Evaluation of seed treatments for the control of *Colletotrichum lindemuthianum* and *Pseudomonas savastanoi* pv. *phaseolicola* in organic production of bean: establishing test prerequisites

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Abstract

***Colletotrichum lindemuthianum* and *Pseudomonas savastanoi* pv. *phaseolicola* cause anthracnose and halo blight, two economically important seed borne diseases of beans. Sanitation of seed can be an effective method to reduce and eliminate the presence of these pathogens. However, in organic agriculture, there are only limited seed treatments available, most of them being physical. Biological seed treatments, including three commercial formulations of bacterial and/or fungus consortia and two natural compounds against *Colletotrichum lindemuthianum* and *Pseudomonas savastanoi* pv. *phaseolicola* in beans are being developed. The methods included the evaluation of the inoculation rate of a *C. lindemuthianum* strain in beans cultivars, the effect of biological seed treatments on the germination of bean seeds, and DNA extraction methods used for bean seeds. Obtaining seeds with an adequate infection rate and extracting high quality DNA from bean seeds have proved to be difficult. Our study presents several challenges that restrict the development of seed treatments.**

Keywords: seed production, organic agriculture, organic seed treatment, biological seed treatment, inoculation of pathogen, DNA extraction, biocontrol

INTRODUCTION

Anthracnose, caused by the fungi *Colletotrichum lindemuthianum* is one of the most widespread and economically important diseases of common bean (*Phaseolus vulgaris*) (Bardas et al., 2009). *Pseudomonas savastanoi* pv. *phaseolicola*, the causal agent of halo blight, is a serious seed borne pathogen of beans that occurs worldwide and can reduce yield and quality (Bradbury, 1986).

For anthracnose, as well as for halo blight, infected seeds represent the major mode of survival and transport of the pathogen (Tinivella et al., 2009) and the best method of control is therefore to use disease-free seeds. Unfortunately, infection cannot always be avoided and seed treatments can be used to reduce the amount of seed borne inoculum. In organic farming, effective seed treatment methods are urgently needed, since the lack of efficiency in preventing the plant disease epidemic caused by seed borne pathogens cannot be later compensated with management practices using chemical plant protection products.

The use of warm water (50°C for 10 min) as a seed treatment has already been recommended for the control of seed borne diseases in organic bean production (Herforth-Rahmé, 2019), including for the control of *C. lindemuthianum*. Several non-chemical methods of seed treatment for vegetables are being developed or are under study, including seed coating using biocontrol agents and natural compounds (Mancini et al., 2016). Seed treatment applications might need a deeper penetration in the seed to control seed borne pathogens for which a surface disinfection is not enough, risking internal damage to the seed and affecting the germination capacity (Spadaro et al., 2017).

To develop and validate seed treatments, infected seeds are needed. However, sourcing seed with even a reasonable level of infection can be difficult, especially in a quantity that allows for many tests. Hence, it is necessary to produce infected seed through artificial inoculation. Current methods consist of inoculation of adult plants (an inoculum solution is

sprayed on leaves or a drop is deposited on a cut on the stem), soaking seeds in inoculum suspension and exposing seeds to colonies of the developing fungi/bacteria (Machado et al., 2004). With artificial inoculation, pathogen inoculum would only adhere to the outer surface of the seed, which could facilitate the disinfection with seed treatments. In addition, it is necessary to check whether the inoculation method gives reproducible results under the same conditions for each host-pathogen combination, since *C. lindemuthianum* and *P. savastanoi* pv. *phaseolicola* present high variability and bean cultivars differ in their levels of susceptibility/resistance.

In order to assess whether or not the seed treatment was effective in reducing the presence of the pathogen, seeds have to be subjected to seed health testing. Seed incubation methods can be used (Tsedaley, 2015). For *C. lindemuthianum*, seeds are incubated on moist blotter papers and identification is based on symptoms observed (ISTA, 2022). Molecular techniques and especially polymerase chain reaction (PCR) can result in a higher level of sensitivity and specificity (Tsedaley, 2015). Isolation of a suitable quantity and quality of DNA is a critical step in PCR pathogen detection. However, seeds possess many compounds that effectively interfere with DNA isolation and subsequently with downstream procedures.

This work describes the steps to develop such seed treatment methods including the evaluation of the inoculation rate of a *C. lindemuthianum* strain in three beans cultivars, the effect of biological seed treatments on the germination of bean seeds, methods of DNA extraction from bean seeds and all the prerequisites necessary for such study.

MATERIALS AND METHODS

Procurement of cultivars

Common bean (*Phaseolus vulgaris*) seed lots were acquired according to the following selection criteria: cultivar with known resistance or susceptibility to the specific pathogen, a standard reference, cultivars with naturally infected as well as healthy seed lots from two organic seed producers. Six different bean cultivars were acquired as described in Table 1.

Table 1. Common bean cultivars obtained.

Cultivar	Suplier	Pathogen
Purple Teepee	Bingenheimer Saatgut AG	No pathogen detected
La Victoire	Sativa Rheinau AG	No pathogen detected
Maxi	Bingenheimer Saatgut AG	No pathogen detected
Helda	Bingenheimer Saatgut AG	No pathogen detected
Purple Teepee	Bingenheimer Saatgut AG	*C. lindemuthianum* detected
Borlotto Mercato OL	Sativa Rheinau AG	*P. savastanoi* pv. *phaseolieola* detected
Canadian Wonder	Sativa Rheinau AG	*P. savastanoi* pv. *phaseolieola* detected

Pathogen strains

A *C. lindemuthianum* reference strain was sourced from INRAE - France. The *C. lindemuthianum* C531 strain was isolated from natural populations of *Phaseolus vulgaris* and was grown in petri dishes at 20°C on potato dextrose agar medium (39 g L^{-1}; Duchefa, Haarlem, The Netherlands). The *P. savastanoi* pv. *phaseolieola* strain 1448AN race 6 was acquired from the CIRM CFBP Collection for Plant associated Bacteria - France and maintained on King's B (KB) medium at 25°C.

Seed treatments

The BRESOV project partner Itaka International Ltd. provided 5 formulated products based on microorganisms or natural compounds. An additional seed treatment based on acetic acid was also included in our tests. The proposed seed treatment method consists in soaking seeds in freshly prepared solutions for a specific period of time (Table 2). A water bath operating at 50°C for 10 min is a physical seed treatment method used by certain organic

seed producers. Two controls were included, soaking the seeds for 10 min in sterile water and seeds not subjected to any seed treatment. After treatments, seeds were spread out in a filter paper container and left to dry on a laminar flow cabinet for at least 2 h.

Table 2. Seed treatments proposed.

Treatment	Type	Description
Itaka product 1	Biocontrol agent	Soaking in biocontrol agent formulation at 10% for 10 min
Itaka product 2	Biocontrol agent	Soaking in biocontrol agent formulation at 10% for 10 min
Itaka product 3	Biocontrol agent	Soaking in biocontrol agent formulation at 10% for 10 min
Itaka product 4	Natural compound	Soaking in natural compound formulation at 1% for 10 min
Itaka product 5	Natural compound	Soaking in natural compound formulation at 1% for 10 min
Acetic acid	Organic acid	Soaking seeds in acetic acid at 5% for 30 min
Warm water	Physical	Warm water bath at 50°C for 10 min
Control – sterile water	-	Method control, soaking seeds in sterile water for 10 min
Control – no treatment	-	Non-treatment control

Inoculation assay

Several methods for the inoculation of *C. lindemuthianum* were tested based on research done on *Colletotrichum lupini* (Alkemade et al., 2021) and a common protocol that was developed in the project BRESOV (BRESOV, 2019). Seed inoculation was done by soaking seeds in an inoculum solution and exposing seeds to fungal colonies on a growing medium. Plant inoculation was done by spraying the inoculum suspension on leaves or injecting inoculum on a wound of the stem of 14-day-old seedlings.

For the seed soaking assay, ten days old *C. lindemuthianum* cultures were flooded with 3 mL of sterile water and the spores were scraped from the plates using a plate spreader. The number of spores was counted using a hemocytometer and the inoculum concentration adjusted to 10^5 spores mL^{-1} before inoculation. Thirty seeds of the cultivars 'Purple Teepee', 'Maxi' and 'La Victoire' were soaked for 10 min in 10 mL of inoculum, each. A fresh spore suspension was used for each bean cultivar. For a given treatment replicate, 10 inoculated seeds were incubated in a polystyrene moist chamber box containing double sheets of paper towels which have been soaked with distilled water. Seeds were covered with one sheet of paper towel soaked in distilled water. Around 50 mL of distilled water was used to wet all 3-paper towels in each box. The percentage of infected seeds was determined by incubating the replicates of 10 seeds in a BOD incubator (WTW, Weilheim, Germany) at 20°C for 7 days. Seeds were examined according to the Rules for the Detection of *C. lindemuthianum* in bean seeds from the International Seed Testing Association (ISTA) (ISTA, 2022). Inoculum was also sprayed on leaves and injected in stem to check for the pathogenicity of the strain on the cultivar.

Currently, seed inoculation by means of exposing the seeds to *C. lindemuthianum* colonies on media is being tested. Fungal cultures were grown in PDA-mannitol agar plates, adjusted to an osmotic potential of 1 MPa by adding 74.69 g L^{-1} D-mannitol, following the method described in Machado et al. (2004).

Seed germination assay

The effect of the different seed treatments on the germination of the 'Maxi' cultivar was assessed. For a given treatment replicate, 50 seeds were incubated for 9 days following the same method as for the inoculation assay. Seedlings were examined according to the Rules from the International Seed Testing Association (ISTA) (ISTA, 1993).

DNA isolation from fungal cultures and seeds

To isolate DNA from *C. lindemuthianum* fungal colonies, a small piece of mycelium (approximately 2×2 mm) was harvested from colonies and ground in a high-speed tissue lyser (FastPrep-24®, M.P. Biomedicals, OH, USA) together with 0.25 mL 0.5 mm zirconia beads (Roth

N034.1) and 0.5 mL TE 1× (10 mM Tris, 1 mM Na2EDTA, pH 8.0). After centrifugation and clarification, the DNA was collected and kept at -20°C.

Real time PCR (qPCR) amplification was done with the genomic DNA isolated from the fungal colonies, specific primers previously described in the literature for the detection of *C. lindemuthianum* were used (Gadaga et al., 2018; Halvorson et al., 2021). The amplification was performed using a Rotor Gene Q cycler (Qiagen, Hilden, Germany) with initial condition at 95°C for 3 min, followed by 95°C for 10 s, 55°C for 10 s, 72°C for 20 s, 40 cycles, and at 72°C for 5 min. DNA quality was checked in 2% agarose gel and 1xTAE buffer solution (40 mM Tris-acetate, pH 8.0, 20 mM sodium acetate, 1 mM EDTA). The samples were stained with GelRed® (Biotium, Freemont, USA) and visualized with an UV transilluminator (Azure Biosystems, Dublin, USA).

To isolate DNA from seeds, seed samples were ground into a fine powder in a ball mill (MM 200, Retsch, Haan, Germany) for 30 s at 30 Hz. Two different DNA methods were tested for the isolation of DNA from the bean seed fine powder, namely the modified CTAB (Byrne et al., 2001) and Quick-DNA Plant/Seed DNA MiniPrep kit (Zymo Research, Irvine CA, USA) methods.

RESULTS AND DISCUSSION

Inoculation methods

The cultivars 'Purple Teepee', 'La Victoire' and 'Maxi' were inoculated with *C. lindemuthianum* C531 strain by soaking the seeds in inoculum. Cultivars presented different infection rates based on the symptoms observed on the seeds following the ISTA rules (Figure 1). 'Purple Teepee' had a susceptible reaction for 40% of the seeds, while 'La Victoire' and 'Maxi' 16.7 and 23.3%, respectively. Plant inoculation done by spraying inoculum on leaves of 14-day-old seedlings showed also compatibility of the *C. lindemuthianum* C531 strain with all three cultivars. 'Purple Teepee' showed again more susceptibility than the other two cultivars. We were not successful in obtaining plants with anthracnose symptoms when injecting inoculum on stem in 14-day-old seedlings.

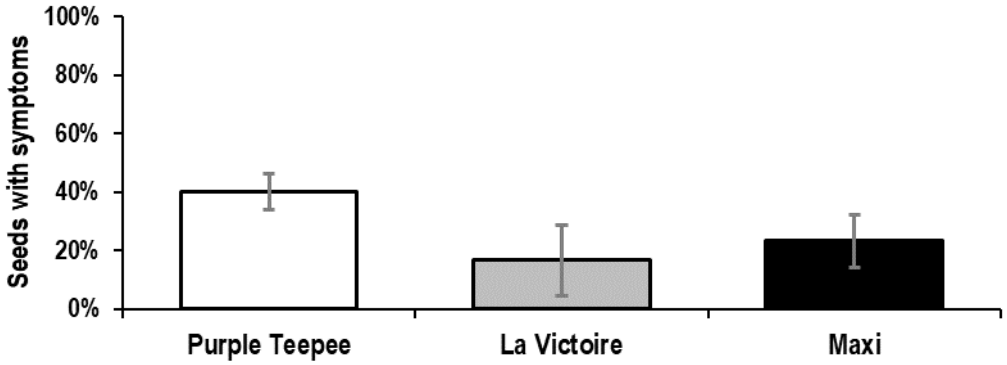

Figure 1. Mean ± SE of the percentage of seeds with *C. lindemuthianum* symptoms on for bean cultivars 'Purple Teepee', 'La Victoire' and 'Maxi' (3 replicates of 10 seeds each per cultivar). Symptoms were evaluated 7 days after seed soaking in spore suspension 10^5 spores mL^{-1} of *C. lindemuthianum* C531 strain.

The method of inoculation by soaking seeds in inoculum presented disadvantages. The pathogen seed infection rate obtained was too low for proceeding with the studies of efficacy of seed treatments. Ideally, in a susceptible cultivar a 100% inoculation of seeds could be expected. A higher spore concentration in inoculum or even longer time of inoculation can be attempted to increase the infection rate. Unfortunately, due to the size and volume of bean seeds a large volume of inoculum is required and longer inoculation time can increase the degradation of the seed.

We are currently evaluating the effectiveness and the viability of seed inoculation by

exposing the seeds to *C. lindemuthianum* colonies. At this time, it is unclear whether this method requires a lower volume of inoculum compared to the soaking method. Other researchers have reported higher percentage of seedlings with pathogen lesions with this method (Machado et al., 2004).

Detection of *C. lindemuthianum* in bean seed

The incubation method proposed by ISTA for the detection of *C. lindemuthianum* in bean seeds was accepted as a mean to determine the infection status of seeds. This method relies on the symptom expression. Positive results with clear evidence of the presence of the pathogen were observed (Figure 2). On the other hand, the seed incubation method requires a great amount of labor and space and is time consuming. It is difficult to perform large experiments because moist chambers boxes are space-consuming and they need minimum relative humidity and temperature control throughout the whole duration of the test. Incubation of bean to detect anthracnose symptoms on the seeds take at least 7 days to be completed.

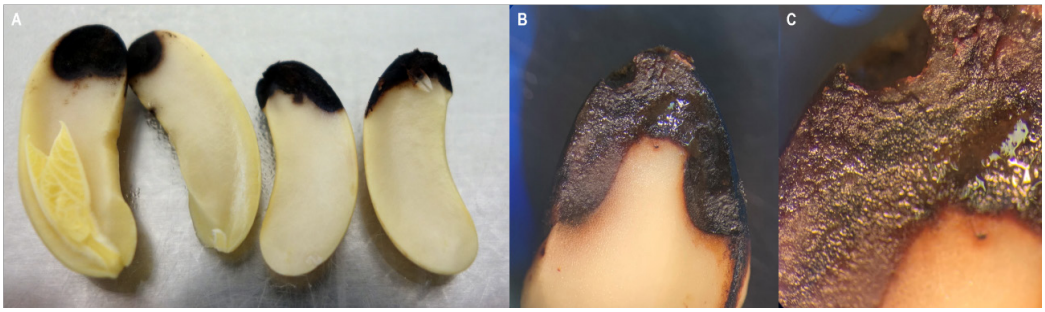

Figure 2. Examples of uncoated bean seeds showing symptoms of *C. lindemuthianum* after 7 days incubation in moist chamber. A) Symptoms are displayed as dark spots; B) 16× magnification, lesions extend into the cotyledons; C) 40× magnification, sunken spots and spore masses.

Molecular detection of *C. lindemuthianum* in bean seed

Many difficulties were encountered when using both tested methods for DNA isolation from seeds. Until now, we were not able to isolate high quality DNA from bean seeds to proceed with qPCR for pathogen detection. DNA precipitation with the modified CTAB method resulted in brownish pellets and during purification with the Quick-DNA Plant/Seed DNA MiniPrep kit the DNA biding silica columns clogged. Both issues may indicate the presence of contaminants in our samples. Several tests are currently being done to try to optimize DNA extraction and isolation from bean seeds. Different grounding methods, including freeze-drying the seeds prior to grounding, varying sample volumes, varying extraction from 10 to 80 mg of seed, and different lysis levels of samples are currently being tested. de Sousa et al. (2015) stated that DNA could not be extracted effectively from bean seeds when the gridding method was not proper. The presence of compounds within the seeds can also inhibit the DNA amplification (Mancini et al., 2016).

Despite that, the qPCR assay with pure cultures of *C. lindemuthianum* amplified a unique DNA fragment of approximately 100 bp and no PCR product yield from negative control. The primers successfully amplified target DNA from all 3 *C. lindemuthianum* samples that were evaluated (*C. lindemuthianum* C531 reference strain and two other isolated strains).

Except for the difficulties in the isolation of DNA from bean seed, the molecular diagnosis proved applicable for detection of seed borne pathogens, due to its speed (completed within 3 to 4 h) and specificity (primers can be designed to amplify nucleic acids of specific pathogens). Equivalent findings of other authors endorse this recommendation (Chen et al., 2007).

Effect of seed treatments on seed germination

The effect of warm water, acetic acid, natural compounds and biocontrol agents used as seed treatments were studied on a seed lot of 'Maxi' with a 90% germination rate (Figure 3). Immersion of 'Maxi' bean seeds in different seed treatments did not affect the germination rate compared to seeds that did not receive treatments (untreated control and sterile water control). Seeds were not damaged by any of the treatments.

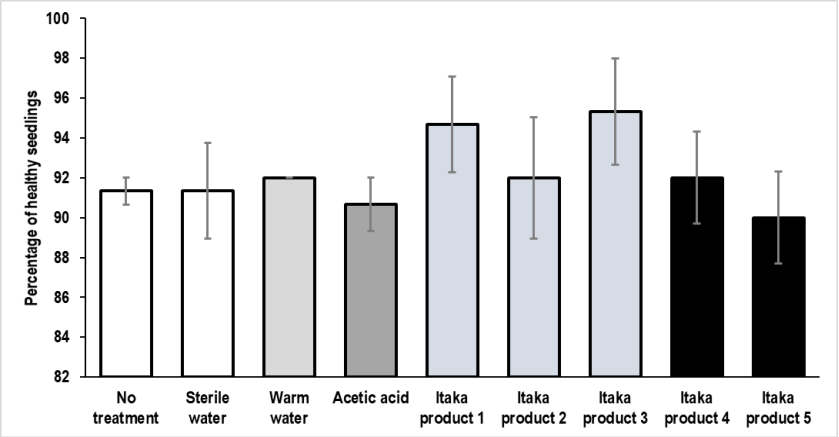

Figure 3. Mean ± SE of the percentage of healthy seedlings obtained with seed treatments with warm water, acetic acid, natural compounds and biocontrol agents (3 replicates of 50 seeds each per cultivar).

Germination rate was determined by the number of normal seedlings according to the ISTA rules for seedling evaluation. This methodology, however, is not enough to evaluate the vigour of the seedlings. Apparently, seed treatment with one of the natural compounds resulted in seedlings with slower hypocotyl development when compared to untreated controls. Van Der Wolf et al. (2008) cited that for certain natural products with a concentration higher than 1%, negative effect on seed germination of vegetable seeds can be observed.

In the upcoming steps, similar tests will be performed on seeds from different cultivars. Afterwards treatments will be applied on artificially inoculated seeds and validated on naturally infected seed lots.

CONCLUSIONS

To summarize, the results of our study indicate that several challenges limit the development of seed treatments in organic bean production. These include sourcing natural seeds with adequate and homogenous infection rate. For this reason, it was decided to perform the tests on artificially inoculated seeds and then validate the most promising seed treatment methods on naturally infected seed lots. However, for *C. lindemuthianum* the inoculation itself has proven to be complex. Several host-pathogens combinations need to be accessed and infection rates obtained are lower than desired.

Detection of pathogen infection in seeds employing the incubation methods has been proven feasible, but still demands a lot of resources and time. Application of qPCR is suitable for molecular seed borne pathogen detection. For the common-bean, DNA extractions of seeds are laborious and need further adjustment before they can be used in routine tests.

A combination of seed borne pathogen detection by means of incubation methods and molecular diagnosis combined would provide more rigorous results. Randomly grouping of seeds in subsamples for molecular detection within a large sample of seeds could make for a good compromise to detect infection levels.

The next steps of this research will be to verify that none of the included seed treatments negatively impact the germination of the remaining cultivars, fine-tune the detection and quantification of seed borne pathogens in treated seed lots and finally to validate successful

treatments on naturally infected seed lots. Following a similar procedure, seed treatments against *Pseudomonas savastanoi* pv. *phaseolicola* will be also tested and validated.

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