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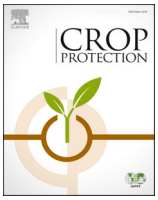

The potential of alternative seed treatments to control anthracnose disease in white lupin

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ABSTRACT

White lupin (*Lupinus albus* L.) is a promising crop to meet the rising global demand for plant-based protein. The seed-borne pathogen *Colletotrichum lupini*, however, threatens lupin cultivation worldwide. Seed dressings using synthetic fungicides were shown effective to reduce infection levels, but their negative environmental impact and exclusion from organic production calls for more sustainable solutions. In this study, a total of eleven different alternative seed treatments were tested in field trials in Switzerland between 2018 and 2021. Treatment types consisted of: hot water, steam, electron, long-term storage, elevated partial pressure of oxygen (EPPO), vinegar, plant extracts and biological control agents (BCAs). The BCAs were tested for potential antagonistic activity against *C. lupini* during white lupin infection under controlled conditions prior to field trials. Long-term storage and vinegar treatments successfully reduced disease incidence and increased yield to levels similar to those observed for certified seeds, without significantly affecting germination rate. Although promising, effectiveness of these treatments needs further validation. Four BCAs showed significant disease reductions under controlled conditions. Besides lowering disease severity, two BCAs also reduced *C. lupini* DNA in stem tissue. These reductions, however, were not observed in the field, highlighting the importance of field validations. The treatments identified in this study provide a solid basis for the development of sustainable and effective seed protection strategies in white lupin to control *C. lupini* successfully.

1. Introduction

White lupin (*Lupinus albus* L.) is a grain legume known for its high protein content (31–39%), nutritional value and its rare capability of forming specialized cluster roots that can mobilize poorly available phosphorus and drastically improve nutrient acquisition (Wolko et al., 2011). Since the development of sweet, low alkaloid varieties (Kroc et al., 2017), white lupin has received increasing attention from the food and feed industry (Lucas et al., 2015). As the demand for animal protein is projected to double by 2050 (FAOSTAT, 2021; Westhoek et al., 2011), the demand for plant-based protein is expected to rise as well. To meet this increasing demand, white lupin grown in temperate regions could be a sustainable substitute for imported soybean (*Glycine max*). One of the main threats limiting cultivation is anthracnose disease, caused by the seed- and air-borne ascomycete *Colletotrichum lupini* (Talhinhas et al., 2016). The current global outbreak is caused by a highly aggressive and genetically uniform group (II) of strains (Alkemade et al., 2021b). *C. lupini* is presumed to be a hemibiotrophic pathogen (Dubrulle et al., 2020), colonizing the host endophytically and causing the typical disease symptoms of stem and pod twisting (Alkemade et al., 2021b; Talhinhas et al., 2016). This is followed by the formation of necrotic lesions containing orange masses of conidia (acervuli) which are rain-splash dispersed within the crop, leading to secondary infections (Thomas and Sweetingham, 2004; White et al., 2008). Infected seeds are

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the primary source of inoculum, and small amounts of infected seeds (0.1%) can cause total yield loss (Thomas and Sweetingham, 2004). As infected seeds are likely to be symptomless they are the most important vehicles for spreading aggressive *C. lupini* strains across the world.

Current disease control mainly relies on planting pathogen-free seeds and foliar fungicide application (White et al., 2008). Pathogen-free seeds are produced under strict phytosanitary control in environments unfavorable for the disease (N. Harzic, pers. comm.) and PCR-based detection methods have been developed to determine seed infection levels (Kamber et al., 2021; Pecchia et al., 2019). Seed treatments with fungicides, such as thiram (which is now banned in the EU), can reduce inoculum viability and transmission (Talhinhas et al., 2016; Thomas et al., 2008). Most fungicides, however, are considered problematic due to their environmental impact and are not applicable for organic cultivation systems. Alternative seed treatments showed to be successful against fungal seed-borne pathogens in various vegetable crops (Mancini and Romanazzi, 2014). Seed treatments through dry-heat (Falconí and Yánez–Mendizábal, 2016) and UV processing (Falconí and Yánez-Mendizábal, 2018) showed to reduce *C. lupini* infection in Andean lupin under controlled conditions but hampered germination rate after 12 h at 65 °C dry heat or at UV doses of 86·4 kJ m—2 and higher. Dry heat (Thomas and Adcock, 2004) and long-term storage (Thomas and Sweetingham, 1999) showed promising results in reducing *C. lupini* infection in blue lupin under field conditions and reduced germination was only observed after 7 days at 70 °C dry heat. As oxidation plays a major role in seed deterioration during storage (Groot et al., 2015), artificial seed aging through storage under an elevated partial pressure of oxygen (EPPO; Groot et al., 2012) might reduce pathogen viability through this deterioration. Hot water seed treatments have been effective against many seed-borne pathogens (Sharma et al., 2015), and proved to reduce *C. kahawae* (Mangwende et al., 2020) and *C. nymphaea* (Yamagishi et al., 2015) viability in eucalyptus and celery seeds, respectively. The mustard powder based product Tillecur® and thyme oil showed promising results against *C. lindemuthianum* in bean (Tinivella et al., 2009). Although these treatments are promising, none have been tested adequately on white lupin in the field so far.

Biological control agents (BCAs) also offer great potential to control seed-borne disease (Mancini and Romanazzi, 2014; Rocha et al., 2019; Tinivella et al., 2009). In Andean lupin, seed treatments with the widely used BCA *Bacillus subtilis* effectively reduced anthracnose incidence (Yánez-Mendizábal and Falconí, 2018) and induced system resistance (Yánez-Mendizábal and Falconí, 2021) under controlled conditions without hampering germination rates. The bacterial endophyte *Paraburkholderia phytofirmans* PsJN can improve vigor and biotic and abiotic stress tolerance in plants (Esmaeel et al., 2018), and is able to colonize white lupin (Kost et al., 2014). Seed treatment with bacterial free-cell filtrate of *Streptomyces griseoviridis* was effective in reducing *C. lupini* incidence in lupin *in vivo* (Mandrik et al., 2007). Seed treatments with *Pseudomonas fluorescens* reduced anthracnose disease in common bean under field conditions (Amin et al., 2014). The fungal mycoparasite *Clonostachys rosea* was effective as seed treatment against *C. acutatum* in blue berry in the field (Verma et al., 2006)and against *C. lindemuthianum* in bean under controlled conditions without affecting germination (Tinivella et al., 2009). *Trichoderma* spp. are known as potent fungal BCAs (Sharma and Gothalwal, 2017) and acted antagonistic on soybean infecting *C. truncatum* (Begum et al., 2010), and reduced incidence of *C. truncatum* in chili (Yadav et al., 2021) and *C. lindemuthianum* in common bean (Amin et al., 2014), respectively. Taken together, alternative seed treatments show potential to reduce *C. lupini* incidence in white lupin, but systematic research is required to identify a treatment against this notorious pathogen which is effective under field conditions.

This study aimed to identify sustainable alternative seed treatments to reduce anthracnose disease in white lupin. Prior to field trials, six different bacterial and fungal BCAs were screened under controlled conditions. Four BCAs and seven other alternative seed treatments (including hot water, steam, electron, long-term storage, EPPO, vinegar and plant extracts) were tested on infected seeds under field conditions in Switzerland. This was done in an attempt to identify sustainable seed treatments that could considerably improve seed health and disease management of *C. lupini* in white lupin.

2. Materials and methods

2.1. Pre-screen biological control agent treatments

Biological control agents (BCAs) were tested under controlled conditions prior to field trials in order to identify possible antagonistic effects against *C. lupini*. Six BCAs were selected: four bacterial species (*Pseudomonas fluorescens* G308 [Pflu-G308], *Bacillus subtilis* HG77 [Bsub-HG77], *Paraburkholderia phytofirmans* PsJN [Pphy-PsJN] and *Streptomyces griseoviridis* [Mycostop®]), and two fungal species (*Clonostachys rosea* [Prestop®], *Trichoderma asperellum* [T-Gro]; Table S1). BCA efficacy was tested on stem wound inoculated plants under controlled conditions in a growth chamber (25 °C, 16-h light and 70% relative humidity) as described in Alkemade et al. (2021a), applying 5 μl of *C. lupini* (strain JA01) spore suspension (10^5 spores/ml) on 14 day old seedlings grown in pots. This stem wound assay has been shown suitable to identify field-relevant resistance against *C. lupini*. Certified (disease free seeds produced under fungicidal control) white lupin seeds of cv. Feodora, obtained from Jouffray-Drillaud (Cissé, France), were used. Naturally-infected seeds could not be used to test non-biological treatments as they showed no or only minor disease symptoms in young seedlings under controlled conditions (data not shown). Two different application methods, seed dressing and stem wounding, were tested (Table S1). For stem wounding, 5 μl of BCA suspension was mixed with 5 μl of *C. lupini* spore suspension prior to inoculation. For seed dressing, seeds were soaked for 30 s in 5 ml BCA suspension prior to sowing. As positive control the *C. lupini* spore suspension was mixed with distilled water and as negative control inoculations were performed with distilled water. Experiments were performed in a randomized complete block design with a minimum of five biological replicates (blocks) per experiment and were repeated at least twice. Anthracnose severity was assessed at 3, 7 and 10 days post inoculation (dpi) using a 1 (healthy) to 9 (dead) scale referred to as disease severity index (DSI) to calculate the standardized area under disease progress curve (sAUDPC) as described in Jeger and Viljanen-Rollinson (2001). At 10 dpi lesion size was determined.

2.2. Seed treatments under field conditions

Eleven seed treatments with a total of twenty-one different conditions were tested on infected seeds of white lupin cv. Feodora harvested from field plots with a mean DSI of 7 (Table 1). The seed infestation is based on the DSI of the parental plants which correlates with pathogen DNA levels in the resulting seeds (Kamber et al., 2021). Six of the treatments were also tested on certified seeds. Untreated infected and certified seeds were used as controls. Hot water and steam treatments were performed at Sativa Rheinau (Rheinau, Switzerland), see Table 1. Thyme oil emulsion (0.1%) and table vinegar (pH = 3, acetic acid 5%) were applied by soaking seeds for 30 min and re-drying overnight at room temperature. Tillecur® (Biofa, Münsingen, Germany), a mustard-based product, was applied as powder on the seeds according to manufacturer's instructions. For the long-term storage treatment, highly infected seeds of cv. Amiga (field resistance level and genetically highly similar to cv. Feodora (Alkemade et al., 2021a; Hufnagel et al., 2021)) harvested in 2016 from plots with a mean DSI of 7 were used. Seeds were stored for four year at room temperature in the dark. Elevated Partial Pressure of Oxygen (EPPO) was applied for 2 weeks at 20 MPa air (which includes 4.2 MPa partial oxygen pressure) to seeds equilibrated at a relative humidity of 50% to mimic dry ageing according to Buijs et al. (2020). Electron treatments were performed at Evonta-Service GmbH (Radeberg, Germany) with different penetration depths and intensities

Table 1
Overview of treatments on white lupin seeds to control anthracnose disease under Swiss field conditions.

Treatment	Description	Year	n	E	Source/company
Inf_Seed	Infected seeds	2018, 19, 20, 21	35	7	Infected field (DSI 7) harvest (seven months old)
Cert_Seed	Certified seeds	2018, 19, 20, 21	32	7	Jouffray-Drillaud, Cissé, FR
Hotwater_1	55 °C/5 min	2018	4	2	Sativa, Rheinau, CH
Hotwater_2	55 °C/10 min	2019, 20	14	4	Sativa, Rheinau, CH
Hotwater_3	65 °C/10 min	2020	8	2	Sativa, Rheinau, CH
Hotwater_4	68 °C/5 min	2020	8	2	Sativa, Rheinau, CH
Steam_1	63 °C/4.5 min	2018	4	2	Sativa, Rheinau, CH
Steam_2	68 °C/4.5 min	2018, 19	10	4	Sativa, Rheinau, CH
Steam_3	75 °C/2 min	2019, 20	14	4	Sativa, Rheinau, CH
Steam_4	80 °C/4.5 min	2020	8	2	Sativa, Rheinau, CH
Steam_5	80 °C/5 min	2020	8	2	Sativa, Rheinau, CH
Storage	4 years/room temperature	2020	8	2	Infected fieldharvest, Mellikon, CH (2016, DSI = 7[a])
EPPO	2 weeks, 20 MPa air pressure	2021	3	1	WUR, Wageningen, NL
Thyme	Thyme oil, 0.1%/ 30 min	2019	6	2	Thymian Thymol bio, Primavera, Oy-Mittelberg, DE
Tillecur®	Mustard based powder, 1 kg/100 kg seeds	2018	4	2	Biofa, Münsingen, DE
Vinegar	Acetic acid 5%, 30 min	2020, 21	11	3	Coop, CH
E6	Penetration depth 1, intensity 2	2020	8	2	Evonta-Service GmbH, Radeberg, DE
E7	Penetration depth 2, intensity 1	2020	8	2	Evonta-Service GmbH, Radeberg, DE
E9	Penetration depth 3, intensity 1	2020	8	2	Evonta-Service GmbH, Radeberg, DE
E11	Penetration depth 4, intensity 1	2020	8	2	Evonta-Service GmbH, Radeberg, DE
Bsub-HG77[b]	0.25 OD^{c}_{600}/1 h	2019	6	2	Hohenheim University, DE
Pphy-PsJN[b]	0.25 OD^{c}_{600}/1 h	2019	3	1	Austrian Institute of Technology, AT
Mycostop®[b]	1 kg/150 kg seeds	2021	3	1	Verdera, Espoo, FI
Prestop®[b]*	5 kg/ha (5 g/L), 3 times	2021	3	1	Verdera, Espoo, FI

n: total number of replicates, E: total number of environments, EPPO: elevated partial pressure of oxygen, E# = electron treatment. a: DSI = disease severity index. b: for detail see Table S1 c: Concentrations measurement of the optical density at 600 nm OD_{600}, * Foliar application.

(actual conditions of the electron treatment settings are IP protected by Evonta-Service GmbH). Bsub-HG77 and Pphy-PsJN were applied by soaking seed for 1 h in a bacterial solution of an optical density (OD) of 0.25 measured at 600 nm, seeds were re-dried overnight at room temperature. Mycostop® was applied as powder on the seeds according to the manufacturer's instructions. Prestop® was foliar applied three times; first at plant emergence, a second time 10 days after plant emergence, and a third time at flowering according to the manufacturer's instructions.

Field trials were conducted between 2018 and 2021 in six-row plots following a randomized complete block design in Feldbach (47°14′20.0"N, 8°47′18.8"E), Mellikon (47°34′05.3"N 8°21′19.3"E) and Leibstadt (47°36′02.8"N 8°11′35.2"E) in Switzerland as described in Alkemade et al. (2021a) and Alkemade et al. (2022). Seed density was 65 seeds/m^2. Total number of replicates per treatment is stated in Table 1 and plot and field sizes are given in Table 2. Seed germination was assessed by counting germinated plants in two times 1 m per plot, 30 days after sowing. Anthracnose disease assessments were performed on plot level using a 1 to 9 DSI as described in Alkemade et al. (2021a), with "1" (0%) being completely healthy and "9" (>61%–100%) completely diseased. Disease assessments were performed three to four times per growing season (60, 80, 100 and 120 days after sowing). The sAUDPC was calculated to assess and compare disease progression. Yield (dt ha^{-1}) was assessed at harvest in mid- or late-August. The sAUDPC, yield and germination rate are visualized in relation to the untreated (infected) seed control.

2.3. DNA extraction and qPCR

For the four BCAs that significantly reduced disease symptoms in the pre-screen (Bsub-HG77, Pphy-PsJN, Mycostop® and Prestop®), quantitative real time PCR (qPCR) was performed to quantify *C. lupini* DNA in stem tissue 1 cm above the inoculation site at 10 dpi. A total amount of 50 mg of thinly sliced stem tissue was harvested and stored at −20 °C prior to usage. Genomic DNA was extracted following a cetyltrimethylammonium bromide (CTAB) extraction protocol described by Kamber et al. (2021). *C. lupini* DNA was quantified by performing qPCR on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using the primers, GAPDH_F "5′-CCCACGGCAAAAGAGTCAGA-3'" and GAPDH_R "5′-CGGCTGTTTCGGCATGATTG-3'", and a fluorogenic hydrolysis probe GAPDH_P "5′-FAM6-CGTCGTGTCATTACAACAAGCC-3'" as described in Kamber et al. (2021). Each 20 μL reaction consisted of 1 μL of DNA template, 300 nM of primers GAPDH_F and GAPDH_R, 100 nM of probe GAPDH_P, and 10 μL of KAPA PROBE FAST qPCR Master Mix 2X (Kapa Biosystems Pty, Cape Town, South Africa). The amplification conditions were: 45 cycles with denaturation at 95 °C for 5 s and annealing and elongation at 69 °C for 20 s after an initial denaturation of 3 min at 95 °C using a S1000 Thermal Cycler (Bio-Rad, Hercules, California, United States). Threshold was automatically determined by Rotor-Gene Q Series Software 2.3.1. *C. lupini* DNA quantity was expressed as 45 minus obtained cycle numbers until threshold (CT) values. The limit of detection (LoD) was priorly determined as Ct 38 via 10-fold dilutions of *C. lupini* DNA. The LoD represents the lowest concentration that is measurable and produces at least 95% positive replicates (Forootan et al., 2017).

Table 2
Mean sAUDPC and yield under Swiss field conditions.

Location	Year	DSI[a]		sAUDPC[b]		Yield (dt ha^{-1})		Field information	
		Mean	SE	Mean	SE	Mean	SE	Plot size	Total field size
Feldbach	2018	5.0	0.28	4.3	0.28	43.9	7.2	1.5 × 2.7 m	81 m^2
	2019	4.7	0.34	4.4	0.34	7.9	3.5	1.5 × 2.7 m	146 m^2
	2020	4.9	0.21	5.6	0.21	3.9	0.9	1.5 × 2.7 m	292 m^2
Mellikon	2018	4.8	0.30	4.1	0.30	19.8	3.6	1.5 × 5 m	180 m^2
	2019	5.0	0.28	5.8	0.28	3.9	0.8	1.5 × 5 m	405 m^2
Leibstadt	2020	4.9	0.17	5.2	0.17	8.6	1.2	1.32 × 3.5 m	333 m^2
	2021	5.2	0.50	5.1	0.50	1.5	0.5	1.32 × 3.5 m	97 m^2

[a] Disease severity index at final scoring (120–135 days after sowing).
[b] Standardized area under the disease progress curve. SE indicates standard error of the mean.

2.4. Data analysis

Statistical analyses were performed with R 4.0.3 (R Core Team, 2020) using the packages lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017) and emmeans (Lenth et al., 2020). It followed a mixed linear model with treatment as fixed factor and environment, environment x treatment, and replicated block nested in environment as random factors, after confirming the assumptions of normality of residuals and homogeneity of variance. To achieve a normal distribution, data were transformed with a square root (lesion size (controlled conditions; CC), yield, germination rate) or log10 (sAUDPC (CC)) transformation. Yield and germination rate data were normalized to the "infected seed" control for each environment. Data are presented as estimated least-squares means using the aforementioned mixed model. Mean separations between treatments and the non-treated control were analyzed using Dunnett's test ($p \leq 0.05$). Graphical representation were created using the R package ggplot2 (Wickham et al., 2016).

3. Results

3.1. Four BCA treatments selected for field trials

The BCA pre-screen showed significant disease reduction for the treatments Bsub-HG77, Pphy-PsJN, and Prestop® when applied through wound inoculation and Mycostop® through seed dressing for sAUDPC and/or lesion size compared with the untreated control (Fig. S1). Therefore, these four treatments were selected for the main experiment under field conditions. Additionally, quantification of *C. lupini* DNA 1 cm above the point of inoculation showed a significant reduction for Bsub-HG77 (45-CT = 8.8, $P < 0.001$) and Prestop® (45-CT = 10.8, $P < 0.001$) compared with the control (45-CT = 15.9; Fig. S2). Seed dressing with Prestop® reduced the germination rate by 70% (Table S2), and was therefore foliar applied according to manufacturer's recommendations in the field trials.

3.2. Seed storage and vinegar reduce anthracnose incidence under field conditions

The mean DSI of non-treated certified seeds at the end of the growing season (120–135 days after sowing) ranged from 4.7 to 5.2 over the different environments (Table 2). The seed treatments long-term storage and vinegar significantly reduced mean sAUDPC in the field, 4.22 ($P = 0.021$) and 4.46 ($P = 0.049$), respectively, compared to the mean sAUDPC for untreated infected seeds (5.37; Fig. 1) and are similar to the mean sAUDPC observed for certified seeds (4.46, $P > 0.05$). Artificial seed aging through elevated partial pressure of oxygen (EPPO) showed a

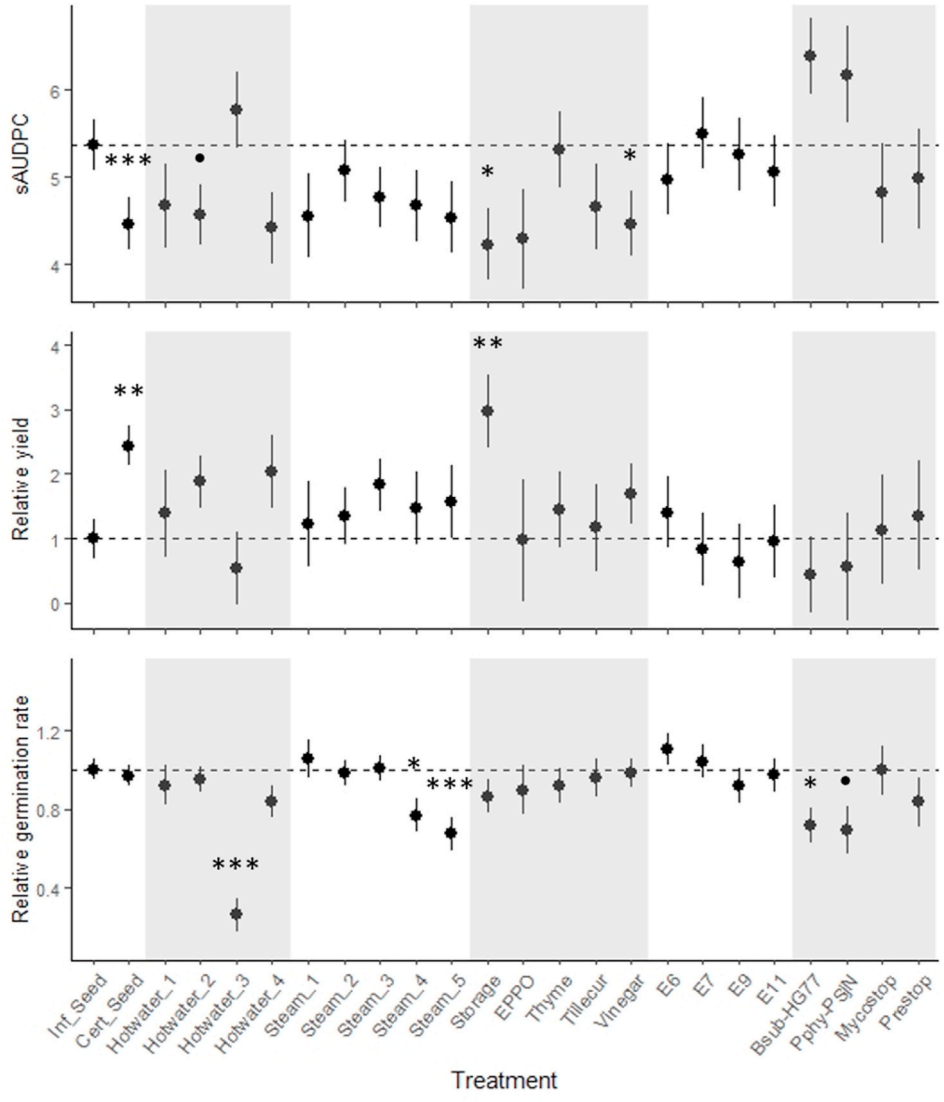

Fig. 1. Seed treatment effects on with *Colletotrichum lupini* infected white lupin seeds tested under field conditions in Switzerland. Disease incidence is expressed the standardized area under the disease progress curve (sAUDPC). Yield (dt ha^{-1}) and germination rate are expressed as relative to untreated infected seeds. Thick black dots indicate estimated means and error bars indicate standard error of the estimated mean. Dotted line indicates mean of the control. • $P \leq 0.1$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, difference with control (Dunnett's test). See Table 1 for description of treatments.

mean sAUDPC of 4.28 but was not significantly different from the infected control ($P = 0.56$). The mean disease reduction observed for hot water treatment 2 tended towards significance (4.56, $P = 0.067$).

The mean yields were highly variable between environments, ranging from 1.5 to 43.9 dt ha^{-1} (Table 2). Therefore, treatment yield means are expressed as relative to the infected (Fig. 1) or certified control (Fig. S3). Long-term storage almost tripled yield (2.96, $P = 0.004$) compared to the infected untreated control and was similar compared to relative yields observed for certified seeds (2.4, $P = 0.83$). None of the treatments showed a significant reduction in disease incidence or increase in yield compared to certified seeds. When applied on certified seeds, the different treatments did not result in any additional reduction in disease severity compared to the certified seed control (Fig. S3). Hot water treatment 3 and steam treatment 4, however, caused almost complete yield loss.

3.3. Seed treatments can affect germination rates

On infected seeds, the seed treatments hot water 3 (0.26, $P < 0.001$), steam 5 (0.67, $P < 0.001$), Bsub-HG77 (0.72, $P = 0.016$) and steam 4 (0.77, $P = 0.04$) significantly reduced germination rate relative to untreated infected seeds (Fig. S3). Especially hot water treatment 3 (65 °C/10 min) strongly affected germination, reducing the mean germination rate by 74%. None of the other tested treatments significantly affected seed germination but Pphy-PsJN (0.69, $P = 0.09$) showed a reduction. Interestingly, none of the treatments on infected seeds that affected germination rate resulted in yield loss. In contrast, on certified seeds, hot water 3 and steam 4 strongly reduced seed germination by 84% (0.16, $P < 0.001$) and 88% (0.12, $P < 0.001$), respectively. This explains the yield reductions for these treatments in Fig. S3. Contrary to their effects on infected seeds, applications of the BCAs Bsub-HG77 and Pphy-PsJN on certified seeds did not affect germination.

4. Discussion

Long-term storage (four years) of infected seeds at room temperature resulted in the strongest reduction in disease severity and increase in yield of all treatments whilst germination rates were not affected. Yields highly varied across environments and might be explained by environmental factors such as rainfall and temperature, with 2018 being hotter (18.4°) and dryer (267 mm) on average compared to 2019 (16.4 °C and 572 mm) during the growing season (Agrometeo, 2020). It should be taken into consideration that the used long-term stored infected seeds were from the cultivar Amiga, which despite being highly similar to the control cv. Feodora, could have influenced the results. Long-term storage was also shown by Thomas and Sweetingham (1999) and Cwalina-Ambroziak and Kurowski (2005) to significantly reduce *C. lupini* infection already after more than six months of storage. This suggests that *C. lupini* structures (conidia or mycelia) cannot survive for long times in or on white lupin seeds. Major disadvantages of long-term storage, however, are the long duration and required storage facility, making it very costly on a large scale. Soaking seeds in vinegar (5% acetic acid) for 30 min also resulted in a clear reduction of disease incidence and did not affect germination rates. Vinegar has been used as seed treatment against common bunt (*Tilletia caries*) and Fusarium graminearum in wheat, is considered cost effective and has been approved for usage in the organic sector (Gao et al., 2020). Acetic and other acids at concentrations of 2.5% or higher reduced seed-associated bacteria (Van der Wolf et al., 2008), indicating that certain acid concentrations can reduce seed-borne pathogen viability without hampering seed germination. The effect on *C. lupini* viability of long-term storage and seed treatments such as acid, heat, UV, and fungicides (Falconí and Yánez-Mendizábal, 2018; Falconí and Yánez–Mendizábal, 2016; Thomas and Adcock, 2004; Thomas and Sweetingham, 2003), could indicate colonization of the seed coat rather than the embryo or endosperm (Shade et al., 2017), as observed for other *Colletotrichum* species (Begum et al., 2008; Harman, 1983). In tomato seeds, however, *C. coccodes* mycelium was observed on both the seed coat and within the embryo (Ben-Daniel et al., 2010).

Under controlled conditions, the bacterial agents Bsub-HG77 and Pphy-PsJN, and the commercial products Prestop® and Mycostop successfully reduced anthracnose disease. While Bsub-HG77 and Prestop® both show direct antagonistic effects, Pphy-PsJN did not reduce *C. lupini* DNA levels suggesting a different mode of action. As *P. phytofirmans* PsJN is known to induce resistance in different plant species (Esmaeel et al., 2018) and can successfully colonize white lupin under controlled conditions (Kost et al., 2014), our observation suggests that induced resistance might also play a role against *C. lupini* in white lupin. While direct antagonistic effects of some BCAs were seen during the pre-screen under controlled conditions, disease-suppressive effects could not be confirmed when seed applied and tested under field conditions. Treatment of infected seeds with Prestop®, Bsub-HG77 and Pphy-PsJN showed reduced germination rates. The effectiveness of BCA treatments under field conditions have often been reported to be highly variable due to e.g. environmental factors (Ojiambo and Scherm, 2006). Changing application conditions, such as concentration and exposure time, might strongly influence disease reducing capacity and germination rate.

Overall, long-term storage and vinegar treatments, showed similar performance compared to certified seeds, making them promising tools to improve seed health of infected seeds. Treating certified seeds did not reduce disease or improve yield but could hamper germination rates, indicating that treatment of certified seeds does not give additional benefits. Exploring the optimal storage conditions could shorten required storage time to reduce *C. lupini* viability, whilst the optimal acidity and soaking duration could improve vinegar treatment effectiveness. Especially for BCA treatments, different concentrations, modes of application or combining BCAs could improve effectiveness, but more research is required to transfer observed controlled condition effects to field conditions. Altogether, this study contributes to the development of treatments with BCAs, acetic acid or through long-term storage, to sustainably improve seed health in white lupin cultivation and provide additional tools to control anthracnose disease.

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Author contribution

JAA, PH, CA and MMM conceived the original idea for this study. PH, CA and MMM acquired the funding for this project. JAA and CH conducted experiments and JAA took the lead in manuscript writing. JAA and CH analyzed the data. CA planned and performed the field trials and provided seeds. AL provided assistance and maintained the field trials. RTV provided BCA strains. SPCG provided EPPO treated seeds. JAA designed the figures and tables with input from PH, MMM, RK, SPCG, RTV and MRF, who also contributed to data interpretation and provided critical feedback that shaped the final version.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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