ETH Zurich
Department of Environmental Systems Science
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Efficacy of non-synthetic seed treatments against anthracnose (*Colletotrichum lupini*) in white lupin

Master's Thesis

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MASTERARBEIT

Forschungsinstitut für biologischen Landbau
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Master Thesis at FiBL Plant Breeding Group

Title	**Molecular characterisation and control of *Colletotrichum lupini*, the causal agent of anthracnose disease, in white lupin (*Lupinus albus*)**
Context	Swiss agriculture is highly dependent on importing protein crops, mostly soybean, from outside Europe. For the organic sector in particular, a more sustainable and local production of legumes is urgently needed. FiBL supports the organic grain legume production in Switzerland focussing research on pea, faba bean, soybean and lupin. Lupin is tolerant to cool spring and dry summer conditions and accumulates nitrogen in the soil leaving a fertile, well-structured soil for the following crop. It is also known to offer nourishment for bees and other insects and produce protein rich seeds for animal feed and human consumption. One of the three commonly cultivated lupin species in Europe, the white lupin (*Lupinus albus*), is well suited for the majority of Swiss soils. However, it is currently not grown due to a risk of anthracnose infection, caused by *Colletotrichum lupini* (Nirenberg *et al*., 2002). The fungus is transmitted via the seed and can cause substantial or near-total yield loss. In 2014, FiBL has started lupin variety field trials to promote lupin growing in Switzerland and initiate a breeding programme for anthracnose resistance.
Procedure/ Method	The aim of this project is to reduce the sources of primary field infection and identify tolerant breeding material. A qPCR-based detection methods has been developed that can now be used to address the following topics: • Lifecycle of *C. lupini* during the growing period of white lupin • Evaluation of different seed treatment methods for their effectiveness to reduce pathogen infection • Develop and apply a screening system to identify white lupin genotypes with tolerance against *C. lupini*
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Abstract

White lupin *(Lupinus albus*) is an interesting crop for use as food, feed, forage or cover crop. However, its cultivation is currently limited because of its high susceptibility to the seed-borne pathogen *Colletotrichum lupini*, causal agent of lupin anthracnose. Twenty-eight seed treatments were studied here for their efficacy against lupin anthracnose, consisting of 4 hot water, 5 steam, 4 dry heat, 5 electron, 2 plant-based and 2 microbial treatments, as well as 6 controls. Experiments were divided into a germination assay and a pot-based disease assessment experiment. Treatment effects were studied by visual assessments of plants, culture-based incubation of plant tissue and quantitative polymerase chain reaction (qPCR)-based detection of *Colletotrichum* spp. in plants. Only the sodium hypochlorite control significantly impaired germination rate, normal germination rate and early vigour of seedlings. Culture-based incubation of epicotyl samples from 1.5-week old seedlings revealed significant treatment effects on the overall seed microbiome. No significant treatment effects were observed for plant vitality scores, percentage of diseased leaves and plant biomass at harvest. *Colletotrichum* spp. was detected in epicotyl samples of 1.5-week old seedlings and in shoot samples of 7.5-week old plants, but no significant treatment effects were observed. Absence of treatment effects could be due to insufficient power of the tested treatments, or to insufficient pathogen levels in the plants. No characteristic anthracnose symptoms were observed, and it is possible that the initial seed inoculum level was too low to lead to sufficient disease pressure in the plants. Nevertheless, the steam treatments significantly reduced the overall seed microbiome, and no *Colletotrichum* spp. was detected in seedlings or grown shoots, indicating a potential efficacy of the steam treatments against lupin anthracnose. The treatments dry heat 75°C/5h, electron at penetration depth 3 and intensity 3, and thyme oil also showed slight indications of efficacy against lupin anthracnose.

Summary

Introduction

Lupins are beneficial plants for soil fertility and structure, and can be used as food, feed, forage and cover crops (Wolko et al., 2011). Among the agriculturally important lupin species, white lupin (*Lupinus albus*) is the one with the highest yield potential, but also with the highest susceptibility to anthracnose (Römer, 2007). Lupin anthracnose is a fungal disease caused by the ascomycete *Colletotrichum lupini* (Nirenberg et al., 2002), and it constitutes the main challenge to lupin cultivation worldwide, particularly in humid regions that favour occurrence and propagation of the disease (Talhinhas et al., 2016). It is a seed-borne disease, and seeds represent the main source of inoculum in a crop stand (Talhinhas et al., 2016). To reduce the seed inoculum levels, seeds can be treated post-harvest by physical or chemical methods. A number of seed treatments have already been studied against lupin anthracnose, but no clear effective treatment has yet been identified (see for example Lindner et al., 1999; Thomas and Adcock, 2004; Waldow et al., 2006; Weimer, 1952). In plants, lupin anthracnose can be studied by visual assessments, as well as microscopic and molecular methods. A quantitative polymerase chain reaction (qPCR) protocol for the detection of lupin anthracnose was recently developed (Szuszkiewicz, 2016), but has not yet been used to detect and quantify *C. lupini* in grown plants.

Objectives

This study was conducted with the following objectives:

a) To compare known and as of now untested non-synthetic treatments of lupin seeds regarding their efficacy against *C. lupini*
b) To assess the practical relevance of the most promising seed treatments
c) To identify a suitable method for the detection of *C. lupini* occurrence in plants

Materials and Methods

A total of 28 treatments were studied, consisting of hot water (4 conditions), steam (5), dry heat (4), electron (5), plant-based (2) and microbial (2) treatments, as well as 6 controls (non-treated, non-treated and not *Rhizobium*-inoculated, electron treatment control, systemic fungicide, sodium hypochlorite, and certified seed control). The experiments were divided into two main parts: a germination assay and a pot-based disease assessment experiment ("pot experiment"). The germination assay was used to determine germination and normal germination rate. Additionally, epicotyl pieces were sampled from seedlings to detect whether *Colletotrichum* spp. was present using culture-based and molecular methods. In the pot experiment, germination rate, normal germination rate and early vigour were recorded for all treatments. Ten selected treatments were visually assessed 4, 5, 6 and 7.5 weeks after sowing. For these treatments, fresh and dry weights were recorded 7.5 weeks after sowing, and shoot samples were taken for molecular detection of *Colletotrichum* spp. Data was statistically analyzed for treatment effects and differences between treatments and the non-treated control.

Results

Only the sodium hypochlorite control significantly reduced the overall germination rate, the normally germinated proportion and seedling vigour by 21.1%, 48.6% and 41.5% respectively, compared with the non-treated control. Molecular analysis of the sampled epicotyl pieces showed *Colletotrichum* spp. absence for several treatments, including the non-treated control. Culture-based incubation of the epicotyl pieces revealed the seed microbiome to be reduced by two hot water, four steam, three electron and the thyme oil treatment. The strongest effect was shown by the treatments steam 63°C/270s and electron penetration depth 2 and intensity 4, both reducing the seed microbiome by 68.8%. Vitality scores in the pot experiment decreased over time with all treatments, but no significant differences to the non-treated control were detected on any of the four assessment time points. However, thyme oil and steam 75°C/120s showed slightly elevated vitality scores in the beginning of the experiment. Similarly, the percentage of diseased leaves and the percentage of dead and diseased leaves increased over time, but no significant differences to the non-treated control were observed on any of the four time points of assessment. No significant differences to the non-treated control were observed regarding fresh or dry biomass at harvest. Molecular analysis of the shoots did not detect *Colletotrichum* spp. in the treatments steam 68°C/270s and 75°C/120s, dry heat 75°C/5h and electron penetration depth 3 and intensity 3.

Discussion

In the pot experiment, no significant treatment effects were detected. This could either indicate that none of the tested treatments had a significant effect on *C. lupini*, or that the pathogen was not present at sufficient levels to lead to a strong disease outbreak and for the treatments to show a significant effect. Since no characteristic disease symptoms were observed in the pot experiment, and also due to an observed variation in the negative controls, it might be that the initial inoculum level of the seeds was lower than expected. Molecular characterization of the seed batch had revealed *Colletotrichum* spp. presence in all tested seeds, however this was not necessarily viable fungus. Seed storage is known to reduce inoculum levels (Weimer, 1952), which might have occurred in the seeds used in this experiment.

Nevertheless, individual analysis of each treatment category indicated that some treatment conditions within a category had stronger effects on the overall seed microbiome and did not show presence of *Colletotrichum* spp. for the categories hot water, steam, electron and plant-based. Comparison of all treatment categories showed that the steam treatments generally seemed to have the highest indications of efficacy against *Colletotrichum* spp., since significant effects on the seed microbiome, slightly elevated vitality scores and absence of *Colletotrichum* spp. from seedling epicotyl samples and grown shoot material were observed The treatments heat 75°C/120s, electron penetration depth 3 and intensity 3 and thyme oil also showed slight promising effects. Previous studies comparing different treatment categories mostly identified hot water treatment as the most effective treatment method (Lindner et al., 1999; Nawrath and Vetter, 2002), which was to the contrary of the observations made in this study.

The high germination rates found with all of the seed treatments tested in this study might indicate a good field emergence under favourable conditions at sowing (Kolasinska et al., 2000; Kulik and Yaklich, 1982). Under less favourable conditions, other seed vigour tests are however

better suited for prediction of field emergence than the germination tests conducted here, and such tests would be useful to get a better understanding of the practical relevance of the treatments (Kolasinska et al., 2000; LaDonne, 1989). Commercial applications can already be found for steam and electron treatments, and these treatments can therefore already be used in practice. However, electron treatment of seeds is not permitted according to the regulations for organic agriculture in Switzerland (Bio Suisse, 2018), meaning that the steam, dry heat and thyme oil treatments currently have the highest practical relevance for the organic agriculture sector in Switzerland.

Visual disease assessments of plants did not show significant treatment effects in this experiment, while the culture-based incubation of epicotyl samples showed treatment effects on the overall seed microbiome. Molecular detection of *Colletotrichum* spp. in plants using qPCR revealed presence of the fungus in selected samples, but overall a high variation in the qPCR technical replicates was observed.

Conclusion and outlook

No treatment showing a significant efficacy against *Colletotrichum* spp. was identified in this study. The absence of significant treatment effects could either be due to insufficient power of the included seed treatments against lupin anthracnose, or to insufficient levels of pathogen in the experiment, as storage of the seeds might have led to reduction of inoculum levels in the used seeds. Nevertheless, the steam treatments seemed to indicate slight control of *Colletotrichum* spp. without impairment of seed vigour, and the same was true to a lesser extent for the treatments dry heat 75°C/5h, electron penetration depth 3 and intensity 3, as well as thyme oil. Application of these treatments on strongly infected seeds is needed to determine whether a significant efficacy against lupin anthracnose can indeed be achieved.

Practical relevance was considered using the germination rates found here, which might serve as predictor for field emergence under favourable conditions. Further seed vigour tests could serve as predictors for field emergence under less favourable conditions. Practical aspects such as existing commercial applications of steam and electron treatments, as well as legal status of electron treatments in Swiss organic agriculture were rapidly assessed, but it would be interesting to further compare costs of the different treatments, as well as to determine potential commercial applications of thyme oil and dry heat treatments.

No conclusions could be drawn regarding method best suited for detection of lupin anthracnose in plants, primarily due to a high variation in results obtained with qPCR technical replicates of plant samples. Before such conclusions can be drawn, it is first necessary to determine where this variation between the technical replicates originated, for example by conducting further trials with strongly infected seeds to determine whether too low concentrations of *C. lupini* led to the observed variation.

1. Introduction

1.1 The plant genus *Lupinus*

The plant genus *Lupinus*, commonly known as lupin, encompasses a large variety of more than 300 leguminous species (ILDIS, 2018). Its three centres of diversity are spread over the American continent as well as around the Mediterranean and northern and eastern Africa. Lupins are grown as food, feed, forage and cover crops, and are also popular ornamental plants due to their large and colourful racemes. Main lupin production occurs in Oceania (75.3% of worldwide production), followed by Europe (17.6%) and the Americas (5.1%; Lucas et al., 2015). In 2014, the main European producers were Poland, followed by the Russian Federation and Germany, while in the Americas, Chile and Peru were the main producers of lupin crops (FAOSTAT, 2014). Historically, worldwide lupin production peaked in the 1990s, principally due to a large increase in Australian acreage. Since then worldwide production has experienced a strong decline, explained by the spread of diseases – mainly anthracnose but also Fusarium wilt – as well as competition with other crop imports (Wolko et al., 2011).

As members of the legume family, lupins undergo a symbiotic relationship with nitrogen-fixing bacteria. The bacteria found in lupin root nodules mainly belong to *Bradyrhizobium* sp. (*Lupinus*) (Jordan, 1982), however rhizobia of different genotypes have been identified that could belong to various *Bradyrhizobium* species (Jarabo-Lorenzo et al., 2003). The lupin root system is strong and widely ramified with a large taproot and specialized cluster roots. The taproot and deep side roots enable the lupin plants to access deep soil layers, ensuring an efficient water uptake under drought conditions. The deep penetration into the soil is additionally beneficial for soil structure, since the roots can break up soil compaction. Using specialized cluster roots, lupins exudate citrate into the soil to mobilize phosphorous and other elements (Fernández-Pascual et al., 2007). Lupins are found across a wide range of environments – however, in general, they can be characterized as calcifugal plants that grow best on well-drained soils and prefer acid to neutral soil pH conditions (Fernández-Pascual et al., 2007; Wolko et al., 2011).

When lupins are grown as a food crop, grains are the part of the plant harvested for consumption. Like other food legumes, lupins produce antinutritional compounds, which need to be considered with regards to human consumption. The main antinutrients present in lupin grains are alkaloids. These are toxic for a number of herbivores as well as for humans, while at the same time possessing antiviral, antibacterial and antifungal properties (Wink, 2005). Lupins can be classified as sweet or bitter according to the amount of alkaloids present in their grains: at an alkaloid level below 0.05%, lupins are considered sweet, even though for human consumption a level below 0.02% is required (Römer, 2007). Regarding other antinutritional compounds such as lectins, phytates, protease inhibitors or trypsin and chymotrypsin inhibitors, these are all either absent in lupins or present at low or very low levels, below those found for example in soybean (summarized in Wolko et al., 2011). Unlike many other food legumes, lupin grains therefore do not need to be heat treated in order to deactivate compounds that would otherwise reduce protein digestion.

The three lupin crops with the largest agricultural importance are white (*L. albus*), yellow (*L. luteus*) and blue (*L. angustifolius*) lupin. They differ regarding yield potential, requirements to

water, soil and climate, as well as their susceptibilities to fungal or viral diseases (Wolko et al., 2011). All three are annual crops for which sweet variants have been produced by breeding, for example the cultivar Feodora in white lupin.

1.2 White lupin (*Lupinus albus*)

Among the agriculturally used lupin species, white lupin is the one with the highest yield potential, lying between 20 and 60 dt/ha (Römer, 2007). With a vegetation period between 140 and 175 days depending on the cultivar, *L. albus* requires more time until maturity compared with both *L. luteus* and *L. angustifolius* (Römer, 2007). Additionally, it has the highest requirements regarding soil fertility and water requirements (Wolko et al., 2011). White lupin can be grown on soils ranging from mildly acidic to mildly calcareous and requires a cool to relatively warm climate during growth, while being able to tolerate some frost (Wolko et al., 2011). As a member of the lupin genus, *L. albus* produces cluster roots, enabling it to access bound soil phosphorus more efficiently than soybean (Watt and Evans, 2003). White lupin is mainly self-pollinating, although from 5 to 10% insect-mediated outcrossing rates have been reported (Faluyi and Williams, cited in Huyghe, 1997). White lupin has a diploid chromosome number of 50 (Wolko et al., 2011) and a 2C DNA content of 1.16 ± 0.044 pg (Naganowska et al., 2003).

The protein content in white lupin seeds ranges from 33 to 47% (Desmaison et al. and Petterson and Mackintosh, cited in Huyghe, 1997) and the amino acid profile of white lupin seeds has been found to equal or surpass the ideal pattern of amino acid requirements of the Food and Agriculture Organization of the United Nations (Muzquiz et al., 1989). The oil content of white lupin seeds varies between 6 and 13% (Beneytout et al., cited in Huyghe, 1997), the majority of this being unsaturated fatty acids (Muzquiz et al., 1989).

The main challenge to a more widespread white lupin cultivation is the high susceptibility of this crop to anthracnose disease (Jacob et al., 2017). In fact, among the three agriculturally most important lupin crops, white lupin has the highest susceptibility to anthracnose (Wolko et al., 2011). Resistance to anthracnose is one of the focus points of current breeding programs (Wolko et al., 2011), and differences in disease occurrence between breeding lines and reference varieties have already been reported (for example by Jacob et al., 2017).

1.3 The fungal genus *Colletotrichum*, causal agent of anthracnose

The fungal genus *Colletotrichum* is part of the Ascomycota group and contains over a hundred nomenclatured species (Cannon et al., 2012). Several of these are plant pathogens of major economic importance (Dean et al., 2012), causing a disease known as anthracnose on a wide array of plant hosts. Besides lupin, hosts of *Colletotrichum* include fruit crops, for example strawberry, mango and banana, but also grasses such as maize, sorghum, or sugarcane can be affected. Spread of the disease occurs either via seed material – some species being seed-borne pathogens – or from fungus surviving saprophytically on dead plant material in the soil (Cannon et al., 2012). Infection of a plant starts with the fungus using specialized turgor-driven structures called appressoria to penetrate the host cuticula (Deising et al., 2000). The fungus then establishes itself within the plant and enters a biotrophic phase which remains externally symptomless and can be of varying duration (Cannon et al., 2012). This is followed by a necrotrophic phase, in

which characteristic necrotic lesions can emerge on leaves, stems, flowers and fruit. If these lesions contain sporulating fungus, the disease can be spread further via rain splash or air dispersal (Nicholson and Moraes, 1980). However, as several *Colletotrichum* species also exist as symptomless endophytes in plants (see for example Joshee et al., 2009; Rojas et al., 2010; Yuan et al., 2009), isolating *Colletotrichum* from living plant tissue does not necessarily indicate a plant pathogenic action of the fungus.

The *Colletotrichum* genus is divided into nine major clades, with a few small clusters and isolated species remaining (Cannon et al., 2012). One of these clades is the acutatum clade or *C. acutatum* species complex. It is composed of 30 closely related species, several of which are responsible for disease occurrence in many economically relevant crops (Cannon et al., 2012; Sreenivasaprasad and Talhinhas, 2005). This includes the species causing anthracnose in lupin, *C. lupini*.

1.4 The case of lupin anthracnose and its causal agent *Colletotrichum lupini*

The causal agent of lupin anthracnose has previously been known under various names: *Gloesporium lupinus* (Bondar, cited in Nirenberg et al., 2002), *C. gloeosporioides* with its associated teleomorph *Glomerella cingulata* (Weimer, cited in Nirenberg et al., 2002), and later *C. acutatum* (Sreenivasaprasad et al., 1994). Nirenberg et al. (2002) reclassified the causal agent of anthracnose in lupin as a new species, *C. lupini*, and this denomination has since been validated and is the common nomenclature used today (Damm et al., 2012). Besides defining *C. lupini* as a new species, Nirenberg et al. (2002) also distinguished between two varieties of this species, *C. lupini* var. *lupini* and *C. lupini* var. *setosum*. However this distinction was not validated by later reviewers (Damm et al., 2012).

The first recorded observation of lupin anthracnose occurred on blue lupin in 1939, in the state of Florida, USA (Weimer, cited in Weimer, 1952). With lupin developing into a more and more popular winter cover crop in the subsequent years, the disease spread across the South-eastern USA (Weimer, 1952). However it was not until a few decades later that lupin anthracnose gained worldwide importance when it was first recorded in South America and Europe, followed by its appearance in regions with lupin cultivation all over the world (summarized by Talhinhas et al., 2016). The disease predominantly emerged in regions with wet climates but was not limited to these: in drier regions of the Australian continent, mostly Western Australia, it also became a serious threat to lupin production (Cowling et al., 1999). As of now, lupin anthracnose is still distributed across the whole world, and it is regarded as a major limiting factor for lupin production worldwide (Talhinhas et al., 2016).

Soon after its occurrence first being reported, lupin anthracnose was identified as a seed-borne disease (Decker, cited in Talhinhas et al., 2016). The pathogen can be found both on the seed surface as well as underneath the seed coat (Cwalina-Ambroziak and Tomasz, 2004; Kreye and Niepold, 2007), and transmittal via seed material constitutes the main source of inoculum in a lupin stand (Sweetingham, cited in Talhinhas et al., 2016). Later studies showed that under favourable climatic conditions, an infection rate of 0.1% of the seeds was sufficient to lead up to 50% harvest loss (Gondran, cited in Thomas and Sweetingham, 2004). The optimal growing temperature for *C. lupini* has been reported to be around 25°C in laboratory conditions (Nirenberg et al., 2002), while on the field warm temperatures, rainfall and wind favour anthracnose occurrence and spread (Römer, 2007; Thomas and Sweetingham, 2004).

Seedlings emerging from infected seeds can already display disease symptoms such as delayed emergence or lesions on plant tissue (Feiler and Nirenberg, 2004). Overall, characteristic symptoms of anthracnose on lupin plants include necrotic lesions on leaves, stems, flowers and fruit, as well as hypocotyl elongation; tilting over of seedlings; twists and distortions of the stems, petioles and whole shoot; wilting of the leaves; malformations on leaves and pods; and bending over of the whole plant from lesions on the stem (Feiler and Nirenberg, 2004; Römer, 2007). Infected lupin plants showing sporulating lesions may serve as secondary source of infection for other plants in the crop stand, and other plant species may also serve as pathogen reservoirs for lupin anthracnose (summarized by Talhinhas et al., 2016). Likewise, lupins may act as inoculum reservoirs for other types of anthracnose (Talhinhas et al., 2016). As mentioned in the previous section, anthracnose typically starts with a biotrophic phase and only later moves into a necrotrophic stage. When looking at the specific case of lupin anthracnose, little is however yet known about the disease cycle and the specific interactions between pathogen and host plant.

1.5 Current control of lupin anthracnose

The use of clean seeds, crop rotation and breeding of resistant cultivars constitute the primary means of preventing occurrence of the disease in a lupin stand (Talhinhas et al., 2016). If production of *Colletotrichum*-free seed material is impossible, for example in a given geographic area where the climatic conditions favour anthracnose development, seeds can be treated post-harvest to reduce seed inoculum levels. Various fungicides have already been studied regarding their applicability as seed dressings against *C. lupini,* for instance by Römer et al. (1999) or Thomas and Sweetingham (2003). As a result, various synthetic fungicides, for example thiram (Thomas and Sweetingham, 2003), exist and have been registered as seed treatments against anthracnose in lupin. In conventional agriculture their use is often recommended to reduce disease occurrence (see for example Jung, 2016; Marceau and Ledu, 2013). In organic agriculture, where the use of synthetic fungicides is not an option, physical treatments or plant or microbial products with fungicidal properties are commonly used as seed treatments. A number of non-synthetic seed treatments have already been studied in lupin against anthracnose. These include treatment with hot water, steam, dry heat, electrons, as well as various microorganisms and plant products (see following section). Besides active treatment against *Colletotrichum*, seed anthracnose infection levels can also be decreased by storage (Weimer, 1952).

1.6 Seed treatments against lupin anthracnose

Seed treatments can be categorized into physical or chemical treatments, depending on their mode of action. Physical seed treatments include treating seeds with hot water, steam, dry heat or electrons, while with chemical treatments the seeds are treated with fungicidal synthetic, plant-based or microbial compounds.

1.6.1 Physical seed treatments

Hot water treatment of seeds – just like other thermal methods such as steam and dry heat – eliminates or reduces pathogens by applying heat in a temperature-time regimen which is lethal to

the pathogens, while only slightly injuring the plant (Black et al., 2006). Seeds are treated with hot water by submerging them into heated water baths, which afterwards requires renewed drying of the seeds (Grondeau and Samson, 1994). The potential of hot water as a method to combat *C. lupini* in lupin seeds has been studied almost since discovering that the disease was seed-borne (Weimer, 1952). Overall, temperatures between 50 and 60°C and treatment durations between 5 and 60 minutes have already been examined by various authors (Lindner et al., 1999; Nawrath and Vetter, 2002; Vetter, 2006; Weimer, 1952). While the first examiners concluded that the impairment of germination after hot water treatment was too severe for this method to be of practical relevance (Weimer, 1952), subsequent studies found mixed effects on germination, with in general a good efficacy against anthracnose (Lindner et al., 1999; Nawrath and Vetter, 2002; Vetter, 2006). However hot water treatment has also been qualified as a labour-intensive and energy-consuming method (Lindner et al., 1999). Nevertheless, brochures from organisations committed to the valorisation of lupin cultivation repeatedly mention treatment of seeds with hot water at 50°C for a duration of 30 minutes against anthracnose (Kaufmann et al., 2009; Römer, 2007), indicating that this method might be somewhat established in practice. Besides lupin anthracnose, hot water has also been studied as a seed treatment method against *C. nymphaea* in celery (Yamagishi et al., 2015) and is recommended against *C. lindemuthianum* in bean (Jahn et al., 2007).

Steam treatment of seeds relies on the same mode of action as hot water treatment, but is able to selectively heat only the external seed layers (Black et al., 2006). This way, higher temperatures and shorter durations can be applied than when submerging seeds in hot water, and the treated seeds do not require renewed drying after being treated (Grondeau and Samson, 1994). As of now, only one study has examined the efficacy of steam seed treatments against lupin anthracnose (Nawrath and Vetter, 2002). While this study found no impairment of seed germination, anthracnose levels in plants grown from the treated seeds ranged from 65 to 100%. This high remaining level of infection might be due to the fact that only treatments at high temperatures – between 150°C and 250°C – and a very short treatment duration of 15 seconds were included in the study, which might not be the adequate conditions to combat lupin anthracnose in seeds.

Along with hot water, dry heat is a treatment method which has already been studied since the early 1950es (Weimer, 1952). To apply the dry heat, seeds are usually placed in ovens or oven-type structures, but dry heat application using solar heat has also already been tested (reviewed by Grondeau and Samson, 1994). Compared to treatment with hot water, which is a better heat conductor than air, dry heat treatment requires longer times of exposure (Grondeau and Samson, 1994). In lupin, treatment temperatures between 50 and 80°C and treatment durations between 2 hours and 8 days have already been examined in various studies (Falconi and Yanez-Mendizabal, 2016; Thomas and Adcock, 2004; Weimer, 1952). These studies identified several treatment conditions that completely eliminated lupin anthracnose from the treated seeds. While Weimer (1952) found no negative effects on germination, and Falconi and Yanez-Mendizabal (2016) found treatment effects ranging from impairment to improvement of germination, indications on seed germination were sometimes omitted by Thomas and Adcock (2004), leaving some open questions on the effect of dry heat treatments on lupin seed vigour.

Electron seed treatments encompass a process of first generating and then applying low-energy electrons on seeds (Weidauer, 2015). As they penetrate the seed coat, the electrons gradually lose their energy by collision processes, until all of the energy is spent. The electron's penetration

depth into the seed is thus regulated by their initial energy. Besides the penetration depth, the applied electron dose – also called intensity – is the second decisive characteristic of an electron treatment. Both the penetration depth and the electron dose can be regulated in the process of electron generation (Weidauer, 2015). The biocidal effect of the electron treatments arises when the low-energy electrons encounter biomolecules, for instance DNA, leading to the formation of transient anions and subsequent breaking of the chemical bonds in the molecules (Sanche, 2009). Treatment of lupin seeds with low-energy electrons was first studied by Lindner et al. (1999), where the included treatments showed no impairment of germination but also a very low efficacy of inoculum reduction. Nawrath and Vetter (2002) later included various electron treatments in their study, concluding that this was one of the most promising treatment methods against anthracnose in lupin seeds. Despite this favourable conclusion, electron treatments were not included in the follow-up steps of that study (Vetter, 2006).

1.6.2 Chemical seed treatments

Various synthetic fungicides have already been applied as seed treatments against lupin anthracnose. Thomas and Sweetingham (2003) compared twenty-one fungicides on their efficacy against lupin anthracnose, identifying thiram as the most effective of the tested products. Further synthetic seed dressings include carbendazim with 8-Hydroxyquinoline copper (II) or with iprodione – corresponding to the product Rovral UFB – (Gondran and Pacault, cited in Talhinhas et al., 2016), as well as a mixture of fludioxonil, cyprodinil and tebuconazole, known under the commercial name Solitär (Römer et al., 1999). In addition to these fungicides, the products Mandat (iprodione and triticonazole) and Prelude UW (carboxin and prochloraz) have also already been used as controls in studies on seed treatments against lupin anthracnose (Lindner et al., 1999). However in most of these studies, a complete elimination of lupin anthracnose from the synthetic fungicide controls was not observed (Lindner et al., 1999; Nawrath and Vetter, 2002; Thomas and Adcock, 2004; Vetter, 2006).

Plant-based seed treatments generally rely on the innate antifungal properties of certain plant extracts. Treatments that have been studied against lupin anthracnose include a mixture of plant extracts of stinging nettle and meadow horsetail, the mustard powder-based product Tillecur, and the plant-strengthening product Sojall-Vitana (Nawrath and Vetter, 2002; Vetter, 2006). Of these three treatments, only Tillecur reduced the anthracnose occurrence in lupin plants. Tillecur and thyme oil were identified as effective seed treatments against *C. lindemuthianum* in bean, and thyme oil also proved effective against *Ascochyta* spp. in pea seeds (Tinivella et al., 2009). Various plant extracts, among which garlic extract, were observed to reduce anthracnose disease in bean and cowpea seeds (Masangwa et al., 2013). Thyme oil has also been studied against *Alternaria* spp. in carrot seeds, where this treatment showed a similar efficacy to some experimental microorganisms (Koch et al., 2010). A mixture of savoury and thyme essential oils proved effective against Fusarium wilt in basil seeds (Lopez-Reyes et al., 2016), and thyme oil also reduced fungal infection rates of wheat seeds after direct and indirect treatment application (Anzlovar et al., 2017). Thyme oil therefore appears to have effective antifungal properties, however its efficacy has not yet been tested against lupin anthracnose.

Microbial seed treatments against pathogens can be based on competitive interactions between the microorganisms and the pathogen, resistance induction in the host, or production of antifungal metabolites by the beneficial microorganisms. Against lupin anthracnose, products containing

Pseudomonas chloraphis (Cedomon) and nitrogen-fixing bacteria (TRF-FU-EB) have already been tested (Nawrath and Vetter, 2002), but neither one showed a good efficacy of anthracnose reduction. The beneficial microorganisms *Trichoderma* and *Pseudomonas* have been tested against *C. truncatum* in soybean and showed some promising effects (Begum et al., 2010), while *Bacillus subtilis* provided effective disease control against *C. gloeosporioides* in chilli seeds (Narasimhan and Shivakumar, 2015). Additionally, a strain of *Clonostachys* provided some control of Ascochyta blight on pea, and *Bacillus subtilis, Pseudomonas putida* and *Fusarium oxysporum* proved effective against bean anthracnose (Tinivella et al., 2009).

1.7 Methodologies for detection of *Colletotrichum lupini*

In order to assess the effects of seed treatments on lupin anthracnose, to identify inoculum levels of lupin seeds, but also to generally study the pathogen-host interactions between *C. lupini* and lupin plants, reliable methodologies for detection of *C. lupini* in seeds and plants are needed. Since the outer discoloration of lupin seeds does not correlate with the level of *C. lupini* contained in the seeds (Feiler and Nirenberg, 2004*)*, microscopic or molecular methods seem best fitted to provide information about the level of inoculum in the seeds. A culture-based seed incubation method was developed in 1998 (Feiler and Nirenberg) using the appearance of *C. lupini*-specific appressoria to detect pathogen presence. Although this method is relatively easy to set up and does not necessitate making a microscopic preparation to inspect fungal spores, it takes three weeks until the final assessment can be done and germinating seeds may disrupt the experimental set-up (Nawrath and Vetter, 2002; Szuszkiewicz, 2016).

To allow for a more rapid pathogen detection, a *Colletotrichum*-specific polymerase chain reaction (PCR) protocol was developed (Niepold, 2003) and later used to detect pathogen presence in growing lupin plants (Kreye and Niepold, 2007). Furthermore, a quantitative PCR (qPCR) based detection and quantification method of *C. lupini* on lupin seeds was recently developed (Szuszkiewicz, 2016), allowing for the quantification of pathogen inoculum in seed and plant tissue. The developed qPCR protocol is however not specific for *C. lupini,* but instead also amplifies DNA from other species contained in the *C. acutatum* complex. Furthermore, no distinction between dead and alive fungus can be made.

1.8 Open questions

Taking all of this into account, several open questions about lupin anthracnose remain: is there a non-synthetic seed treatment that can effectively control anthracnose in lupins? When comparing different treatment categories – hot water, steam, dry heat, plant-based, ... –, which one shows the highest control of *C. lupini*? Can as of now untested treatment methods such as thyme oil or *Bacillus*-preparations reduce anthracnose inoculum in lupin seeds? If effective treatments are identified, are they applicable in practice? And furthermore, which detection method is most sensitive for detection of viable *C. lupini* from seeds or in plants?

2. Objectives

The study described here was conducted with the following objectives:

a) To compare known and as of now untested non-synthetic treatments of lupin seeds regarding their efficacy against *C. lupini*

b) To assess the practical relevance of the most promising seed treatments

c) To identify a suitable method for the detection of *C. lupini* occurrence in plants

Out of these objectives, the following research questions were formulated:

Which seed treatments could significantly reduce the amount of viable *C. lupini* in seeds?

Did any seed treatments negatively affect seed germination?

How did the most promising of the tested seed treatments compare regarding practical considerations such as potential field emergence and commercial applicability?

When comparing visual disease assessments of plants, culture-based incubation of plant tissue and qPCR of plant tissue, which method had the highest sensitivity of *C. lupini* detection in plants?

3. Materials and methods

All sterile work was carried out in an ENVAIR eco safe Basic Plus workbench (ENVAIR, Germany). Ultrapure water was obtained from a PURELAB flex 3 unit (ELGA, Switzerland).

3.1 Overview of experimental design

The experiment conducted in this study consisted of two main parts, a seed germination assay and a pot-based disease assessment experiment (designated "pot experiment"). Both the germination and the pot experiment were conducted with four replications and a randomized complete block design with replicates as blocks. A total of 28 seed treatments were studied, as summarized in **Table 1** (page 16). Of these, only 27 were studied in the germination assay, since the control not inoculated with rhizobia was not included.

3.2 Plant material

Two different white lupin seed batches were used, as described in **Table 2** (page 17). The trial-saved seeds, harvested from a FiBL field trial in 2017, were used for all the treatment applications. The certified seeds were used as certified seed control. Prior to the beginning of the experiments, DNA was individually extracted from 24 trial-saved seeds (see section 3.6.2) and qPCR was performed on the extracted DNA (section 3.6.3) to determine the seed inoculum level with *Colletotrichum* spp.

3.3 Seed treatments

All products used for the application of the treatments are shown in **Table 3** (page 17). The hot water and steam treatments were applied at Sativa Rheinau AG (Sativa), a Swiss producer of organic and biodynamic vegetable seeds, which uses seed treatment techniques to optimize seed health of the produced material. The electron treatments were applied by EVONTA-Service GmbH (Evonta), a German company specialized in the treatment of seeds with electrons against plant diseases.

At the beginning of the trial, seeds were randomly assigned to four batches for the four experimental replicates. Each batch was then further sub-divided into sub-batches, corresponding to the treatments. Treatments were applied on the entire sub-batches except for the microbial treatments, for which treatments were separately applied on randomly selected seeds from the sub-batches directly before use in an experiment. For all treatments except the dry heat and electron conditions, treatments were applied on the experimental replicates individually.

3.3.1 Hot water treatments

Hot water treatments were applied using a prototype device of Sativa. For the treatment application, the water was heated to the desired temperature and the seeds to be treated were placed inside a cloth bag. This bag was attached to the lid of the device and the lid then placed on

Table 1 Overview of applied seed treatments. Twenty-eight treatments were assessed in total, consisting of 22 non-synthetic seed treatments and 6 controls. Abbreviations show the treatment names used hereafter.

Treatment category	Treatment condition	Abbreviation	Comment
Hot water	50°C, 5 minutes 50°C, 30 minutes 52°C, 10 minutes 55°C, 10 minutes	HW-50C-5min HW-50C-30min HW-52C-10min HW-55C-10min	
Steam	60°C, 270 seconds 63°C, 270 seconds 68°C, 270 seconds 70°C, 180 seconds 75°C, 120 seconds	S-60C-270s S-63C-270s S-68C-270s S-70C-180s S-75C-120s	
Dry heat	60°C, 24 hours 70°C, 12 hours 75°C, 5 hours 80°C, 3 hours	DH-60C-24h DH-70C-12h DH-75C-5h DH-80C-3h	
Electron	Penetration depth 2, intensity 2 Penetration depth 2, intensity 3 Penetration depth 2, intensity 4 Penetration depth 3, intensity 2 Penetration depth 3, intensity 3	E-PD2-int2 E-PD2-int3 E-PD2-int4 E-PD3-int2 E-PD3-int3	2 = medium 3 = high 4 = very high
Plant-based	Tillecur, 20% (w/v) Thyme oil, 0.1%	Tillecur ThymeOil	
Microbial	RhizoVital 42, 0.2% Mix of 3 *Bacillus* strains	Rhizovital Bacillus-Mix	
Controls	No treatment No treatment + no inoculation Electron control Wakil XL, 44% Sodium hypochlorite, <5% Certified seed	NoTreatment NoInoculation E-control WakilXL NaOCl CertifiedSeed	Non-treated seeds (negative control) Non-treated and non-inoculated seeds (negative control) Non-treated seeds, electron treatment control (negative control) Synthetic systemic fungicide (positive control) Surface sterilization (positive control) Certified seeds (positive control_

Table 2 Description of used plant material. Trial-saved seeds were used for the non-synthetic seed treatments; certified seeds were used as certified seed control.

Name	Variety	Year of harvest	Producer	Origin	Comment
Trial-saved seeds	Feodora	2017	FiBL	Feldbach (ZH), Switzerland	Seeds were harvested from different plots of a field trial and mixed together; on average the harvested plots were given a disease score of 5
Certified seeds	Feodora	2015	Jouffrai-Drillaud	France	Seeds certified to be free of anthracnose

Table 3 Overview of products used for seed treatments.

Product name	Active ingredient(s)	Concentration used	Producer
-	*Bacillus subtilis BC1, Bacillus subtilis BC2, Bacillus megaterium*	> 10^6 spores per *Bacillus* strain	Feldsaaten Freudenberger GmbH & Co. KG, Germany
RhizoVital 42	*Bacillus amyloliquefaciens*	0.2%, > 10^6 spores	Andermatt Biocontrol AG, Switzerland
Thymian Thymol bio	Thymol	0.1%	Primavera Life GmbH, Germany
Tillecur	Mustard powder	20%	Biofa AG, Germany
Wakil XL	Cymoxanil, fludioxonil, metalaxyl-M	44%	Syngenta AG, Switzerland
M-Classic Javel-Wasser Natur	Sodium hypochlorite	< 5%	Migros, Switzerland

top of the device, making sure that the seeds were immersed in the water. The seeds were left in the water for the appointed time of the treatment condition and then removed. While the seeds were undergoing treatment, the temperature was monitored repeatedly and observed to revolve maximally by 0.1°C around the desired value. After taking the seeds out of the water, they were spread out on nets and air-dried overnight at room temperature, with pump-driven air circulation turned on for three intervals of two hours length.

3.3.2 Steam treatments

Steam treatments were applied using a prototype device of Sativa. To apply the steam treatments, the steam was heated to the desired temperature and the speed of the device's conveyor belt was set to the desired frequency. For the seeds to remain under the device's steam hood for a total of 270, 180 or 120 seconds, the frequency of the belt was set to 15.0, 23.0 or 32.3 Hz respectively. The seeds were then spread out on the conveyor belt and passed under the steam hood. During

this time the temperature was monitored repeatedly and observed to revolve by maximally 0.3°C around the desired value. After the seeds had come out from under the hood, they were spread out on nets and air-dried overnight at room temperature, with pump-driven air circulation turned on for three intervals of two hours length.

3.3.3 Dry heat treatments

Dry heat treatments were applied in a BD 53 microbiological incubator (BINDER, Germany). The incubator was heated to the desired temperature of the treatment condition, and the seeds were then placed inside on sheets of parchment paper. The duration of the treatment was set with the internal timing function of the incubator. After the period of treatment application had ended, the seeds were left to cool at room temperature.

3.3.4 Electron treatments

The seeds were sent to Evonta by mail, where the treatments were applied. The treated seeds were then sent back to FiBL.

3.3.5 Microbial treatments

For the Rhizovital treatment, a 0.2% solution was made from the RhizoVital 42 stock solution (> $2.5x10^{10}$ *B. amyloliquefaciens* spores/ml), so that the diluted solution contained at least $1x10^{7}$ spores/ml of *B. amyloliquefaciens*. The seeds were soaked in the solution for 10 minutes, after which they were spread out in an open Petri dish and left to dry overnight under sterile conditions.

Before making a mixture of the three *Bacillus* strains, the solutions of *B. subtilis* BC1, *B. subtilis* BC 2 and *B. megaterium* were verified to contain each at least $1x10^{6}$ spores/ml using a haemocytometer. The three *Bacillus* strains were then mixed at equal volumes, and the seeds were soaked in this mixture for 10 minutes. The seeds were then spread out in an open Petri dish and left to dry overnight under sterile conditions.

3.3.6 Plant-based treatments

A 0.1% thyme oil emulsion (Tinivella et al., 2009) was prepared by emulsifying the oil in a 0.1% Tween 20 (ITW Reagents, United States of America) solution made with ultrapure water. The seeds were placed into closed bottles, after which the emulsion was added until all the seeds were completely submerged. The bottles were placed on a shaker at room temperature and shaken for 30 minutes at 120 rpm. The seeds were then washed three times with tap water and left to dry overnight on filter paper under sterile conditions.

Tillecur was applied by making a 20% (w/v) suspension of the powder in ultrapure water (Tinivella et al., 2009). The seeds were dipped into the suspension and the mixture was rapidly stirred until all of the seeds were evenly coated. The seeds were then spread out on nets and left to dry overnight under sterile conditions.

3.3.7 Controls

The non-treated control consisted of seeds not subjected to any seed treatment, but originating from the same seed batch and stored under the same conditions as the treated seeds. A non-inoculated control was included in the pot experiment, consisting of non-treated seeds that were not inoculated with rhizobia prior to sowing. The electron control consisted of non-treated seeds that were sent to Evonta and back along with the seeds for the electron treatment.

For the systemic fungicide control, seeds were put into a 44% (w/v) solution of the product Wakil XL (10% cymoxanil, 50% fludioxonil, 17.5% metalaxyl-M), at a ratio of 4.5 µl solution per gram seed material according to the approved dosage instructions in Switzerland and the manufacturer's instructions (BLW, 2018; Syngenta, 2018). The mixture was rapidly stirred until all seeds were evenly coated, after which the seeds were spread out on a net and left to dry overnight under sterile conditions. The surface sterilization control was carried out by soaking the seeds for 10 minutes in a < 5% solution of sodium hypochlorite (Szuszkiewicz, 2016). As with the systemic fungicide, the seeds were then spread out on a net and left to dry overnight under sterile conditions. The certified seed control consisted of seeds certified to be anthracnose-free.

3.4 Seed germination assay

3.4.1 Experimental set-up

A sheet of filter paper was placed into a container with a pleated paper sheet on top. For a given treatment replicate, 50 randomly selected seeds were placed into the pleats of the pleated paper. The container was then installed on a tray filled with water, according to a randomized complete block design with replicates as blocks. The trays were put into GroBank BB-XXL.4 (CLF Plant Climatics, Germany) growth chambers and kept at 20°C under 12h/12h light/dark conditions.

3.4.2 Assessments

Seed germination rate was determined after 12 (replicates 1 and 4) or 13 (replicates 2 and 3) days. Emerging seedlings were categorized into normal or abnormal seedlings according to ISTA regulations (ISTA, 1991). Seedlings were considered normal if they were completely intact with all essential structures well developed, in proportion and healthy; if they exhibited a balanced development with only slight defects such as limited damage on the leaves or roots with half or more of the tissue functioning normally; or if they were affected by secondary infection.

To detect whether *C. lupini* was present above the cotyledons after 1.5 weeks, ten seedlings were randomly selected from the seedlings in the germination assay for each treatment replicate. Approximately 2-mm long pieces of the epicotyl were sampled from these. The epicotyl samples were then vertically cut in half under sterile conditions using a previously sterilized scalpel. One half of each sample was collected in a single "Universal" Extraction Bag (Bioreba AG, Switzerland) per treatment replicate and frozen at -20°C for subsequent DNA extraction (see

section 3.6.2) and molecular detection of *Colletotrichum* spp. (see section 3.6.3). The remaining epicotyl halves were plated onto growth medium as described in section 3.6.1 to assess occurring fungal growth by counting and morphological categorization. DNA was extracted from two samples per replicate for each fungal category and presence of *Colletotrichum* spp. was assessed molecularly (sections 3.6.2 and 3.6.3 respectively).

3.5 Pot-based disease assessment experiment

3.5.1 Experimental set-up

Seeds of each treatment replicate were randomly selected and inoculated with HiStick® L rhizobia (BASF, Germany) by mixing both in a glass container. Six seeds per replicate were sown in Einheitserde Classic potting soil (Einheitserde Werkverband e.V., Germany) on multi-pot trays, leaving one buffer row between two adjacent treatments. The treatments were arranged as a randomized complete block design, with replicates as blocks. The trays were placed in a climate chamber and kept at 22°C under 16h/8h light/dark conditions on sodium vapour lamps and 70% air humidity. Germination rate (normal and abnormal, see section 3.4.2) and early vigour were assessed after 12 (replicates 1 and 2) or 8 (replicates 3 and 4) days.

After 13 (replicates 1 and 2) or 9 (replicates 3 and 4) days, three randomly selected plants of the treatments ThymeOil, Bacillus-Mix, HW-55C-10min, DH-75C-5h, S-68C-270s, S-75C-120s, E-PD2-int4, E-PD3-int3, WakilXL and NoTreatment were transferred into larger pots. Treatments were arranged as a randomized complete block design, with replicates as blocks, and the three plants of each treatment replicate were randomly distributed within a block.

3.5.2 Assessments

The pot plants were assessed at 4, 5, 6 and 7.5 weeks after sowing. On each time point of assessment, each plant was assessed individually according to the criteria shown in **Table 4** (page 21). At 7.5 weeks, shoots and roots were harvested and weighed. Shoot material was cut with sterile scissors into small pieces and mixed thoroughly to obtain a representative subsample for subsequent DNA extraction (section 3.6.2) and molecular detection of *Colletotrichum* spp. (section 3.6.3). Remaining roots and shoots were dried at 60°C for three days to determine dry weights.

3.6 Experimental procedures

3.6.1 Plating of epicotyl samples on growth medium and morphological categorization of fungal colonies

After cutting the epicotyl samples in half under sterile conditions, one half from each sample was placed on a Petri dish containing selective PDA medium (see **Table 5**, page 22), so that five epicotyl halves fitted on one plate. Per treatment replicate, two plates were thus filled with epicotyl samples. The plates were incubated for 6 days at 20°C and then assessed. Epicotyl

samples with fungal growth were counted. Fungal colonies growing from the plated epicotyl halves were categorized into morphological groups according to colour, form and surface structure. The categorizations were based on observations from the plate tops and bottoms.

Table 4 Parameters assessed on plants in pot experiment.

Parameter	Description	Scoring range	Explanation of scoring
Vitality score	Overall assessment of plant vitality and disease progression	1-9	9=completely healthy plant; 8=healthy plant, no dead leaves but at least one leaf starting to display disease symptoms; 7=overall healthy plant but several leaves starting to display disease symptoms and few dead leaves possible; 6=overall still healthy plant but with several diseased and dead leaves; 5=plant starting to appear overall diseased but still several healthy leaves; 4=sick plant but with some leaves still more healthy than diseased; 3=sick plant but several leaves still alive; 2=sick plant but at least one leaf alive; 1=dead plant
Number of leaves on plant	Number of individually discernable leaves on plant	Count	-
Number of diseased leaves	Number of leaves showing symptoms of disease (yellow colouring, wilting, drying out) or of general plant weakening (mining flies, virus)	Count	-
Number of dead leaves	Number of leaves with all sub-leaves dried out or fallen off and with leaf stem completely bent over	Count	-
Mining flies	Presence of mining flies on any part of the plants	Yes/no	-
Virus	Plant affected by virus	Yes/no	-
Flowering	Plant flowering or flowering already occurred	Yes/no	-

Table 5 Composition of used growth media and buffers.

Name	Ingredients	Preparation
Growth media		
PDA	18.5 g PDA (Roth AG, Switzerland) 0.5 ml 20 mg/ml Tetracycline hydrochloride (Roth AG, Switzerland) Water	PDA was put into a 750 ml Schott flask and water was added until the 500 ml mark. The solution was stirred with a magnetic stirrer and then autoclaved in a Microjet microwave sterilizer (Enbio Technology Sp., Poland). The solution was again rapidly stirred and then cooled down to 60°C in a water bath. After cooling, tetracycline was added, the solution was stirred again, and the medium was poured into sterile Petri dishes under sterile conditions.
Buffer solutions		
CTAB (1x)	6.05 g TRIS (Roth AG, Switzerland) 40.91 g NaCl (Roth AG, Switzerland) 9.31 g Na_2EDTA (Roth AG, Switzerland) 10.0 g CTAB (Roth AG, Switzerland) 5.0 g PVP K25 (Roth AG, Switzerland) 5N HCl Ultrapure water	CTAB and PVP K25 were added to approximately 300 ml ultrapure water, followed by the addition of TRIS, NaCl and Na_2EDTA. The volume of the solution was adjusted to 500 ml, and the solution was stirred overnight with a magnetic stirrer. If necessary, the pH of the solution was adjusted to 8.0 using 5N HCl.
TAE (50x)	121 g TRIS (Roth AG, Switzerland) 28.6 ml 1M acetic acid 50 ml 0.5M Na_2EDTA (Roth AG, Switzerland) Ultrapure water	All ingredients were dissolved in 300 ml ultrapure water under constant stirring. After dissolving, the volume of the solution was adjusted to 500 ml with ultrapure water.
TE (100x)	12.10 g TRIS (Roth AG, Switzerland) 3.70 g Na_2EDTA (Roth AG, Switzerland) 0.1N HCl Ultrapure water	TRIS and EDTA were dissolved in approximately 80 ml of water. The pH was adjusted to 8.0 with 0.1N HCl while stirring, and the volume of the solution was increased to 100 ml.

3.6.2 DNA extractions

From seeds
DNA extractions from seeds were performed using a Quick DNA Plant/Seed Miniprep Kit (Zymo Research, Germany) according to the manufacturer's instructions.

From plant material
The total number of plant samples for DNA extraction was counted and the necessary amount of CTAB extraction buffer (composition see **Table 5**) was determined: 4 ml per bag containing the epicotyl samples, and 5 ml per bag containing the shoot material of the plants from the pot experiment. Beta-mercaptoethanol was added freshly to the CTAB buffer until a concentration of

0.2% β-mercaptoethanol. The necessary amount of extraction buffer was then added to each extraction bag. The plant samples were homogenized with a HOMEX 6 unit (Bioreba AG, Switzerland). One ml of each plant sample extract was transferred into a screw cap microtube and incubated at 65°C for 1 hour. After cooling, 0.8 ml Roti-C/l chloroform (Roth AG, Switzerland) was added in the hood, and the samples were vortexed three times at full speed for 2 seconds. The samples were then centrifuged for 2 minutes at 20'000 g, and 0.8 ml of the clear supernatant from each sample was transferred into a new tube containing 0.6 ml isopropanol (Roth AG, Switzerland). The samples were mixed by inverting the tubes upside down four to five times, after which they were incubated at room temperature for 1 hour. This was followed by centrifugation for 2 minutes at 20'000 g. The supernatant was discarded from each sample and the remaining DNA pellet was washed once with 0.8 ml 70% ethanol. The samples were centrifuged for 1 minute at 20'000 g, the supernatant was again carefully discarded, and any remaining ethanol was evaporated by placing the open tubes for 5 to 15 minutes on a heat block at 70°C. The DNA pellets were then dissolved in 100 µl TE 0.1x (composition see **Table 5**, page 22**)**. Before qPCR analysis, the DNA samples were placed for 12 minutes on a heat block at 50°C to solubilise any unsolved DNA. DNA concentration was assessed using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Switzerland). In selected cases, the DNA quality was additionally analysed by gel electrophoresis at 120V in TAE buffer (composition see **Table 5**, page 22) on a 1.5% agarose gel.

From fungus on growth medium
Circular pieces of 2-5 mm diameter were cut from the growth medium where the fungus of interest was growing. Cutting was performed under sterile conditions. The agar pieces were put into screw cap microtubes, and 0.25 ml of 0.5 mm Zirconia/Glass-Beads® were added to the tubes with 0.5 ml of TE 1x buffer (composition of buffer see **Table 5**, page 22). The tubes were placed at 95°C for 10 minutes, and the contents of the tubes were homogenized while still hot in a FastPrep-24 homogenizer (MP Biomedicals, United States of America) at 6 m/s. The tubes were then centrifuged for 1 minute at 20'000 g, and the undiluted supernatant was used to perform qPCR analysis.

3.6.3 Quantitative PCR

Quantitative PCR was performed following the protocol developed by Szuszkiewicz (2016), involving the use of a TaqMan® probe containing a locked nucleic acid (LNA) modification. The total reaction volume of the qPCR was 10 µl, with 1 µl of DNA sample and 9 µl master mix, containing the enzyme mix, primer pair, TaqMan® probe and ultrapure water. Two different enzyme mixes were used over the course of the experiment: the SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, United States of America) to analyze the inoculum level of the trial-saved seed batch, and for all subsequent qPCR analyses the enzyme mix KAPA PROBE FAST Universal (Kapa Biosystems, United States of America). Used reagent concentrations and sequences of the primers and TaqMan® probe are shown in **Table 6** (page 24). All reactions were performed on a Rotor-Gene Q MDx (QIAGEN, Germany*)* thermocycler with two technical replicates per DNA sample. Prepared standards with known amounts of spores were included in each qPCR run to calculate a standard curve from which to determine *Colletotrichum* spp. concentrations in the samples of interest. The temperature settings of the reaction were 3 minutes at 95°C for enzyme activation, followed by 45 cycles of 5 seconds at 95°C for denaturation of double-stranded DNA and 20 seconds at 60°C for primer annealing and DNA elongation. The

qPCR curves were analyzed using the Auto-Find Threshold function of the Rotor-Gene Q software to determine cycle threshold values and corresponding *C. lupini* spore equivalent (SporeEq) concentrations.

Table 6 Concentrations and sequences of qPCR reagents used. F=forward, R=reverse, P=probe. Bold letters show the location of a locked nucleic acid (LNA) modification.

Reagent	Concentration in qPCR reaction	Sequence
SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, United States of America)	1X	-
KAPA PROBE FAST Universal 2X qPCR Master Mix (Kapa Biosystems, United States of America)	1X	-
Forward primer Clup01_F	300 nM	5'-AGC-ACC-GCT-TGG-TTT-TGG-G-3'
Reverse primer Clup01_R	300 nM	5'-GGG-GTT-TTA-CGG-CAA-GAG-TCC-3'
TaqMan® probe Clup01_LNA_P	100 nM	5'-FAM6-CCT-**TGA**-AGG-TAG-TGG-CGG-ABHQ1-3'
Ultrapure water	-	-

3.7 Data analyses

All data was analyzed by linear modelling of the response variables as a function of treatment and replicate. The residuals of the linear models were visually controlled for normal distribution by inspecting the normal Q-Q and residuals vs. fitted plots. If this condition was not met, the data was transformed until the residuals followed a normal distribution (arcsine-transformed for percentage data, log-transformed for SporeEq data or rank-transformed in case other transformations did not result in normally distributed residuals). The linear models were then analyzed by analysis of variance (ANOVA). If a significant treatment effect was found, Dunnett's many-to-one comparisons test at $p<0.05$ was carried out to compare all treatments to the non-treated control (Dunnett, 1955). Additionally Dunnett's critical distance between means was calculated for selected parameters using the critical values indicated by Dunnett (1964). Data from the pot experiment was analyzed after removing all plants that showed symptoms of viral disease.

Statistical analysis was performed using the software R-Studio (version 1.1.442; RStudio-Team, 2016) and R (version 3.3.3; R Core Team, 2017). The R packages "plyr" (Wickham, 2011) and "multcomp" (Hothorn et al., 2008) were used for data analysis, and the package "ggplot2" (Wickham, 2016) for data visualization. An example of R code used for data analysis is shown in **App. Table I**.

4 Results

4.1 Molecular characterization of trial-saved seeds

Out of the 24 tested seeds, 21 gave a positive signal for *Colletotrichum* spp. for both qPCR technical replicates, and the remaining 3 seeds gave a positive signal for *Colletotrichum* spp. for one out of the two technical replicates. Also considering the samples where only one of the two technical replicates gave a signal, the average *Colletotrichum* spp. concentration was $3.1x10^4$ ($7.0x10^4$) spore equivalents (SporeEq)/100 mg dried seed. Two seeds had a concentration of more than $2.0x10^5$ SporeEq/100 mg seed, while on the other side two seeds had a concentration of less than $5.0x10^2$ SporeEq/100 mg seed. The remaining 20 seeds had a concentration of approximately $1.0x10^4$ SporeEq/100 mg seed.

4.2 Germination assay

4.2.1 Germination rate

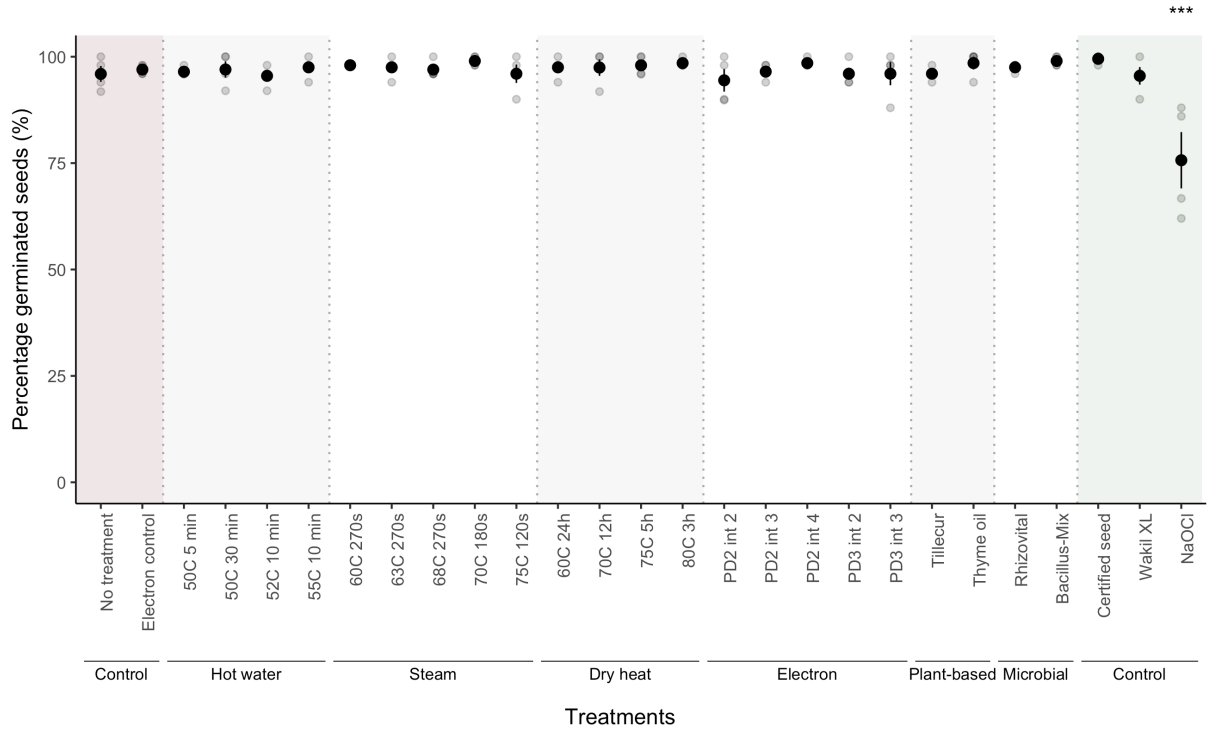

Figure 1 Mean percentage of germinated seeds of different treatments after 1.5 weeks. Stars at the top of the plot show significant differences to the NoTreatment control (Dunnett's test; '***' = p<0.001, '**' = p<0.01, '*' = p<0.05, '.' = p<0.1). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.

The seed germination rates of all seed treatments after 1.5 weeks are shown in **Figure 1** (page 25). The NaOCl treatment significantly ($p<0.001$) reduced the seed germination rate by 21.1% compared with the NoTreatment control (96.0%). All other treatments did not significantly differ from the NoTreatment control and ranged between 94.5% and 99.5%. The normal germination rate as a percent of the germinated seedlings is depicted in **Figure 2**. With a normal germination rate of 44.7%, the NaOCl treatment significantly reduced ($p<0.001$) the normal germination rate by 48.6% compared to the NoTreatment control (86.9%). The hot water treatment HW-50C-30min (62.9%) did not significantly reduce the normal germination rate compared with the NoTreatment control, but was tending toward significance ($p=0.0701$).

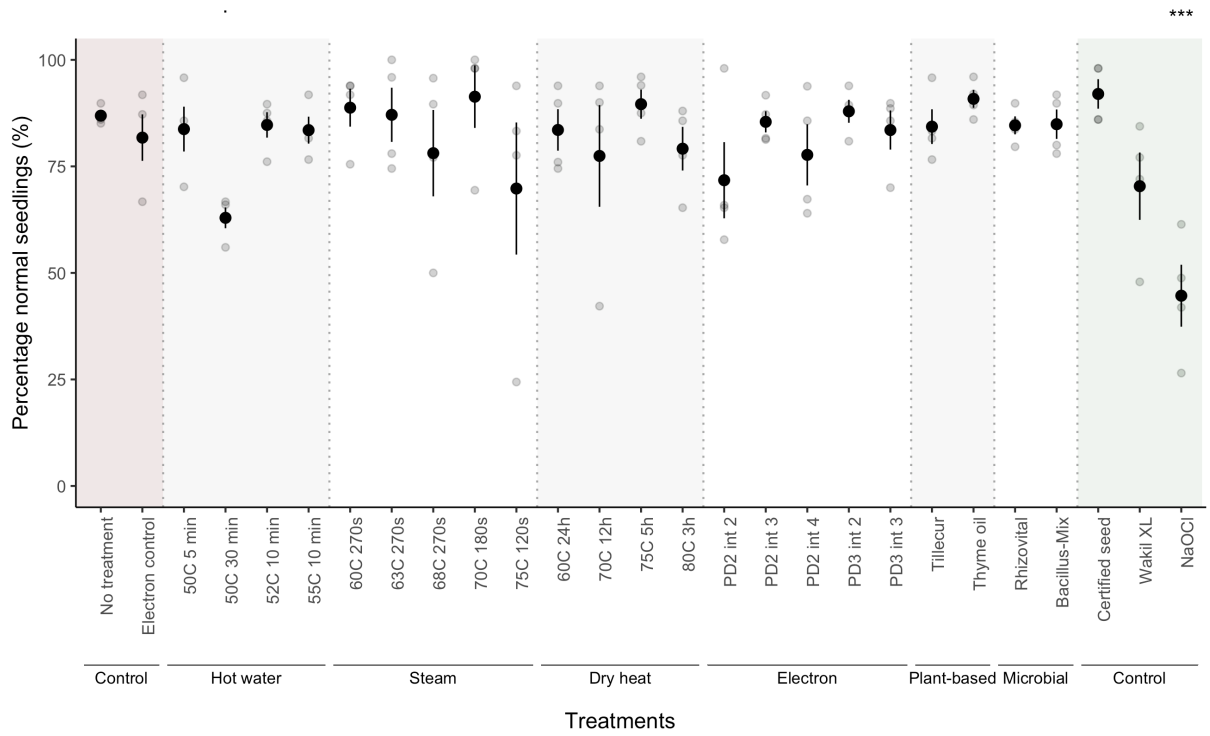

Figure 2 Mean normally germinated seeds of different treatments after 1.5 weeks as percentage of germinated seeds. Stars at the top of the plot show significant differences to the NoTreatment control (Dunnett's test; '***' = $p<0.001$, '**' = $p<0.01$, '*' = $p<0.05$, '.' = $p<0.1$). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.

4.2.2 Detection of fungi in epicotyl samples after 1.5 weeks

Molecular detection of *Colletotrichum* spp. in epicotyl samples

Figure 3 (page 27) shows the concentrations of *Colletotrichum* spp. SporeEq/10 halves of epicotyl samples collected from 1.5 week-old seedlings. When analyzing the extracted DNA

using qPCR, it happened repeatedly that one of the two qPCR technical replicates gave a signal for *Colletotrichum* spp., while the other technical replicate did not. To analyze the data, it was therefore necessary to generate two datasets: one containing only the concentrations when both technical replicates had given a signal, and one dataset containing all the concentrations, regardless of the number of technical replicates giving a signal. The log transformations of these two datasets are depicted in different colours in **Figure 3**.

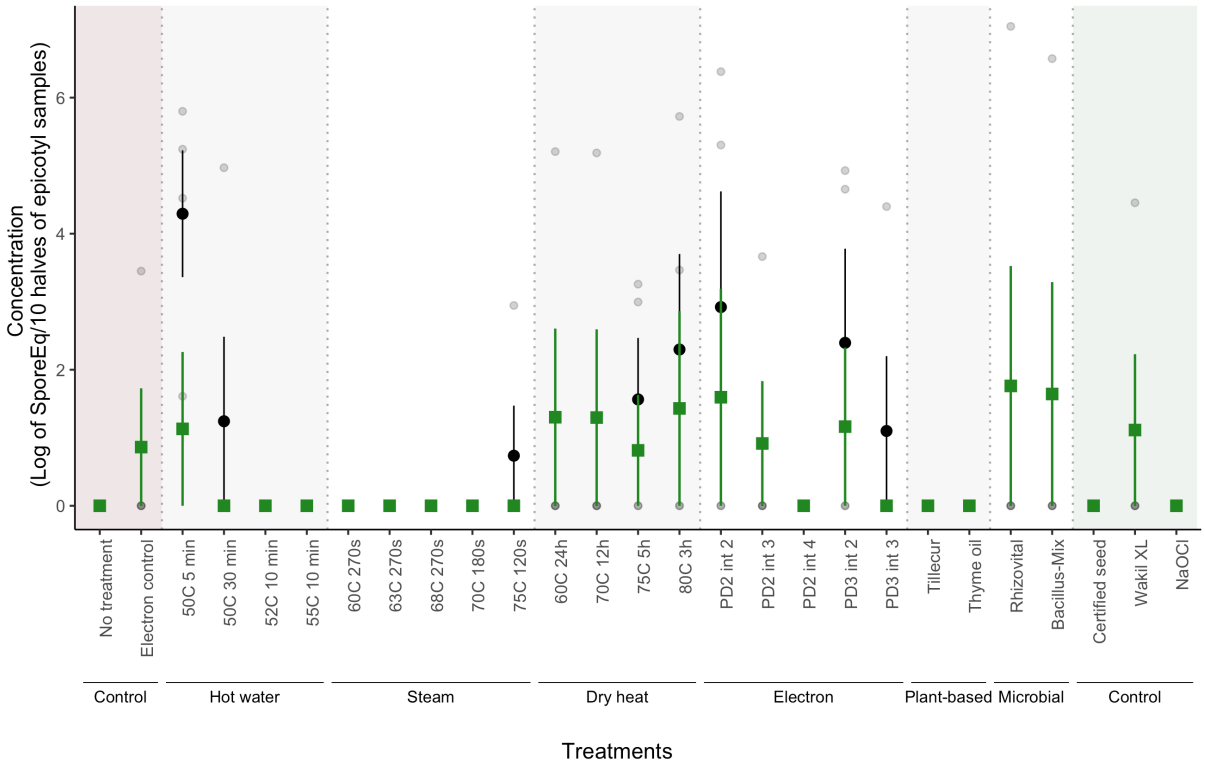

Figure 3 Mean concentrations of *Colletotrichum* spp. SporeEq/10 pooled epicotyl halves of different treatments. Green squares show the mean treatment concentrations when both technical replicates of the qPCR gave a signal. Black points show the mean treatment concentrations taking into account all concentrations, regardless of the number of technical replicates giving the signal. The residuals of the statistical model used for analysis were not satisfyingly normally distributed, which is why no information on significant differences between treatments is indicated. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.

Colletotrichum spp. was not detected in the NoTreatment control with either one of the two datasets. Signals from both technical replicates were obtained for the electron control; the hot water treatment HW-50C-5min; all four of the dry heat treatments; E-PD2-int2, E-PD2-int3 and E-PD3-int2; both microbial treatments; as well as the systemic WakilXL control. Taking into account signals from one single replicate as well, *Colletotrichum* spp. was detected in three additional treatments (HW-50C-30min, S-75C-120s and E-PD3-int3). Over all concentrations,

mean treatment concentrations ranged from 4.5 SporeEq/10 epicotyl halves (S-75C-120s) to 287.3 SporeEq/10 epicotyl halves (Rhizovital). *Colletotrichum* spp. was not detected with the treatments HW-52C-10min and HW-55C-10min; S-60C-270s, S63C-270s, S-68C-270s and S-70C-120s; E-PD2-int4; Tillecur and ThymeOil, and in the NoTreatment, CertifiedSeed and NaOCl controls. Statistical analysis of the concentrations was done on the rank and log-transformed concentrations of both datasets. However even after transforming the data, the residuals did not satisfyingly follow a normal distribution (see **App. Figure I** for residuals with and without rank transformation of dataset with all concentrations). Taking into account all concentrations, regardless of the number of technical replicates giving the signal, the hot water treatment HW-50C-5min significantly increased the *Colletotrichum* spp. concentration by 153.0 SporeEq/10 epicotyl halves when compared to the NoTreatment control. However since this was based on a model where the assumption of normal distribution of the residuals was not satisfyingly met, the significance of the difference was not indicated in **Figure 3** (page 27)

Fungal growth from epicotyl pieces on growth medium

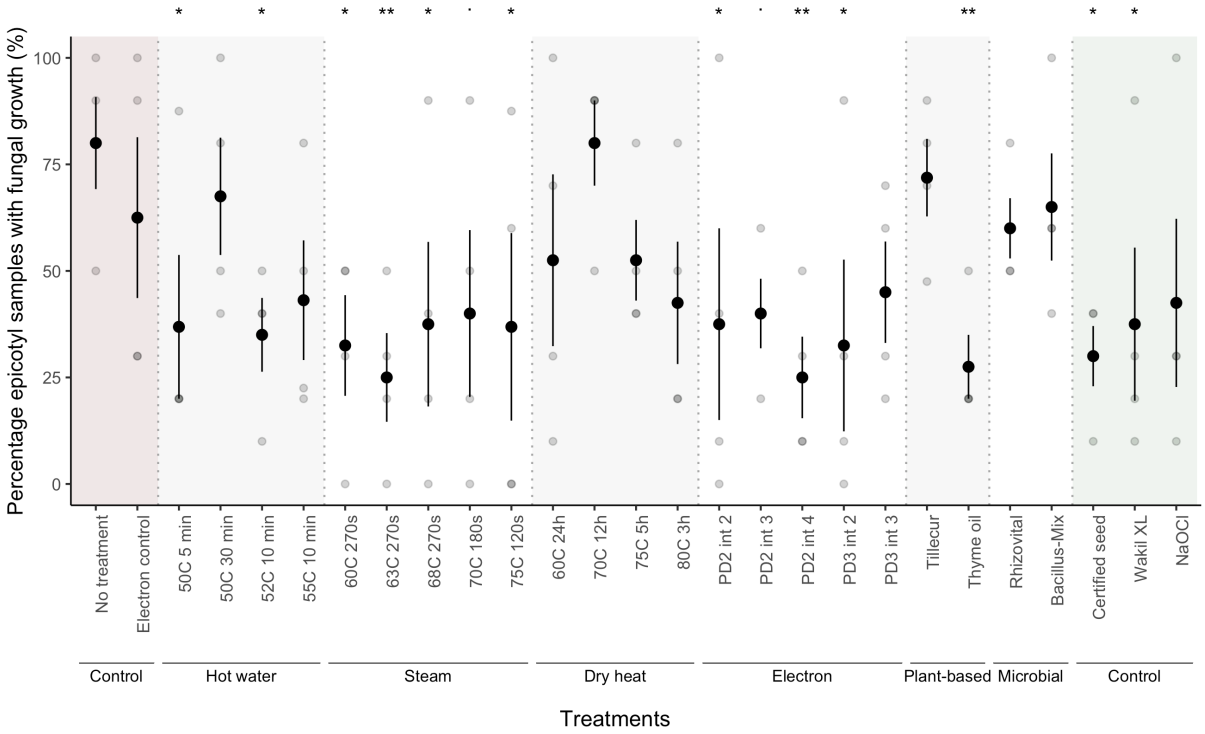

Figure 4 Mean percentages of epicotyl samples on growth medium that displayed fungal growth for different treatments. Stars at the top of the plot show the significant differences to the NoTreatment control (Dunnett's test; '***' = p<0.001, '**' = p<0.01, '*' = p<0.05, '.' = p<0.1). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.

Figure 4 (page 28) shows the mean percentages of epicotyl pieces put on growth medium which displayed fungal growth after incubation for 6 days at 22°C. None of the treatments showed a complete absence of fungal growth from the epicotyl pieces: the mean percentages of epicotyl pieces with fungal growth ranged from 25% to 80%. In total, 12 treatments – HW-50C-5min, HW-52C-10min, S-60C-270s, S-63C-270s, S-68C-270s, S-75C-120s, E-PD2-int2, E-PD2-int4, E-PD3-int2, ThymeOil and the CertifiedSeed and WakilXL controls – significantly ($p<0.05$ or less) reduced the fungal growth compared to the NoTreatment control. In addition, the differences between S-70C-180s and E-PD2-int3 and the NoTreatment control were tending toward significance. In other words, only the dry heat and the microbial treatments did not show any treatment conditions that significantly differed from the NoTreatment control for the percentage of epicotyl samples displaying fungal growth. The significant reductions in percentage samples with fungal growth compared to the NoTreatment control (80%) ranged from 53.2% (S-68C-270s, E-PD2-int2 and WakilXL) to 68.8% (S-63C-270s and E-PD2-int4).

The fungi growing on the plates were sorted into categories based on their morphological attributes. A total of 14 different categories were observed (**Figure 5**). All categories were analyzed with qPCR to determine whether *Colletotrichum* spp. was present. Only one fungal category, category D, gave a positive signal. This category was observed once in the entire experiment (i.e. on one epicotyl sample of 1080) for the treatment HW-50C-30min.

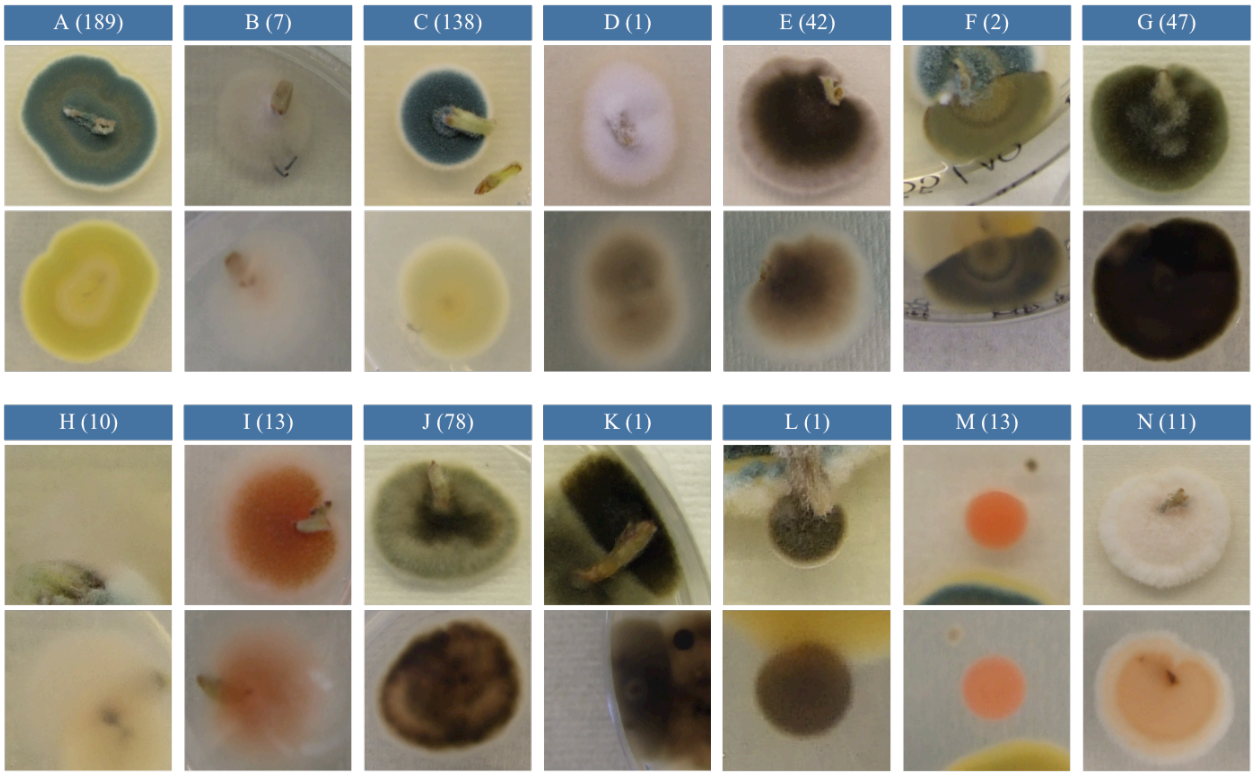

Figure 5 Representative images of the fungal categories observed on the plates containing the epicotyl samples. Each category was assigned a letter of the alphabet, as indicated above the pictures. The number in brackets gives the number of times the respective fungal category was observed in the entire experiment. Top pictures show the fungi as seen from the plate tops, bottom pictures as seen from the plate bottoms.

4.3 Pot-based disease assessment experiment

4.3.1 Germination rates and early vigour

To compare the germination assay to the pot experiment, germination rate and normal germination rate were recorded 1.5 weeks after sowing (**App. Figure II** and **App. Figure III** respectively). None of the treatments significantly reduced or improved the germination rate compared with the NoTreatment control (87.5%). The NaOCl control significantly ($p<0.01$) reduced the normal germination rate by 38.6% compared with the NoTreatment control (95.8%).

At the time point when germination was assessed, each treatment was additionally given an early vigour score (**Figure 6**, page 30). The NaOCl control (mean early vigour score of 3.8) significantly reduced ($p<0.01$) the vigour score by 41.5% compared to the NoTreatment control (mean early vigour score of 6.5).

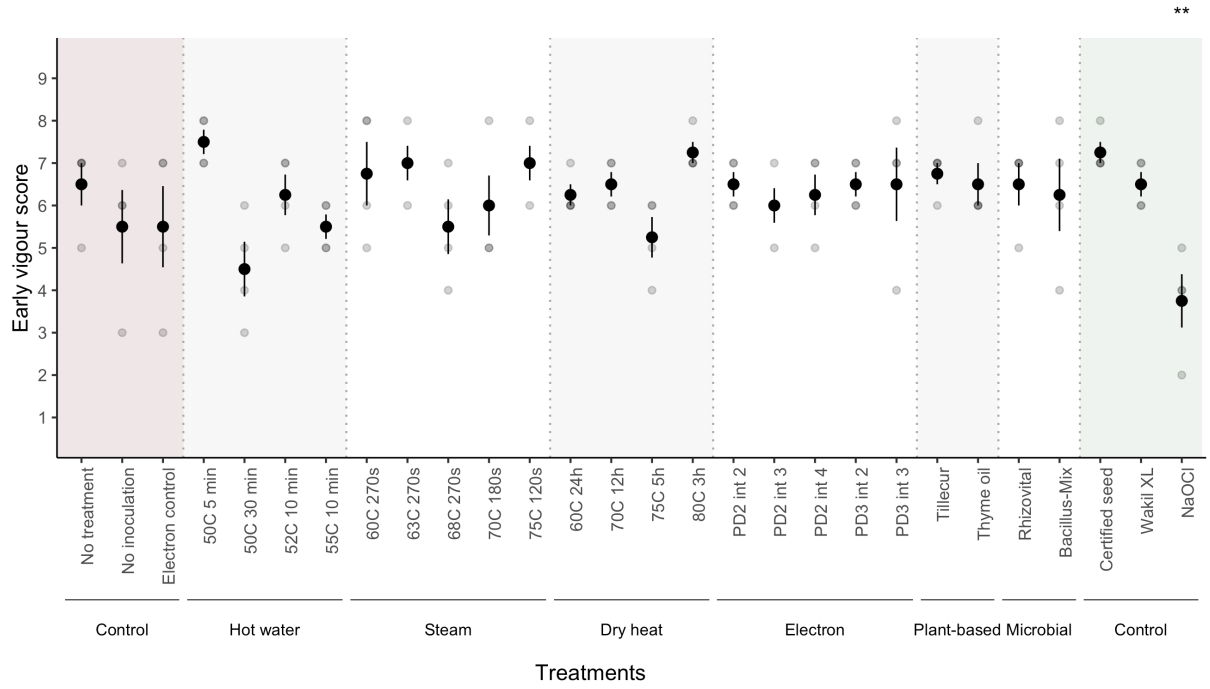

Figure 6 Mean early vigour scores of different treatments scored 1.5 weeks after sowing. Stars at the top of the plot show the significant differences to the NoTreatment control (Dunnett's test; '***' = $p<0.001$, '**' = $p<0.01$, '*' = $p<0.05$, '.' = $p<0.1$). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.

4.3.2 Vitality scoring

Mean vitality scores over time are shown in **Figure 7** (page 31). The same information is shown in **App. Figure IV**, with the four assessment time points separated into different panels to

provide more detail. The NoTreatment control had a vitality score of 6.3 at the beginning of the experiment (4 weeks), and a vitality score of 4.7 at the time point of harvest (7.5 weeks). Statistical analysis was done individually on each assessment time point. No significant differences between treatments and the NoTreatment control were found on any of the time points of assessment. Additionally, the vitality scores of the four assessment time points were averaged over time for each treatment replicate (**App. Figure V**), but no significant differences were detected between treatments and the NoTreatment control.

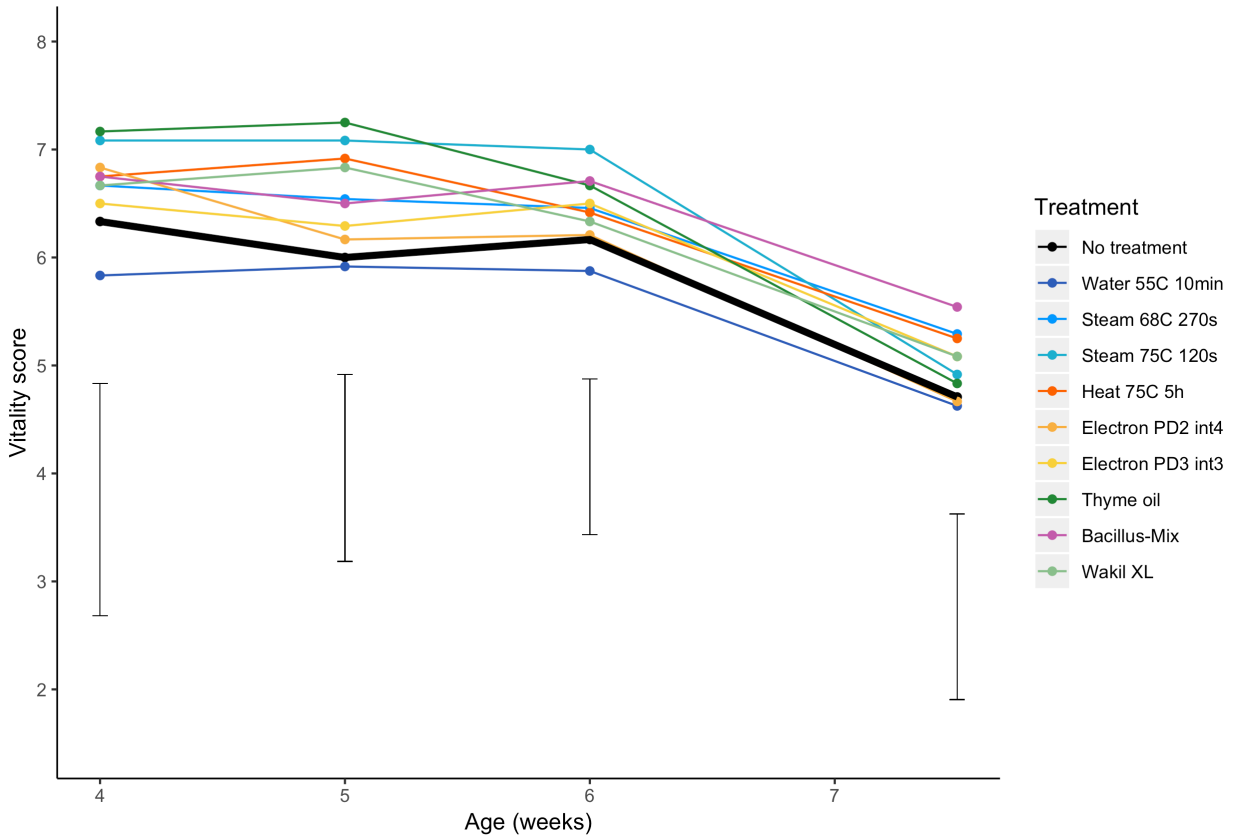

Figure 7 Mean vitality scores of different treatments over time. Possible scores range from 9 (completely healthy plant) to 1 (dead plant). Treatments are indicated in colour and the black bold line shows the NoTreatment control. Bars indicate Dunnett's critical distance between means for each time point of assessment.

4.3.3 Percentage of leaves with disease or weakening symptoms

The progression of the diseased leaves (percentage of total leaves), along with the diseased and dead leaves (percentage of total leaves) over time, are shown in **Figure 8** (page 32). The same data is shown with the four time points of assessment separated into different panels for more detail in **App. Figure VI**. Over the course of the experiment, the percentage of diseased leaves in the NoTreatment control moved from 35.0% (4 weeks) to 86.9% (7.5 weeks). The percentage of dead and diseased leaves of the NoTreatment control moved from 0% (4 weeks) to 31.2% (7.5 weeks). Time points of assessment were analyzed individually, but none of the treatments

showed a significant difference to the NoTreatment control on any of the four time points. The percentages of diseased and of dead and diseased leaves were additionally averaged over the four assessment time points per treatment replicate (**App. Figure VII**), but no significant differences were detected between treatments and the NoTreatment control.

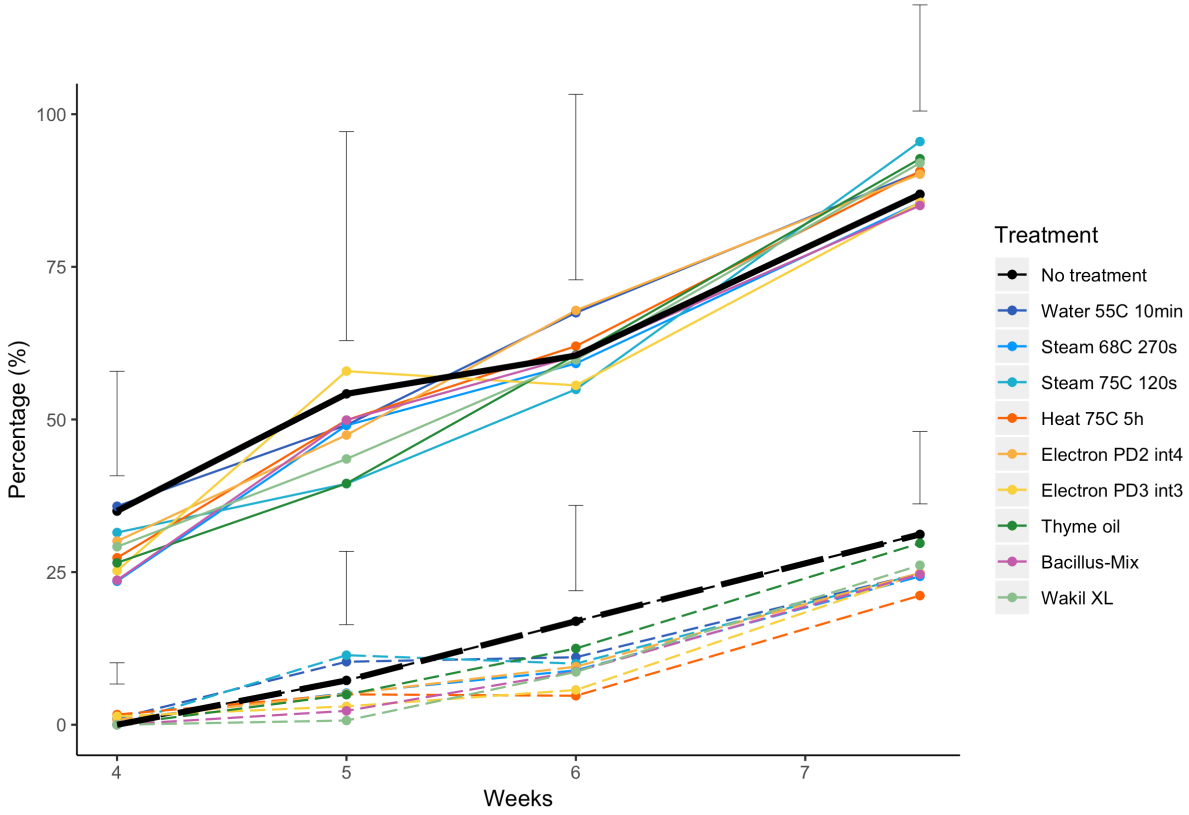

Figure 8 Mean percentages of diseased and of dead and diseased leaves of different treatments over time. Treatments are indicated in colour, the black bold lines show the NoTreatment control. Solid lines show the diseased leaves as percentage of total leaves, dashed lines show the dead leaves as percentage of total leaves. Bars indicate Dunnett's critical distance between means for each time point of assessment.

4.3.4 Plant biomass at harvest

Figure 9 (page 33) shows the mean shoot dry weights of all seed treatments in the pot experiment 7.5 weeks after sowing. The NoTreatment control had a mean shoot dry weight of 2.8 g, and none of the treatments significantly differed from the NoTreatment control. The mean fresh weights of shoots and roots, dry weights of roots, ratios of fresh and dry shoot to root biomass as well as the mean water content of the shoots are shown in the appendix (**App. Figure VIII** to **App. Figure XIII** respectively). None of these parameters showed a significant difference between the treatments and the NoTreatment control.

Roots were controlled for inoculation with rhizobia during harvest, and nodules were detected on the roots of all plants. Flowering started 6 weeks after sowing, and 70.8% of the plants (86 plants) had flowered after 7.5 weeks.

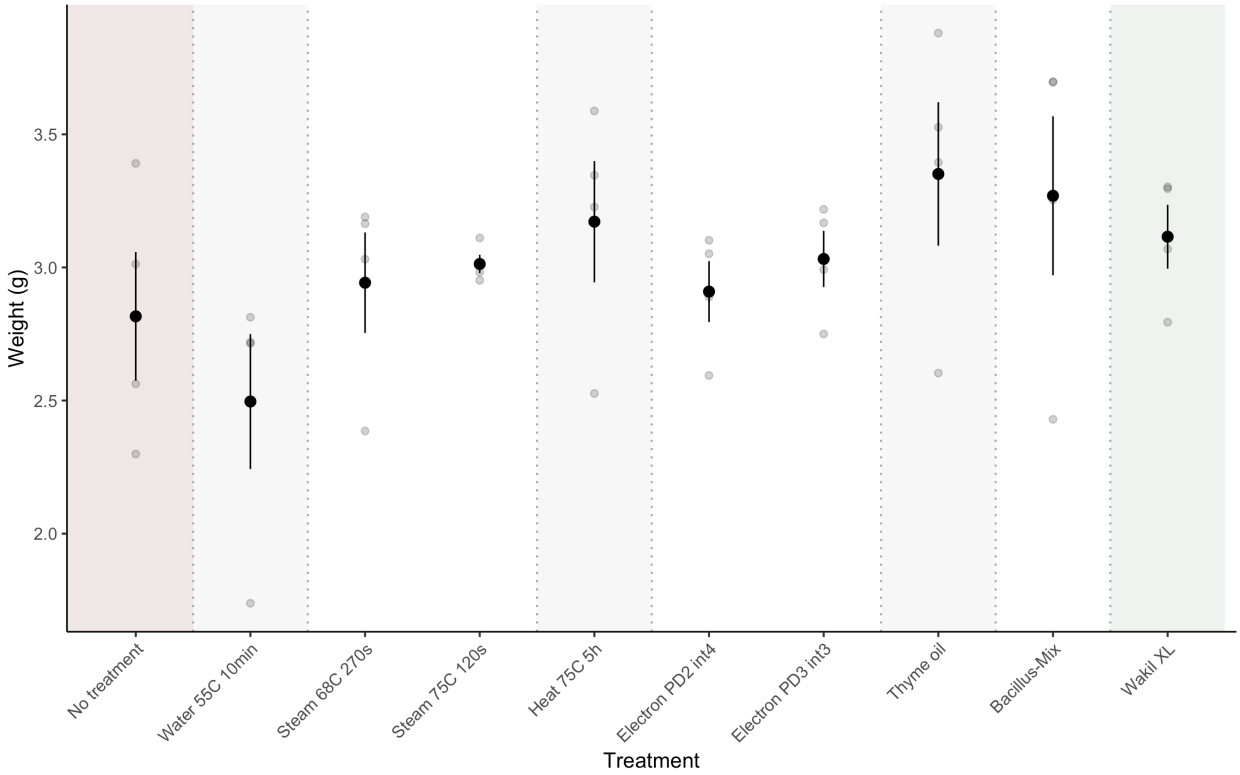

Figure 9 Mean shoot dry weights of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.

4.3.5 Molecular detection of *Colletotrichum* spp. in harvested shoot material

The concentrations of *Colletotrichum* spp. SporeEq/g fresh shoot material as determined by qPCR are shown in **Figure 10** (page 34). Analogous to what was observed in the qPCR analysis of the epicotyl DNA samples (section 4.2.2), various shoot samples collected from the 7.5-week old plants in the pot experiment gave a positive qPCR signal for *Colletotrichum* spp. with one qPCR technical replicate, while the other technical replicate did not give a signal. It was therefore again necessary to construct two datasets for analysis, depending on the number of technical replicates giving the signal. The two log-transformed datasets are displayed in different colours in **Figure 10** (page 34). *Colletotrichum* spp. was only detected with both qPCR technical replicates with the treatment E-PD2-int4. Taking into account all concentrations, irrespective of the number of underlying technical replicates giving the signal, mean treatment concentrations ranged from 0.5 (BacillusMix) to 9079.5 (NoTreatment) SporeEq/g fresh shoot material. *Colletotrichum* spp. was not detected with the two steam treatments S-68C-270s and S-75C-120s, the dry heat treatment DH-75C-5h and the electron treatment E-PD3-int3. Statistical analysis of the concentrations was done on the rank and log-transformed concentrations of the dataset containing all values, irrespective of the underlying number of technical replicates giving the signal. However even after transformation, the residuals did not satisfyingly follow a normal

distribution (**App. Figure XIV**), and none of the treatments showed a significant difference to the NoTreatment control.

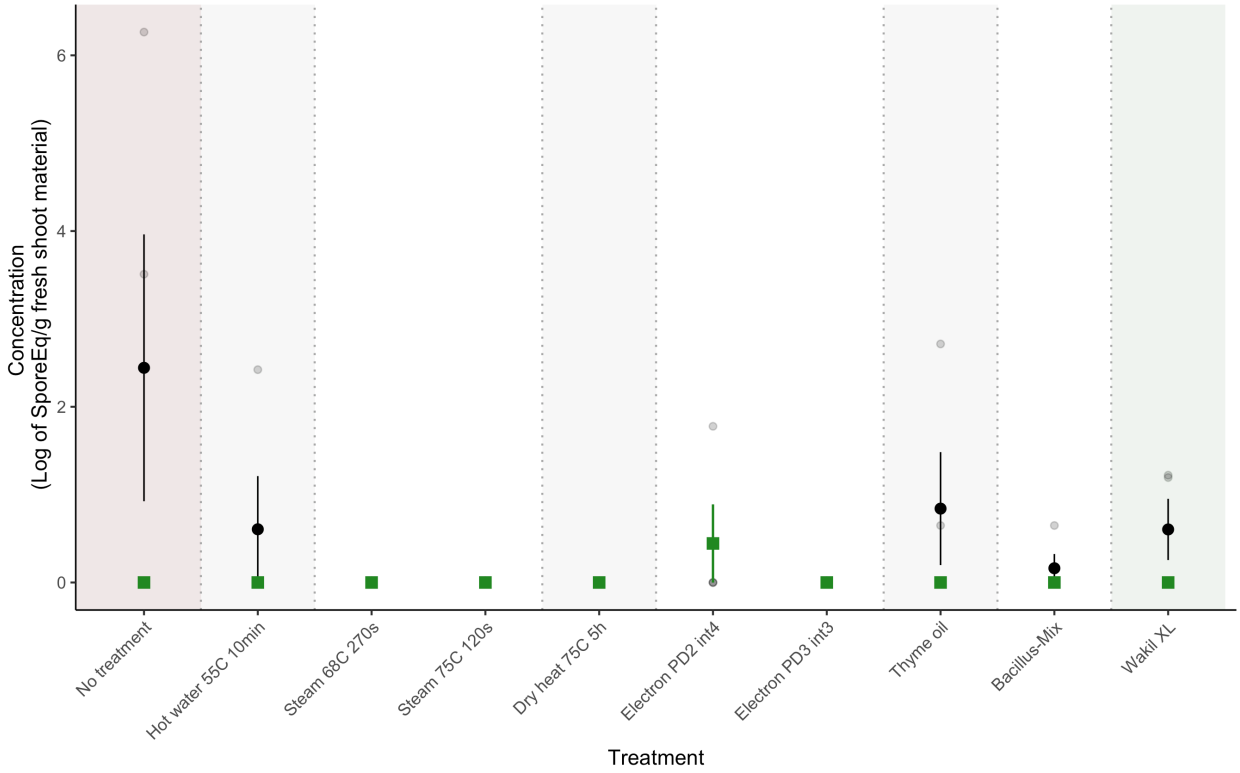

Figure 10 Mean log-transformed concentrations of *Colletotrichum* spp. SporeEq/g fresh shoot material of different treatments in pot experiment, harvested 7.5 weeks after sowing. Green squares show the mean treatment concentrations when both technical replicates of the qPCR gave a signal. Black points show the mean treatment concentrations taking into account all concentrations, regardless of the number of technical replicates giving the signal. The residuals of the statistical model used for analysis were not satisfyingly normally distributed, which is why no information on significant differences between treatments is indicated. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.

5 Discussion

5.1 General discussion of experiments

Germination and normal germination were assessed after 1.5 weeks in both the germination assay and in the pot experiment, with concurrent observations from both these experiments. Epicotyl samples were taken from seedlings to detect whether *Colletotrichum* spp. was already present above the cotyledons after 1.5 weeks. *Colletotrichum* spp. was detected by qPCR with several of the treatments, but no signals were detected in the non-treated control. Furthermore, several samples only gave a signal with one of the two technical replicates in the qPCR runs. The absence of *Colletotrichum* spp. from the non-treated control and the general fluctuation in qPCR signals could indicate that 1.5 weeks is simply too soon for the fungus to be consistently established in the epicotyl. Another explanation for the observed variability could also be an underlying variation in initial inoculum of the seeds. Characterization of the seed batch used for treatments indicated *Colletotrichum* spp. presence in all of the tested seeds – although detection of DNA by qPCR does not necessarily mean that the fungus is viable. The same epicotyl samples used for molecular detection of *Colletotrichum* spp. were also put on growth medium, and various morphological categories of fungal growth were observed. However, only one of these categories, which was seen only on one out of 1080 epicotyl pieces, contained *Colletotrichum* spp. Again, the failure to detect *Colletotrichum* spp. could indicate that the fungus was not present in the epicotyl pieces – either because the time point of assessment was too soon, or because not enough pathogen was present in the seeds – or that it was present, but could not grow on the growth medium due to low competitiveness compared to other microorganisms of the plant microbiome. Since some qPCR signals for *Colletotrichum* spp. were detected, albeit not in the non-treated control, this would hint towards the pathogen being present but not growing on the plates due to low competitiveness. The assessment of the fungal growth from epicotyl pieces showed that some of the treatments had a significant effect on the general plant microbiome, since significant reductions in fungal growth occurred. In the pot experiment, no significant differences to the non-treated control were detected for any of the assessed parameters. This could mean that either there was not enough pathogen in the plants for the treatments to have an effect on it, or *C. lupini* was present, but the treatments did not have enough of an effect to show differences to the control. Although symptoms of plant weakening and unspecific disease symptoms such as yellowing, drying out and falling off of leaves were seen, no characteristic anthracnose symptoms – lesions with sporulating fungus or bending over of the stems – appeared. This supports the hypothesis that *C. lupini* was not present at a high enough level to lead to a strong anthracnose outbreak. Alternatively, the growth conditions might not have been optimal for growth of *C. lupini* in the plants, or the experiment might have been stopped before the disease could really break out. Overall, it therefore seems as though the disease was missing or not strong enough when the plants were assessed, and what little was present might not have been enough for the seed treatments to show an effect. The initial inoculum level in the seeds was tested by qPCR, and all of the tested seeds gave a signal for *Colletotrichum* spp. However, this was not necessarily viable fungus. The seeds used for the treatments had been harvested from a field trial in 2017, from plots where anthracnose disease had been present. It is known that storage leads to a reduction in seed inoculum levels: Weimer (1952) reported that approximately half the fungus in infected seeds had died by the planting time of the harvest year. It is therefore well possible that the viable *C. lupini* in the seeds progressively diminished from the time of

harvest until the seeds were used in this experiment, and that the initial inoculum level in this experiment was much lower than first expected.

5.2 Effects of seed treatments on germination and anthracnose occurrence

5.2.1 Looking at treatments within a treatment category

Hot water treatments

None of the hot water treatments studied here reduced the germination rates compared to the non-treated control, and only HW-50C-30min showed a tendency to reduce the normal germination rate. For HW-50C-5min, this coincides with findings from literature, as Weimer (1952) found a 98% germination rate with these same conditions. However, reported germination rates for the other hot water treatments differ from what was observed here. For HW-50C-30min, germination rates ranging from 20% (Weimer, 1952), to approximately 60% in a field trial with blue lupin (Vetter, 2006), to more than 80% both under laboratory and field conditions (Nawrath and Vetter, 2002) have been reported. No specifications on normal germination rate were found. This wide range in reported effects of HW-50C-30min on germination might be reflected here in the tendency toward reduced normal generation observed. Weimer (1952) also reported a germination rate of 48% for HW-52C-10min, compared to 96% in the non-treated control. Seeds in the study of Weimer (1952) were pre-soaked for 1 hour at 45°C and given a post-treatment immersion in cold water. On the other hand, neither Nawrath and Vetter (2002) nor Vetter (2006) – who reported germination rates closer to the ones found here – included either one of these two steps. Should the discrepancies between reported and observed germination rates thus be due to differences in application protocol, the pre-soaking or the post-immersion in cold water could be likely explanations for these discrepancies. Since the pre-soaking control of Weimer (1952) did not show reduced germination, the post-treatment immersion in cold water could be the reason for impaired germination in that study. Of the four hot water treatments tested here, *Colletotrichum* spp. was detected in 1.5-week old epicotyl samples with HW-50C-5min and HW-50C-30min. Furthermore, HW-50C-30min did not show as high an effect on the seed microbiome as the other hot water treatments, and it was the only treatment where a colony identified as *Colletotrichum* spp. was observed. Finally, HW-50C-5min slightly improved the early vigour of 1.5-week old seedlings, while the other three hot water treatments slightly reduced it – HW-50C-30min showed the highest reduction. Overall, HW-52-10min and HW-55C-10min thus seemed to show the highest indication of efficacy against *Colletotrichum* spp. in 1.5-week old seedlings, with unclear effects of the two treatments at 50°C. Previous studies have reported a decrease in anthracnose occurrence from HW-50C-5min (68%) to both HW-50C-30min (0%) and HW-52C-10min (0%; Weimer, 1952), or a slight decrease in seed infection from treating at 50°C for 20 minutes to 30 minutes (Nawrath and Vetter, 2002). On the other hand, Lindner et al. (1999) observed an increase in anthracnose occurrence from treatment at 50°C for 10 minutes to 50°C and 20 minutes. In literature as well, there thus appear to be conflicting findings on the efficacy of disease suppressiveness when treating at identical temperatures but different durations. Indeed, while treating for a longer duration might have a stronger effect on the pathogen, it might also more strongly impair the plant itself, which could then again favour the pathogen. Furthermore, it is interesting to note that, while the treatment HW-50C-30min was the seed treatment most often mentioned across publications and studies on lupin anthracnose (Kaufmann et al., 2009; Nawrath and Vetter, 2002; Römer, 2007; Vetter, 2006), it did not

compare very well to the other treatments studied here regarding both germination and efficacy of *Colletotrichum* spp. suppressiveness in 1.5-week old seedlings.

Steam treatments

Germination rate was not impaired by any of the five tested steam treatments, but the normal germination showed a tendency toward being reduced by S-68C-270s and S-75C-120s in the germination assay, and by S-60C-270s and S-70C-180s in the pot experiment. The only other study investigating steam seed treatments against lupin anthracnose also reported no impairment of germination rates by the treatments (Nawrath and Vetter, 2002). However the treatment conditions in that study – temperatures from 150°C to 250°C, tested for a duration of 15 seconds – were very different from the ones included here (Nawrath and Vetter, 2002). Heller (2009) tested steam on carrot seeds at 65°C for durations up to 120 seconds, and found germination rates between approximately 70% and 90%. The germination rates found here were higher, even though higher temperatures and longer durations were also included. This might be because lupin seeds can support stronger treatment conditions than carrot seeds due to their thick seed coat (Perissé and Planchuelo, 2004). Fungal growth from 1.5-week old epicotyl samples was reduced in all the steam treatments – significantly in all but S-70C-180s –, and *Colletotrichum* spp. could only be detected in S-75C-120s. Early vigour was slightly reduced by S-68C-270s and S-70C-180s. Over the first 1.5 weeks, all steam treatments except S-75C-120s thus seemed to indicate a good efficacy of *Colletotrichum* spp. suppression, and S-63C-270s was the only treatment not giving any indication of impairment of normal seed germination. Two steam treatments were included in the long-term pot experiment: S-68C-270s and S-75C-120. While on the first three assessment time points, S-75C-120s consistently received a higher vitality score than S-68C-270s, the opposite was true on the last assessment time point. Very similar fresh and dry weights of shoots and roots were observed after 7.5 weeks, and *Colletotrichum* spp. was not detected in 7.5-week old shoots from either one of the treatments. Differences between treatments were observed in the shoot water contents: shoots of S-75C-120s contained slightly more water than shoots of S-68C-270s, indicating a slightly more desiccated plant with the latter condition. Overall, S-75C-120s thus seemed to perform better in the pot experiment than S-68C-270s. It is also noteworthy that S-75C-120s was the only steam treatment for which *Colletotrichum* spp. was detected in 1.5-week old epicotyls, but no signal appeared in 7.5-week old shoots. Heller (2009) observed a general reduction of fungal contamination of carrot seeds after steam treatment: treatment at 65°C for 90 and for 120 seconds led to 0% fungal contamination. This coincides with the overall low qPCR signals for *Colletotrichum* spp. and reduction of fungal growth from epicotyl samples observed here. However, a complete eradication of fungal growth from epicotyl samples was not observed for any treatment, even though higher temperatures and longer durations than in the study of Heller (2009) were studied. This might again be due to reasons of seed morphology: lupin seeds have a thick seed coat, and while similar treatment conditions might thus lead to less impairment of germination, they might also have a weaker effect on the seed microbiome.

Dry heat treatments

All dry heat treatments maintained the germination rate at the level of the non-treated control. The normal germination rate was slightly reduced by DH-70C-12h and DH-80C-3h in the germination assay, and by DH-75C-5h in the pot experiment. While no germination rates were found in literature for the exact conditions tested here, seeds treated at 60°C, 70°C or 80°C for one week had germination rates of 87%, 91% and 32% respectively (Thomas and Adcock, 2004). Such a drop in germination rate with temperatures above 70°C was not observed here, and

Weimer (1952) also reported germination rates of 90% for seeds treated at 75°C for both 4 and 7 hours. The strong reduction of germination observed by Thomas and Adcock (2004) with the treatment at 80°C is likely due to the very long duration of the treatment, while a duration of 3 hours did not lead to germination impairments in the study described here. None of the treatments showed a significant reduction in fungal growth from epicotyl samples compared to the non-treated control, although slight reductions were observed with DH-60C-24h, DH-75C-5h and DH-80C-3h. *Colletotrichum* spp. was detected in epicotyl samples of all four treatments, with the lowest concentration found in DH-75C-5h (taking into account only samples where both qPCR technical replicates gave a signal). Early vigour was slightly reduced by DH-70C-12h. Overall, the dry heat treatments did therefore not impair germination, but also indicated a low efficacy of *Colletotrichum* spp. reduction. In literature, reductions of anthracnose infection levels were observed after treating seeds at 70°C for 7 hours (22% infection), as well as at 75°C for 4 and 7 hours (0% infection for both durations; Weimer, 1952). On the other hand Thomas and Adcock (2004) only noted a slight reduction in infection level with the treatment condition DH-60C-24h, with some inoculum still present even after treating for 7 days or more at that temperature. Reductions of seed infection levels were also reported following treatment at 70°C and 80°C (Thomas and Adcock, 2004), but the reductions depended on the initial infection levels of the seeds. In the seeds with the higher initial inoculum, complete elimination of the pathogen was observed between 2 and 4 days treatment at 70°C. In the seeds with the lower initial inoculum, complete elimination of the pathogen occurred between 8 and 24 hours treatment at 70°C, and between 4 and 8 hours treatment at 80°C.

Electron treatments

None of the electron treatments reduced the germination rate, while E-PD2-int2 and E-PD2-int4 slightly reduced the normal germination rate in the germination assay. In previous studies, emergences between 79% and 92.8% have been reported following electron treatment of lupin seeds (Lindner et al., 1999; Nawrath and Vetter, 2002). However, since the exact parameters of the electron treatments tested in this study are currently not known, no definite comparisons to previous studies can be made. All electron treatments showed a reduced fungal growth from epicotyl samples, with E-PD3-int3 being the only treatment where the difference to the non-treated control was not or did not tend to be statistically significant. However, *Colletotrichum* spp. was detected in epicotyl samples with all treatments except E-PD2-int4. The early vigour scores with all electron treatments remained unchanged to the non-treated control. In the long-term pot experiment, E-PD3-int3 generally received slightly higher vitality scores than E-PD2-int4. E-PD3-int3 showed slightly higher final fresh and dry weights of shoots and roots than E-PD2-int4, and also had a higher mean water content in the shoots at the time point of harvesting. Furthermore, no *Colletotrichum* spp. was detected in the 7.5-week old shoots with E-PD3-int3, while some fungus was detected with E-PD2-int4. Overall, E-PD3-int3 therefore seemed to indicate the best efficacy of disease suppressiveness, suggesting that a higher penetration depth might be more important to reduce *C. lupini* concentrations than a higher treatment intensity. This agrees with the findings of Lindner et al. (1999), who reported an increase in anthracnose occurrence with increasing treatment dosage (i.e. intensity) but identical voltage (i.e. penetration depth). On the other hand, Nawrath and Vetter (2002) observed an increase in anthracnose occurrence with increasing voltage (i.e. penetration depth), but identical dosage (i.e. intensity), which was not observed in this experiment.

Plant-based treatments

Neither Tillecur nor thyme oil negatively affected the seed germination rates, however both treatments slightly reduced the normal germination rate in the pot experiment. For Tillecur, this agrees with previous reported germination rates from literature, where field emergences between 89% and 91% in lupin (Nawrath and Vetter, 2002), as well as emergences of 88.0% in bean and 90.7% in pea (which corresponded to a tendency toward reduced emergence; Tinivella et al., 2009) were observed. For thyme oil, emergences of 88.0% in bean and 96.0% in pea (which corresponded to a tendency toward reduced emergence; Tinivella et al., 2009) with 0.1% thyme oil have been reported, but also germination rates of 50% in wheat seeds directly treated with 0.05% thyme oil, and almost 0% germination with a thyme oil concentration of 0.2% (Anzlovar et al., 2017). However, when wheat seeds were treated indirectly with the essential oil, germination rates were close to 100% with thyme oil concentrations up to 2% (Anzlovar et al., 2017). In both the study described here and the study of Tinivella et al. (2009), seeds were treated directly with the thyme oil, but no strong impairments of seed reduction were observed. Again, these differences might be due to differences in seed coat thickness or morphology between legumes and wheat. Although only thyme oil reduced the amount of fungal growth from epicotyl samples, *Colletotrichum* spp. signals were not detected with either one of the two treatments. Furthermore, early vigour was maintained with both plant-based treatments. After 1.5 weeks, both treatments thus seemed to indicate effective reduction of *Colletotrichum* spp. in the seedlings, with thyme oil showing the stronger effect on the overall plant microbiome. In the study of Tinivella et al. (2009), Tillecur significantly reduced the diseased plants as percentage of the emerged plants and increased the healthy plants as percentage of total seeds sown in bean, but showed no significant effects in pea. On the other hand, thyme oil significantly reduced the diseased plants as percentage of the emerged plants in bean, but this effect was not significant anymore when compared to the total seeds sown, due to a reduced germination rate (Tinivella et al., 2009). In pea, thyme oil significantly reduced the disease index and increased the healthy plants as percentage of total seeds sown (Tinivella et al., 2009). Over the entire study, thyme oil therefore demonstrated a wider effect on seed-borne pathogens than Tillecur (Tinivella et al., 2009), which might be reflected here in the stronger effect on overall plant microbiome. In the study of Anzlovar et al. (2017), significant reductions of infection rates where also observed after thyme oil treatment. At a same essential oil concentration, the reduction was stronger with direct than with indirect treatment application (Anzlovar et al., 2017), however with a direct application of 0.2% thyme oil, an infection rate of approximately 20% was still detected.

Microbial treatments

Like the plant-based treatments, neither the treatment with RhizoVital 42 nor with the mixture of three *Bacillus* species showed a negative effect on germination rate. In the pot experiment, both treatments led to a slightly reduced normal germination rate. The active ingredient of RhizoVital 42 is the bacterium *Bacillus amyloliquefaciens*, while the *Bacillus*-mix treatment consisted of two *B. subtilis* strains and one strain of *B. megaterium*. For seed treatment with *B. subtilis*, emergence rates from 77.3% to 94.0% have been reported in bean seeds (the reduction compared to the non-treated control was significant for one of four tested strains; Tinivella et al., 2009), and from 96.7% to 98.0% in pea seeds (Tinivella et al., 2009). In the experiment described here, neither of the microbial treatments led to a significant reduction of fungal growth from the epicotyl pieces, and *Colletotrichum* spp. was detected in the epicotyls with both treatments. Early vigour was not affected by either one of the treatments. Overall, neither of the microbial treatments therefore seemed to indicate effective reduction of *Colletotrichum* spp. inoculum levels, and no differences between the two treatments were detected. RhizoVital 42 had been

applied on seeds at a concentration of 0.2%. After treatment application, it was noted that the producer recommends mixing the stock solution in only a small amount of water, and apply the product on seeds that way (Andermatt Biocontrol, 2018). Unfortunately, the reference for using a 0.2% concentration could not be traced back. It must therefore be concluded that the Rhizovital treatment was conducted with an inadequate *B. amyloliquefaciens* concentration, and this might explain the low efficacy of the treatment against *Colletotrichum* spp. in this study. In bean, three of four tested *B. subtilis* strains significantly reduced the diseased plants (as percentage of emerged plants) and significantly increased the healthy plants (as percentage of seeds sown), while one strain significantly decreased the healthy plants (as percentage of seeds sown) when compared to the non-treated control (Tinivella et al., 2009). In pea, one of the tested *B. subtilis* strains significantly increased the disease index, and two of the strains significantly reduced the healthy plants (as percentage of seeds sown; Tinivella et al., 2009). In that study, several *B. subtilis* strains thus seemed to have a good efficacy against bean anthracnose, but showed no efficacy against Ascochyta blights on pea. However, not all strains showed the same disease-suppressing efficacy, and concentrations of at least $1x10^9$ spores/ml were used (Tinivella et al., 2009), compared to at least $1x10^6$ spores/ml in this study. The low efficacy of the *Bacillus*-mix treatment might therefore also be due to an insufficient spore concentration, or to an insufficient disease-suppressing efficacy of the included *Bacillus* strains.

Non-inoculated and electron controls (negative controls)

Neither the non-inoculated nor the electron control significantly differed from the non-treated control for any of the parameters assessed in this experiment. Nevertheless, the non-inoculated control showed a slight increase in germination rate in the pot experiment, and the electron control exhibited slightly less fungal growth than the non-treated control in the culture-based assessment of the epicotyl samples. Also, *Colletotrichum* spp. was detected in epicotyls from the electron control, while none was detected in the non-treated and non-inoculated controls. Both the electron and the non-inoculated control slightly reduced the early vigour of 1.5-week old seedlings. Although the differences between electron and non-treated control were not significant, the variation in observed treatment effects (detection of *Colletotrichum* spp. in epicotyls with one treatment but not the other, slight reduction in fungal growth from epicotyls with electron control) points toward an underlying variation in seed inoculum of non-treated seeds. This is also supported by the observations from the non-inoculated control (slightly higher germination rate, slightly reduced early vigour), since absence of rhizobia should not show an effect 1.5 weeks after sowing (de Lorenzo et al., 1998).

Certified seed, systemic fungicide and surface sterilization controls (positive controls)

The sodium hypochlorite treatment – intended as a surface sterilization control – was the only treatment to significantly reduce the germination rate in the germination assay. The normal germination rate was significantly reduced by sodium hypochlorite in both the germination assay and the pot experiment, and Wakil XL (systemic fungicide control) slightly reduced the normal germination rate in the germination assay. Fungal growth from epicotyl pieces was significantly reduced in the certified seed control, as well as by Wakil XL. With sodium hypochlorite, the fungal growth was also reduced, but the difference to the non-treated control was not significant. *Colletotrichum* spp. was only detected in epicotyls with the Wakil XL treatment. Early vigour was significantly reduced by sodium hypochlorite. After 1.5 weeks, the certified seed control was therefore the only positive control that showed no presence of *Colletotrichum* spp. and at the same time maintaining high seed vigour. In the pot experiment, Wakil XL showed a slightly higher vitality score than the non-treated control for all time points of assessment, but

Colletotrichum spp. was detected in 7.5-week old shoots. Similar to previous studies on seed treatments against lupin anthracnose (Lindner et al., 1999; Nawrath and Vetter, 2002; Thomas and Adcock, 2004; Vetter, 2006), no complete elimination of *Colletotrichum* spp. was therefore obtained with the systemic fungicide control. The sodium hypochlorite control was initially intended as surface sterilization control, since *C. lupini* can be present both on the seed surface and underneath the seed coat (Cwalina-Ambroziak and Tomasz, 2004; Kreye and Niepold, 2007). No *Colletotrichum* spp. was detected with sodium hypochlorite, but it was the only treatment to significantly impair seed vigour. This was probably due to the long time (10 minutes) for which the seeds were soaked in the sodium hypochlorite solution (concentration <5%). This protocol for treatment application was taken from Szuszkiewicz (2016), however in that study the surface sterilization was used for subsequent culture-based seed incubation, and not for use of seeds in a pot experiment. Other studies report surface sterilization of legume seeds with sodium hypochlorite for durations and concentrations ranging from 30 seconds in a 0.1% solution (lupin seeds; Cwalina-Ambroziak and Tomasz, 2004), to 3 minutes in a 5.25% solution (soybean seeds; Begum et al., 2010), 5 minutes in a 10% solution (lupin seeds; Bancel and Citharel, 1997), 15 minutes in a 10% solution (lupin seeds; Sánchez-Pardo and Zornoza, 2014) or 20 minutes in a 20% solution (lupin seeds; Annicchiarico and Alami, 2012). However, these protocols always included a thorough rinsing or soaking step of seeds in water after treatment with sodium hypochlorite. As such a step was not mentioned in the protocol used in this study (Szuszkiewicz, 2016), the sodium hypochlorite treatment tested here was likely too strong and had a stronger effect than only surface sterilization.

5.2.2 Comparison of treatment categories

When looking at the fungal growth from epicotyl pieces, all treatment categories except dry heat and microbial exhibited significant reductions compared with the non-treated control. Molecular detection of *Colletotrichum* spp. revealed presence of the fungus in 1.5-week old seedlings with all categories except steam and plant-based. In the long-term pot experiment, the hot water treatment consistently received slightly lower vitality scores than the non-treated control, while all other treatments – except E-PD2-int4 – consistently received slightly higher vitality scores than the non-treated control. Particularly the microbial treatment (*Bacillus*-mix), the dry heat treatment (DH-75C-5h) and the steam treatment S-68C-270s were always among the treatments with the highest vitality scores. The steam treatment S-75C-120s and the thyme oil treatment had the highest vitality scores at the beginning of the experiment but exhibited a drop in vitality scores on the last assessment time point. Molecular detection revealed *Colletotrichum* spp. presence in shoots of all treatments except the two steam treatments, the dry heat treatment and the electron treatment E-PD3-int3. Overall, it therefore seemed like the steam and plant-based treatments showed the greatest indication of effect on *Colletotrichum* spp. in 1.5-week old seedlings (no signal in qPCR), while in 7.5-week old shoots, the steam treatments and the dry heat treatment showed the highest indication of efficacy against *Colletotrichum* spp. (no signal in qPCR) with the strongest maintenance of plant vigour (slightly higher vitality scores than non-treated control). Additionally, the electron treatment E-PD3-int3 seemed to suppress *Colletotrichum* spp. (no signal in qPCR), while *Bacillus*-mix and to a lesser extent the thyme oil treatment seemed to positively impact plant vitality scores, even though *Colletotrichum* spp. was detected in the shoots. Several studies have already compared different seed treatment categories for their efficacy against lupin anthracnose. For instance Nawrath and Vetter (2002) compared hot water, electron, plant-based and microbial treatments, and identified one hot water treatment,

treatment with Tillecur and the electron treatments as effective methods to combat lupin anthracnose. Lindner et al. (1999) compared hot water and electron treatments, and concluded that hot water was effective but impractical, while electron treatment showed low efficacy against lupin anthracnose. Waldow et al. (2006) tested warm and hot water, as well as plant-based and microbial treatments – among which Tillecur, various plant extracts and oils, as well as a *B. subtilis*-based product –, but found that no treatment was effective against lupin anthracnose. Although previous comparisons of treatment categories therefore seem somewhat conflicting, hot water repeatedly appeared as one of the most effective treatments. This was to the contrary of what was observed in this study, since in the long-term pot experiment the hot water treatment had the lowest vitality scores of all and *Colletotrichum* spp. was detected in 7.5-week old shoot material. It is noteworthy that in the pot experiment, the dry heat treatment seemed to be one of the most effective treatments against *Colletotrichum* spp., while in 1.5-week old seedlings none of the dry heat treatments had seemed to have an effect on the fungus. Detection of *Colletotrichum* spp. in an early stage of the plant development might therefore not necessarily indicate low efficacy of the respective seed treatment. The steam treatments fared well over the entire course of the experiment and constitute the treatment category that showed the overall strongest indication of efficacy against *Colletotrichum* spp. without impairment of plant vigour. Thyme oil also seemed promising in seedlings and in the early assessment time points of the pot experiment, but less so at the time point when plants were harvested. *Colletotrichum* spp. was also found absent from seedlings and shoot material with treatments of the electron category. All in all, the steam, dry heat and electron treatments, as well as treatment with thyme oil, therefore seemed to indicate promising effects against *Colletotrichum* spp.

5.3 Practical relevance of steam, dry heat, electron and thyme oil treatments

Germination

A first look at practical relevance of the tested treatments was taken here by assessing whether any treatments impaired the germination rate. Both in the germination assay and in the pot experiment, no such impairment was observed with any of the treatments using steam, dry heat or electrons or thyme oil. In soybean, Kulik and Yaklich (1982) found a significant correlation between sand bench test and field emergence. Another study in common bean revealed a significant correlation between standard germination test and field emergence with temperatures at sowing between 9 and 15°C, with the first count of the germination test showing a higher correlation with field emergence than the last count (Kolasinska et al., 2000). However, both correlations were not significant anymore at sowing temperatures below or above this intermediate temperature range, and the authors concluded that only conductivity testing of seed leachate could predict field emergence regardless of the soil temperature at sowing (Kolasinska et al., 2000). This confirmed what had been found in a previous study on emergence of pea seeds (LaDonne, 1989). The high germination rates found here could therefore indicate a high potential field emergence under favourable conditions at sowing. However, under less favourable conditions, the germination rates found here might not adequately predict field emergence. Other assays, for example the conductivity test of seeds, might give further indications on potential field emergence of the treated seeds.

Comparison with FiBL field trial 2018

Several of the treatments assessed in this study were also included in a FiBL field trial taking place over the summer 2018 (FiBL, 2018). These were the steam treatments S-63C-270s and S-

68C-270s, the hot water treatment HW-50C-5min and the plant-based treatment Tillecur. Among these, the two steam treatments are of particular interest here, in light of the results from this study indicating a slight effect of the steam treatments against lupin anthracnose. Treatments were applied on seeds of the white lupin cultivar Feodora from two different seed batches: trial-saved seeds from the FiBL field trial 2017 – corresponding to the same seeds used here for the treatment applications – and certified anthracnose free seeds from France, harvested in 2017. Although the data from the field trial has not yet been statistically analyzed, a first look at the field emergence values indicated no reduction of emergence by either of the two steam treatments compared to the non-treated controls. A first look at the disease assessment data indicated a reduction of the disease score with the treatment S-63C-270s in the trial-saved seeds, and to a lesser extent also with S-68C-270s. In the certified seeds, S-63C-270s showed a higher disease score than the non-treated certified control, while S-68C-270s did not seem to differ from the non-treated certified control. All in all, a first assessment of the field emergence data seems to support applicability of the steam treatments in the field. It is too soon to say whether the steam treatments had a statistically significant effect on the disease scores. However, similar to the experiments described in this study, there seemed to be some indications of an effect of the steam treatments on anthracnose severity in the field.

Commercial treatment applications

Commercial applications can currently be found for steam and electron treatments. The Swedish ThermoSeed technology offers steam seed treatments on an industrial scale, with a reported capacity of 200 tons seeds treated per day (Wilbois et al., 2007). For electron treatment of seeds, commercial applications using the e-ventus® technology can achieve throughput rates ranging from 3 to 5 tons per hour for cereal grains (EVONTA, 2018). While for commercial application of dry heat treatment, the main consideration would presumably be the availability of a commercial-scale oven-type structure permitting precise control of time and temperature parameters, for thyme oil treatment the main consideration would likely be the generation of large quantities of essential oil emulsions, as well as the costs for the essential oil.

Legislation

A final consideration for the practical relevance of the seed treatments against lupin anthracnose is their admission for use in organic agriculture, or lack thereof. While physical seed treatments such as mechanical or thermal treatments can be applied in Swiss organic agriculture, electron treatment of seeds is currently not permitted (Bio Suisse, 2018). This differs from the situation in the European Union, where the use of ionizing radiation is forbidden for treatment of organic food, feed, and raw materials used in organic food or feed (EU Council Regulation 834/2007, Art. 10), but not for seed material (discussed for example by Speiser et al., 2017).

5.4 Comparison of methods to assess occurrence and virulence of *C. lupini*

In this study, visual disease assessment of plants, culture-based incubation of plant tissue and molecular detection of *Colletotrichum* spp. in plants using qPCR were used to study the effect of seed treatments on lupin anthracnose. Visual assessment of plants revealed no differences between treatments, neither by vitality scoring of plants, counting of diseased leaves or assessment of biomass at harvest. Culture-based incubation of 1.5-week old seedlings and characterization of fungal growth showed significant differences between treatments. *Colletotrichum* spp. was however seen only once in the entire experiment, probably due to a low

competitiveness of the fungus compared with other fungi of the plant microbiome. The culture-based incubation of plant tissue therefore served more as a general indication of treatment effects on the overall seed microbiome. This method was also quite time-intensive, since each fungal growth was categorized morphologically, and each category was then separately sampled for molecular detection of *Colletotrichum* spp. In order to simply assess whether *Colletotrichum* spp. is present, it might be more adequate to simply sample all fungal growth occurring on a plate at once. This would however prevent precise indications on percent samples affected. Using the molecular qPCR-based detection method, fluctuation in qPCR results was observed due to a repeatedly occurring non-uniformity of the technical replicates. This was not observed during analysis of the fungal categories from the growth medium, indicating that this issue might be due to interactions between plant and fungal DNA, or due to the DNA extraction protocol from the plants. Alternatively, *C. lupini* might have been present at very low doses in the plants, although this does not explain why sometimes a strong signal was detected with one technical replicate, and no signal in the other. Nevertheless, some *Colletotrichum* spp. was detected both in 1.5-week old seedlings and in 7.5-week old shoots, and it was therefore at least possible to identify certain treatments for which no *Colletotrichum* spp. was detected at all. Overall, the observed fluorescence in the qPCR runs performed here seemed to be lower than that reported by Szuszkiewicz (2016), even though the same reagents and protocol were used. The difference in fluorescence can be seen in the difference in qPCR threshold values used for analysis of the fluorescence curves: Szuszkiewicz (2016) reports a threshold value of 0.075 or 0.05, while the threshold values used here were determined automatically and ranged around 0.01. In addition to these three methods of assessment, the culture-based seed incubation method developed by Feiler and Nirenberg (1998) had been tested in a pre-trial leading up to this study. This method was however not included in the main study, as it seemed to give very variable results on inoculum levels in a same seed batch: analysis of the trial-saved seeds – which were the seeds used here for the treatment applications – sometimes revealed *C. lupini*-specific appressoria in certain experimental runs, while in other runs nothing was observed (data not shown). Since *Colletotrichum* spp. had been detected in all seeds using molecular qPCR-based characterization, it was judged that the seed incubation method was not very reliable. However, it could also be that the culture-based seed incubation method in fact reflected a more realistic inoculum level of the seed batch than the molecular characterization, as the seed incubation method only shows viable fungus, which is not the case for qPCR. Nevertheless, the culture-based seed incubation method was deemed impractical due to the long time until final assessment is possible, as well as experimental complications arising with germinating seedlings being displaced on the plate or disrupting the growth medium (as also observed by Nawrath and Vetter, 2002; Szuszkiewicz, 2016). Development of a viability qCPR protocol (reviewed for exmaple by Fittipaldi et al., 2011) for detection of viable *C. lupini* would be an interesting method to specifically detect live fungal DNA in seeds.

6 Conclusion and outlook

This study was conducted with the aims to compare non-synthetic seed treatments for their efficacy against lupin anthracnose, to assess the practical relevance of the most promising seed treatments, and to identify a suitable method for the detection of *C. lupin* in plants.

None of the studied treatments significantly differed from the non-treated control for any of the remaining parameters assessed here, except for the effect on overall seed microbiome, which was significantly reduced by several treatments. The absence of significant treatment effects could either be due to the treatments not having a sufficiently strong effect on lupin anthracnose, or to the disease not being present at sufficient levels in the experiment. As no characteristic anthracnose symptoms were observed, and due to the variability observed in the non-treated controls, it might be that the initial seed inoculum level was too low or too variable across seeds to reliably show treatment effects. Nevertheless, the steam treatments seemed to indicate slight control of *Colletotrichum* spp. without impairment of seed vigour, and the same was true to a lesser extent for the treatments dry heat 75°C/5h, electron penetration depth 3 and intensity 3, as well as thyme oil. In order to determine whether these treatments really are effective at controlling *C. lupini*, the next step would be application of the treatments on seeds known to be strongly infected with viable *C. lupini*.

None of the treatments with steam, electron, dry heat or thyme oil impaired the seed germination in the germination assay or in the pot experiment conducted here, which might indicate a good emergence in the field under favourable conditions at sowing. However, further seed vigour tests are needed to predict potential field emergence under less favourable conditions. Conductivity testing of seed leachate would be a possible method for this. Commercial applications of steam and electron treatments currently exist, meaning that both of these treatments can already be used in practice. However, as electron treatment of seeds is not allowed according to Swiss organic agriculture regulations, the non-synthetic seed treatments with the greatest practical relevance to the Swiss organic agriculture sector are the steam, dry heat and thyme oil treatments. It was not possible to further dive into questions of practical relevance within the scope of this study, but it would be interesting to compare costs of the different treatments, as well as to determine in what way commercial application of thyme oil and dry heat treatments could be performed.

Comparison of visual disease assessment of plants, culture-based incubation of plant tissue and molecular detection of *Colletotrichum* spp. in plants using qPCR revealed no best suited method for detection of lupin anthracnose in plants. While the culture-based seed incubation of plant tissue served as an assessment of treatment effects on the overall seed microbiome, it was not suited to specifically assess treatment effects on lupin anthracnose. Some *Colletotrichum* spp. was detected using qPCR of epicotyls and of shoot samples, but a high variation in the technical replicates of the qPCR was observed. Before conclusions on the suitability of the methods can be drawn, it is first necessary to determine where this variation between the technical replicates stemmed from. If the variation was due to too low concentrations of *C. lupini* in the plant material because of insufficient initial inoculum levels, further trials with strongly infected seeds might shed some light on this matter.

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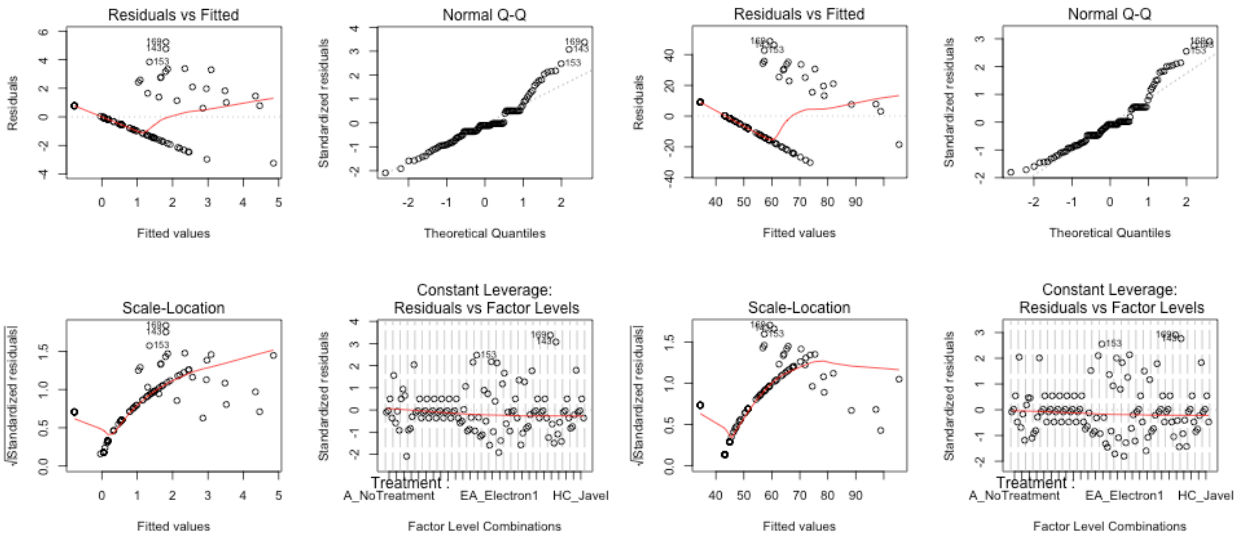
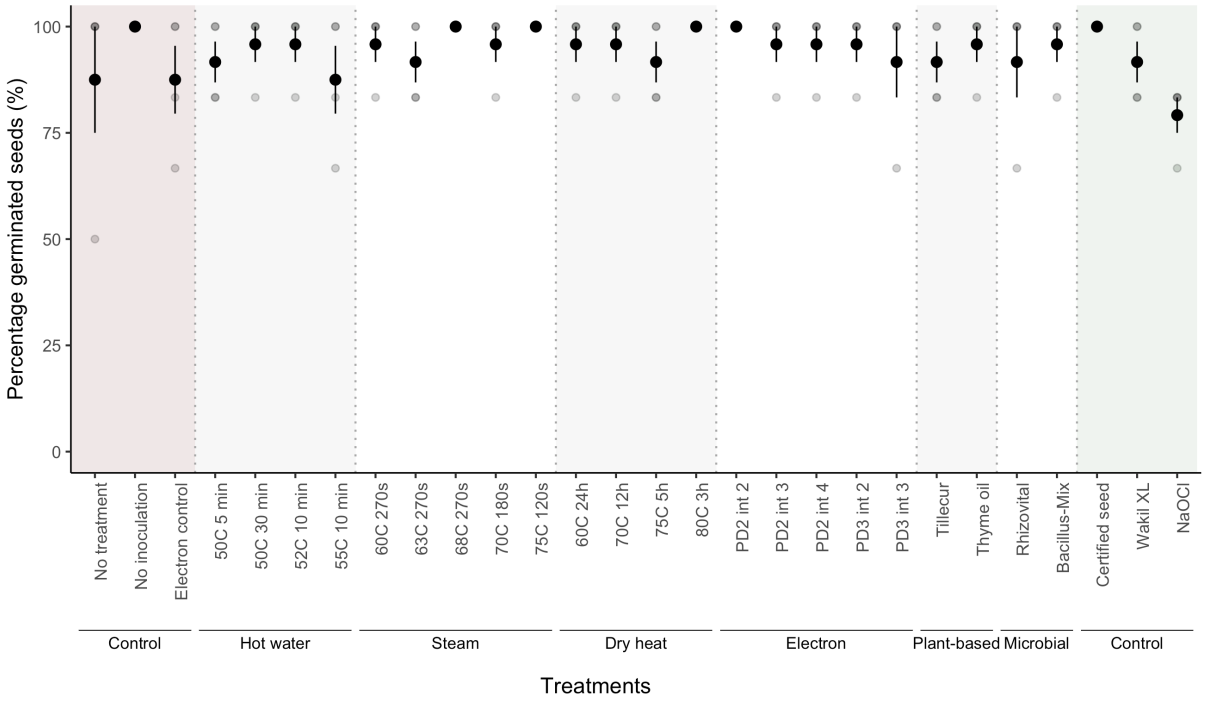
Appendix

Step	R code	Comment
1	lm1 <- lm(Parameter ~ Treatment + Replicate, Dataset)	Linear modelling of parameter as a function of the factors treatment and replicate, all contained in dataset
	plot(lm1)	Visual assessment of distributions of residuals from linear model
	anova(lm1)	Analysis of variance of linear model
2	summary(glht(lm1, linfct=mcp(Treatment="Dunnett")))	Dunnett's many-to-one comparisons test comparing all treatments to non-treated control (first treatment in dataset)
3	df.res <- df.residual(lm1)	Degrees of freedom of residuals; df.res is 27 with 10 treatments and 4 replicates
	dun.t <- 2.88	$t_{Dunnett}$ critical value for two-sided comparisons between 9 treatments and a control for a joint confidence coefficient of P = 95% with 27 degrees of freedom of residuals (interpolated between 24 and 30 degrees of freedom)
	ss.res <- deviance(lm1)	Sum of squares of residuals
	n <- 4	Sample size; n is 4 with 4 replicates
	dun.dis <- dun.t * sqrt((2 * (ss.res / df.res)) / n)	Calculation of Dunnett's critical distance between means

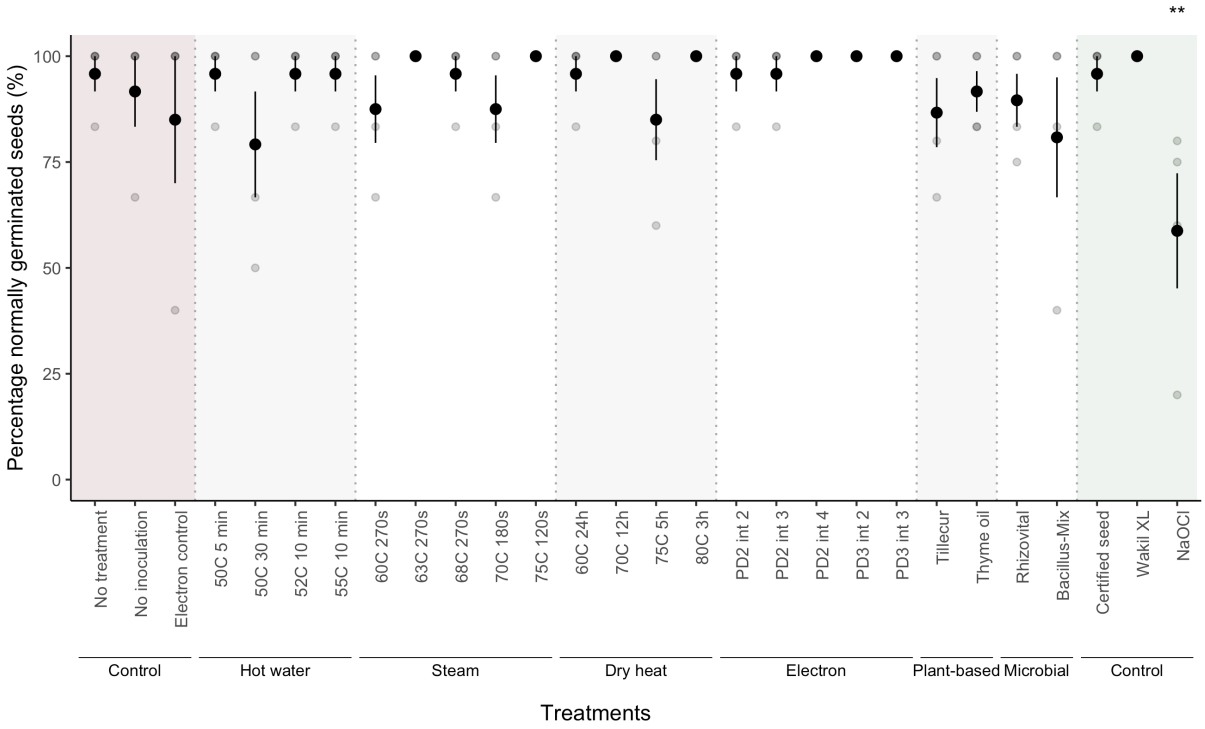
App. Table I Example of R code code used for data analysis. Step 1 was performed for all analyzed parameters and corresponds to linear modeling of parameter as a function of treatment and replicate, followed by assessment of distribution of residuals and analysis of variance (ANOVA). Step 2 was only performed if a significant treatment effect was observed with ANOVA, and corresponds to Dunnett's many-to-one comparisons test. Step 3 finally corresponds to calculation of Dunnett's critical distance between means, which was used in the long-term pot experiment (with 10 treatments including control and 4 replicates).



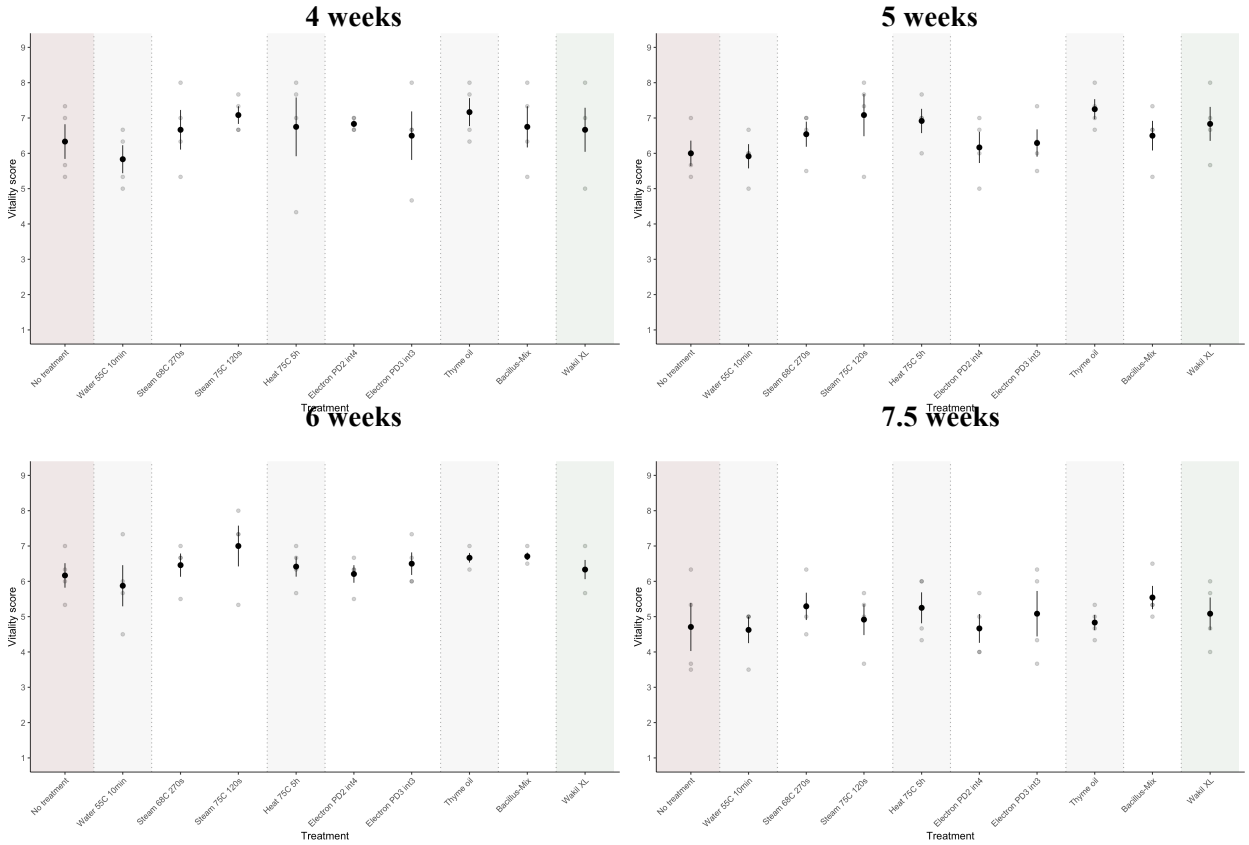
App. Figure I Distribution of residuals from linear models (~ Treatment + Replicate) with log-transformed (left) and rank and log-transformed (right) concentrations of *Colletotrichum* spp. in epicotyls of 1.5-week old seedlings.



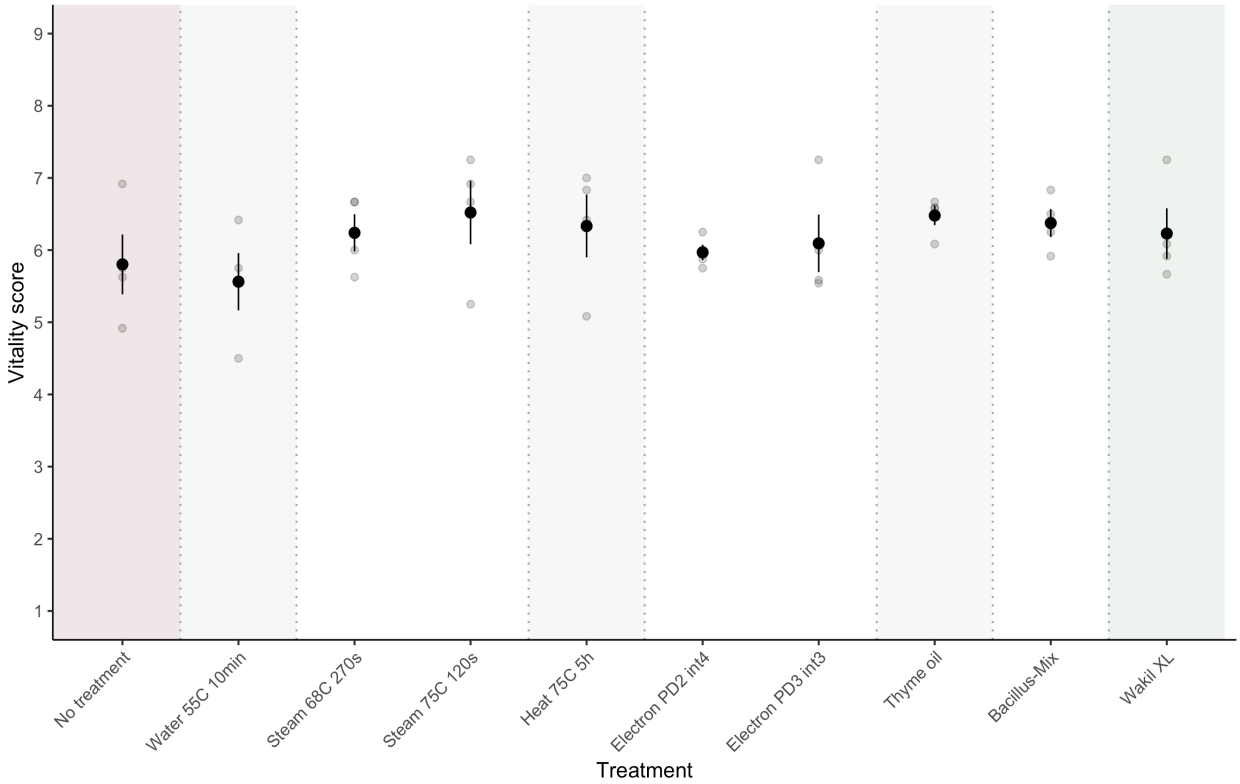
App. Figure II Mean percentage of germinated seeds of different treatments in pot trial after 1.5 weeks. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.



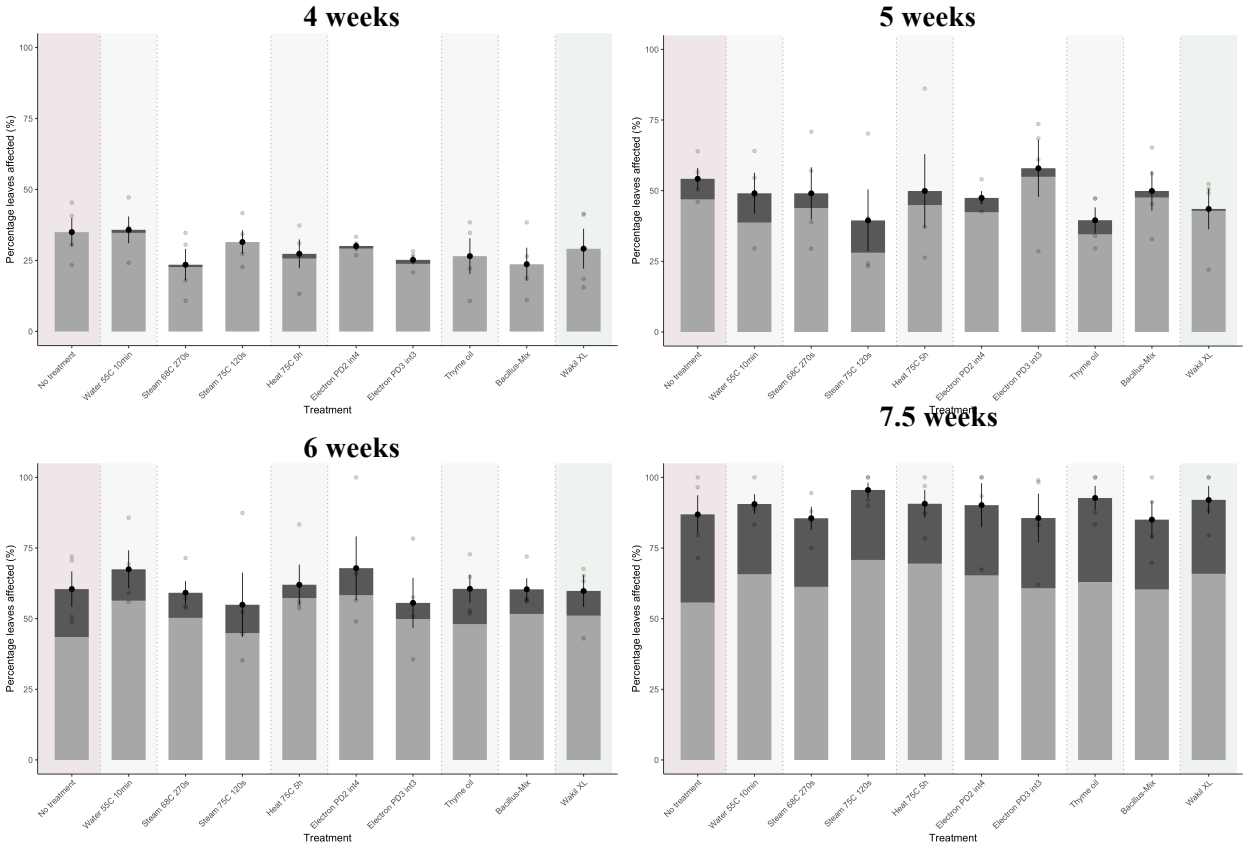
App. Figure III Mean normally germinated seeds of different treatments in pot experiment after 1.5 weeks as percentage of germinated seeds. Stars at the top of the plot show significant differences to the NoTreatment control (Dunnett's test; '***' = p<0.001, '**' = p<0.01, '*' = p<0.05, '.' = p<0.1). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative controls, green background the positive controls, and grey and white backgrounds delimit the non-synthetic treatment categories.



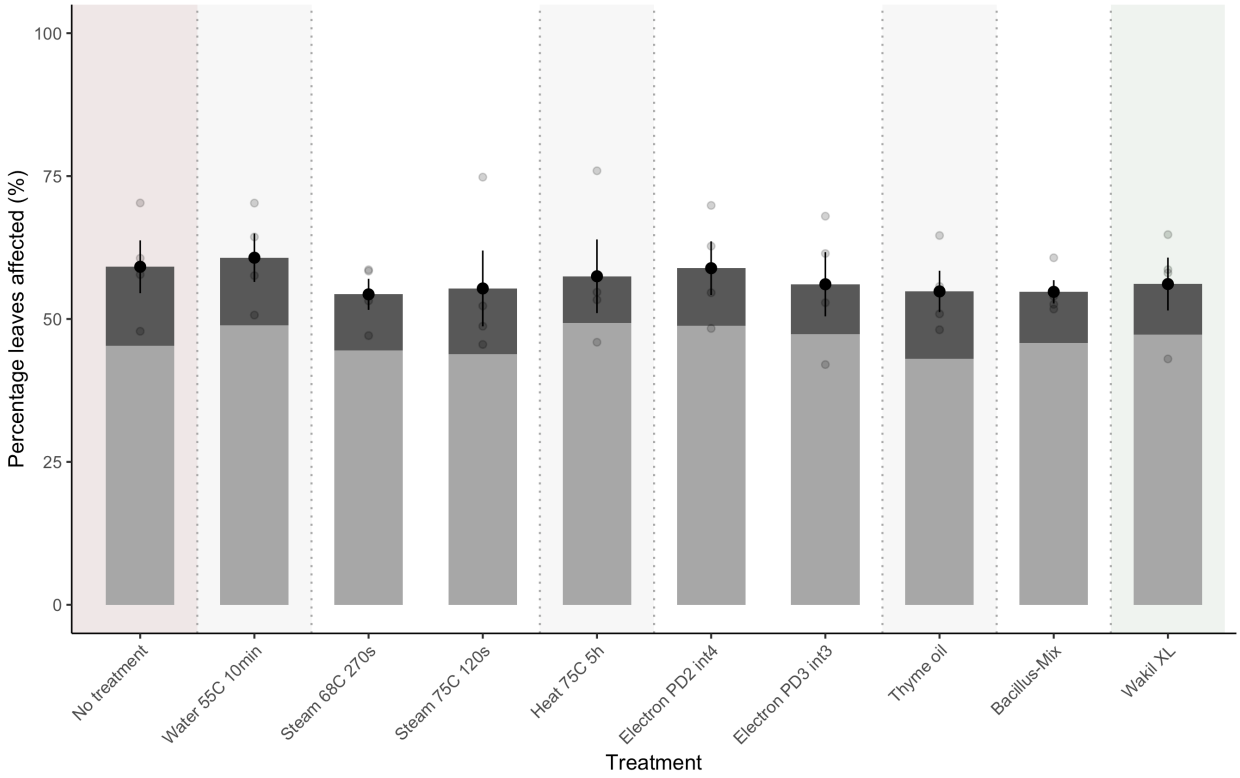
App. Figure IV Mean vitality scores of different treatments for the four time points of assessment. Possible scores range from 9 (completely healthy plant) to 1 (dead plant). Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red backgrounds show the negative control, green backgrounds the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



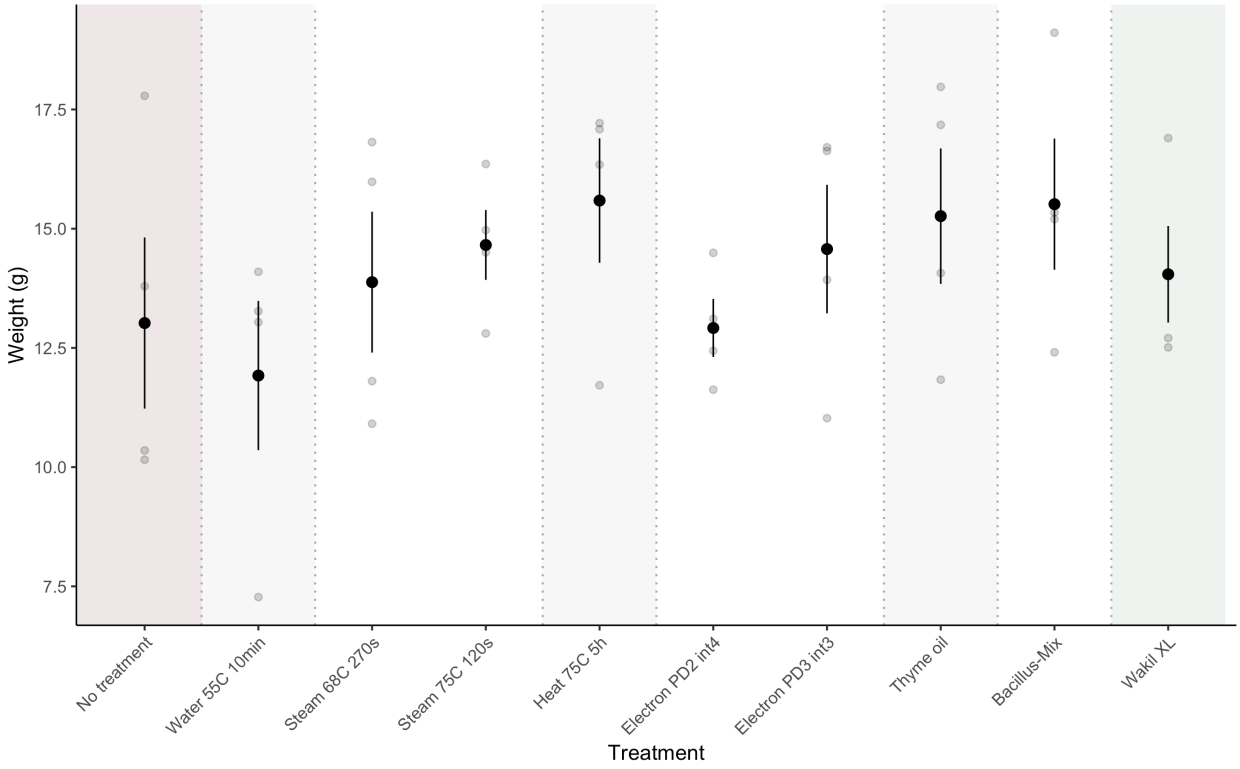
App. Figure V Mean vitality scores of different treatments in pot experiment averaged over the four assessment time points. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



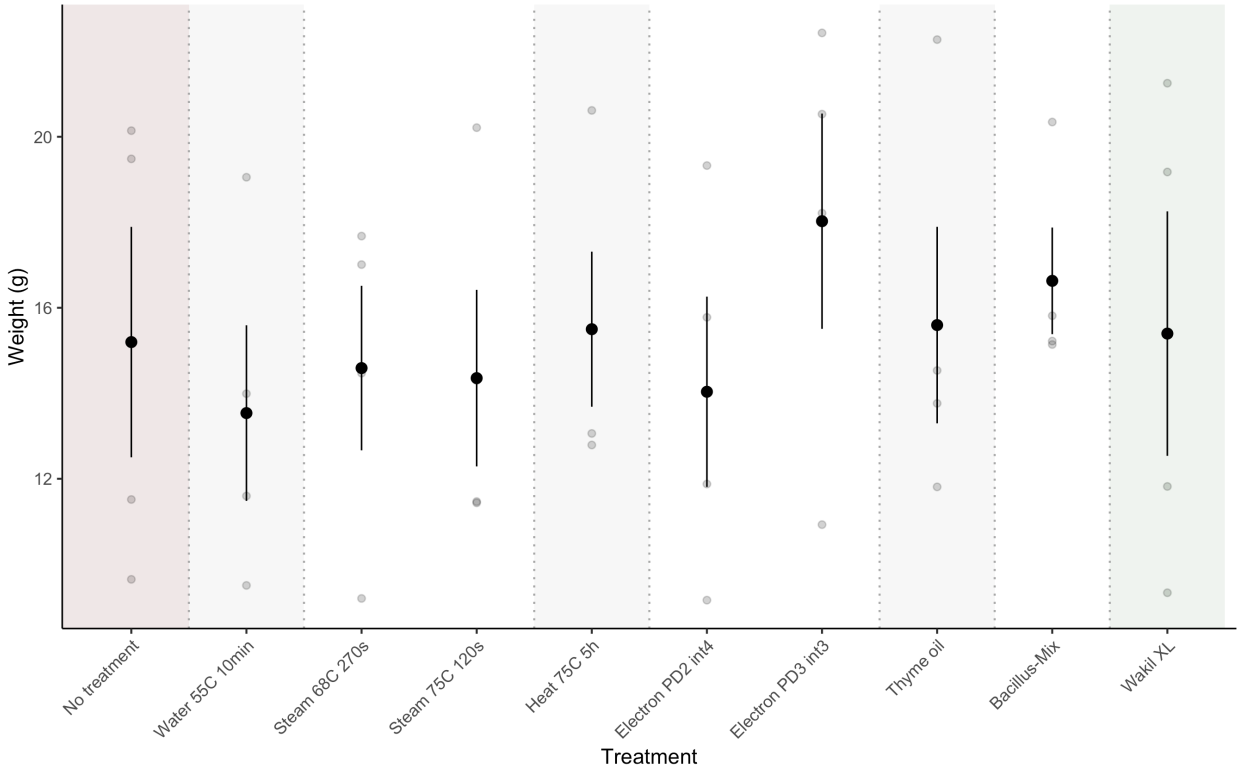
App. Figure VI Mean percentages of diseased leaves of different treatments for the four time points of assessment. Light grey areas in the bars show the percentage of leaves that were diseased but alive, dark grey areas in the bars show the percent of diseased and dead leaves. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red backgrounds show the negative control, green backgrounds the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



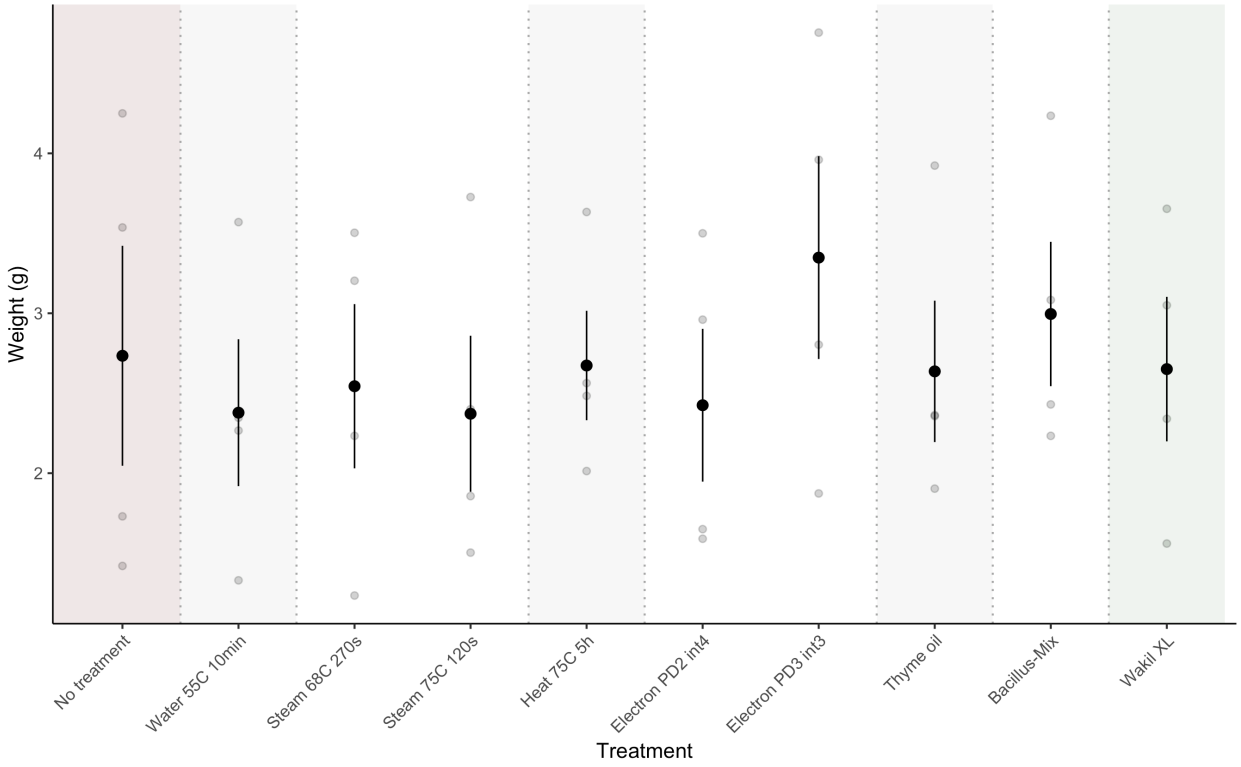
App. Figure VII Mean percentage of diseased leaves of different treatments averaged over the four assessment time points per replicate. Light grey areas in the bars show the percentage of leaves that were diseased but alive; dark grey areas in the bars show the percentage of diseased and dead leaves. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



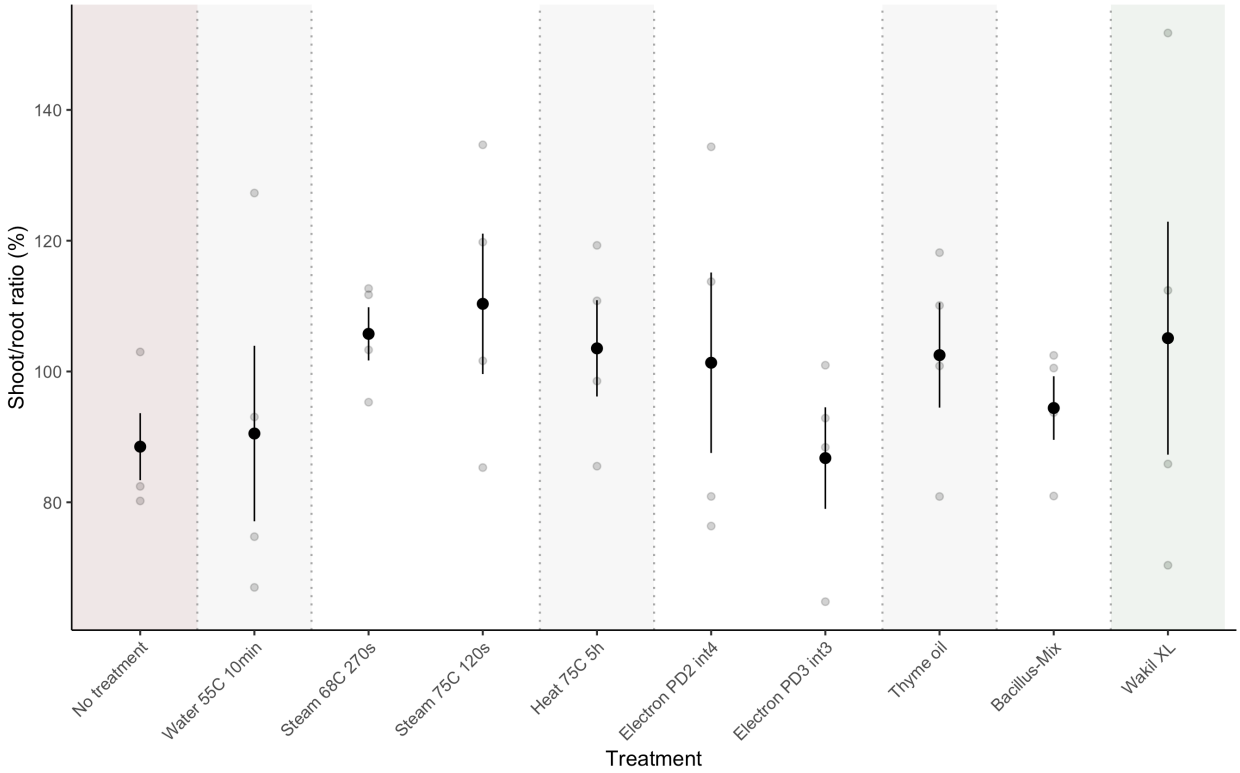
App. Figure VIII Mean shoot fresh weights of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



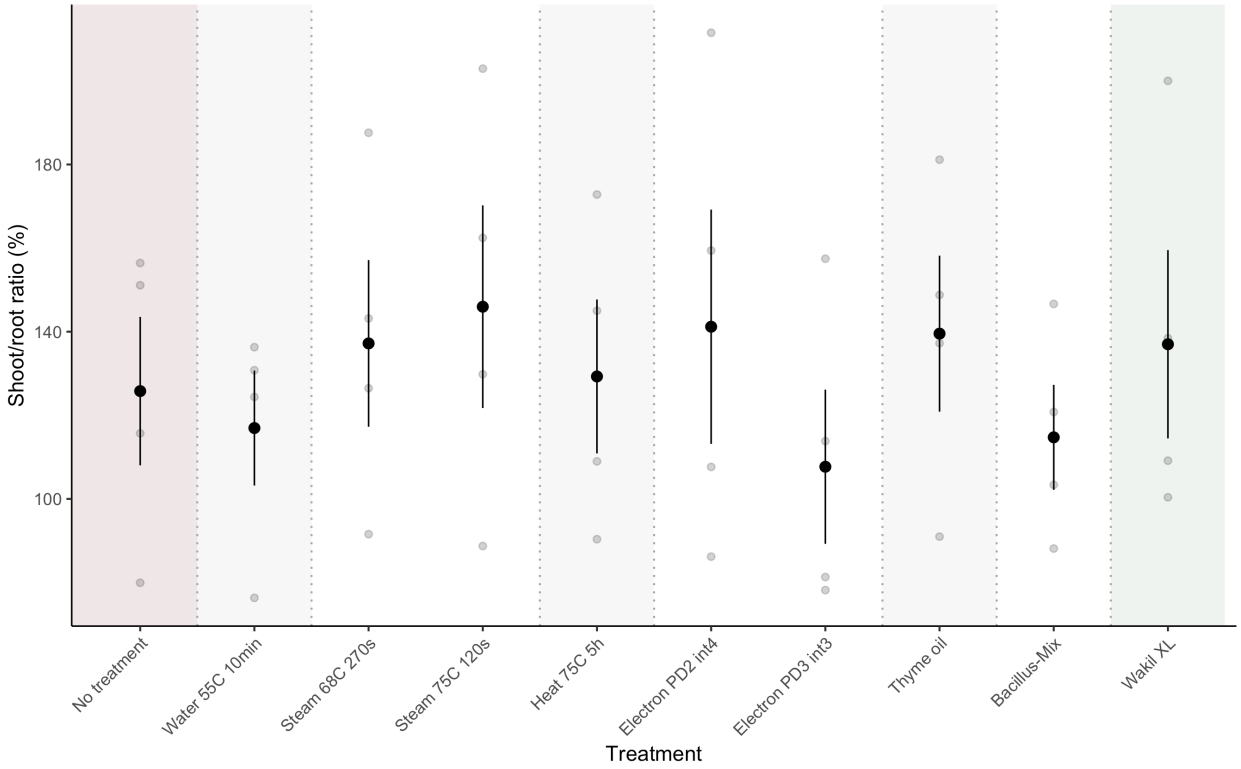
App. Figure IX Mean root fresh weights of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



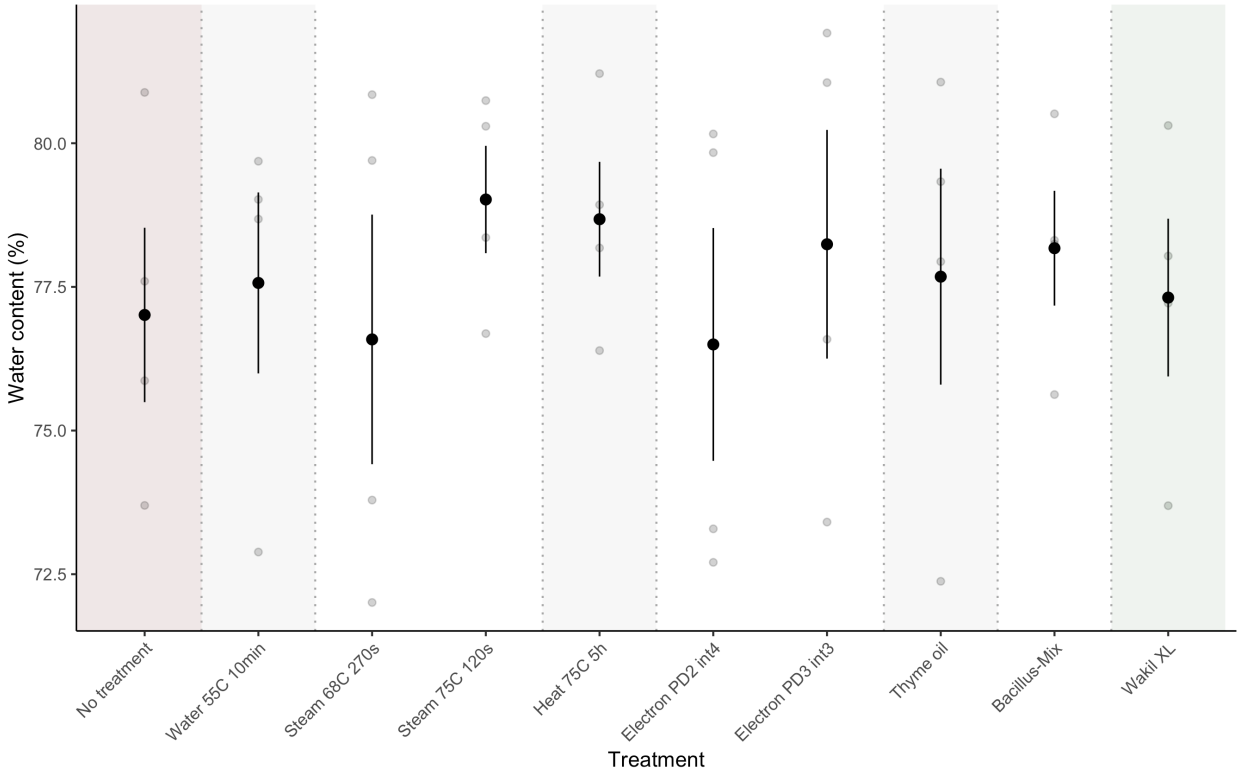
App. Figure X Mean root dry weights of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



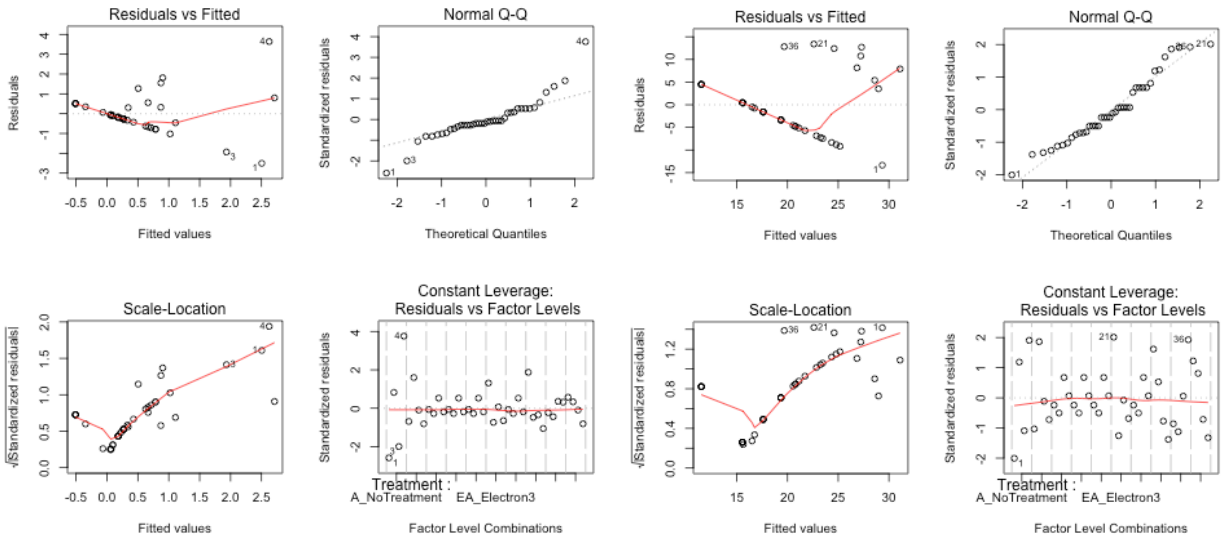
App. Figure XI Mean shoot to root ratios of fresh biomass of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



App. Figure XII Mean shoot to root ratios of dry biomass of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



App. Figure XIII Mean shoot water contents of different treatments in pot experiment 7.5 weeks after sowing. Error bars show the standard error of the means, grey dots show the values of each replicate (n=4). Red background shows the negative control, green background the positive control, and grey and white backgrounds delimit the non-synthetic treatment categories.



App. Figure XIV Distribution of residuals from linear models (~ Treatment + Replicate) with log-transformed (left) and rank and log-transformed (right) concentrations of *Colletotrichum* spp. in 7.5-week old shoots from pot experiment.