptoms of late blight disease on tomato. Top left: lesions of the stem. Top right: Sporulation of the pathogen on a young tomato leaf. Bottom left: Necrotic lesion on leafs and bottom right: Lesion of a tomato fruit (USA-blight, 2018).

Infected plants suffer from reduced photosynthetic leaf area, defoliation, reduced plant vigor, loss of fruits and seeds, which ultimately leads to the death of the plant (Nelson, 2008). The disease cycle restarts through the sporulation of 2N sporangia, which then again release zoospores that are easily transmitted via air. The disease is usually not able to continue if the temperatures climb above 35°C. Nevertheless, the pathogen is able to survive in plant tissue and can therefore, once the abiotic factor become more favorable, reemerge (Nowicki, Kozik, & Fooland, 2013). Under the right environmental conditions the pathogen has a short regeneration time and the infection cycle can restart in 5-7 days (Nowicki et al.,, 2012).

1.3 Anthracnose and black dot disease

Green house production of vegetables is often limited to a few cultivars such as tomatoes. The production system also does not allow a flexible crop rotation compared to outdoor cultivation. These two factors enhance the chance of the development and establishment of soil borne pathogens such as *Colletotrichum coccodes* (Andermatt Biocontrol , 2017).
The pathogen is defined as an imperfect fungus, which belongs to the *Melanoconiales* (Lee & Hilton, 2003).Possible hosts are mostly Solanaceae like tomatoes, potatoes, paprika and egg plants (Foolad, Ashrafi, & Merk, 2008).
Temperature optimum for the germination of conidia lays at 22°C, whereas temperatures around 7° C prevent conidial germination. The highest culture growth rate can be observed at 25-31°C with an optimum around 28°C (Lee & Hilton, 2003). *C. coccodes* can cause two different kind of diseases on tomato. One is the black dot on the roots the other anthracnose of the fruit (Dillard & Cobb, 1998).

Fig. 4: Tomato roots with sclerotia visible as black dots on the left. On the right, young tomato roots with sclerotia (Michel & Terrettaz, 2011).

Garibaldi et al. (2008) describe the symptoms of black dot root rot on tomato as deteriorations of both old and new roots (see Fig. 4). The infected parts show signs of large necrosis and the tissue turns black with the possibility of cracks. The lesions are covered with small black sclerotia, which according to Dillard and Cobb (1998) gave the disease the name black dot. Garibaldi et al. (2009) claim that the disease is especially recognizable on older roots, leading to gray-black tissue. The pathogen can strongly reduce the plants ability to absorb water and nutrient, leading to wilt and deficiency symptoms (Michel & Terrettaz, 2011).
As mention above, the pathogen can also cause anthracnose. Most production systems in Switzerland do not promote the development of this particular disease but it is described as a

devastating ailment affecting mostly processing tomatoes in the United States, where it can result in crop losses up to 70%. Favorable condition, such as regular rainfall, warm temperatures and high humidity can lead to the appearance of symptoms 5-6 days after inoculation (Dillard & Cobb, 1998) (Byrne, Hausbeck, & Hammerschmidt, 1997). In this case, the first visible symptoms on the fruit are small sunken and dark lesions leading to a larger soft and sunken area on the tomato (see Fig. 5 below). Black acervuli can be observed just beneath the skin tissue of the fruit. As the disease continues to progress, the lesions continue to mature and the stomata of the acervuli develop setose sclerotia. These scleortia enable the pathogen to overwinter and survive in soil (Dillard & Cobb, 1998). New studies indicate that the pathogen is able to survive up to 8 years in soil (Michel & Terrettaz, 2011).

Fig. 5: Tomato fruit showing symptoms of anthracnose caused by *C. coccodes.*

Even though the pathogen has not been associated with foliar blight on tomato, it can infect the foliage, leading to small necrotic spots. This infection might not cause significant damage to the foliage itself, but through sporulation, a potential inoculum for the nearby fruits could develop (Byrne J. et al.,, 1998).

As mentioned by Michel (2011) in the previous chapter 2.1, the Swiss tomato production is manly done in green houses. This means that the problem of *Colletotrichum coccodes* is, or could become, relevant for the Swiss production system.

Furthermore the soil born pathogen can be considered an interesting test-object for this Master's-thesis due to its capacity of infecting both potato and tomato plants. Lee and Hitlon (2003) mention that the isolates found on potato could not be distinguished morphologically from those taken from tomato plants.

1.4 Management methods of *P. infestans* and C. *coccodes*

P. infestans, being the hazardous pathogen that it is, is the topic of many research projects, including studies on new control measures against it. Today's disease management practices include cultural practices to reduce inoculum, such as crop rotation, irrigation techniques (Heller, 2004), but also the application of chemical fungicides and resistance breeding (Nelson, 2008) (Nowicki et al., 2012). Especially the usage of chemical control substances has been increased. The demand for protectant like chlorothalonil or systemic fungicides has grown over the last few years. However according to Nowicki (2012) chemical treatments can be ineffective in certain circumstances, for example if the abiotic factors seem to be highly advantageous for the development of the pathogen. In addition, the abundant application of substances like phenylamide resulted in a higher selective pressure on the oomycete, leading to de development of resistant isolates in some countries. Moreover, the genetic variations between the different strains allowed the pathogen to overcome resistant plants (Nowicki, Kozik, & Fooland, 2013). Concerns about chemical products regarding their safety and negative impacts on both humans and the environment become more and more concrete, leading to the withdrawn of different products from the agricultural sector. The tendency towards stricter regulation and instruction could be observed in the last few years (Bundesrat, 2017). Therefore, not only the sustainable agriculture sector needs to develop new strategies where *P .infestans* is concerned but it is a pressing issue for the whole sector as well. As an additional strategy, Foolad et al. (2008) suggest that foliar wetness should be reduced by usage of soil directed irrigation techniques to diminish disease pressure. They further stress the potential of disease forecast systems. Nelson (2008) advises further to combine crop rotation with non-susceptible host plants, usage of selected with good crop sanitation to achieve an integrated pest management.

Dillard and Cobb (1998) list multiple application of protestant fungicides as a possible control measure against *C. coccodes*. They further suggest that well drained land, elimination of host weed and crop rotation can decrease the disease pressure. They also mention the usage of tolerant and resistant cultivars as a possible strategy against the pathogen. Experimental studies executed by Garbialdi et al. (2008) were not able to confirm that rootstock grafting as a single measurement is able to control the pathogen. Only the chemical soil fumigation showed promising results. Since these products are not approved for the usage in Switzerland, and as for now, there are no systemic fungicides against the disease, other solutions are required. Unfortunately, the host range of the disease includes several cultivars, which are often cultivated in greenhouses. Therefore, Michel and Terrettaz (2011) state, that crop rotation is not an effective strategy but measurers regarding hygiene are. They however imply that the cultivation in soil could reduce the danger of inoculum (Michel & Terrettaz, 2011).

1.5 Bacteria associated with entomopathogenic nematodes

The bacteria of the genera *Xenorhabdus* spp. are gram-negative *Entorobactericeae* that are often associated with the soil-living, entomopathogenic nematodes *Heterorhabidis* spp., of the families *Steinernematidae* (Ng & Webster, 1997).

The bacteria are motile due to petrichous rods. They are further described as porogenous and approximately 0.8-2.0 by 4.0 to 10 micrometers in length. They are facultative anaerobic and nutrition is chemoorganotrophic (Thomas & Poinar, 1997).

The bacterial symbionts are released into the host by the infective juvenile stage of the nematodes, which carry them in a vesicle in their intestine. This way the nematode allows them, to be transported into the insect and provides the bacteria protection against the immune responses of the victim. Once the juvenile has penetrated the host, it releases the symbionts into its hemocoel. The bacteria reimburses its symbiotic partner by providing nutrients, producing antimicrobial substances and maintaining favorable conditions for the entomopathogens reproduction. This collaboration results in the death of the host insect within 48 hours after the infection of the pathogen. Even after the death of the host, the two symbionts keep growing and reproducing. Once a new generation of juveniles emerge, including the bacterial symbiont, a new infection cycle can start (Hu & Webster, 2000) (Boemare, Akhurst, & Mourant, 1993).

In addition to influencing environmental conditions inside insect hosts, the symbionts have also the ability to make different artificial produced media suitable for the reproduction of the entomopathogenic nematodes. This is an important step for the mass production of the nematodes, used for the control of insect pests in agriculture (Akhurst R. J., 1980).

The bacteria, naturally only occurring in the intestine of their symbionts, can be cultured artificially both *in vitro* on solid media or in liquid fermentation (Shapiro-Ilan, Bock, & Hotchkiss, 2014).

The species of *Xenorhabdus* can occur in two forms of colonies. They differ from each other by form, color and their ability to absorb bromothymol, which only the primary form is capable of. So far, only the first form has been isolated from infective nematodes. R. J. Arkhurst (1980) states that the unstable first form produced the secondary form, and that the primary form, unlike the secondary form, is able to produce antibiotic metabolites. The effect of such secondary compounds can be observed when infected insects do not decay, as seen in other cases, due to the antibiotics preventing the development of other competing microorganisms (Hu & Webster, 2000) (Ng & Webster, 1997). Different biological qualities can be associated with metabolites, and many are of importance to the agricultural, nutritional and pharmaceutical sector (Gokulan & Kahre, 2014). These biologically active compounds, or secondary metabolites, can be found in the supernatant of the respective bacterial solution

(Böszörményi, et al., 2009). These secondary metabolites are not essential for cell growth, but play an important function regarding the survival strategies of the organisms. They are usually synthesizes when the organism is stressed, due to environmental factors, lack of nutrition or other conditions limiting its development (Gokulan & Kahre, 2014).

So far, different compounds with antibiotic activity produced by *Xenorhabdus* spp. have been identified, including indoles, xenorhabdins, xenocoumacin, nematophin, didithiolopyrrolones and several others depending on the species and strain of the bacteria (Fang, Li, & Zhang, 2011). Findings by Sigh et al. (2015) state that the production of antimicrobials are highly influenced by environmental conditions. Additional factors such as cell density and intercellular signaling influence the time of synthesis and the produced levels of antimicrobial metabolites as well. Studies showed that the antibiotic activity of *Xenorhabdus nematophila* grown in Grace's insect medium were significantly reduced compared to LB broth, stressing the fact that synthesis of secondary metabolites can be influenced by abiotic factors (Singh, et al., 2015). Furthermore Maxwell et al. (1993) define the availability of nutrients as a limiting factor for the production of antibiotics, whereas heat stability was confirmed.

Different authors suggest that the antibiotic compounds synthesized by *Xenorhabdus* spp. show antagonistic effects against different plant pathogenic fungi (Shapiro-Ilan, Bock, & Hotchkiss, 2014). Trials conducted by Akhurst (1982) where different primary forms of *Xenorhabdus* were capable to inhibit the development of different microorganism such as *Escherichia coli*, *Bacillus thuringiensis* etc. confirm this hypothesis. The secondary form however were not able to suppress the growth of the tested organism. In addition, the study indicates that, different bacterial strains produced different antibiotics, which could lead to strains having a specific host-pathogenicity.

Ng and Webster (1997) studied the organic metabolites which phase one of *Xenorhabdus bovienii* strain produced and could detect antimycotic traits against pathogenic fungi *Phytophthora infestans, Pythium ultimum* and *Rhizoctonia solani*. Tests on agar showed, that the bacteria suppressing proprieties were most effective against *P. infestans* leading again to the assumption, that the produced metabolites showed selectivity against specific pathogens. In pot-trials the antimycotic metabolites were applied directly on the leaves of potato plants. The results showed a reduction of 24% of blight symptoms on treated plants. They further stated that control croups treated with only the antimycotic metabolites showed signs of phytotoxic damages on the plant, which increased with higher concentration. According to Fang (2011), the leaves would be able to keep growing, hence recover from the toxic effect.

In 2009, Böszörményi et al. tested different strains of *Xenorhabdus* against *Erwinia amylovora* and *Phytophthora nicotianae* and came across antagonistic effects against both plant pathogens.

Unpublished data based on different experimental trials conducted at ZHAW, Switzerland delivered further evidence that *Xenorhabdus* strains have different capability to suppress microbial growth. A screening assay tested various *Xenorhabdus* strains on their antagonistic effect against *Botrytis cinerea*, *Rhizoctonia solani* and *Phytophthora infestans* (Müller, 2013). In a second screening assay the proprieties of 25 *X. bovienii* strains and their effect on *P. infestans* were of utmost interest. Some of the tested strains were able to inhibit mycelial growth in both an *in vitro* confrontation assay and an *in vivo* detached leaf assay. The trials suggest that especially the strains *X. bovienii n.d* isolated from the nematode *Steinernema faltiae* and *X. bovienii FR44* isolated from *Steinernema intermedium* (#34 and #45 number according to the ZHAW collection) showed potential as an antagonist. In the confrontation assay strain #34 and #45 were able to reduce the development of the oomycete. In both cases the supernatant was more effective than the bacterial solution, leading to a stronger inhibition of mycelial growth. The bacterial solution and supernatant of strain #34 were able to completely suppress the development of *P. infestans* in the detached leaf assay. Strain #45 showed antagonistic proprieties regarding both bacterial solution and supernatant as well, but the effect was effective compared to strain #34. A pot-trial, testing the effect of bacterial solution and corresponding supernatant , led to promising results, suggesting that the strain *X. bovienii FR44* isolated from *S. feltiae* has antagonistic proprieties *in planta* as well. In both treatment groups the reduction in sporangia formation as well as the delay of the infection could be observed (Walch, 2016). On one hand competition for limiting nutrients between the pathogen and the bacteria could explain the reduction of disease pressure, on the other hand secondary antibiotics produced by the antagonist could be the causing factor as well (Singh, et al., 2015). According to Fang et al. (2011), *X. bovienii* produces two classes of antibiotics including indoles and dithiolopyrrolones,as well as exo- and endochitinase, leading to a possible suppression of *P. infestans.* The compound indole is known to possess both anti-.oomycetal and antifungal activity, the first being stronger than the latter. The dithiolopyrrolones poses antifungal activity as well as against gram-positive bacteria, whereas *X. bovienii's* chitinase seems to have antimycotic activity against the conidial germination and the elongation of germ tubes of *B. cinerea*. Ganh et al. (2011) assume that the antagonistic effect of different *X. bovienii* strains is caused due to different combinations of indole derivatives and dithiolopyrrolones, even though the specific mode of action of the bacteria against different plant pathogens is not yet known.

In addition to applying the solution directly to the plant Fang (2011) suggest that the soil could be treated as well, reducing the source of initial inoculum regarding annual epidemics. On the other hand concern have been voiced regarding possible negative consequences of such treatments on microorganism living in the soil due to its possible toxicity. (Maxwell, Chen, Webster, & Dunphy, 1993).

1.6 Aim of this study

The purpose of this study was to examine two bacterial strains #34 and #45 and their metabolic products regarding their effect on the two plant pathogens *Colletotrichum coccodes* and *Phytophthora infestans.* While the main focus was on the latter, a possible dual effect was also of interest.

Based on insight gained in previous studies conducted at ZHAW (unpublished data by Walch (2016) and Müller (2013)) where a large number of different strains were tested on their antagonistic role against the LBD, two strains that showed promising results were chosen for further testing.

The study based on the following hypotheses:

- **Hypothesis 1:** The bacterial strains #34 and #45 have an antimicrobial effect against the pathogens *Phytophthora infestans* and *Colletotrichum coccodes.*
  Prediction: Both strains are able to suppress the development and growth of both pathogens.
- **Hypothesis 2:** The antimycotic activity is strain specific.
  Prediction: The strains do not show the same antagonistic effectiveness regarding the pathogen.
- **Hypothesis 3:** The suppressing effect of the stains remains similar in different trail systems.
  Prediction: A strain that shows promising results in one trial will lead to similar results in another trail setting, furthermore if a trial is repeated, similar result are obtained.

In a first step, the two strains were tested on their suppressing effect against both pathogen by applying an *in vitro* confrontation assay. Only after several pre-trials, establishing a successful *in planta* infection protocol with *P. infestans* the pot-trials were planned, Based on the results of the pre-trials and the confrontation assay, different pot-trials were conducted to study the interaction between the two strains and *P. infestans in vivo.*

2 Material and method

2.1 Isolation pathogens and bacteria strains

To study the interaction between *Phytophthora infestans* and *Xenorhabdus bovienii* the *P. infestans* isolate #1-isolation fies, was applied. The pathogen had been isolated on the campus Grüental in Wädenswil, Switzerland.
For the duration of the trials, *P. infestans* was kept on V8 agar (recipe see appendix A).
For the different experiment, spore-solution made from 3-week-old *P. infestans* was required. The pathogen was therefore propagated every week, which would guarantee a viable solution at all times. For this purpose, a sterile cork borer was used to cut out a small plug of agar and mycelium. With a sterile needle the plugs were transferred to the middle of the new petri-dish containing the V8 agar. The plates were then sealed with Parafilm and stored in an incubator (Binder) in the dark at 18°C.

The soil born pathogen *Colletotrichum coccodes* was provided by the research institute Agroscope in Wädenswil. For the multiplication of the fungus, the same procedure as for *P. Infestans* was applied. The only difference was that malt agar was used instead of V8 (recipe see appendix A) based on the recommendation from Agroscope. To promote the pathogens development the petri-dishes were put in an incubator at 23°C. They were kept there until the fungus had overgrown the whole surface of the agar. Subsequently they were stored in a refrigerator at 5°C.

Before the first trial started the bacterial strains #34 and #45 were kept in a freezer at -86°C for storage. They were kept in tubes containing standard broth and 30% glycerol. Once single bacterial colonies were needed for the first experiment, the tubes were taken out of the freezer and defrosted. After they reached room temperature, they were vortexed (Heraeus® Multifuge 1S).for 15 minutes at 3200 rmp. Working in the sterile bench, using a single-use sterile inoculation loop a small amount of solution was dispersed on different petri-dishes containing standard agar (recipe see appendix A). The plates were then sealed with parafilm and put in an incubator at 23°C. Once the bacteria had developed the petri-dishes were transferred to a refrigerator were they were retained at 5°C.

2.2 *In vitro* confrontation assay

The first trial consisted in an *in vitro* confrontation assay where the two stains of bacterial antagonists were confronted with either *Phytophthora infestans* or *Colletotrichum coccodes.* The confrontation assay was set to have 14 different treatment groups as shown in table 1 below.

Table 1: List of all treatment groups used in the *in vitro* confrontation assay and their composition.

ID Nr.	Treatment Group=Agar+component	Code	Agar	Nr. of repetition
1	Control group Phy+sterile water	C-Phy	V8	7
2	Control group Coc+sterile water	C-Coc	Malt	7
3	Control group Xen #34 bacterial suspension	C-Xen#34B	St	9
4	Control group Xen #34 supernatant	C-Xen#34P	St	9
5	Control group Xen # 45 bacterial suspension	C-Xen#45B	St	9
6	Control group Xen # 45 supernatant	C-Xen#45S	St	9
7	Xen #34 bacterial suspension+Phy	Xen#34B+Phy	V8	9
8	Xen #34 supernatant+Phy	Xen#34S+Phy	V8	9
9	Xen # 45 bacterial suspension+Phy	Xen#45B+Phy	V8	9
10	Xen # 45 supernatant+Phy	Xen#45S+Phy	V8	9
11	Xen #34 bacterial suspension+Coc	Xen#34B+Coc	Malt	9
12	Xen #34 supernatant+Coc	Xen#34S+Coc	Malt	9
13	Xen # 45 bacterial suspension+Coc	Xen#45B+Coc	Malt	9
14	Xen # 45 supernatant+Coc	Xen#45S+Coc	Malt	9

For this experiment, three different types of agar were needed. For each treatment group 100 ml agar was prepared separately in a 250ml Schott bottle. For the treatments regarding *P. infestans*,V8 agar was produced whereas for *C. coccodes* malt ,and for the control group of *X. bovienii* Standard medium was required.

To prevent thickening of the agar the bottles were kept in a water bath at approximately 45°C for the duration of the experiment. To obtain the needed bacterial suspension and the corresponding supernatant 4 single cell colonies of both strains were added separately to 4 Falcon tubes containing 35 ml standard broth. For the duration of the incubation time of 24 hours the Falcon tubes were put on an elliptical lab shaker.

All tubes were then centrifuged for 15 minutes at 3200 rmp. Afterward the supernatant was poured into a new Falcone tube. To each Falcon tube containing the bacteria, 5 ml 0.9% NaCl solution was added and with help of the vortex stirred.

The optical density (OD) of the bacterial solution was measured at 600 nm.

To determine the OD NaCl solution was used as a reference value. For the bacterial solution an OD 0.625 was targeted. This was done by adding stepwise 0.9% NaCl solution.
For each treatment group different components were added to the corresponding agar as previously seen in table 1. A ratio of 9:1 of medium and solution was obtained by adding 11.1 ml solution to 100 ml agar. 11.1 ml sterile water was added to V8 agar for the Phy control group and to malt agar for the Coc control group. For the bacterial control groups and the treatment groups 11.1 ml bacterial suspension of one strain or the respective supernatant was added to the medium.
To prevent the agar from thickening during the preparation of the various solutions, the Schott bottle was again kept in a water bad and put on a heated magnetic stirrer. By doing so, the temperature of the liquid was kept at approximately 45°.
With a pipette 10 ml of agar were transferred to the petri-dish. For each treatment 9 petri-dishes were prepared the same way. They were kept in the sterile bench to allow cooling. Once the agar had thickened and dried the pathogens were applied to the medium. The pathogens were added to the petri-dishes the same way as described in chapter 2.1. In case of *P. infestans* the plugs were produced out of 3 week fungus, whereas the plates containing *C. coccodes* were prepared 4 weeks before the confrontation assay. The plugs of pathogens were taken from one petri-dish per pathogen and put in the center of the new plate. To obtain an exact position a template with a pre-marked position was put under the petri-dishes as documented in figure 6.

Fig. 6: Plates put on the template to ensure a uniform position of the pathogen for all plates.

As a last step, the plates were sealed with parafilm M and stored in an incubator without light at 18°C for 8 days.

To quantify the growth of the pathogens, the mycelium was traced with a permanent marker at 2, 4, 6 and 8 days after inoculation (Fig. 7) The area was then measured using a planimeter (Tamaya® Planix 5). Each line was measured 3 times and then the arithmetic mean was calculated.

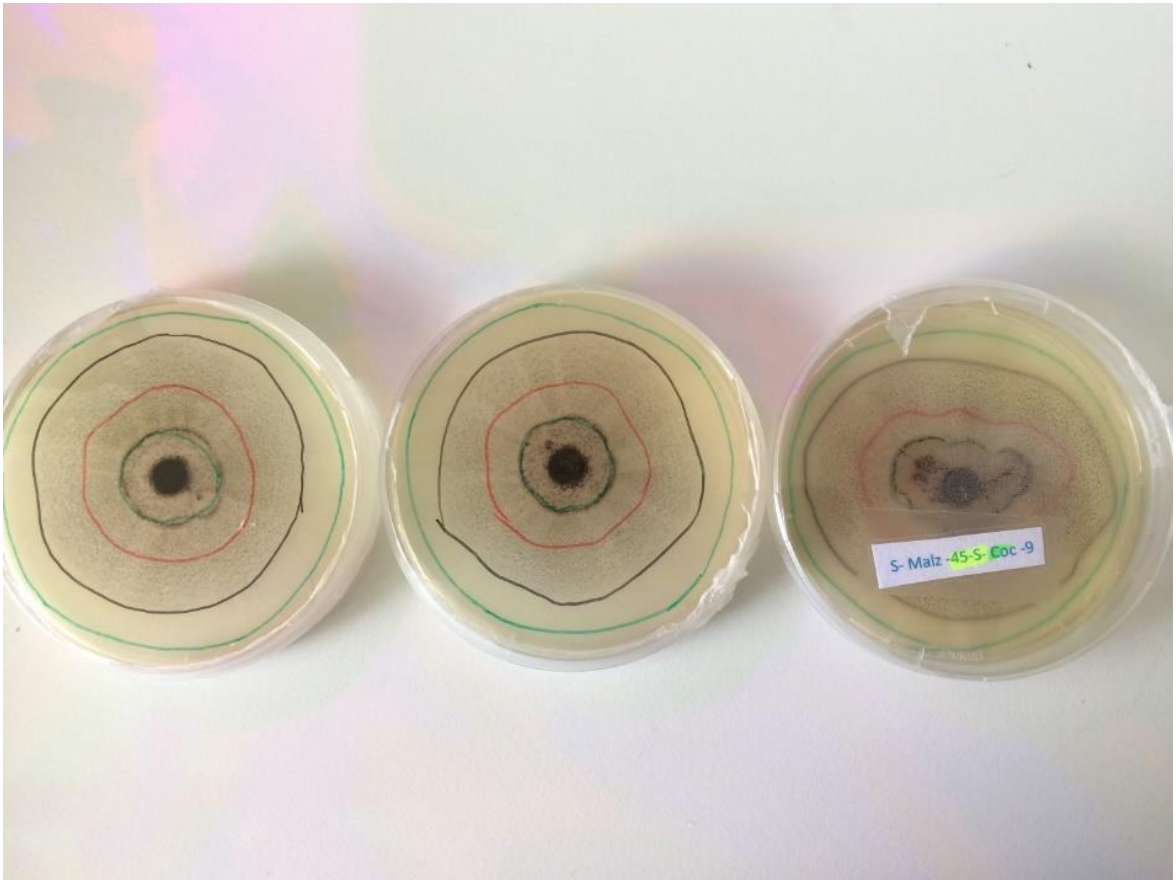

Fig. 7: The growth of the pathogen was traced after every two days with a permanent marker in the back of the plate.

Once the raw data was gathered, normality was tested using a Shapiro-Wilk test. Homoscedasticity was tested with both Levene's Test and Barlette Test. If the requirements of normal distribution and equal variance were fulfilled a one-way Anova was applied with a post hoc pair-wise t test. If the needed assumption were not met, a non-parametric Kruskal-Wallis test was used and for pairwise comparison a pairwise Wilcox rank sum test. For all tests alpha was 0.05.

2.3 Pre-trials infection protocol *P. infestans*

For the pot-trial to produce significant results, a successful infection protocol of *Phytophthora infestans* on tomato was important. Thus, several pre-trial were conducted to establish the parameters and factors that would lead to a successful infection and development of the pathogen.
While certain parameters were adjusted for the different experimental settings, some aspects were deliberately kept the same for all pre-trials as visible in the table 2 below.
Parameters like humidity, hours of light and temperature were adopted from previous trials conducted at ZHAW. This would allow to compare the new results with the data already at hand.

Table 2: List of parameters that remained the same over all 3 pre-trials.

Fixed parameters	Description
Cultivar	Roter Gnom(Syngenta)
Temperature	18°C
Light hours	14 h
Humidity growing chamber	80%
OD bacterial solution	0.625
Concentration spore-solution	Ca. 5000 spores/milliliters

The following settings remained the same for all 3 pre-trials:
For all experiments, the used tomato cultivars were Roter Gnom from Syngenta. As of shortly they have been withdrawn from the market due to their high susceptibility to *Phytophthora infestans* which makes them perfect test objects for this Master's thesis. The tomato plants were potted separately in 9cm plastic pots and filled with soil (Floradur from Floragard ®) and kept at room temperature under artificial light for 14 hours per day.

Regarding the treatment compilation, each trial consisted of at least 2 treatment groups and a control group. Each group contained at least 6 tomato plants that were put into different plastic tray and covered with a plastic cover. In a plastic measuring cup a logger (Elipro) was put and added to one of the trays to control de development of the air humidity and the temperature inside the covers. For all three experiments, the plants were sprayed with tap water until run-of. In addition, the insides of the covers were sprayed and water was poured into the trays.
The control groups were only sprayed with tap water, whereas the treatment groups were treated additionally with the pathogen.
For all pre trials, the spore solution was prepared the same way. Working in the sterile bench, using a pipette, 10 ml of sterile pure water was added to the petri-dish containing

three week old *P. infestans*. Subsequently a microscope slide was dipped in 75% ethanol and sterilized by heating it with the Bunsen burner. After waving it for at least one minute, to insure the glass cooled down, it was used to softly scrape of the mycelium. Afterwards the solution was poured through sterile glass funnel filled with glass wool into a sterile spray bottle (50ml). Once the spore solution was obtained, its concentration was determined using a microscope and a Neubauer improved cell counting chamber. Following this, the solution was kept in a refrigerator at 5°C to promote the development of zoospores. The concentration of the spore solutions used in the three pre-trials was approximately 5000 spores/milliliters. After applying the spore solution the plants were kept in a growing chamber at 18°C to promote the development of zoospores. The humidity was fixed at 80%, which was the highest setting that the growing chambers would allow. The settings for the photoperiod were 14 hours of light and 10 hours of darkness.

2.3.1 First pre-trial

In a first setting, the mother plants were used. These plants had been cultivated from seeds 3 moth beforehand. The plants were put on trays with a metal frame that could be covered with a plastic tent(see Fig. 8 below)., to increase the humidity. It also served to prevent the infection of the control group with the pathogen, since all trays were allocated in the same growing chamber (Fitotron® SGC120) After inoculation with *P. infestans* the plants were kept in the growing chambers for 10 days with the standard settings for light, humidity and temperature. 10 days after inoculation all plants were visually analyzed for symptoms of LBD.

Fig. 8: Plastic trays with metal frame, covered the plants with a plastic tent.

2.3.2 Second pre-tiral

7 days prior the start of the pot trial tomato cuttings of the cultivar Roter Gnom were prepared by cutting of the main shoot of the mother plant. They were then put into 9 cm plastic pots filled with seeding compost. For acclimation, the cuttings were put into the growing chambers where they were exposed to 14 hours of artificial light, 23 °C and 60% humidity.
The decision to use cuttings was based on pre-trials conducted by the author of this thesis, that showed that freshly produced cuttings were more susceptible to the pathogen.
For the infection trial, the trays with the metal frame and the plastic cover were used again. Since the first 24 hours are crucial for the development of the pathogen, after the inoculation the plants were kept in darkness. The absence of light would decrease the transpiration of the water film on the leaves, hence promoting the development of the oomycete and ultimately allow the pathogen to penetrate the tissue. After that, the settings for humidity and light were the same as described in chapter 2.3. The plants were visually analyzed after 14 days.

2.3.3 Third pre-trial

In the third experiment, the goal was to increase the humidity, thus promoting a faster development of the pathogen. This was done by using smaller plastic trays with no framework (Fig. 9). Cuttings, which had been produced 7 days earlier the same way as seen in chapter 2.3.2, were inoculated with *P. infestans* and each tray was put in a plastic bag, which was then sealed by cord. By downsizing the plastic trays, the air volume of the infection-tent was reduced, which should allow to reach a humidity up to 100%. The rest of the trial settings were kept as seen in the second pre-trial bevor. 10 days after trial start, the plants were again visually analyzed. As an additional control measure, after the termination of the experiment samples of the visible mycelia were studied under the light microscope to identify the pathogen.

Fig. 9: Tomato cuttings covered directly with the plastic bag compared to the trays with metal frame.

2.4 *In planta* pot trial

2.4.1 Pot trial bacterial strain # 34

To be able to study the effect of the bacterial antagonist on *P. infestans in planta* a pot trial based on the gained knowledge from the pre-trials were conducted.
For this, 6 different treatment groups were put to use as described in table 3 below. For the following pot trials two growing chambers were at disposal, limiting the number of replicas to three per treatment.

Table 3: List of all treatment groups used in the *in planta* pot trial with bacterial strain #34, their abbreviation and the number of replica including the quantity of used plants.

ID Nr.	Treatment	Code	Nr. of replica	Plants per replica
1	Control-H2O	K-H20	3	3
2	Control bacterial solution #34	K-B	3	3
3	Control supernatant# 34	K-S	3	3
4	Control Phy	K-Phy	3	3
5	Combined bacterial solution #34+Phy	T-B-Phy	3	3
6	Combined supernatant #34+Phy	T-S-Phy	3	3

Each replica consisted of three plats put into one plastic tray. The bacterial solution, supernatant as well as the spore-solution of Phy were prepared as described in chapter 2.2. and chapter 2.3.2. The bacterial solution was deluded to an OD= 0.625 and the spore-solution was as mentioned in chapter 2.3 approximately 5000 sp/ml.
The plants were treated with tap water in the same way as described in chapter 2.3. The Phy-control-group was sprayed with 20 ml of spore-solution per plant on the backside of the leaves. The H20-control-group was sprayed with tap water in the same way.
The control-groups consisting of the bacterial solution or the supernatant were sprayed with 20 ml of solution. The treatments containing the antagonist as well as the pathogen were sprayed with both solutions, first the antagonist then the pathogen.
The general experimental settings were the same as in the third pre-trial, since it had resulted in the most effective disease development. Hence, the trays were covered with plastic bags and sealed with a cord. They were then put inside the growing cabinet and kept again at 18°C, the first 24 hours without light and afterwards 14 hours of artificial light per day at a humidity rate of 80%.

To diminish the possibility of inhomogeneity regarding abiotic factors inside the growing chambers a random block design was applied, meaning that the trials were randomly distributed in the two growing chambers (Fig. 10).

Fig. 10: Growing chamber showing the different treatment.

As an additional control measure, the spore-solution was also applied on V8 agar, and the bacterial solution and supernatant on their respective standard agar (4 repetition for each treatment). The petri-dishes were sealed with parafilm and two per treatment were put in each of the growing chambers.

After 10 days, the plants were visually analyzed. Since plant cuttings had been used it was possible to count the total number of leaves per plant and the percentage of infected leaves. The statistical analysis was conducted the same way as illustrated in chapter 2.2.

2.4.2 Pot trial bacterial strain #45

The procedure for the *in planta* pot trail studying the effect of the bacterial strain #45 was exactly the same as described in chapter 2.4.1. The following table 4 shows the used treatment groups and their abbreviation.

Table 4: List of all treatment groups used in the *in planta* pot trial with bacterial strain #45, their abbreviation and the number of replica including the quantity of used plants.

ID Nr.	Treatment	Code	Nr. of replica	Plants per replica
1	Control-H2O	K-H20	3	3
2	Control bacterial solution #45	K-B	3	3
3	Control supernatant n# 45	K-S	3	3
4	Control Phy	K-Phy	3	3
5	Combined bacterial solution #45+Phy	T-B-Phy	3	3
6	Combined supernatant #45+Phy	T-S-Phy	3	3

2.4.3 Combined pot trial bacterial strain#34

In a third pot trial two different proprieties of the strain #34 were tested. The decision to reuse the #34 strain was based on its the promising results it had produced in the earlier conducted pot trial as well as previous experiments conducted at ZHAW. One question to be answered was, if the time the bacteria had to multiply while on the lab shaker, had an influence on its suppressing qualities. For this, the single cell colonies, once added to the sterile tubes filled with standard-broth, were kept on an elliptic lab shaker for 48 hours instead of 24 hours. In a second step, the bacterial solution and its supernatant were produced as described in chapter 2.1, with an OD=0.625.

Since both bacterial solution and supernatant showed qualities that inhibited mycelial grow, as seen in the previous experiments, a combined solution might result in an even higher antagonistic effect. To test this hypothesis, the bacteria-broth solution was prepared as mentioned in chapter 2.1. Once the supernatant and the bacterial solution were separated, the supernatant was used to delude the bacterial solution to the point of an OD=0.625. This combined solution was then used to treat the respective treatment groups.

All three solution, bacterial suspension, supernatant and combined solution were tested together. The trial setting was the same as illustrated in the previous chapter. Only the number of treatment groups differed from the other pot trials as seen in table 5 below.

Table 5: List of all treatment groups used in the *in planta* pot trial bacterial strain #45, their abbreviation and the number of replica including the quantity of used plants.

ID Nr.	Treatment	Code	Nr. of replica	Plants per replica
1	Control-H2O	K-H20	3	3
2	Control bacterial solution #34 48h	K-B48	3	3
3	Control supernatant# 34 48h	K-S48	3	3
4	Control bacterial solution+ supernatant	K-BS	3	3
5	Control-Phy	K-Phy	3	3
6	Combined bacterial solution #34 48h +Phy	T-B-Phy	3	3
7	Combined supernatant #34 48h +Phy	T-S-Phy	3	3
8	Combined bacterial solution+ supernatant+Phy	T-BS-Phy	3	3

3 Results

3.1 Results *in vitro* confrontation assay

3.1.1 Confrontation assay *Xen* vs. *Phytophthora infestans*

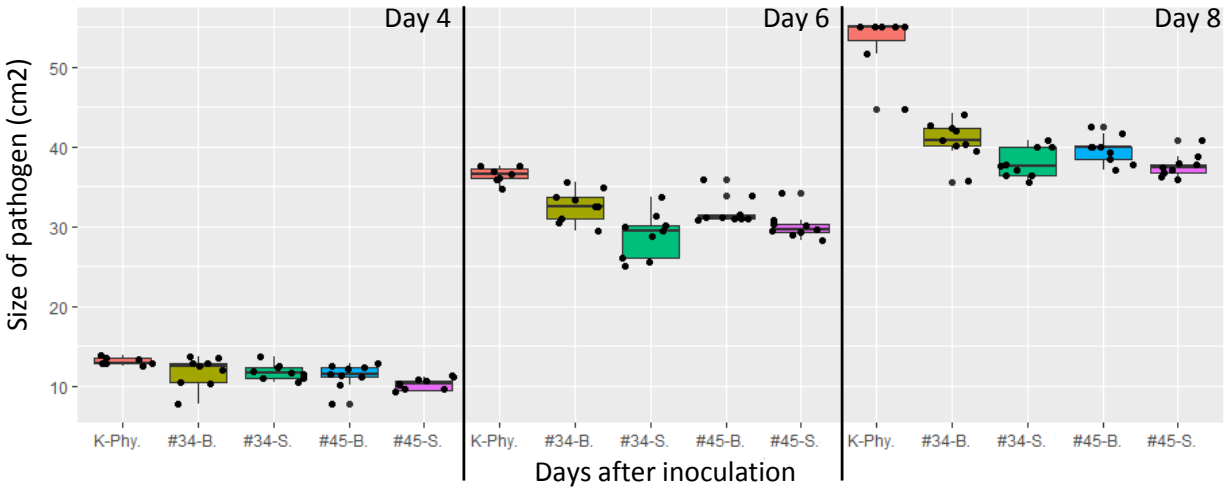

Fig. 11: Results of the *in vitro* confrontation assay *P. infestans* vs. the bacterial solution and supernatant of two different *X. bovienii* stains showing the mean of the measured area in cm2 per treatment at 4,6,8 days after inoculation. Control group K-Phy containing only the pathogen, #34-B=Bacterial solution of strain 34.
#34-S= Supernatant of strain #34. #45-B=Bacterial solution of strain #45, #45-S=Supernatant of *Xen*. #45

The first confrontation assay studying the interaction between the two bacterial strains and *P. infestans* showed promising results (Fig. 11, for raw data see appendix B, for statistical analysis appendix E). In the first days after inoculation, only small differences between the treatment group and the control group could be detected, but as the trail went on, they grew more prominent. At day 4, only the bacterial solution of the strain #45 and its supernatant showed significant differences (P=0.031 and P==0.01) regarding the control group with only *P. infestans.* Furthermore the difference between the #34 supernatant and the #45 supernatant were significant, but the area where #34-S had been applied was not significantly smaller than the control group K-Phy.

Six days after inoculation all treatment showed significant deviations in measured areas compared to the control group. The highest variation could be found between K-Phy and the bacterial solution of both strains. Furthermore both supernatants exhibited strong suppressing effects against the pathogen with a P-value of 0.01. Differences between the treatment groups were only significant between #45-B and #45-S (P=0.032).

The variations after 8 days were even more distinct between the control group K-Phy and all other treatment groups. The bacterial solutions and supernatants of both strains were able to inhibit the development of the pathogen. Differences regarding the treatments were not significant.

3.1.2 **Confrontation assay *Xen* vs. *Colletotrichum coccodes***

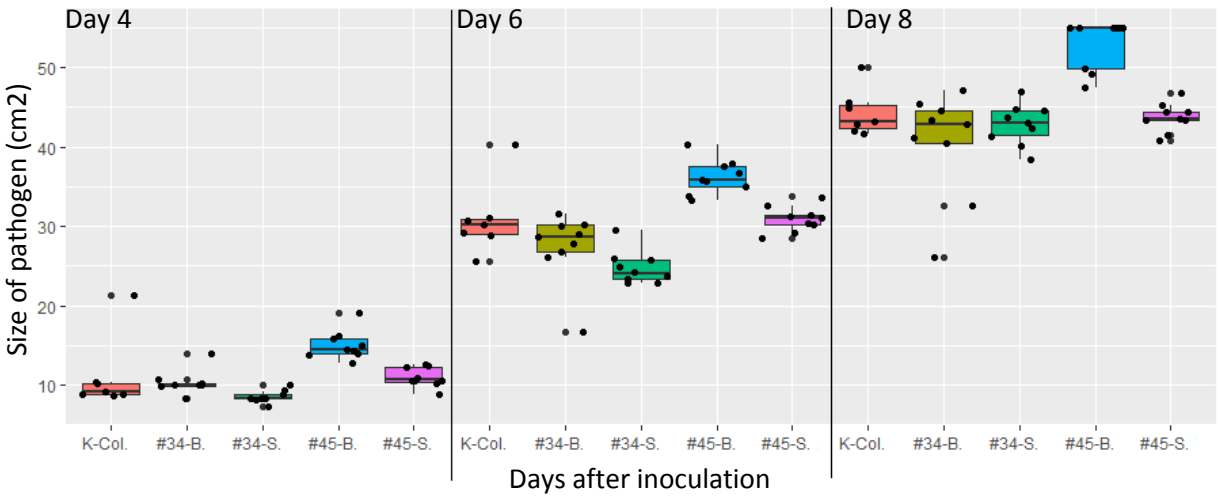

Fig. 12: Results of *in vitro* confrontation assay *C. coccodes* vs. the bacterial solution and supernatant of two different *X. bovienii* stains showing the mean of the measured area in cm2 per treatment at 4,6,8 days after inoculation. Control group K-Phy containing only the pathogen, #34-B=Bacterial solution of strain 34. #34-S= Supernatant of strain #34. #45-B=Bacterial solution of strain #45, #45-S=Supernatant of *Xen*. #45

The results of the confrontation assay between *Xen.* and *Colletotrichum coccodes* showed no reduction regarding mycelial growth of the pathogen (Fig. 12, for raw data see appendix B, for statistical analysis appendix E).

Four days after trial start the only significant differences occurred between the treatment groups them self. The measured area in presence of #45-B was significantly larger compared to the treatment group #34-B (P=0.0069), #34-S (P= 0.0034) and #45-S (P=0.00041) but not in comparison to the control group.

At day 6, the mycelial growth of *C. coccodes* was significantly smaller in presence of #34-S then with no antagonist. The treatment consisting of #45-B showed again a larger area than all other treatments, but without a significant P-value regarding the control group K-Col.

On the last day no antagonistic effect of *X. bovienii* could be detected. Once more the only distinction with a significant P-value occur between #45-B and 34-S (P=0.0032), both bacterial solution and supernatant of the #34 strain (P=0.0032) and the control group. Instead of diminishing mycelia growth, the pathogen showed a larger area with the bacterial solution than without. Furthermore, the variation within a treatment group has to be defined as rather high preventing a significant.

3.2 Results pre-trials infection protocol *P. infestans*

3.2.1 Pre-trial 1

After 10 days, the experiment resulted in a rather moderately successful infection (Fig 13 below). Even though in the first treatment group all plants showed symptoms of late blight disease the opposite was the case for the second group. Here no signs of the pathogen could be detected. The control group treated with tap water showed no signs of *Phytophthora infestans* infestation as well. The humidity rate inside the plastic tent was in average 82%.

Fig. 13: Upper corner left: Control group treated with only tap water showing no sign of infestation after 10 days. Upper corner right: Plants sprayed with *P. infestans* spore solution, with no visible signs of LBD.

3.2.2 **Pre-trial 2**

After 10 days, the control group showed no sign of LBD but both treatment groups did (see Fig. 14). Although each plant showed visible symptoms, the infection developed only slowly and with a mild disease pressure.
With help of the plastic cover it was possible to increase the humidity, resulting in rates between 85,6% and 89,6%.

Fig. 14: Control group inoculated with *P. infestans* after 14 days showing symptoms of LBD.

3.2.3 **Pre-trial 3**

After only 6 days, all plants of the treatment group showed clear signs of LBD whereas the control-group showed none (visible in figure 15 below).

Fig. 15: The treatment group on the left inoculated with *P. infestans* showed clear signs of LBD whereas the control group on the right, treated only with tab water, showed no such signs.

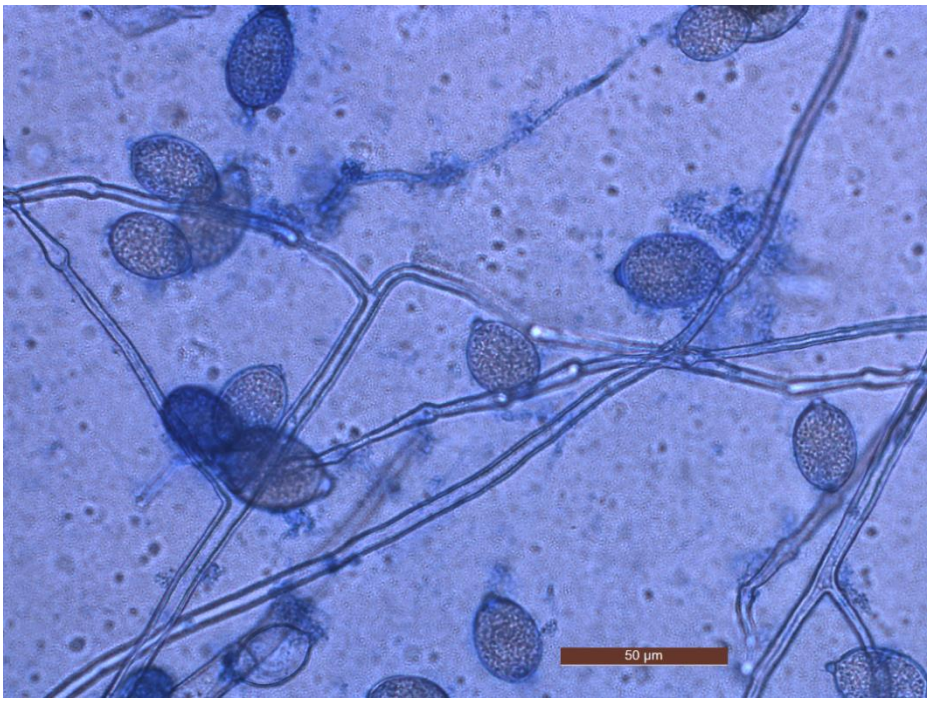

Fig. 16: The lemon shaped sporangia visible under the microscope, a clear signs that the disease symptoms were caused by *P. infestans*

The microscopic control of the visible fungus confirmed that *P. infestans* was the causing agent for the symptoms. As seen in figure 16 the typical lemon shaped sporangia associated with *P. infestans* could be clearly detected.

Results pot trial

3.2.4 Pot trial bacterial strain #34

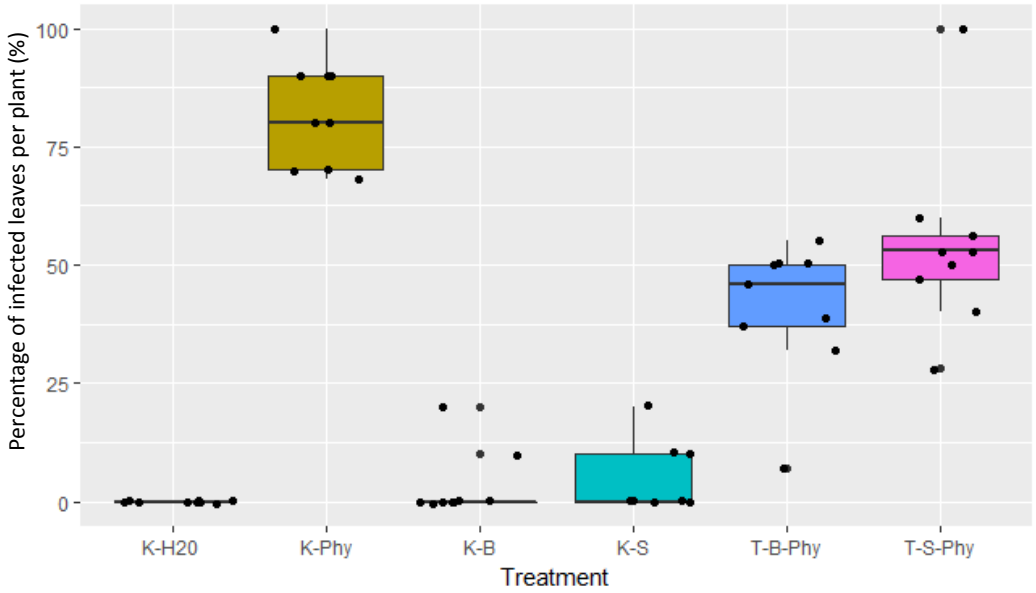

Fig. 17: Results pot trial with *X. bovienii* strain #34 vs. *P. infestans* after 10 days showing the percentage of infected leaves per plant. The four control group consisted of K-H20= only treated with tap water, K-Phy= Control group that was inoculated with the pathogen, K-B= plants only treated with bacterial solution and K-S= only supernatant was applied. Combined treatment included T-B-Phy= Plants sprayed both with spore solution and bacterial solution, T-S-Phy= Treatment of plants with supernatant and spore solution.

The pot trial with the bacterial strain #34 as an antagonist against *Phytophthora infestans* lead to promising results after 10 days. The infection protocol worked out well, leading to an almost complete infection of the control group K-Phy, whereas the control group that had been treated with tap water showed no signs of LBD.

The trial provided evidence of an antimycotic effect of both bacterial solution and supernatant (See Fig: 17. For raw data consult annex B, for statistical analysis appendix E).The percentage of contaminated leaves was significantly higher in case of the pathogen control group K-Phy than for the replicas treated with the bacterial solution and pathogen (P= 0.0031). In addition treating the plants with the supernatant lead to a significant reduction in infected leaves compared to the control group K-Phy (P=0.0261) as well. The treatments with bacterial solution was not significantly better in lowering the infection rate then the supernatant.

The microscopic control affirmed that *P. infestans* was indeed the causing agent for the disease symptoms (see appendix D).

On the control groups for the bacterial solution and the corresponding supernatant, first signs of foliar wilt were visible, but the microscopic control of the leaves showed no infection with the pathogen.

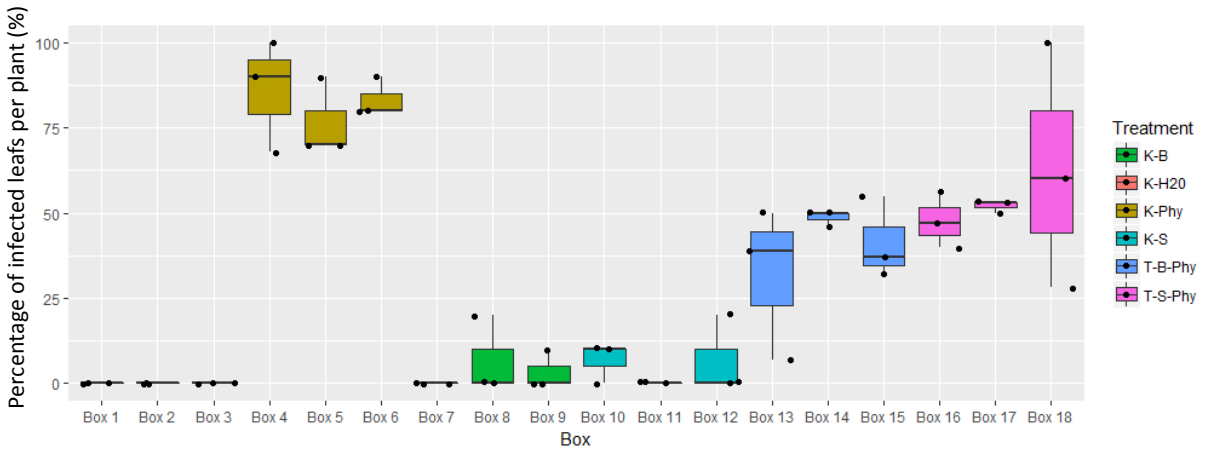

Fig. 18: Treatment listed with corresponding box number they were put in, to test if the allocation of the boxes had an influence on the results.

To see if the position of the trays had an influence on the trial results or if the random block design worked to eliminate the influence figure 18 as seen above was computed.
The results of the control groups K-H20, K-Phy, K-B and K-S strongly indicate that the allocation of the plants inside of the growing chambers had no significant influence on the results. The variation regarding the replicas of the treatments T-B-Phy and T-S-Phy was notably higher in box 13 and 18 in comparison to the other boxes. Nevertheless, it does not indicate that the results were significantly influenced by the allocation of the plants.

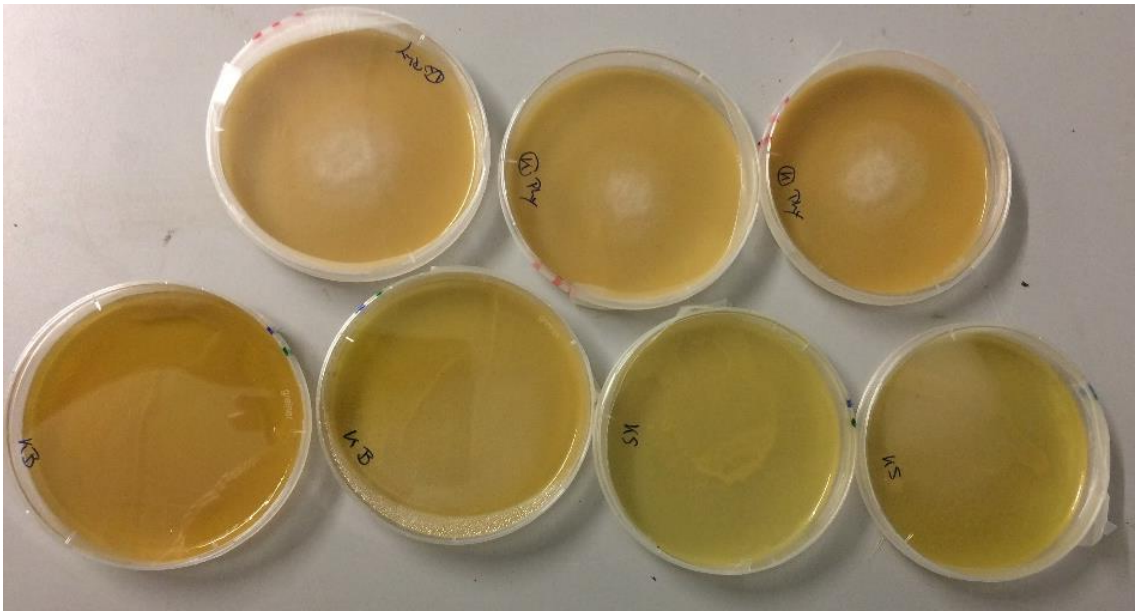

Fig. 19: Control measure, used solutions sprayed on their corresponding agar to monitor their development under ideal conditions.

After 4 days the petri-dishes inoculated with either pathogen, bacterial solution or supernatant showed signs of mycelial grow as well as bacterial growth. Additionally there was no indication of contamination of the applied solutions (Fig. 19).

3.2.5 Pot trial strain bacterial strain #45

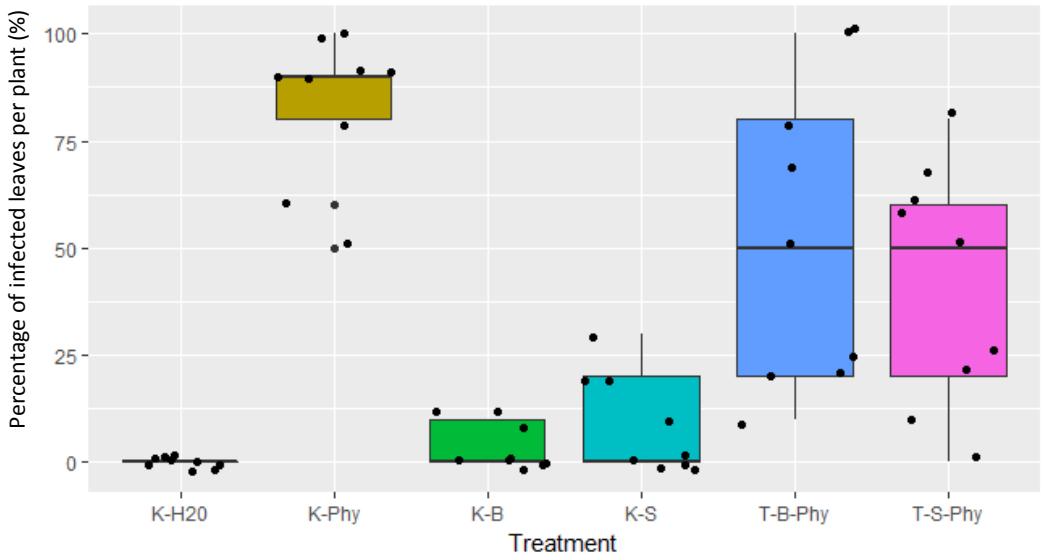

Fig. 20: : Results pot trial with *X. bovienii* strain #45 vs. *P. infestans* after 10 days showing the percentage of infected leaves per plant. The four control group consisted of K-H20= only treated with tap water, K-Phy= Control group that was inoculated with the pathogen, K-B= plants only treated with bacterial solution and K-S= only supernatant was applied. Combined treatment included T-B-Phy= Plants sprayed both with spore solution and bacterial solution, T-S-Phy= Treatment of plants with supernatant and spore solution

In this experiment, where the effect of the bacterial strain #45 was tested, the infection protocol was again successful. The control group containing the tomato plants that were sprayed with tap water showed no signs of LBD, but the pathogen control group clearly did (see Fig: 20. For raw data consult for raw data see annex B, for statistical analysis appendix E).
The application of bacterial solution T-B-Phy resulted in the reduction in infected leaves, but the deviation from the control group K-Phy was not significant. The difference in befallen leaves was only significant regarding the treatment with supernatant T-S-Phy and the control group. *P. infestans* was confirmed as the cause of the LBD symptoms by microscope (see appendix D).
In this trial, the plant with only the bacterial solution or supernatant present, showed again signs of wilt. Also In this case, the microscopic control confirmed the absence of the pathogen.

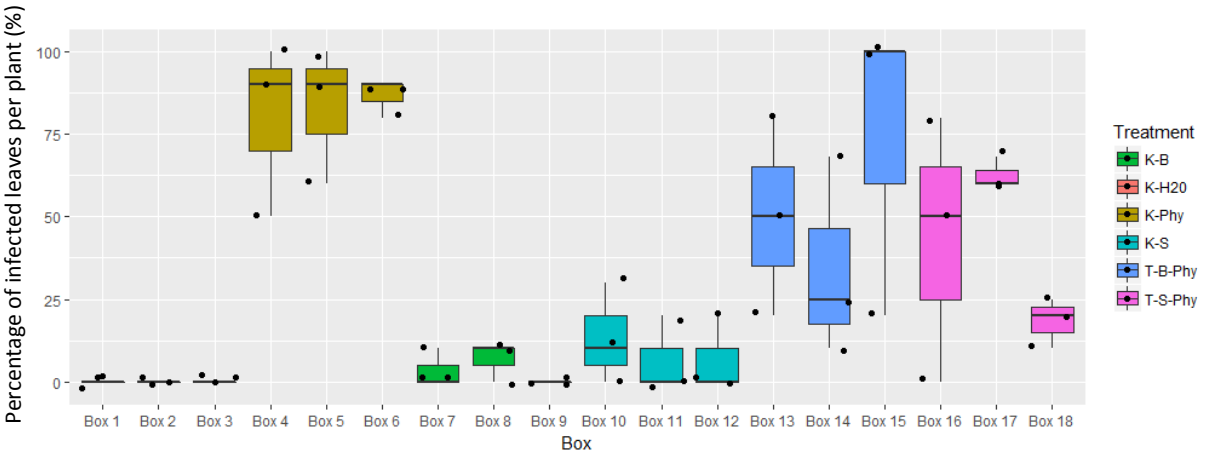

Fig. 21: Treatment listed with corresponding box number they were put in to test if the allocation of the boxes had an influence on the results.

The results within the treatment control groups and the respective boxes were homogeneous for all control groups (Fig. 21). Only the pathogen group K-Phy showed an elevated variance. An influence of the boxes position inside the chamber could not be detected in these cases. This was not the case for the combined treatment groups. The data for both T-B-Phy and T-S-Phy show recognizable differences between the boxes. However, since they also show larger variation within the groups it is not possible to say, if the location of the trays was the influencing factor.

After 4 days it was possible to monitor the development of the pathogen and the two bacterial treatments in their corresponding petri-dishes. Further, there was no sign of contamination of the solutions.

3.2.6 Combined pot trial bacterial strain #34

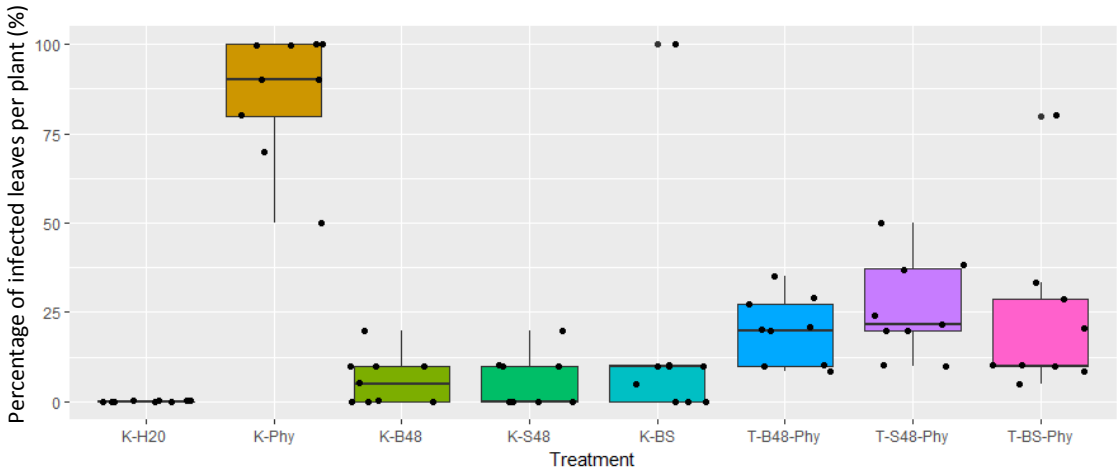

Fig. 22: Results of the second pot trial with *X. bovienii* strain #34 vs. *P. infestans* after 10 days listing the percentage of infected leaves per plant. Treatment groups were T-B48-Phy=Bacterial solution with 48 hours of incubation time and pathogen applied , T-S48-Phy= Supernatant after 48 hours applied and pathogen, T-BS-Phy= Inoculated with pathogen and both bacterial solution and supernatant after 24 hours. The control groups were K-H20= plants treated with tap water, K-Phy= Inoculation with *P. infestans*, K-B48= only bacterial solution applied, K-S48= only supernatant and K-BS= Bacteria solution mixed with supernatant.

The results of this experiment showed significant difference between the various treatments and the control-group inoculated with only the pathogen K-Phy (see Fig. 22. For raw data see annex B, for statistical analysis appendix E). The treatment T-B48-Phy was able to strongly reduce the number of diseased leaves on the plants compared to K-Phy (P=0.0083). The ability to reduce infection could also be associated with the treatment T-S48-Phy (P=0.0093) with no significant difference to the latter. Even the combined solution of bacterial suspension and supernatant decreased the disease pressure compared to the K-Phy (P= 0.016). The differences between the treatments were not significant.

Foliar wilt could be observed once more on the control treatments where only the antagonists were applied.

The presence of the pathogen was again asserted by microscope for the intentionally inoculated groups and refused for the control groups.

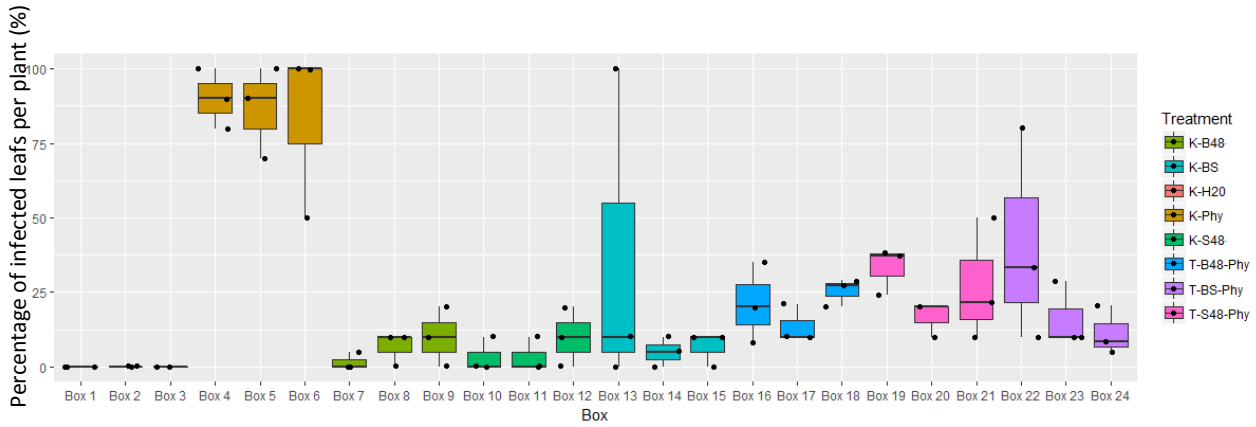

Fig. 23: Treatment listed with corresponding box number they were put in to test if the allocation of the boxes had an influence on the results.

The visual analyzation of the random block design suggests that the location of the different trays per treatment did not influence the results (Fig. 23). Only in case of box 13 and box 22 a higher variance due to one outlier each is recognizable. However, with only three plants per box the results have to be considered rather an indication of a trend and not clear correlations.

The third pot trial included the second control measure as well, with treatment solution that had been applied on agar. The development of the oomycete, the bacterial solution and the supernatant was clearly visible after 4 days, with no indication of contamination.

4 Discussion

4.1 Confrontation assay

4.1.1 *Xenorhabdus bovienii* vs. *Phytophthora infestans*

The results of the confrontation assay *Phytophthora infestans* vs. *Xenorhabdus bovienii* support the hypothesis 1 where *P. infestans* is concerned. Both strains, bacterial solution and supernatant; show signs of antimicrobial effects against the pathogen. Mycelia growth is inhibited, confirming the results of the studies from Ng and Webster (1997) and the trials conducted at ZHAW. The results show no significant difference between the supernatant and the bacterial solution after 8 days, since both are able to hinder the development of the pathogen. This could indicate that bacterial competition with the pathogen, as well as produced metabolites and their proprieties are able to restrain *P. infestans.* Even though all tested solutions exhibit ability to reduce the development of *P. infestans,* none of them seem to be capable to suppress the growth completely. This indicates that the antimicrobial proprieties are in place, but most probably, the procedure of treatment preparation is not yet ideal. One possible aspect could be that after 24 hours on the lab shaker, the bacteria has not developed enough or that the concentration of microbial components is not yet high enough. Furthermore, it should be taken into account, that while the confrontation assay is set to create ideal and favorable conditions regarding the pathogen, this is not the case for the bacterium. Hence, if condition occur that benefit the antagonist, its protective effect might increase.

Hypothesis 2, claiming that the antimycotic activity is strain specific cannot be supported with the results of this trial due to the fact that both strains show antimicrobial activity against *P. infestans.* However, this was to be expected, since the selection of these two strains was based on their ability as antagonists against the oomycete indicated in previous experiments.

Since this was a reproduction of an experiment that already had been computed once at ZHAW, and similar results were gained, hypothesis 3 cannot be rejected. By repeating a trial, the probability of the first results being random, is tested. The results of the confrontation assay verify that there is an antibiotic effect of the two strains against the oomycete and that its effect on the pathogens remains the same in different trials and is hence not caused by chance.

4.1.2 *Xenorhabdus bovienii* vs. *Colletotrichum coccodes*

The in *vitro assay* analyzing the effect of *X. bovienii* on *Colletotrichum coccodes* does not support the hypothesis of an antimicrobial effect of the bacteria against the pathogen (Fig. 24). Only on day 6 the supernatant of strain #34 shows a significantly smaller area of the pathogen. Since the difference after 8 days is no longer significant, this could be discarded as not relevant.
This trial however confirms the second hypothesis, showing that the antagonistic effect of a certain strain can be clearly defined as pathogen specific, which had been indicated by Böszörményi et al. (2009).
Where the hypothesis foresaw the inhibition of the mycelial growth, the opposite can be observed where the bacterial solution of strain #45 had been applied. The treatment facilitates an even larger area than the control-group. Here the possibility occurs that a symbiotic effect between the pathogen and the bacteria exists. It might be that the bacterial solution renders the media even more suitable for *P. infestans* the same way as it is described by Akhurst (1980) for the symbiosis between nematodes and bacteria.

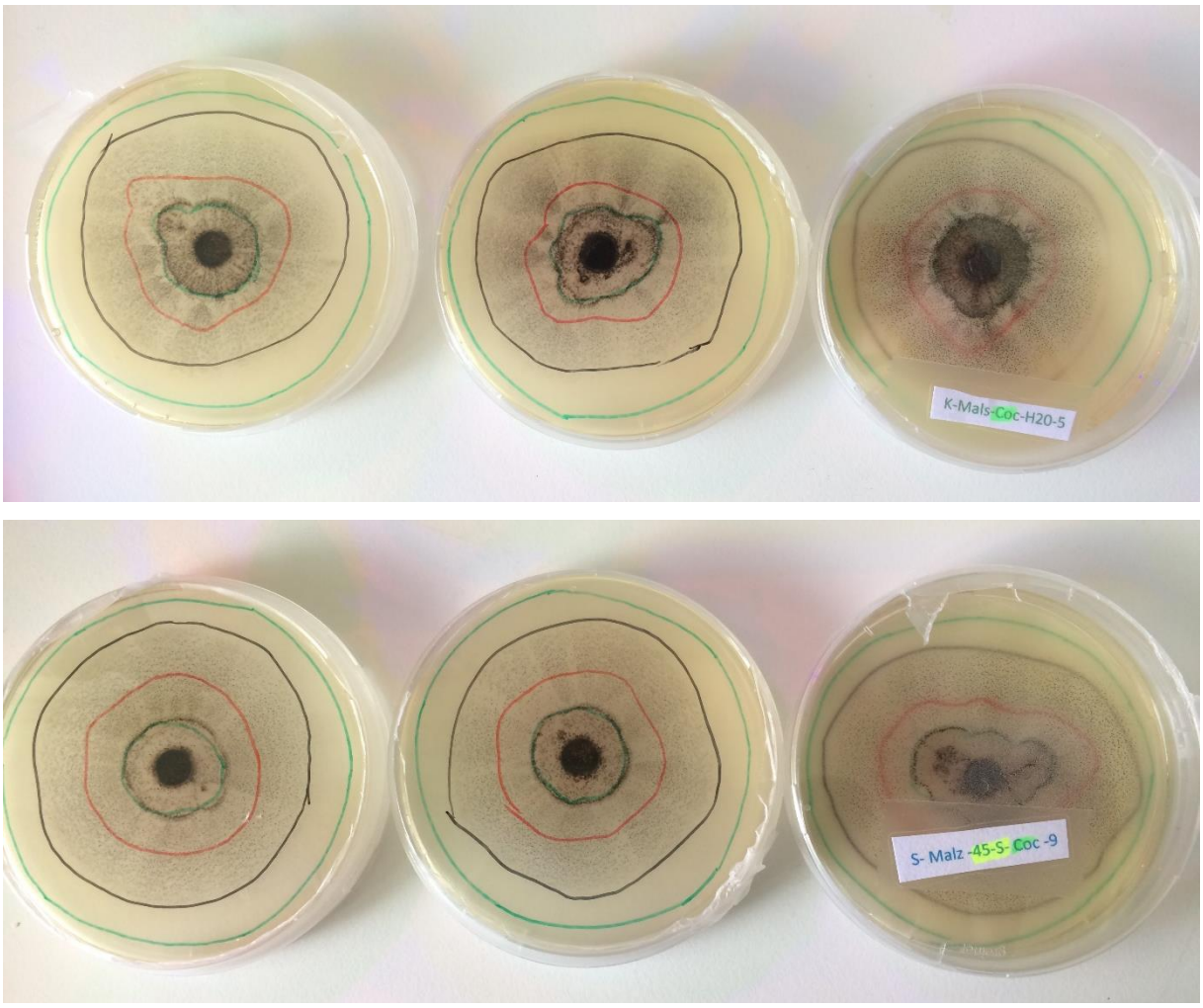

Fig. 24: Three replicas of the control group Pathogen+ sterile water above and three replicas treated with the supernatant of strain #45 and pathogen below. The measured areas are not significantly different.

4.2 Pre-trials infection protocol *P. infestans*

Even though the first replica inoculated with the pathogen showed clear signs of the disease, the infection protocol used for the first pre-trial could not yet be defined as successful. Since the fungus was only able to infest the tomato plants of one out of two replica, the infection protocol had to be further adjusted. As mentioned by Hardham and Blackman (2010) the success rate of infestation depends strongly on the environmental conditions which was obviously not the case in this pre-trial. Even though the plants were sprayed with tap water before inoculation, which should allow the biflagellate zoospores to move and penetrate the leaf tissue as described by Hardham and Blackman (2010), the usage of artificial light shortly after inoculation could have prevented that, due to evaporation of the water film on the leaves. The fact that the humidity rate inside the tents was 82%, which would still allow evaporation, probably added to the fact.

The second pre-trial ended in an infection of all inoculated plants. By keeping the plants in darkens for the first 24 hours, hence preventing evaporation of the water film on the plant tissue seems to indeed promote a first development of the oomycete as Nowicki et al. (2012) pointed out. However, the mild disease pressure had to be adjusted, due to the arising probability of cross infection and diminished plant health caused by the low temperature and the high humidity rate. This aspect was known to be a problem from previous trials done at ZHAW (B. Walch, 2016). With a humidity maximum at 89,6% it is possible, that the rate was not high enough for a fast pathogen development but in the long run, it could have negative impact on the plants. Moreover, the disease pressure might be reduced by the temperature inside the growing chambers, but while Nowicki et al. (2013) state that 12 °C is the optimal temperature for zoosporogenesis of the pathogen, lowering the temperature would lead to an even higher stress rate of the plant.
Consequently a balance between ideal abiotic factors to promote fungal development and condition that would not lead to cross infection and ailing plants had to be found, resulting in a third pre-trial. By increasing the disease pressure caused by a higher humidity rate than in the previous trials, the experiment could be terminated after fewer days, diminishing the influence of temperature and humidity on plant health. The third pre-trial ultimately lead to a successful infection protocol, hence the settings of the growing chamber and the trial setting had been successfully adjusted.

4.3 *In planta* pot trials

4.3.1 Pot trial bacterial strain #45

The pot trial studying the effect of the *X. bovienii* strain #45 validates the hypothesis 1 of antagonistic effects against *P. infestans* once more. The supernatant is capable to reduce the percentage of infected leaves significantly compared to the pathogen control group (Fig. 25). A reduction of symptoms by approximately 40% could be observed. It also confirms the results from (1997) where the metabolites produced by *X. bovienii* could decrease the development of *P. infestans* on potato leaves, even though the reduction rate of 27 % was smaller compared to the results gained in this latest experiment. This is a notable difference, especially considering that NG and Webster (1997) extracted the metabolites from the supernatant of bacteria that were allowed to grow for 94 hours, whereas this experiment used non-filtrated supernatant, which generally exhibit a lower concentration of secondary compounds. Moreover, the supernatant was produced from bacteria that had been incubated in broth for only 24 hours, therefor had less time for development. The difference in effectiveness of treatment could be caused due to the usage of two different strains of *X. bovienii*. As Fang, Li and Zhang (2011) state the produced metabolites and their combination vary depending on the bacterial species or even strain. The bacterial strain #45 either produces a higher number of metabolites in 24 hours of incubation or the combination possesses stronger suppressing abilities. It is also possible that the soy broth used by NG and Webster to culture the supernatant lead to a reduced antibiotic activity compared to the standard broth used in this trial, as it was mentioned by Singh et al. (2015) the broth type can strongly influence the synthesis and activity of metabolites. It is probable that by using standard broth the factors for the development of the bacteria were not ideal, the organism experienced an increased level of stress, leading to the production of a higher number of antibiotics. Since *P. infestans* is an oomycete it is most likely that a high concentration of indole was produced, since this compound is known to possess both anti-.oomycetal activity and according to Fang et al. (2011) *X. bovienii* is able to synthesis it.
The outcome also matches the results of the *in planta* trial by B. Walch (2016) where the supernatant of *X. bovienii* lead to a milder disease pressure. In contrast to Walchs results, here only the plants treated with supernatant show significant reduction in infected leaves, whereas the bacterial solution shows high variability, with no significant deviation from the control group. The same bacterial strain were used for the two trials, but they were isolated from different nematodes. This could indicate that the strains origin effects the mode of interaction between the bacterium and the pathogen. While Sigh et al. (2015) repot that environmental conditions influence the production of antimicrobials, the pot trial suggests that this is valid for the bacteria as well. Since the supernatant of both strains exhibit antagonistic effect, the produced secondary metabolites seem to remain the same, hence are not influenced by the nematode the bacterium was isolated from or the method

that was used to produce them. The variation in efficiency of bacterial solution on the other hand indicates that the strains strategies for interspecies competition differ from one another. In this pot trial the bacteria was not able to prevail over the oomycete, hence the pathogen showed stronger competitive traits in the trial setting that was deliberately design to promote its development. Under environmental condition that are less favorable towards *P. infestans* the bacterial solution might be able to prevail, since the results indeed indicated a suppressing effect, but the differences were not significant compared to the *P. infestans* control group due to its high variance. The fact that the bacterial strain was able to inhibit the development of the pathogen in the confrontation assay further supports this assumption. However based on the results of this pot trial the third hypothesis, stating that the effect of the treatment remains the same in different experimental settings has to be rejected.

However, the number of replica has to be taken into account while making these conclusions. With only 3 replica, even if they each contained 3 plants, some results could still be caused by chance. A higher number of replica would deliver more reliable results, where correlations and tendencies would be more significant.

Fig. 25: One replica consisting of three plant per treatment. Upper left corner K-H20= Control group sprayed with tap water. Upper right corner K-Phy= Control group inoculated with *P. infestans*. Left corner below
T-S-Phy= Plants inoculated with the pathogen and treated with supernatant #45. Right corner below
T-B-Phy= Plants treated with bacterial solution and inoculated with the pathogen.

Fig. 26: Control group treated with supernatant #45 showing signs of wilt and chlorosis.

The detected foliar wilt occurring in the control groups K-S and K-B are consistent with observation from Ng and Webster (1997) (Fig. 26). This supports their hypothesis that the bacterial solution and the supernatant not only have an antagonistic effect on the pathogen but phytotoxic proprieties that can harm the plant as well.

Due to space limitation, it was not possible to monitor the plants once the trial had been terminated, thus it is not possible to determine if the leaves would have been able to recover from the negative effect of the solution as Fang (2011) predicted. This should be further investigated, since the severity of these negative impacts on the cultivar would have to be considered when optimizing such biocontrol agent with the scope to introduce it to the agricultural market.

In addition to foliar wilt, chlorosis could be detected on some leaves. Reason for this could be the high humidity inside the plastic cover, causing damages to the roots and leading to nutrient deficiencies, rather than the influence of the treatment on the plant. As mentioned in chapter 4.2. a high humidity rate is needed for the successful development of *P. infestans* but it can compromise the health of the plants.

4.3.2 Pot trials bacterial strain #34

The first pot trial conducted with the bacterial strain #34 showed promising results. Hypothesis 1 of antimycotic proprieties against *P. infestans* is supported by the outcomes of both bacterial solution and corresponding supernatant. The bacterial solution is able to reduce the percentage of infected leaves in average by 34%, whereas the supernatant lead to a reduction of approximately 27% compared to the *P. infestans* inoculated control group. The results consist with the results from B. Walch (2016) where both treatments showed protective effects against the disease. The fact that the two *in planta* trials resulted in similar outcomes indicates that the two strains have similar characteristics.

Strain #34 shows suppressing antagonistic effects in both the pot trial setting and the confrontation assay, hence supporting the hypothesis 3, that the effect remains the same in different systems. Again the applied treatments resulted in a reduction of disease pressure, but not in a complete oppression of the pathogen. While this consists with the results from NG and Webster (1997), where the disease was not completely suppressed but the number of infected leafs was reduced by 27%, it does not fully match the results of the detached leaf assay by B Walch (2016) where this particular strain was capable of completely suppress the outbreak. The detached leaf assay proves that the bacterial solution, as well as the supernatant, possess the necessary traits to successfully inhibit the pathogen. This leads to the conclusion that the experimental settings applied in this study were not yet able to activate this specific mechanism.

Fig 27: One replica consisting of three plants per treatment. Upper left corner K-H20= Control group sprayed with tap water. Upper right corner K-Phy= Control group inoculated with *P. infestans*. Left corner below T-S-Phy= Plants inoculated with the pathogen and treated with supernatant #34. Right corner below T-B-Phy= Plants treated with bacterial solution and inoculated with the pathogen.

The bacterial solution and the supernatant show no significant difference in their capacity to suppress the pathogen, as already noted in the confrontation assay and the detached leaf assay conducted by B. Walch (2016). This implies that the two strategies of competition and antimycotic metabolites are equally successful against the pathogen regarding this trial. Based on this conclusion, it was deduced that a combined treatment of bacterial solution and supernatant would increase the protective effect, leading to the conduction of a second pot trial with strain #34. Since according to Sigh et al. (2015) different aspects influence the growth of bacteria and the synthesis of metabolites, the influence of prolonged growing time of the bacteria on the lab shaker was of interest as well.

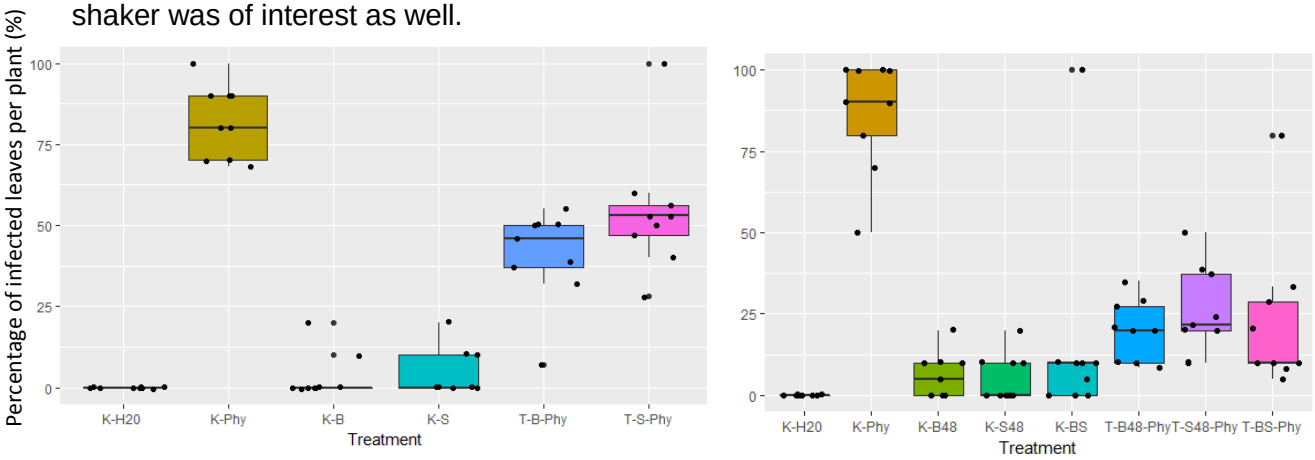

Fig. 27: On the left, results from the first pot trial with strain #34 applied after 24 hours and on the right the combined pot-trial with bacterial solution and supernatant after 48 hours of development and the combined bacterial and supernatant solution

As seen in Fig. 27, all treatments were able to reduce the percentage of infected leaves. The combined treatment of both bacterial solution and supernatant lead to a reduction of nearly 80%. The bacterial solution with 48 hours of development reduced the symptoms by approximately 70 % and the corresponding supernatant by 68.3%. All application showed a higher success rate than the treatments of the first trial.

Since the same bacteria strain was used and the method of preparation was the same, the influence of the incubation time is clearly visible. By doubling the hours the bacteria had to develop on the lab shaker, the number of produced metabolites probably increased, which explains the stronger suppressing qualities the supernatant showed.

As for the bacterial solution, the OD was kept the same as in the first trial, but the longer duration of development could lead to more competitive bacteria. Based on these results, allowing the bacteria to develop for even more than 48 hours could improve the efficiency of the two treatments against plant pathogens even more.

The application of both bacterial solution and corresponding supernatant indicates that when the strategies of both solutions are combined, a higher success rate can be achieved. The results suggest that the different traits of the solutions complement each other. That where the bacterial solution fails to inhibit the pathogen the supernatant does and vice versa. If that were not the case, the result would probably have been the same as in the first *in planta* trial. Based on the outcomes of

the other treatments with 48 hours of incubation, keeping the combined treatment longer on the lab shaker could result in an even higher capability to reduce the disease pressure. As it already was reduced by 80%, this modification could lead to a complete suppression of the pathogen.

The different trials conducted for this thesis, confrontation assay, pot trial with bacterial strain #45 and the two pot trials with strain #34 have in common that *P. infestans* was inhibited by part of the treatments, but none of them was able to completely suppress the pathogen. In addition to the possible adjustments mentioned before, granting the bacterial antagonist a head start by applying it to the medium or plant before inoculation with the pathogen occurs could be a further strategy. By giving it some time to establish and develop and only later infect the test object with the pathogen could facilitate the complete inhibition of the disease.
Based on the potential the treatments showed *in planta* the suggestion made Fang (2011) to reducing the annual inoculum by treating the soil seems feasible as well. But concerns such as Maxwell et al. (1993) voiced have to be considered as well, especially if the antibiotic efficiency of the applied solution is increased. More knowledge about the effect on other microorganism, especially beneficials is needed and the necessity of further studies arises, should *Xenorhabdus bovienii* be used as a biocontrol agent against plant pathogens in the future.

Fig. 28: Control group treated only with the supernatant showing signs of foliar wilt.

The applied solutions did not only lead to the reduction of disease pressure but another side effect was visible as well. All three treatments resulted in some percentage of foliar wilt, but it was not possibly to detect an increase in number compared to the first pot trial. This suggests that toxic effects do not depend on whether the bacteria has 24 hours or 48 hours to multiply, nor does a combined application of both solutions enhance the effect. This conclusion contradicts the findings by NG and Webster (1997), where increasing the concentration of the metabolic

compounds lead to a higher percentage of foliar wilt. It is possible that not all varieties of tomatoes show the same susceptibility against the treatments, hence the different results. The bacterial strain might not only show selectivity against specific pathogens as mentioned by Akhurst (1982) but also the severity of the phytotoxic effects differs according to plant variety. This should be further investigated by applying the same treatment to different varieties of tomatoes or other cultivars leading to new insight into the phytotoxic effect. Is the plant indeed able to outgrow the stressful impact the treatments can have, as mentioned by Fang (2011), or are the negative impact on the cultivar limited to only certain varieties, this are important questions that need to be answered when promoting *X. bovienii* as a biocontrol agent against plant pathogens.

5 Conclusion

The purpose of this study was to further investigate the antagonistic effects of *Xenorhabdus bovienii* against *Phytophthora infestans,* which had already been assessed in previous trials conducted at ZHAW (Walch,2016:Müller, 2013). The different strategies of the bacterial solution and supernatant and their success rate in reducing the disease pressure were of utmost interest. While both bacterial solution and corresponding supernatant were able to inhibit the development of the disease, a complete suppression was not achieved. Still the different trials lead to promising results, highlighting the potential of *X bovienii* as a biocontrol agent against an important plant pathogen such as *P. infestans*, which up to today, causes worldwide economic losses in the agricultural sector (Nowicki, Kozik, & Fooland, 2013). This study confirms that treatments based on *Xenorhabdus* spp., could be viable alternatives to the application of chemical fungicides. New, more sustainable management methods are becoming more important with increasing number of stricter regulation for applications with negative impacts on the environment or human health.

With the newly gained knowledge regarding the mode of action between the bacteria and the pathogen, also new questions arose and with them, the necessity for further experimental studies, especially if the antagonist should be promoted as biocontrol agent. Shapiro-Ilan, Bock and Hotchkiss (2014) recommendet that field testing as well as economical feasibility should be adressed in aditional studies, since the environmental factors, both biotic and abiotic could strongly influence the efficiency of the treatments. The interaction of *X. bovienii* strain #34 and #45 with *P. infestans* on other cultivars such as potatoes should be examined as well. The identification of the secondary metabolites produced by the different strains and their combinstion would allow to further adjust the treatments.

The severity of the phytotoxic effect on cultivars has to be studied in detail, since a successful management method cannot include the reduction of plant health, leaving it more susceptible to other plant pathogens or insect pests. Research on this topic should include the question if higher dosage of secondary metabolites really results in a higher rate of foliar wilt as suggested by NG and Webster (1997) or if the findings from this thesis are more accurate. Additionally the effect on other microorganism should be determined and potential risks, especially to beneficials, should be determined and if possible reduced. This aspect is particularly relevant for sustainable farming, since an intact ecosystem, both in the soil and above ground is crucial for successful.

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8 Appendix

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Appendix A

Agar recipes

Recipe V8 agar

- 200 ml V8 Tomato Jus de Legumes (von Globus)
- 3 g CaC03 (Merck:1.02069 CAS:471-34-1)
- 15 g Agar (Sigma: A7002 CAS: 9002-18-0)
- 800 ml tap water

Autoclave for 15 minutes at 121° C.

Recipe Malt agar

- 15 g Malt (Fluka: 70167)
- 12 g Agar (Sigma: A7002 CAS: 9002-18-0)
- 1000 ml deionized water

Recipe Standard-agar

- 36 g Standard I nutrient agar
- 1000ml deionized water

Recipe Standard I broth

- 25 g Standard I nutrient broth
- 1000 ml deionized water

Annex B

Raw Data

Results confrontation assay *Phytophthora infestans* vs. *Xenorhabdus bovienii*

Table 6: Measured area in cm2 of *P. infestans* depending on the treatment after 4 days, 6 days and 8 days after inoculation.

	Day after inoculation		
Treatment	Dai 4	Dai 6	Dai 8
K-Phy 1	12.8	37.6	44.7
K-Phy 2	13.8	35.9	55
K-Phy 3	13.3	36.6	55
K-Phy 4	13.6	34.7	51.6
K-Phy 5	12.9	36.9	55
K-Phy 6	12.9	37.6	55
K-Phy 7	12.5	36.1	55
#34-B 1	12.9	34.8	42.6
#34-B 2	10.4	30.4	42
#34-B 3	7.7	29.4	35.6
#34-B 4	13.6	32.4	39.5
#34-B 5	12	33.6	42.3
#34-B 6	12.5	33.3	40.1
#34-B 7	10.3	30.9	40.8
#34-B 8	12.9	32.5	40.2
#34-B 9	13.4	35.5	44.1
#34-S 1	12.4	26.1	37.7
#34-S 2	11.6	28.8	35.5
#34-S 3	11.4	31.4	40.7
#34-S 4	10.9	30	39.9
#34-S 5	10.5	25.6	36.4
#34-S 6	13.6	29.5	37
#34-S 7	11.7	30.1	37.5
#34-S 8	11	25	36.3
#34-S 9	12.5	33.6	39.9
#45-B 1	11.5	31	39.9
#45-B 2	12.9	30.8	38.4
#45-B 3	7.8	31.1	37
#45-B 4	12.4	31.1	39.9
#45-B 5	12.5	31.5	41.6
#45-B 6	12.1	35.8	42.4
#45-B 7	10.1	33.8	39.9
#45-B 8	11.1	31	39.3
#45-B 9	11.3	30.9	37.8
#45-S 1	9.2	30.8	36.7
#45-S 2	10.3	30.1	40.8
#45-S 3	11.2	29.3	36.2
#45-S 4	9.5	28.3	37.4
#45-S 5	10.7	29.5	35.8
#45-S 6	9.5	29.6	37.8
#45-S 7	11.2	29	37.8
#45-S 8	10.1	30.3	37.1
#45-S 9	10.6	34.2	38.8

Results confrontation assay *Xenorhabdus bovienii* vs *Colletotrichum coccodes*

Table 7: Measured area in cm2 of *C. coccodes* depending on the treatment after 4 days, 6 days and 8 days after inoculation

	Day after inoculation		
Treatment	Dai 4	Dai 6	Dai 8
K-Col 1	8.8	29.1	42
K-Col 2	9.1	25.6	41.6
K-Col 3	21.3	40.3	50.1
K-Col 4	10.2	30.6	44.8
K-Col 5	8.7	31	45.6
K-Col 6	8.7	28.7	43.1
K-Col 7	10.3	30.2	42.8
#34-B 1	9.9	26	40.4
#34-B 2	8.2	16.7	26
#34-B 3	10	29	32.6
#34-B 4	10.1	30.1	45.4
#34-B 5	9.9	30.2	44.6
#34-B 6	10.7	28.6	43.3
#34-B 7	9.9	27.7	42.8
#34-B 8	13.9	31.6	47.1
#34-B 9	10.1	26.8	41.1
#34-S 1	8.3	24.1	42.4
#34-S 2	7.2	25.8	44.5
#34-S 3	9.2	22.9	43
#34-S 4	8.8	25.9	43.7
#34-S 5	8.3	22.8	40.1
#34-S 6	8.3	23.7	41.4
#34-S 7	8.3	24.9	44.8
#34-S 8	8	23.3	38.4
#34-S 9	10	29.5	47
#45-B 1	12.8	35.7	47.5
#45-B 2	13.8	36.7	49.9
#45-B 3	14.3	33.2	55
#45-B 4	13.9	40.3	55
#45-B 5	15.8	35.9	55
#45-B 6	14.4	33.8	49.2
#45-B 7	19	37.8	55
#45-B 8	15	35	55
#45-B 9	16.1	37.6	55
#45-S 1	8.8	28.4	43.3
#45-S 2	10.5	30.4	43.4
#45-S 3	12.2	31.3	44.3
#45-S 4	10.9	29.1	41.5
#45-S 5	12.5	32.6	46.8
#45-S 6	10.4	31	43.5
#45-S 7	10.6	31.2	40.8
#45-S 8	10.1	30.2	45.3
#45-S 9	12.4	33.7	44.4

Results pot trial *Xenorhabdus* bacterial strain #34 vs. *Phytophthora infestans*

Table 8: Percentage of infected leafs per plant, corresponding median and description of visible symptoms.

Treatment	Replica 1	Replica 2	Replica 3	Symptoms
K-H20 1	0%	0%	0%	No
K-H20 2	0%	0%	0%	No
K-H20 3	0%	0%	0%	No
Median			0%	
K-B 1	0%	20%	0%	Wilt
K-B 2	0%	0%	10%	Wilt
K-B 3	0%	0%	0%	No
Median			0%	
K-S 1	10%	0%	20%	Wilt
K-S 2	0%	0%	0%	No
K-S 3	10%	0%	0%	Wilt
Median			0%	
K-Phy 1	90%	90%	80%	LBD
K-Phy 2	68%	70%	90%	LBD
K-Phy 3	100%	70%	80%	LBD
Median			80%	
T-B-Phy 1	39%	50%	37%	LBD
T-B-Phy 2	50%	46%	32%	LBD
T-B-Phy 3	7%	50%	55%	LBD
Median			46%	
T-S-Phy 1	40%	50%	100%	LBD
T-S-Phy 2	47%	53%	60%	LBD
T-S-Phy 3	56%	53%	28%	LBD
Median			53%	

Results pot trial *Xenorhabdus bacterial strain #45* vs. *Phytophthora infestans*

Table 9: Percentage of infected leaves per plant, corresponding median and description of visible symptoms.

Treatment	Replica 1	Replica 2	Replica 3	Symptoms
K-H20 1	0%	0%	0%	No
K-H20 2	0%	0%	0%	No
K-H20 3	0%	0%	0%	No
Median			0%	
K-B 1	0%	0%	0%	Wilt
K-B 2	10%	10%	0%	Wilt
K-B 3	0%	10%	0%	Wilt
Median			0%	
K-S 1	10%	0%	0%	Wilt
K-S 2	30%	20%	20%	Yellow leaves
K-S 3	0%	0%	0%	No
Median			0%	
K-Phy 1	100%	60%	90%	LBD
K-Phy 2	50%	90%	90%	LBD
K-Phy 3	90%	100%	80%	LBD
Median			90%	
T-B-Phy 1	50%	25%	100%	LBD
T-B-Phy 2	20%	68%	100%	LBD
T-B-Phy 3	80%	10%	20%	LBD
Median			50%	
T-S-Phy 1	80%	60%	20%	LBD
T-S-Phy 2	0%	60%	25%	LBD
T-S-Phy 3	50%	68%	10%	LBD
Median			50%	

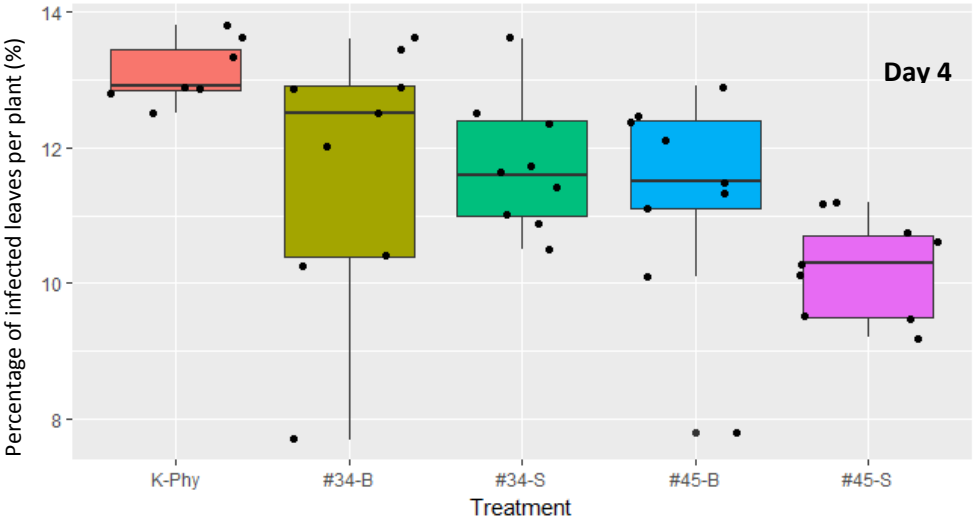
Results combined pot trial *Xenorhabdus* bacterial strain #34 vs. *Phytophthora infestans*

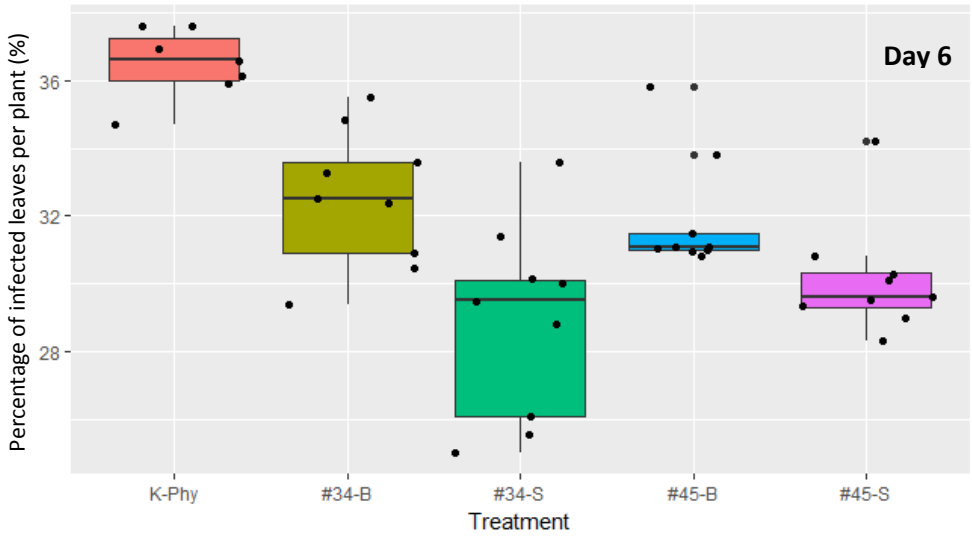
Table 10: Percentage of infected leaves per plant, corresponding median and description of visible symptoms.

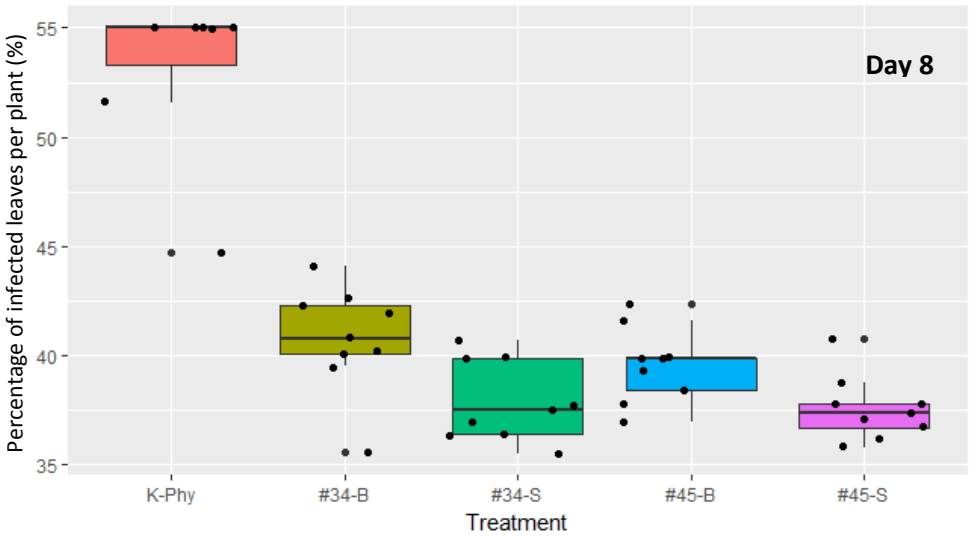
Treatment	Replica 1	Replica 2	Replica 3	Symptoms
K-H20 1	0%	0%	0%	No
K-H20 2	0%	0%	0%	No
K-H20 3	0%	0%	0%	No
Median			0%	
K-Phy 1	100%	90%	50%	LBD
K-Phy 2	80%	100%	100%	LBD
K-Phy 3	90%	70%	100%	LBD
Median			90%	
K-B48 1	5%	10%	20%	Wilt
K-B48 2	0%	10%	0%	Wilt
K-B48 3	0%	0%	10%	Wilt
Median			5%	
K-S48 1	0%	0%	20%	Wilt
K-S48 2	10%	10%	10%	Wilt
K-S48 3	0%	0%	0%	Wilt
Median			0%	
K-BS 1	100%	5%	10%	Wilt
K-BS 2	10%	10%	10%	Wilt
K-BS 3	0%	0%	0%	Wilt
Median			10%	
T-B48-Phy 1	35%	10%	20%	LBD
T-B48-Phy 2	8,3%	21%	29%	LBD
T-B48-Phy 3	20%	10%	27,3%	LBD
Median			20%	
T-S48-Phy 1	38,4%	10%	10%	LBD
T-S48-Phy 2	37,1%	20%	50%	LBD
T-S48-Phy 3	24%	20%	21,7%	LBD
Median			21,7%	
T-BS-Phy 1	80%	10%	20,6%	LBD
T-BS-Phy 2	33,3%	28,6%	5%	LBD
T-BS-Phy 3	10%	10%	8,3%	LBD
Median			10%	

Appendix C

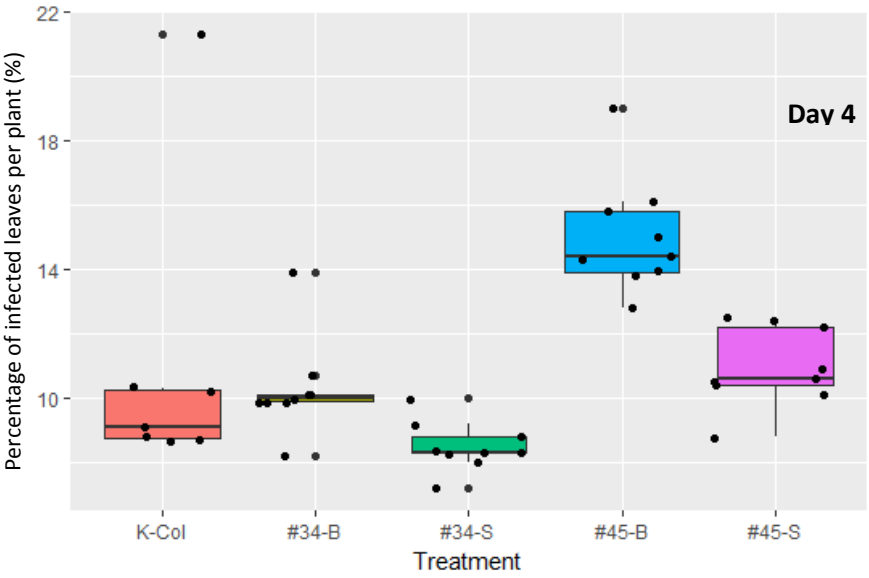
Figures confrontation assay CA-Phy

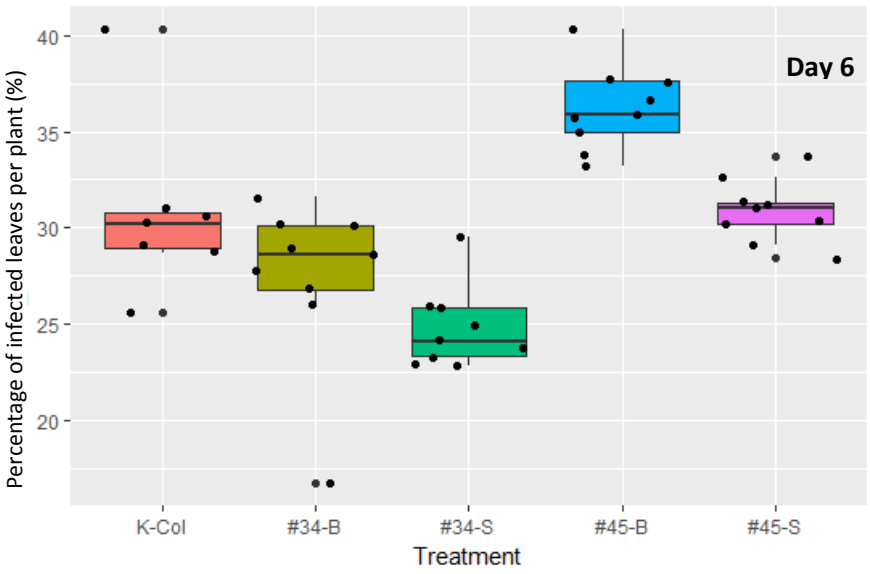


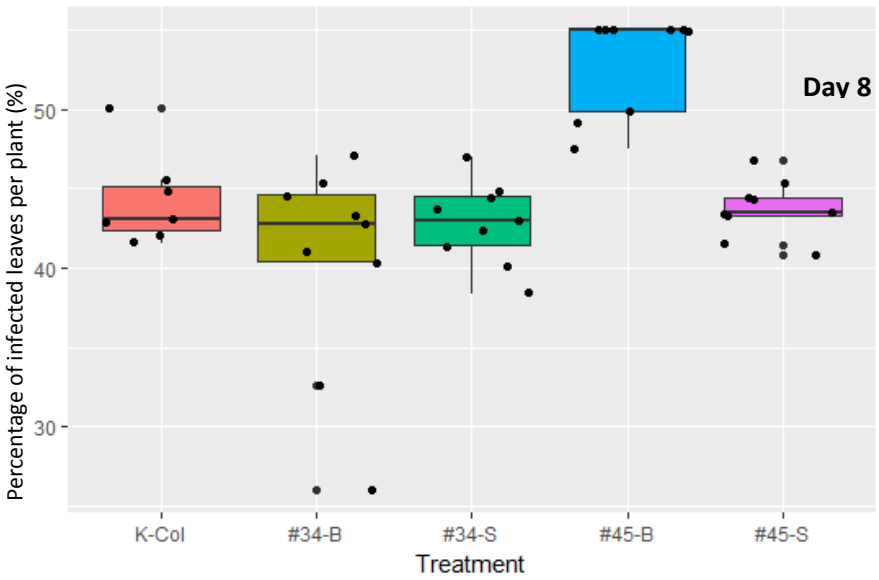




Figures confrontation assay CA-Col

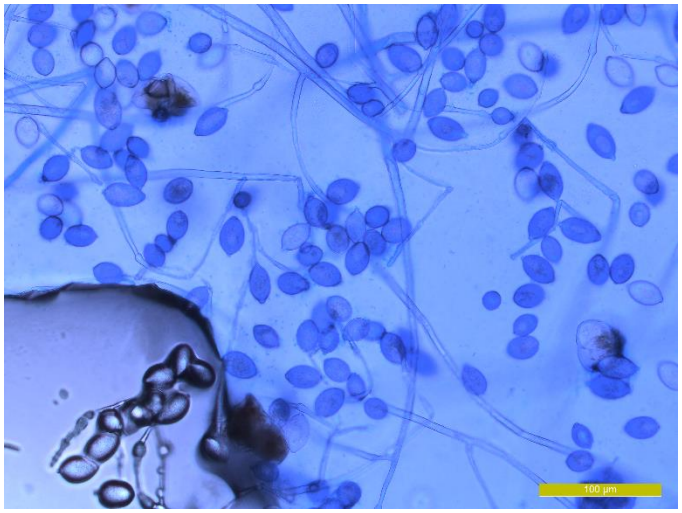




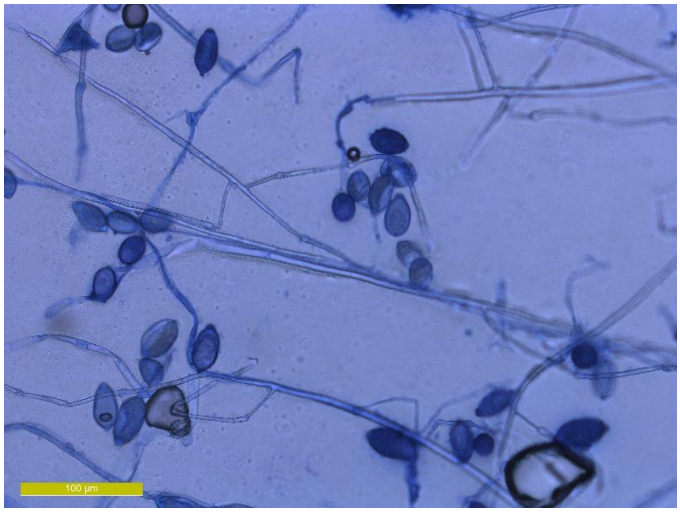


Appendix D

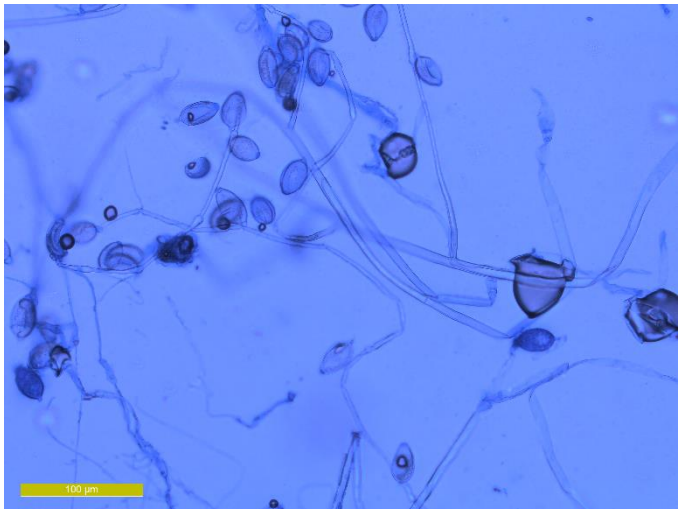
Microscopic control pot trial bacterial strain #34



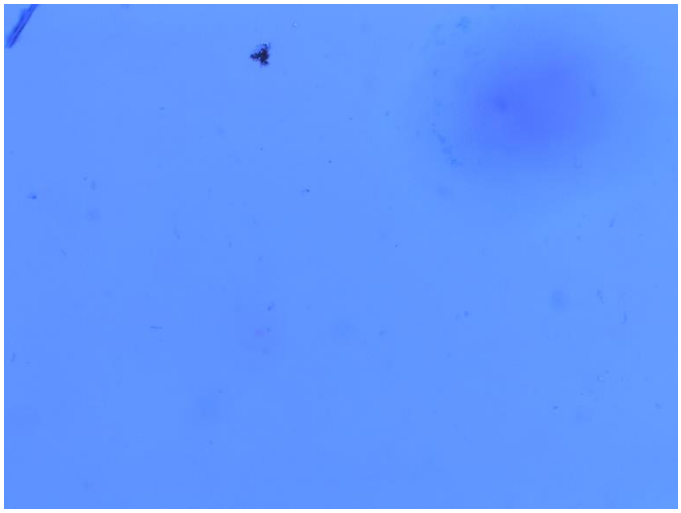
K-Phy



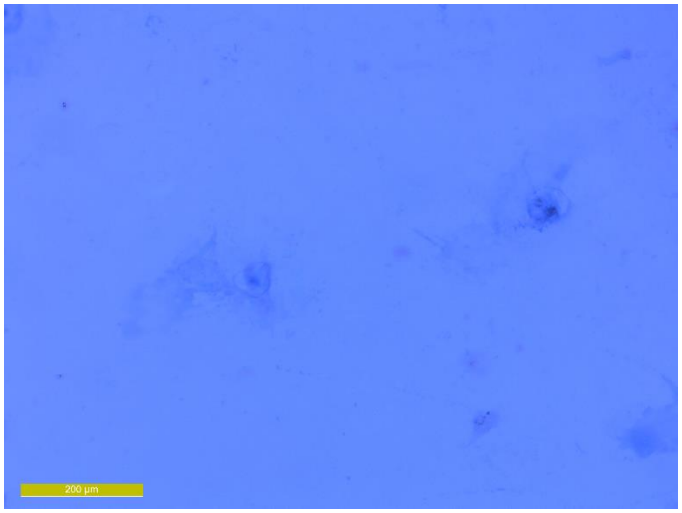
K-B-Phy



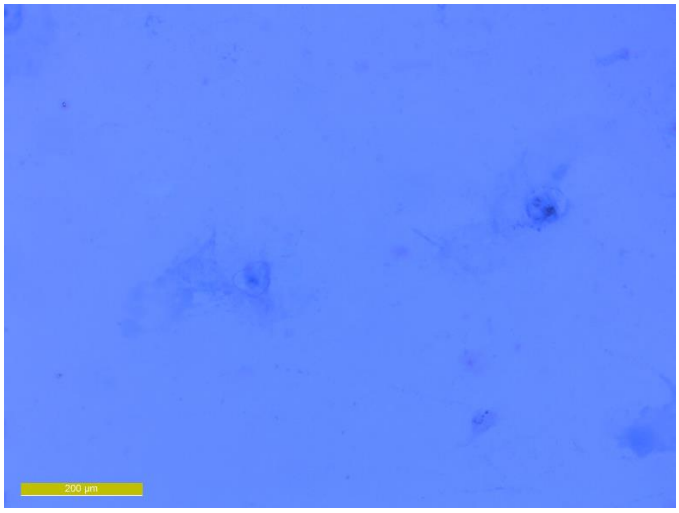
K-S-Phy



K-H20

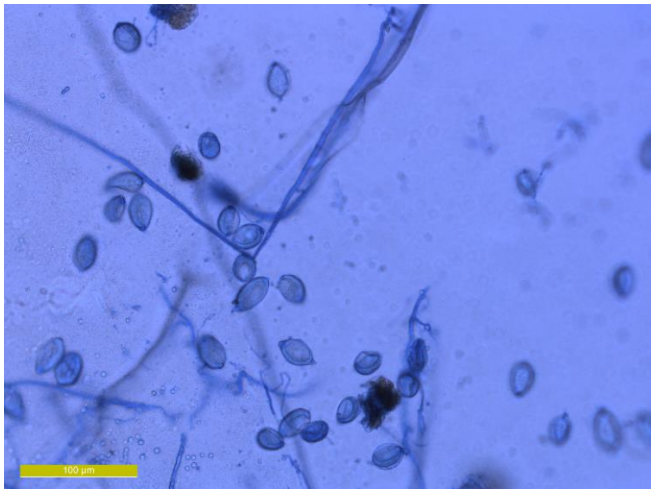


K-B



K-S

Microscopic control pot trial bacterial strain #45



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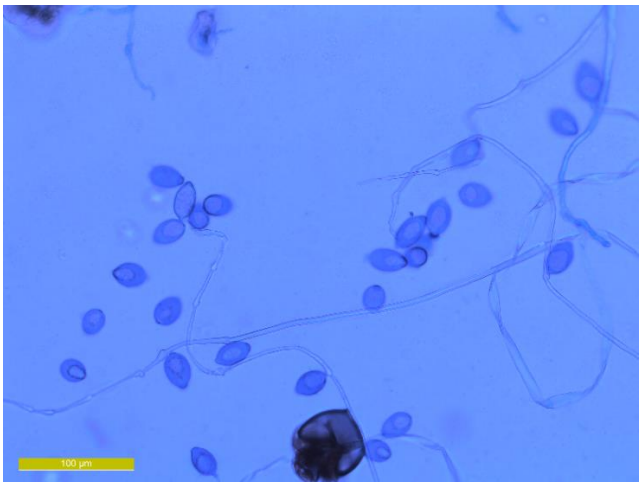

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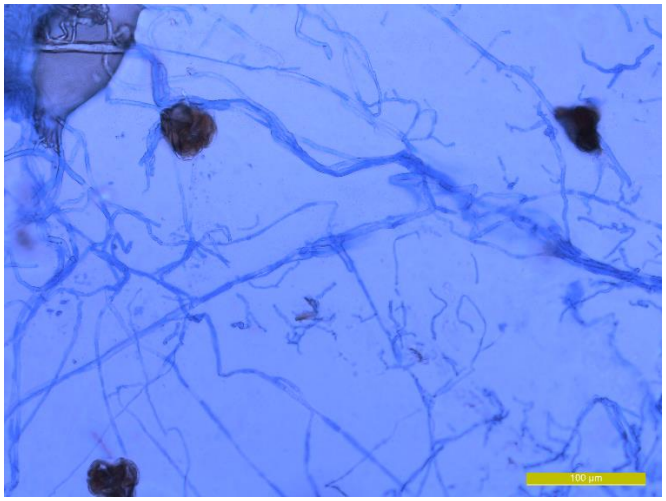

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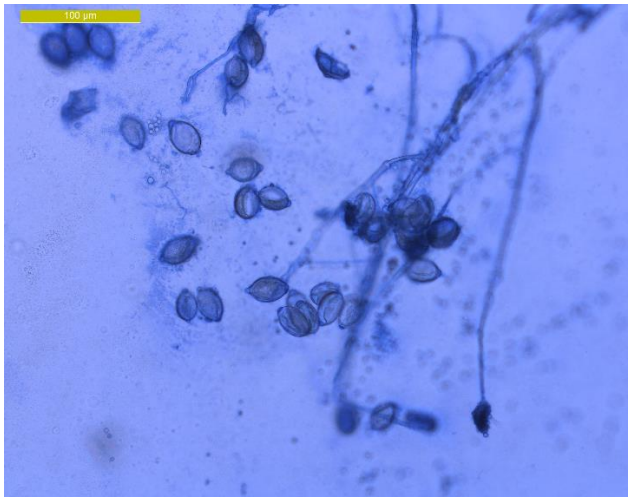
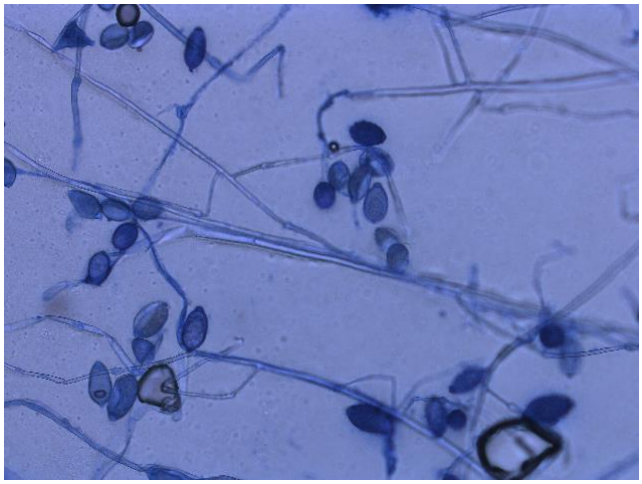
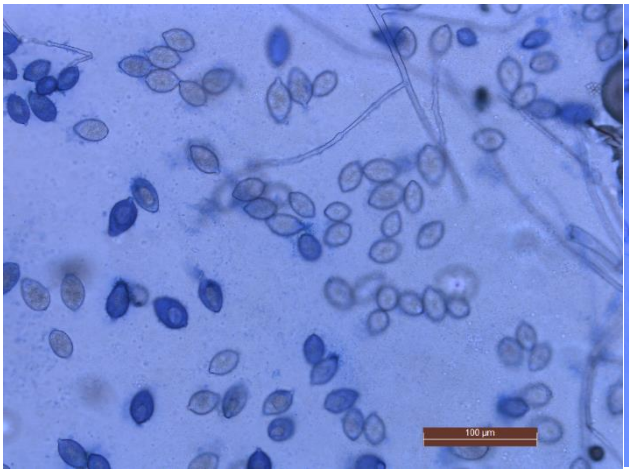


K-S-Phy

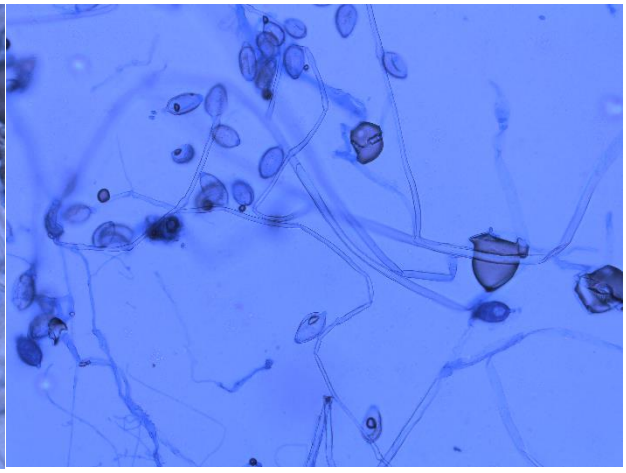


K-B-Phy

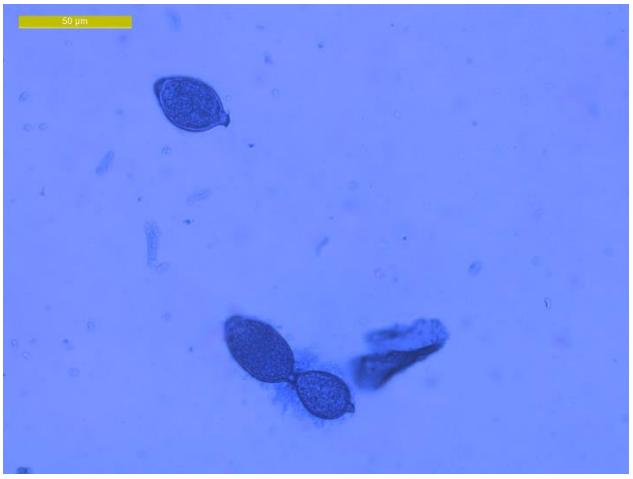
Microscopic control combined pot trial bacterial strain #34



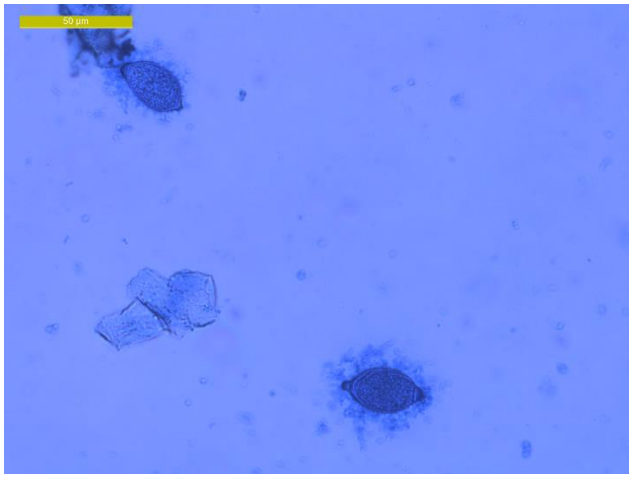
K-Phy



K-BS



K-B



K-S

Annex E

Statistical analysis

Confrontation assay *P. infestans* vs. *X. bovienii*

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.4 and Treatment

      #34-B #34-S #45-B #45-S
#34-S 1.000 -     -     -
#45-B 1.000 1.000 -     -
#45-S 0.232 0.037 0.226 -
K-Phy 0.539 0.056 0.031 0.010
```

```
> with(S_ca, pairwise.wilcox.test(Dai.6, Treatment, "holm"))

        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.6 and Treatment

      #34-B #34-S #45-B #45-S
#34-S 0.071 -     -     -
#45-B 0.853 0.071 -     -
#45-S 0.071 0.853 0.032 -
K-Phy 0.015 0.010 0.012 0.010

P value adjustment method: holm
```

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.8 and Treatment

      #34-B  #34-S  #45-B  #45-S
#34-S 0.0904 -      -      -
#45-B 0.2451 0.2451 -      -
#45-S 0.0904 1.0000 0.1066 -
K-Phy 0.0084 0.0084 0.0084 0.0084

P value adjustment method: holm
```

Confrontation assay *P. infestans* vs. *C. coccodes*

```
> with(S_ca, pairwise.wilcox.test(Dai.4, Treatment, "holm"))

        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.4 and Treatment

      #34-B   #34-S   #45-B   #45-S
#34-S 0.05173 -       -       -
#45-B 0.00694 0.00346 -       -
#45-S 0.24081 0.00694 0.00041 -
K-Col 0.67063 0.11428 0.09891 0.24081
```

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.6 and Treatment

      #34-B   #34-S   #45-B   #45-S
#34-S 0.08577 -       -       -
#45-B 0.00041 0.00041 -       -
#45-S 0.08577 0.00115 0.00066 -
K-Col 0.37093 0.01993 0.08577 0.37093
```

```
> with(S_ca, pairwise.wilcox.test(Dai.8, Treatment, "holm"))

        Pairwise comparisons using Wilcoxon rank sum test

data:  Dai.8 and Treatment

      #34-B  #34-S  #45-B  #45-S
#34-S 1.0000 -      -      -
#45-B 0.0032 0.0032 -      -
#45-S 1.0000 1.0000 0.0032 -
K-Col 1.0000 1.0000 0.0164 1.0000
```

Pot trial bacterial strain #34

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Percent and Treatment

        K-B    K-H20  K-Phy  K-S    T-B-Phy
K-H20   0.3387 -      -      -      -
K-Phy   0.0031 0.0023 -      -      -
K-S     0.6945 0.3075 0.0031 -      -
T-B-Phy 0.0037 0.0023 0.0031 0.0051 -
T-S-Phy 0.0031 0.0023 0.0261 0.0031 0.3075

P value adjustment method: holm
```

Pot trial bacterial strain #45

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Percent and Treatment

        K-B    K-H20  K-Phy  K-S    T-B-Phy
K-H20   0.3032 -      -      -      -
K-Phy   0.0037 0.0022 -      -      -
K-S     0.7795 0.1684 0.0039 -      -
T-B-Phy 0.0054 0.0022 0.3032 0.0349 -
T-S-Phy 0.0264 0.0057 0.0349 0.0890 0.7795

P value adjustment method: holm
```

Combined pot trial bacterial strain #34

```
        Pairwise comparisons using Wilcoxon rank sum test

data:  Percent and Treatment

          K-B48  K-BS   K-H20  K-Phy  K-S48  T-B48-Phy T-BS-Phy
K-BS      1.0000 -      -      -      -      -         -
K-H20     0.1773 0.0798 -      -      -      -         -
K-Phy     0.0079 0.0509 0.0041 -      -      -         -
K-S48     1.0000 1.0000 0.2990 0.0077 -      -         -
T-B48-Phy 0.1005 0.3045 0.0042 0.0083 0.1005 -         -
T-BS-Phy  0.2990 0.9260 0.0042 0.0168 0.2850 1.0000    -
T-S48-Phy 0.0403 0.1787 0.0042 0.0093 0.0403 1.0000    1.0000

P value adjustment method: holm
```

Annex F

Poster

Potential of Xenorhabdus bovienii, symbiont of enpathogenic nematodes, against plant pathogens *Phytophtora infestans* and *Colletorichum coccodes*

Master's thesis Natural Resource Science by Manuela Anele 2018

Life Sciences und Facility Management

Introduction

The purpose of this master's thesis was the examination of the potential of *Xenorhabdus bovienii*, a symbiont of entomopathogenic nematodes, against the plant pathogens *Phytophthora infestans* and *Colletotrichum coccodes*.
Results from previous trial suggest *Xenorhabdus* spp. show antagonistic effects against different plant pathogens such as *Phytophthora infestans, Phytium ultimum* and *Rhizoctonia solani*.
By applying the secondary metabolites produced by *Xenorhabdus bovienii* the symptoms caused by *P. infestans* could be significantly reduced on potato leaves(Ng & Webster, 1997).
A screening assay conducted at ZHAW tested various strains of *X. bovienii* on their abilities to suppress late blight disease caused *by P. infestans* (B.Walch,2016).
Most important hosts of *P. infestans* are crops of the Solanacea family like tomato, nightshade and potato. The pathogen is able to infect all parts of the plant that grow above ground and is not limited to a certain development stage of the host (Nelson, 2008). Every year late blight disease leads to world wild economical losses. If no counter measures are applied, the disease is able to destroy crops in matters of days with the possibility of complete yield loss (Nowicki, Kozik, & Fooland, 2013).

Material and Methods

***In vitro* confrontation assay**
X. bovienii vs. C. coccodes on malt agar
X. bovienii vs. P. infestans on V8 agar
Interaction between the bacteria and the pathogens was monitored by measuring the area of the pathogen after 4,6,8 days after inoculation and comparing it to the bacterium free control group.

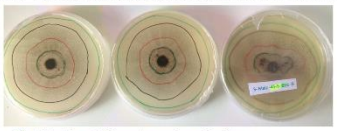


Fig. 1: Petro-dishes with C. coccodes on malt agar after 8 days.

Successful infection protocol P. infestans
3 pre-trial were conducted to establish a successful infection protocol for *P. infestans* on tomatoes.

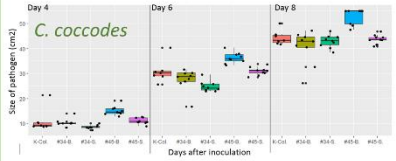
Fig. 2: The treatment group on the left inoculated with *P. infestans* showed clear signs of LBD whereas the control group on the right, treated only with tab water, showed no such signs.

***In planta* pot-trial**
X. bovienii strain #34 and #45 vs. P. infestans
The effect of *X. bovienii* on *P. infestans* was tested *in planta* by inoculating tomato cuttings with both spore-solution and bacterial solution or its corresponding supernatant.
The for the first two trials the bacteria broth was kept on the lap-shaker for 24 hours, for the combined trial it was extended to 48 h.
In addition a combined treatment of bacterial solution and supernatant was tested.
The plants were put in different trays, covered with plastic tents stored in 2 growing number of leafs showing signs of Late blight disease were counted per plant.

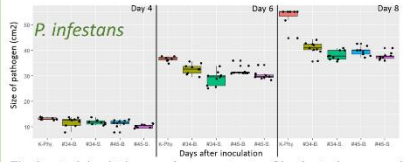
Fig. 3: Tomato cuttings covered directly with the plastic bag on the left, right growing chamber showing the different treatment.

Results

In vitro confrontation assay
X. bonienii vs. P. infestans/ C. coccodes

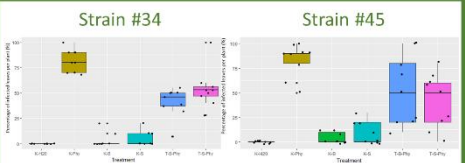


No treatment was able to inhibit the pathogen. The results show no significant suppressive effect of *X. bovienii* on *C. coccodes*.

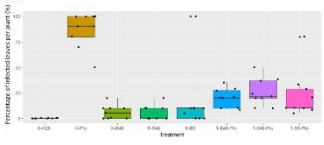


The bacterial solutions and supernatants of both strains were able to inhibit the development of the *P. infestans* significantly. Especially 8 days after inoculation the inhibition was clearly visible.

In planta pot-trial
X. bonienii vs. P. infestans



The bacterial solution (T-B-Phy) of strain #34 with 24h of incubation was able to reduce the percentage of infected leaves by 34 %, the corresponding supernatant (T-S-Phy) by 27% and the supernatant of strain #45 ((T-S-Phy) by 40 %. All treatment control groups showed signs of foliar wilt.



The combined treatment(T-BS-Phy) reduced the numbers of infected leaves by 80%, the bacterial solution (T-B48-Phy) with 48h of incubation by 70 % and its corresponding supernatant (T-S48-Phy) by 68.3%
Again all treatment control groups showed signs of foliar wilt.

Discussion

In the confrontation assay *X. bovienii* showed no suppressing effect *C. coccodes,* confirms the hypothesis that antimycotic activity is strain specific (Böszörményi et al. ,2009) and that the two strains do not possess the needed abilities to suppress this pathogen.
In the pot trials the strains were able to reduce the disease pressure. This was due to different strategies such as competition where the bacterial solution was concerned, and the production of secondary metabolites included in the supernatant.
The efficiency of the treatments was increased when the incubation of the bacteria in the broth solution was extended. The combined treatment with bacterial solution and supernatant resulted in a stronger inhibiting effect as well, indicating that the two strategies complement each other, leading to a more pronounced suppression of the disease.
The different trials conducted for this thesis, confrontation assay, pot trial with bacterial strain #45 and the two pot trials with strain #34 have in common that *P. infestans* was inhibited by part of the treatments, but none of them was able to completely suppress the pathogen. This leafs room for further studies with the scope to improve these treatments.

Fig 7:

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Appendix G

Assigned task

Appendix H

Declaration of originality

Appendix I

Consent for online publication

Erklärung betreffend Einwilligung zur elektronischen Veröffentlichung einer Masterarbeit auf der ZHAW Digitalcollection

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Titel der Arbeit: Potential of *Xenorhabdus bovienii,* symbiont of entomopathogenic nematodes, against plant pathogens of tomatoes

Name der/des Studierenden: Manuela Anele

Name der/des 1. Korrigierenden: esther Fischer

Welche Schlagwörter schlagen Sie für die öffentliche online Suche vor?

- *Xenorhabdus bovienii*
- *Xenorhabdus bovienii*
- *Phytophthora infestans*
- *Colletotrichum coccodes*
- Late blight disease
- Plant pathogen
- Biocontrol
- Biological control measures