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RESEARCH ARTICLE

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Characterisation of *Trichoderma* sp. BI 7376 and *Pseudomonas chlororaphis* subsp. *aurantiaca* BI 7439 as maize seed treatment for commercial traits

Eckhard Koch[a], Tobias Pfeiffer[a], Astrid von Galen[a], Tim Birr[b], Mario Hasler[c], Mathias Kotte[d], Ulf Feuerstein[e], Friederike Meyer-Wohlfarth[f], Petra Zink[a] and Ada Linkies[a]

[a]Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Biological Control, Dossenheim, Germany; [b]Department of Plant Diseases and Crop Protection, Institute of Phytopathology, Faculty of Agricultural and Nutritional Science, Christian-Albrechts-University of Kiel, Kiel, Germany; [c]Variationsstatistik, Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University of Kiel, Kiel, Germany; [d]EVONTA-Service GmbH, Lohmen, Germany; [e]DSV Deutsche Saatveredelung GmbH, Asendorf, Germany; [f]Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Braunschweig, Germany

ABSTRACT

In previous experiments, *Trichoderma* sp. BI 7376 and *Pseudomonas chlororaphis* subsp. *aurantiaca* BI 7439, applied as seed treatments, controlled soilborne *Fusarium culmorum* on maize. The current paper is focused on a deeper characterization of the effects of both strains. The experiments were conducted as pot tests with artificial inoculation of the substrate with *F. culmorum*, or with maize seed lots infected with *Fusarium* spp. When seeds were treated with *Trichoderma* strain BI 7376, *Pseudomonas* strain BI 7439 or with the chemical active ingredient thiram and stored before they were sown in substrate inoculated with *F. culmorum*, the protection by all agents declined. During the storage period of 211 days, the activity of thiram and *Trichoderma* strain BI 7376 dropped by about 38% and 57%, respectively. After 36 days of storage, *Pseudomonas* strain BI 7439 failed to provide any protection, which was obviously related to the observed total loss of viable cells of this agent. Moreover, we observed that both strains protected against soilborne *F. subglutinans* and *F. verticillioides*, showing that their activity was not limited to *F. culmorum* which was used in the previous experiments. Further, experiments with seed lots suspected or known to be infected with *Fusarium* species indicated that *Trichoderma* strain BI 7376 also controlled seedborne inoculum. When electron seed treatment was followed by application of *Trichoderma* strain BI 7376, both seed- and soilborne infections were controlled, showing that the concept of using a combination of a physical seed treatment and microbial antagonists appears feasible.

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CONTACT Ada Linkies ada.linkies@julius-kuehn.de Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Biological Control, Schwabenheimer Str. 101, Dossenheim, Germany

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Introduction

At the germination and seedling stage, maize (*Zea mays* L.) is affected by a number of pathogenic fungi, including different species of *Fusarium,* that can be seed – or soilborne. The disease symptoms include pre- and post-emergence damping-off, low seedling vigour, stunting and discoloration and decomposition of roots and the mesocotyl (Solorzano & Malvick, 2011). Seed treatment with chemical fungicides is still the most practiced method to prevent losses caused by seedling diseases in agricultural crops. In order to overcome, or at least reduce the use of agrochemicals, research efforts are being made to develop non-chemical alternatives for seed treatment, like physical methods (Bänziger et al., 2022; Forsberg et al., 2005; Rahman et al., 2008) and use of microbial agents (Bonaterra et al., 2022; O'Callaghan, 2016; Pereira et al., 2011).

We recently performed a screening of bacteria and fungi, most of them from maize roots, to identify pathogen antagonists that are able to protect germinating maize kernels and seedlings, when applied as seed treatment (Pfeiffer et al., 2021). In the first instance, the screening was performed to find antagonists of *F. culmorum*, and selected antagonistic isolates were also tested against *Rhizoctonia solani* and *Globisporangium ultimum* (syn. *Pythium ultimum*). The most active isolates identified included the fungal isolate BI 7376 and the bacterial isolate BI 7439 which were taxonomically determined as *Trichoderma* sp. (in the following termed *Trichoderma* strain BI 7376) and *Pseudomonas chlororaphis* subsp. *aurantiaca* (in the following termed *Pseudomonas* strain BI 7439), respectively. *Trichoderma* strain BI 7376 protected not only against *F. culmorum* but also against *Rhizoctonia solani*. The anti-*Fusarium* and anti-*Rhizoctonia* activities in maize were recently corroborated (Koch et al., 2022).

One option for the potential commercial use of both isolates would be as stand-alone seed treatments for maize. Alternatively, they could be employed in combination with electron seed treatment or other physical seed treatment methods. Electron treatment uses low energy electrons for the superficial disinfection of seeds. The technology is mainly utilised in Germany, primarily to control common bunt (*Tilletia caries*) on wheat. Like all other physical seed treatments, electron treatment does not control soilborne infections, because it is applied before sowing and only affects pathogens present on or in seeds. Therefore, it appears logical to combine it with microorganisms that have activity against soilborne inoculum (Pfeiffer et al., 2021).

Even if there are currently no registered microorganisms for control of plant diseases in maize, in other crops a number of microorganisms have been commercialised, including some used as seed treatments (Kamilova & de Bruyne, 2013; Köhl & Ravensberg, 2021; Mathre et al., 1999; O'Callaghan, 2016). The development of biocontrol agents from discovery to commercialisation is a complex process that must consider many criteria besides antagonistic activity (Köhl et al., 2011). According to Köhl et al. (2011), commercialisation often fails because screenings for new strains generally focus on biocontrol efficacy and ignore properties that are important for marketing, like appropriateness of the strain for cost-effective production, product shelf life or absence of toxicological concerns.

Due to the encouraging results obtained with *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439 mentioned above, we decided to further characterise the properties and capabilities of these agents. With the focus on practical use, we performed experiments with potting substrate artificially inoculated with *F. culmorum,* or with seed

lots known or suspected to be infected with *Fusarium* species. The experiments mainly addressed properties of the strains important for commercialisation.

At first the required amount of product per unit of seed was determined, which is a key factor for the commercialisation of seed treatment agents. It not only has an effect on the efficacy against the target pathogen but also determines the formulation type of the product, whether the latter can be tightly affixed to the seed and, last but not least, the product price.

We also conducted experiments to characterise the shelf life of seeds treated with *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439. Ability to withstand periods of storage is an important characteristic of biocontrol products based on microbial antagonists (Berninger et al., 2018). Mathre et al. (1999) stated that the marketplace usually demands that products have a shelf-life sufficient to carry them through at least one growing season, and preferably well into the next season. This is also valid for maize seed treatments. Companies take treated, unused seed back and sell it in the following season.

Another set of experiments aimed to characterise the activity spectrum of *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439. During the early stages of identification and development, candidate biocontrol agents are usually tested using single representative pathogen species or isolates. However, certain plant diseases can be caused by more than one pathogen species, and isolates of a given species may differ in aggressiveness. In the case of seedling diseases of maize, multiple species of *Fusarium* are able to infect the germinating seed and developing seedling (Munkvold & White, 2016). It is therefore imperative that seed treatments have activity against several *Fusarium* spp.

In our previous studies *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439 protected against *F. culmorum* in pot experiments with the pathogen inoculum present in the potting substrate, but, for commercial use, seed treatments are also expected to control infections arising from the seedborne inoculum. One of the aims of this study was therefore to test if both agents have activity against seedborne infections. Included in these experiments was a combination treatment, where electron seed treatment was followed by the application of *Trichoderma* strain BI 7376.

The last topic treated was the production of metabolites with antimicrobial activity, which is a common feature of many microorganisms employed in biocontrol. Knowledge about such metabolites is important to understand the mode of action, but the presence of metabolites in commercial formulations may also complicate the registration process. Therefore, an experiment was performed that addressed the production of antimicrobial metabolites by *Pseudomonas* strain BI 7439 and their potential contribution to the anti-*Fusarium* activity of this bacterium.

In summary, the objectives of this study were to elaborate the amount of product needed per unit of seed, to characterise shelf life and activity spectrum of treated seeds, to test the activity against seedborne infections and to assess if metabolites contributed to the activity.

Materials and methods

Maize seeds

The experiments were conducted with seeds taken randomly from five different seed lots of commercial hybrid maize varietes suspected to carry infections with *Fusarium*

spp. (= seed lots 1–5), and with F1 seeds of a commercial hybrid maize variety with 32% infection with *Fusarium* spp. ('2018ZM059'; Drechsel, 2020) (= seed lot 6). Seed lot 6 was produced in the previous season by spraying the silk with a mixture of conidia of *F. culmorum*, *F. verticillioides* and *F. graminearum*.

Seed lots 1 and 2 had been pre-treated with electrons to eliminate superficial *Fusarium*-infections, all other seed lots had not received such pre-treatment. However, in the experiments performed with seed lots 3, 4 and 5, electron treatment was one of the treatments among others. Electron treatment was performed by EVONTA Service GmbH (Lohmen, Germany) using the seed treatment technology `e-ventus´ (for details see Röder et al., 2009). The seed lots used in the experiments are stated in the figure legends in the `Results and Discussion´ section.

Seed treatments

The inoculum concentrations used were based on previous experience as described in Koch et al., 2020; Pfeiffer et al., 2021 and Koch et al., 2022. The material used for seed treatments was a talc formulation of *Trichoderma* strain BI 7376 and the culture suspension of *Pseudomonas* strain BI 7439. In most of the experiments, a treatment with the chemical compound thiram was included using the commercial seed treatment product Thiram SC700 (active ingredient 686 g L^{-1} thiram; Satec HandelsGmbH, Hallwang, Austria).

Preparation of suspensions and formulations

Pseudomonas strain BI 7439

Pseudomonas strain BI 7439 was grown in 300 mL Erlenmeyer flasks filled with 50 mL tryptic soy broth (TSB; Merck, Darmstadt, Germany). The medium was inoculated with a loop-full of bacteria from tryptic soy plates (TSB + 18 g agar L^{-1}), and the flasks were placed for 48 h (in the experiment with different *Fusarium* species and in the experiment for comparative testing of fractions) or for 24 h (in all other experiments) on a rotary shaker (175 rpm at 27°C). The resulting suspension was used for seed inoculation. The number of colony-forming units (CFU) in the 24 h-culture suspensions determined by dilution plating on TSA was 5.8×10^9 mL^{-1}.

Trichoderma strain BI 7376

Conidia of *Trichoderma* strain BI 7376 were produced on grains in aerated 1-L mini-solid-state-fermenters. The fermenters were filled with a mixture of 120 g rice grains and 80 g flaked oat grains boiled for 5 min in de-ionised water. The fermenters were sterilized in an autoclave (121°C, 1 bar, 20 min) and after cooling inoculated by adding 5 mL of a starter culture. The latter was produced by inoculating 50 mL Difco potato dextrose broth (VWR, Darmstadt, Germany) in 250 mL flasks with 2.2×10^7 spores of *Trichoderma* strain BI 7376 and incubation for 96 h at 27°C and 175 rpm. The mycelial dry weight in starter cultures was routinely assessed and generally was about 5 mg mL^{-1}. The fermenters were incubated at 25°C and aerated with humidified compressed air (0.5 bar, 120 L h^{-1}). After 2 weeks, the air supply was increased to 230 L h^{-1} (0.8 bar) and humidification of the air was stopped.

After 21 days of fermentation the process was terminated, and the colonized grains were taken from the fermenters and allowed to dry at room temperature (20–22°C) for 2 days. Conidia were harvested by suction using a mycoharvester (Version 5b; www.dropdata.net/mycoharvester/), weighed and mixed with talcum (Carl Roth, Karlsruhe, Germany) in a ratio 1:9 or 1:1. The spore powder taken directly from the mycoharvester contained 5×10^7 CFU mg^{-1} as determined by plating on potato dextrose agar (Merck, Darmstadt, Germany) supplemented with 50 ppm streptomycin and 20 ppm rifampicin (PDA + A).

Application of seed treatments

Thiram and Pseudomonas strain BI 7439

The thiram-containing liquid chemical reference product Thiram SC700 was applied to maize seeds by shaking in 250 mL polyethylene bottles. A mixture of water and an organic binder was pipetted into the bottles at the rate of 100 µL per 10 g of seeds. After adding the seeds in portions of 20-50 g, the bottles were vigourously shaken to homogenize the mixture. Thiram SC700 was then added at a volume corresponding to 4 g active ingredient kg^{-1} seed. The bottles were shaken for at least 2 min and the seeds poured out and allowed to dry.

Pseudomonas strain BI 7439 was applied by placement of maize kernels for 10 min in the bacterial culture suspension produced as described above. The kernels were then separated from the liquid by passing through a sieve, dried overnight in a laminar flow hood and sown the following day.

Trichoderma strain BI 7376

Fifty grams of seeds were mixed in polyethylene bottles with 1.5 mL of the mixture of binder and water, and 1.4 g of the 1:9 spore-talc mixture described above were added. This resulted in an application rate of 2.8 g *Trichoderma* spores kg^{-1} seed. The seeds were then vigourously shaken, poured on a glass plate and allowed to dry overnight. Application rates of 0.28 and 0.56 g spores kg^{-1} seed were obtained by adding 0.14 or 0.28 g, respectively, of the 1:9 mixture to 50 g seeds. In order to prepare seed lots with 14 and 28 g spores kg^{-1} seed, the volume of the water-binder mixture was increased to 2 mL per 50 g seed, and 1.4 g or 2.8 g, respectively, of the 1:1 spore-talc mixture were added. After vigourously shaking for at least 2 min, the treated seeds were poured out, allowed to dry and sown the following day.

Pot tests

The plant experiments were performed essentially as described previously (Koch et al., 2022; Pfeiffer et al., 2021). Briefly, the tests were run in a plant growth room in 8 × 8 × 8.5 cm plastic pots in a pre-heated (48 h at 60°C) mix of a horticultural substrate (Fruhstorfer Erde Typ P; Hawita Gruppe GmbH, Vechta, Germany) and sand (60:40; w/w). In the experiments for determination of the activity of seed treatments against soilborne *Fusarium* spp., millet seeds colonized by *F. culmorum* VIII18, *F. verticillioides, F. subglutinans* or *F. proliferatum* (Koch et al., 2020) were evenly mixed into the potting substrate at concentrations of 0.5% (w/w). Five maize kernels were sown per

pot and five or seven pots were used per treatment. The data presented are generally from one experiment each, but some are the means of more than one experiment. The details are stated in the figure legends in the `Results and Discussion´ section. After sowing, the pots were placed in a randomized block design in a plant growth room at 20°C and 50–70% relative humidity and covered with an opaque plastic sheet. The latter was removed after 5 days and the pots were exposed to 16 h of light from fluorescent lamps (Photosynthetically active radiation 115 µmol/m^2s). The pots were watered every second day by re-adjusting their weight to the original weight plus 10 g. Two weeks after planting, the number of emerged plants per pot was recorded, plants were cut at the crown, and the above-ground plant dry weight in each pot was determined after drying in an oven for 48 h at 60°C.

Assessment of biocontrol activity and survival of agents after storage

Maize seeds treated with *Trichoderma* strain BI 7376 or *Pseudomonas* strain BI 7439 as described above were placed in Petri dishes stored in a refrigerator at 6°C. For assessment of biocontrol activity against soilborne *F. culmorum,* samples were taken after 2, 35, 77, 119 and 211 days of storage and subjected to the pot test described above, each with three replications with five pots each.

For determination of the number of CFU after storage, 10 g of maize kernels were mixed with glass beads (diameter 5 mm), filled in 250 mL Erlenmeyer flasks and 30 mL of a sterile solution of NaCl (0.9%) were added. After shaking for 1 h on a rotary shaker at 175 rpm, serial dilutions in water were prepared and plated on 0.1 strength TSA (*P. chlororaphis*) or potato dextrose agar (PDA, Merck, Darmstadt, Germany) (*Trichoderma* sp.). After incubation of plates for 2–5 days at 25°C, the number of colony-forming units re-isolated from the seeds was determined and expressed as CFU g^{-1} seeds. Samples were analysed after 2, 36, 108 and 150 days of storage.

Comparative testing of culture suspension, pellet and supernatant of *Pseudomonas* strain BI 7439

In this experiment, the maize kernels were treated by immersion for 10 min in the culture suspension of strain *Pseudomonas* strain BI 7439, or in supernatant and cell suspensions obtained by centrifugation of cultures. The cultures were produced in Erlenmeyer flasks as described above, their volumes were combined and used to prepare four different treatments as follows. Fifty mL suspension were used directly for seed treatment (treatment 1). Another 50 mL were centrifuged for 10 min at 4000 rpm. The supernatant was decanted and made up to 50 mL with TSB (treatment 2). The pellet was washed two times by centrifugation in sterile NaCl (0.9%) and re-suspended in 50 mL TSB (treatment 3). Another 50 mL of culture suspension was centrifuged and the pelleted cells were washed in NaCl as described above, but the pellet was resuspended in 5 mL TSB (treatment 4).

Statistical analysis

The statistical software R (R Core Team, 2022) was used to evaluate the data. The data evaluation for the different trials and experimental questions always started with the

definition of an appropriate linear model, assuming the data to be normally distributed and homoscedastic. These assumptions were based on a graphical residual analysis. If a certain trial consisted of several subtrials, a mixed model (Pinheiro & Bates, 2000) was applied, where the subtrial was considered as a random factor. If the actual (fixed) factors were not orthogonal, a pseudo factor was used, representing the mixture of the actual factors and covering all treatments within the trial (Schaarschmidt & Vaas, 2009). Based on these models, multiple contrast tests (Bretz et al., 2016; Hothorn et al., 2008) were conducted in order to compare the treatments with respect to the experimental question.

Results and discussion

Application rate of Trichoderma strain BI 7376

In order to study the relationship between application rate and efficacy against *F. culmorum*, maize kernels were treated with increasing amounts of spores of *Trichoderma* strain BI 7376 and sown in potting substrate artificially inoculated with *F. culmorum*.

Compared to the healthy control, the number of emerged plants and plant dry weight per pot of the pathogen control were significantly reduced by about 70% (Figure S1 and S2) and 95% (Figure 1), respectively. Compared to the pathogen control, all seed treatments significantly increased both parameters. In the treatments with *Trichoderma* strain BI 7376, the highest plant dry weight was recorded for the two highest application rates. However, application rate and biomass formation were not proportional, an

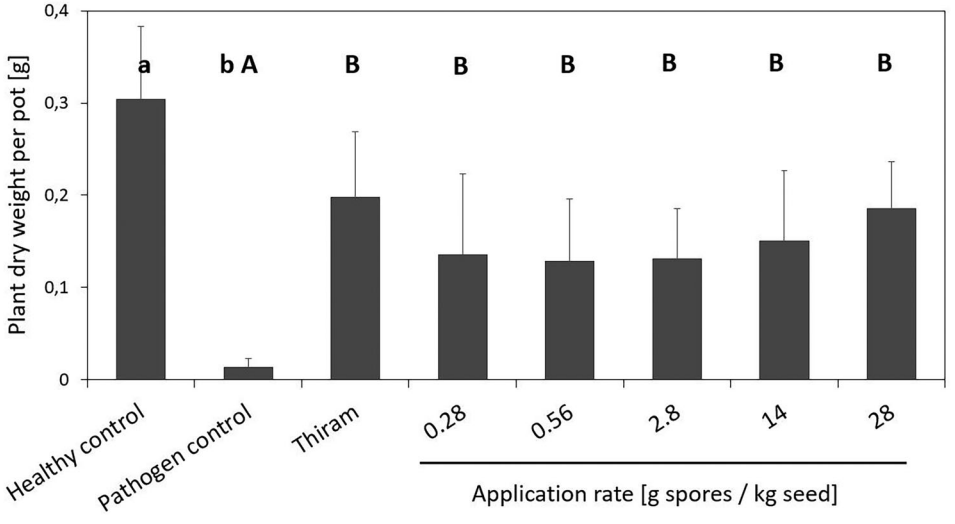

Figure 1. Relationship between application rate of *Trichoderma* strain BI 7376 as seed treatment and biomass of maize seedlings (seed lot 1) in potting substrate artificially inoculated with *F. culmorum*. Means and standard deviation of two independent experiments, each with 5 pots per treatment and 5 seeds per pot, determined 14 days after sowing. The chemical seed treatment thiram was included as a reference. Different lowercase letters indicate a statistically significant difference between the healthy control and the pathogen control. Upper case letters indicate statistically significant differences of the seed treatments to the pathogen control (Tukey test; $p \leq 0.05$).

increase of the application rate by a factor of 100 resulting in a ca. 13fold increase of the plant dry weight per pot.

The number of antagonist propagules needed to obtain an appropriate level of control by biological seed treatments depends on the antagonist, the pathogen and the host plant. Only $> 5 \times 10^3$ conidia per seed of *Clonostachys rosea* were needed to protect barley and wheat against *F. culmorum* (Jensen et al., 2000). For *Trichoderma*, the reported numbers are generally higher. Significant control of *Fusarium* wilt on chickpea and pigeon pea was obtained with spore loads of 7–8 $\times 10^6$ CFU kg^{-1} seed (Khan et al., 2011). According to Mastouri et al. (2010), application of 2×10^{10} CFU kg^{-1} seed of *T. harzianum* strain T-22 resulted in an increase of seedling vigour and amelioration of stress in tomato. For control of *Fusarium* spp. on wheat, 1×10^{10} CFU kg^{-1} seed of *T. asperellum* and *T. harzianum* was optimal (Couto et al., 2021). In the present study we used a talc formulation containing *Trichoderma* conidia produced by solid state fermentation. In a comparable study, the number of conidia in the harvested spore powder was 3.6×10^7 mg^{-1} (Elósegui Claro et al., 2009). We determined 5×10^7 CFU mg^{-1} in the freshly harvested *Trichoderma* spore powder by plating serial dilutions of the sample (not shown). Adopting this value and assuming a 1000-grain weight of 300 g, the application rates of 0.28 - 28 g spores per kg seed employed in our experiment (compare Figure 1) corresponded to 4×10^6 - 4×10^8 CFU of *Trichoderma* per maize kernel. *Trichoderma* spores were washed from seeds that were previously treated with 2.8 g kg^{-1} spores. The spore count revealed 3×10^7 CFU per kernel (not shown), which was in good agreement with the corresponding calculated value (4×10^7 CFU per kernel) for this application rate.

At the highest application rate (28 g spores per kg seed), plant number and plant dry weight were similar in magnitude as after seed treatment with thiram. It is noteworthy that at this high application rate phytotoxic effects were not observed. However, because the highest application rate would likely be problematic regarding cost and technical applicability to the seeds, we chose the application rate of 2.8 g kg^{-1} seed for the further experiments.

Storage test of treated seeds

A large number of shelf-life studies have been performed with *Trichoderma* spp., typically as part of projects developing new methods of production and formulation (Locatelli et al., 2018, and references herein). The term shelf-life generally refers to CFU-numbers in formulations stored in vials or other containers based on colony numbers obtained by dilution plating. In the context of the present study, we use the term `shelf-life´ for the survival of propagules on treated, stored seeds. We also evaluated the storability of treated seeds based on biocontrol efficacy in potting substrate inoculated with *F. culmorum*.

In non-inoculated potting substrate (healthy control; Figure 2(A)), the number of emerged plants remained constant over the whole storage period of 211 days, indicating that storage of the maize kernels at 6 °C had no adverse effect on germinability. In potting substrate inoculated with *F. culmorum,* the plant numbers recorded after two days of storage in the thiram, *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439 treatments clearly exceeded those of the pathogen control (Figure S3). After 77 days of

storage, only the thiram and *Trichoderma* strain BI 7376 treatments had still a considerably higher number of plants than the pathogen control (= seeds not treated) (Figure 2 (A)).

Differently from germinability, plant dry weight per pot of the healthy control declined during storage by about 40% (data not shown), indicating a reduction of

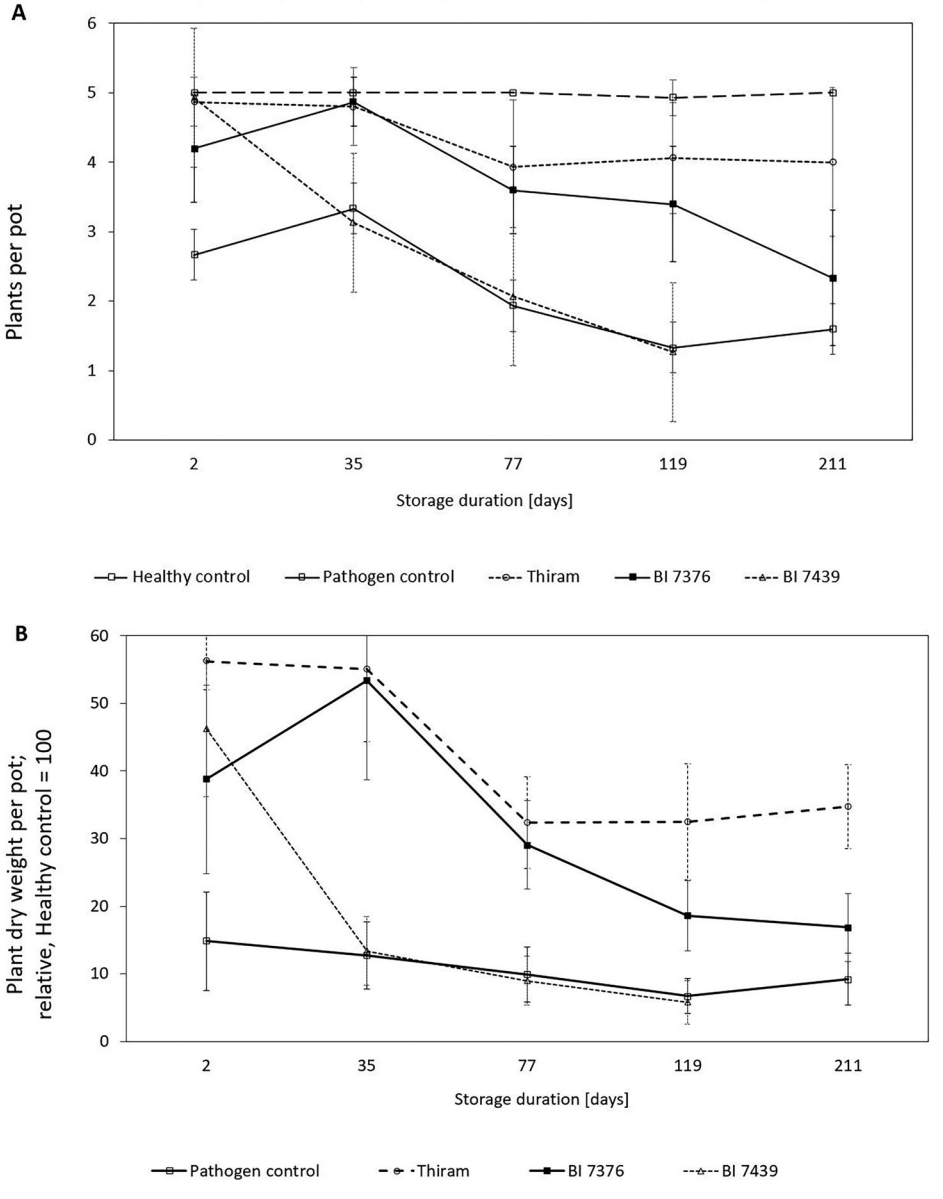

Figure 2. Effect of storage of maize seed (seed lot 2) treated with *Trichoderma* strain BI 7376 (talc formulation) or *Pseudomonas* strain BI 7439 (culture suspension) on (A) number of emerged plants and (B) production of dry matter in potting substrate inoculated with *F. culmorum*. The chemical seed treatment thiram was included as a reference. Means of 3 replications per sampling, each with 5 pots and 5 seeds per pot.

vigour of the aging seeds. It is not known if the reduction was typical for maize seeds or just a property of the seed lot used. The observation nevertheless shows that studies with treated, stored seeds should always consider the seed vigour, since it may have serious implication on biocontrol efficacy. Because of this decline, the changes in plant dry weight recorded during the storage period are expressed in relation to the healthy control (Figure 2(B)). Compared to the latter, the plant dry weight was clearly reduced in the pathogen control at the first sampling. Thiram, *Pseudomonas* strain BI 7439 and *Trichoderma* strain BI 7376 provided a comparatively high initial level of control, although plant dry weight was reduced by about 40–60% relative to the healthy (= non-inoculated) control (Figure 2(B)). During the following storage period, the activity against *F. culmorum* declined. In the case of *Pseudomonas* strain BI 7439 there was a sharp drop during the first 35 days down to the level of the pathogen control. After 211 days of storage, the reduction of plant dry weight for thiram and *Trichoderma* strain BI 7376 was about 38% and 57%, respectively, compared to the plant dry weight determined after 2 days of storage.

The number of CFU of *Pseudomonas* strain BI 7439 and *Trichoderma* strain 7376 on maize kernels was monitored from one day until 150 days of storage. From an initial value of 8×10^6 CFU per kernel, populations of *Pseudomonas* strain BI 7439 dropped to zero after 36 days of storage (data not shown), which explains the observed loss of biocontrol activity.

The drop in CFU of *Pseudomonas* strain BI 7439, which was applied as an unformulated suspension, can be attributed to the sensitivity to desiccation stress of non-sporulating bacteria (Berninger et al., 2018). Arriel-Elias et al. (2018) also reported that a *P. fluorescens* strain reached values close to 0% viability in only 10 days of storage at 8 °C and 28 °C. With specific formulations, improvement of the shelf-life of gram-negative bacteria may nevertheless be possible. For example, use of coconut fiber of high moisture content as carrier sustained the viability of two strains of *P. chlororaphis* for at least 120 days under refrigeration (Corrêa et al., 2015). In peat- and talc-based formulations, *P. chlororaphis* PA23 survived up to 180 days of storage at 28 °C (Nakkeeran et al., 2006). Cedomon and Cerall, an oil- and a water-based formulation, respectively, of *P. chlororaphis* MA342 can be stored for eight weeks at 4–8°C and 3 weeks at room temperature, and seed treated with Cedomon can be stored for up to 1 year (Mehnaz et al., 2016). The latter indicates that the shelf-life of the formulation may diverge from the storability of treated seeds.

The number of CFU per kernel of *Trichoderma* strain BI 7376 remained fairly stable at values around 1×10^7 (data not shown) during the monitored period of 155 days. However, the stable population on seeds contrasted with the observed decline of biocontrol activity of *Trichoderma* strain BI 7376 during storage. A reason could be that germination rates of the aged conidia were higher on PDA than in the seed environment, but other factors like loss of activity during storage, as observed in dual cultures with phytopathogenic fungi (Abdel-Kader et al., 2012; John et al., 2014), may have also played a role.

Biocontrol of different *Fusarium* species

In order to characterize their activity spectrum, *Pseudomonas* strain BI 7439 and *Trichoderma* strain BI 7376 were applied to maize seeds, and the latter were then sown in

substrate inoculated with isolates of three *Fusarium* species, namely *F. subglutinans* (two strains), *F. verticillioides* and *F. proliferatum*.

Of the four isolates tested, only the two isolates of *F. subglutinans* caused a slight, although statistically not significant reduction in germination (Figure S4). All *Fusarium* species visibly impaired plant growth (Figure S5), with the two strains of *F. subglutinans* causing significant reductions in plant biomass (Figure 3). Both strains were similar in pathogenicity, but seed treatment appeared to be more effective in the experiment with *F. subglutinans* strain 2. Nevertheless, increases in biomass following seed treatment were also recorded in the pots inoculated with *F. subglutinans* strain 1. In previous experiments (Koch et al., 2022; Pfeiffer et al., 2021) and in the other experiments of the current paper, *Pseudomonas* strain BI 7439 and *Trichoderma* strain BI 7376 gave reliable control also of a strain of *F. culmorum.* Therefore it appears safe to conclude that both strains are effective against different *Fusarium* species. So far, we have no indication from our work with *Pseudomonas* strain BI 7439 and *Trichoderma* strain BI 7376 that these strains have growth promoting properties. Increases in plant biomass after seed treatment with these strains can therefore only be expected in situations with a sufficiently high infection pressure.

Activity against seedborne inoculum and combinations with electron seed treatment

When seeds of seed lots 3, 4 and 5 with a suspected *Fusarium* infection were sown in non-inoculated potting substrate, electron treatment, treatment with *Trichoderma* strain BI

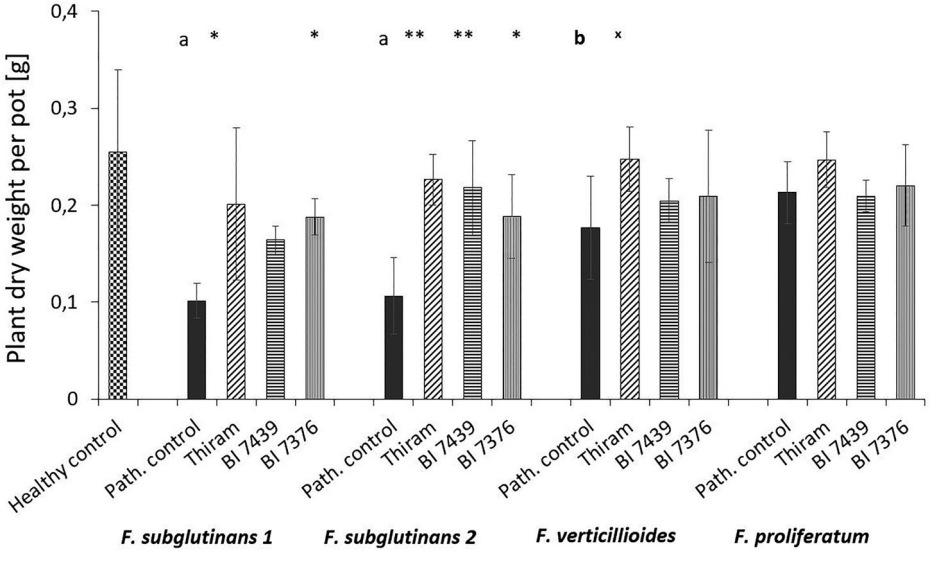

Figure 3. Effect of seed treatment with thiram, *Pseudomonas* strain BI 7439 and *Trichoderma* strain BI 7376 on biomass of maize seedlings (seed lot 1) in potting substrate inoculated with two strains of *F. subglutinans* or one strain each of *F. verticillioides* and *F. proliferatum*. Means and standard deviations of 5 pots per treatment with 5 seeds each. Letters above bars indicate differences of the respective pathogen control to the healthy control (a = $p \leq 0.001$; b = $p \leq 0.1$). Asterisks above treatments indicate statistical differences to the respective pathogen control. (Tukey test; $^{\times}$ $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$;)

7376 and the combined treatment had no effect on the number of emerged plants (Figure S6A), but both increased the dry weight per emerged plant. With seed lot 5, electron seed treatment tended to increase ($p \leq 0.1$) the plant dry weight compared to the untreated control, and significant increases ($p \leq 0.001$) were recorded after application of *Trichoderma* strain BI 7376 and of electron treatment followed by strain BI 7376 (Figure 4 (A)). The increases were less pronounced with seed lots 3 and 4, which apparently reflected a lower degree of *Fusarium*-seed infection of these seed lots compared to seed lot 5 (Figure 4(A)). In the case of seed lot 6, which was highly infected with *Fusarium*, seed treatment with thiram and treatment with *Trichoderma* strain BI 7376 tended to increase plant dry weight. After seed treatment with *Pseudomonas* strain BI 7439, there was no or only a marginal increase of plant dry weight (Figure 5(A)), which may be an indication for a lack of activity of *Pseudomonas* strain BI 7439 against the seedborne *Fusarium*-inoculum. However, more results are needed to confirm this conclusion.

Effects of electron seed treatment against seedborne *Fusarium* infections were recently reported by Drechsel (2020). In her experiments with three maize seed lots (including seed lot 6 used in the present study) carrying seed infections with *Fusarium* spp., electron treatment at different dosages caused a significant reduction of the percentage of seed infection, in case of seed lot 6 from 32% to about 5–10%. When in our experiments seed lots 3, 4 and 5 were sown in potting substrate inoculated with *F. culmorum*, drastic reductions of plant number (Figure S6B) and especially plant dry weight per pot (Figure 4(B)) were recorded. Compared to the pathogen controls, thiram, *Trichoderma* strain BI 7376 and the combination of *Trichoderma* strain BI 7376 with electron treatment caused significant increases of the number of emerged plants and plant dry weight (Figure 4(B)). A very similar pattern was also recorded for seed lot 6. In potting substrate inoculated with *F. culmorum,* plant dry weight dropped significantly. Compared to the pathogen control, treatment with *Trichoderma* strain 7376 and *Pseudomonas* strain BI 7439 tended to increase the plant dry weight (Figure 5(B)).

Physical treatments applied to seeds before sowing cannot protect against soilborne pathogens. This is clearly demonstrated in the experiments with all four seed lots (Figures 4(B) and 5(B), S7) where electron treatment failed to provide any protection (Figure S8). Our findings also show that *Trichoderma* strain BI 7376 can be safely combined with electron seed treatment. There was no indication for adverse effects of the combined treatment, however, synergism between both methods was not observed.

Activity of components of the suspension of *Pseudomonas* strain BI 7439

In order to test if metabolites are involved in the activity of *Pseudomonas* strain BI 7439 against *Fusarium*, we applied the culture suspension, the pelleted bacterial cells as well as the supernatant obtained after centrifugation of the culture to maize seeds that were then sown in potting substrate inoculated with *F. culmorum*.

Despite a high infection pressure in the experiment, all treatments increased the plant biomass significantly and to a comparable level as the chemical thiram, although the activities of the supernatant, the pellet and the concentrated pellet appeared to be more variable than that of the culture (Figure 6). Similar results have been reported for other microbial antagonists.

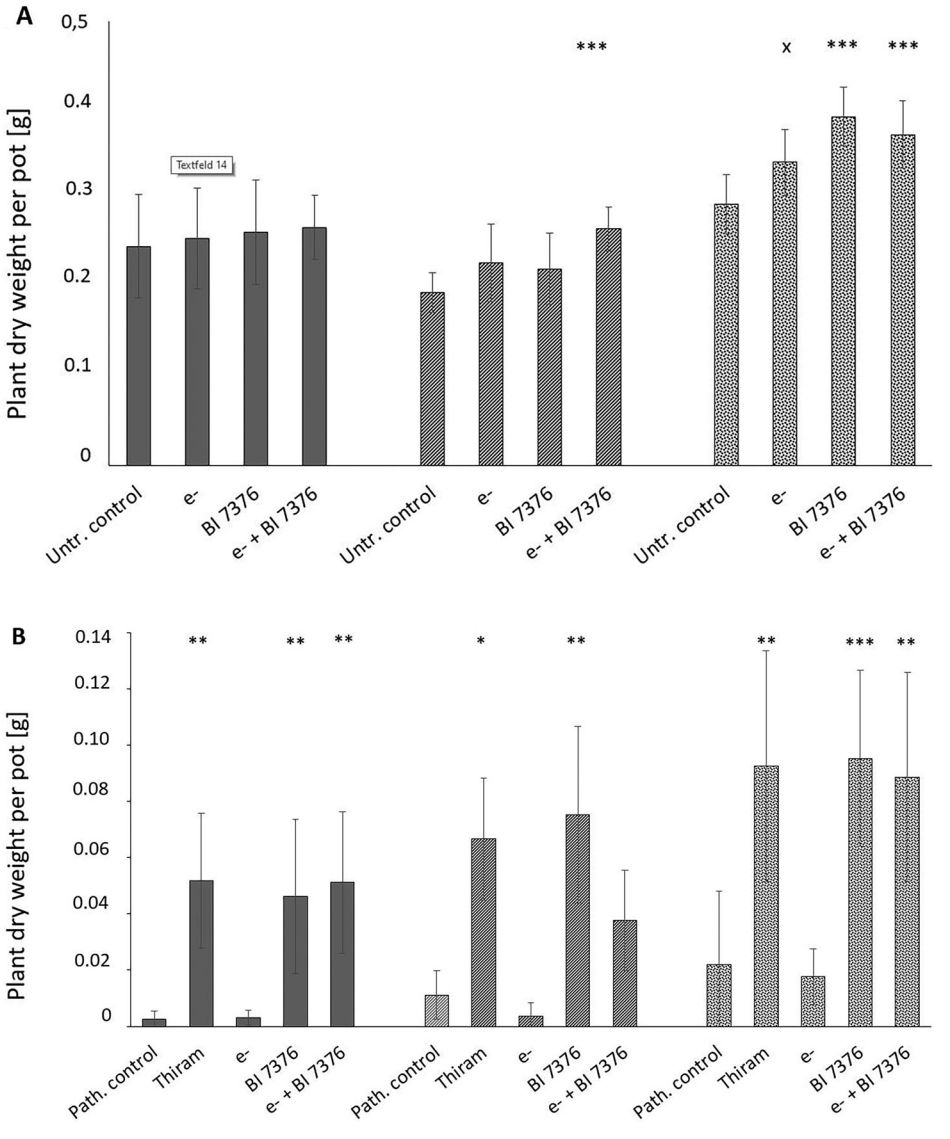

Figure 4. Effect of seed treatment of three seed lots on the biomass of maize seedlings growing in (A) non-inoculated potting substrate or (B) in potting substrate inoculated with *Fusarium culmorum*. Means and standard deviation of 7 pots with 5 seeds each per treatment. Filled bars: seed lot 3 (means of two experiments); hatched bars: seed lot 4 (one experiment); dotted bars: seed lot 5 (one experiment). e-: seeds treated with electrons; BI 7376: seeds treated with *Trichoderma* strain BI 7376; e- + BI 7376: seeds treated with electrons and with *Trichoderma* strain BI 7376. Asterisks above bars indicate statistical differences to the respective Untreated controls (in A) or the respective pathogen controls (in B). (Tukey test; ^{x} $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

For example, *Bacillus amyloliquefaciens* QST713, the active microbial ingredient of the biofungicide Serenade ASO, is known to produce lipopeptide and surfactant antibiotics (Marrone, 2002). From experiments with cell suspensions and product filtrates, Lahlali et al. (2013) concluded that the activity of Serenade against *Plasmodiophora brassicae*

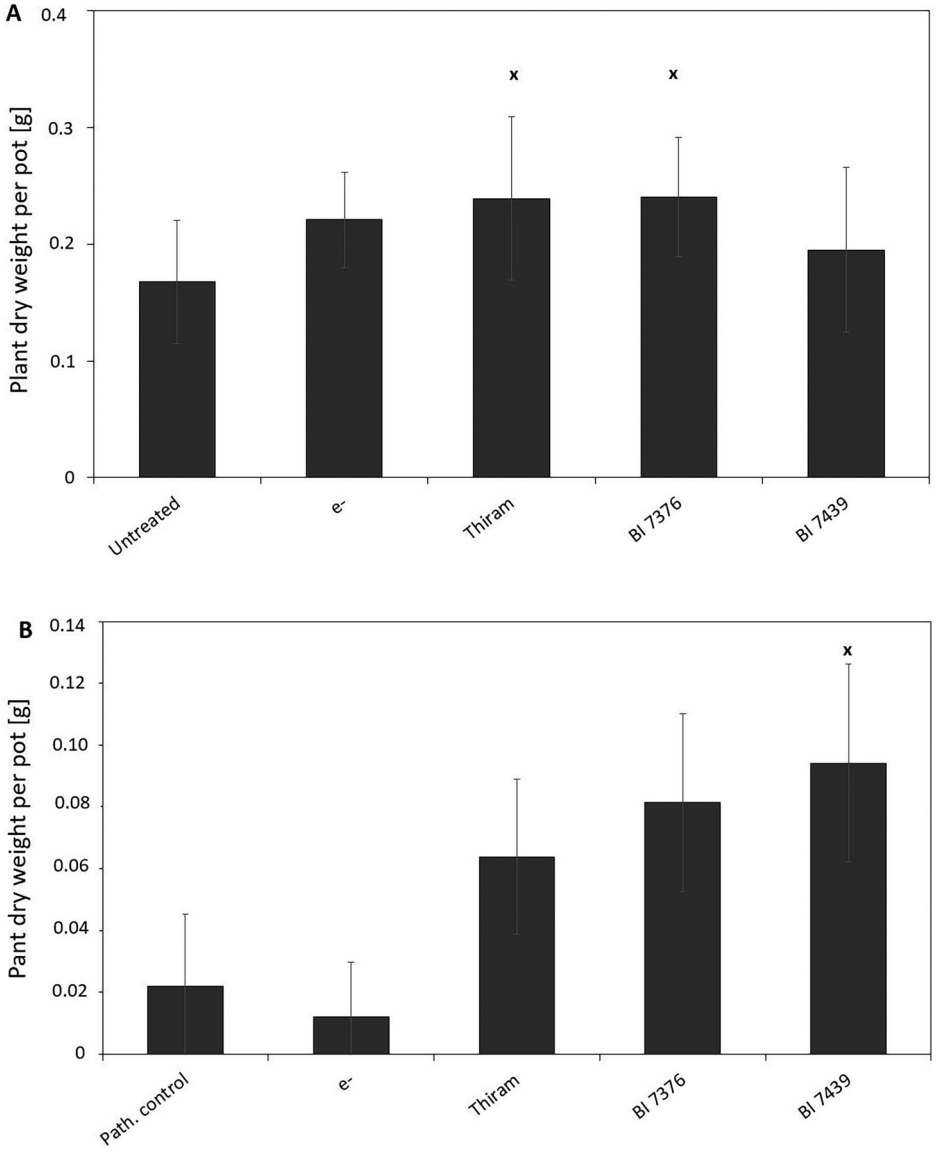

Figure 5. Effect of different seed treatments on biomass of maize seedlings (seed lot 6) growing in non-inoculated potting substrate (A) or potting substrate inoculated with *Fusarium culmorum* (B). e-: seeds treated with electrons; BI 7376: seeds treated with *Trichoderma* strain BI 7376; BI 7439: seeds treated with *Pseudomonas* strain 7439. Means and standard deviation of 5 pots per treatment with 5 seeds per pot. Asterisk above bar indicates the statistical difference to the pathogen control (Tukey test; ^{x} $p \leq 0.1$).

was partially due to the antibiotics in the formulation. Following centrifugation of the product Serenade and of a culture of strain QST713, Stephan et al. (2005) tested the activity of the components against *Phytophthora infestans* in a detached leave assay. Whereas the supernatant of the Serenade suspension and the supernatant of the culture displayed full activity, the pelleted spores did not show any effect.

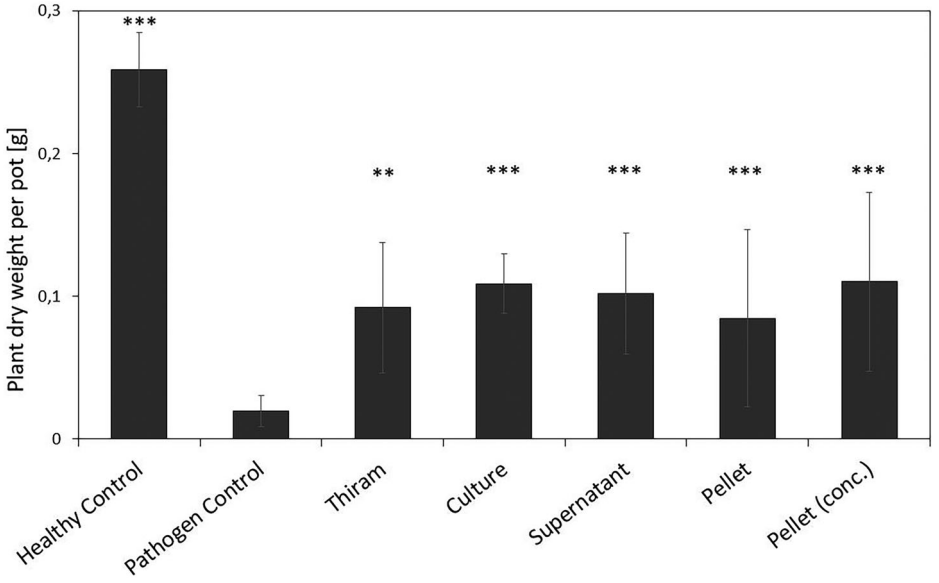

Figure 6. Efficacy of *Pseudomonas* strain BI 7439 as seed treatment against *Fusarium* seedling blight. Before they were sown in potting substrate inoculated with *F. culmorum,* the seeds (seed lot 2) were treated with a shake culture of *Pseudomonas* strain BI 7439 (treatment 1; compare Materials and Methods) or the supernatant (treatment 2), the pellet (treatment 3) or a concentrated pellet obtained from centrifugation of the culture (treatment 4). Means and standard deviation of two separate experiments, each with 5 pots per treatment and 5 seeds per pot. Asterisks above bars indicate statistically significant differences to the pathogen control (Tukey test; *** $p \leq 0.001$)

Pseudomonas chlororaphis and its subspecies are known to produce a large number of antimicrobial metabolites including phenazins, pyrrolnitrin, dialkyl resorcinols, hydrogen cyanide and enzymes (Anderson & Kim, 2018; Arrebola et al., 2019). The observed activity of the supernatant in our study is likely related to these compounds and indicates that they have a role in the activity of *Pseudomonas* strain BI 7439 against *Fusarium* spp. The formation of these compounds should therefore be monitored during the fermentation process as they can possibly be exploited to increase the antifungal activity or even improve the shelf life of the formulation. The downside of this finding, however, is that `relevant metabolites´ must be addressed in the registration process, which may pose a hurdle for commercialisation.

Conclusions

The work reported here was initiated in order to characterize *Trichoderma* strain BI 7436 and *Pseudomonas* strain 7439 regarding traits important for commercialisation. The application rate of 2.8 g kg^{-1} seed determined for *Trichoderma* strain BI 7436 appears reasonable and can be taken as a starting point for further development. The same is true for the shelf life of strain BI 7436 on treated, stored seeds, taking into account that the spores in both experiments were used as mixtures with talc as carrier, without any additives or adjuvants. In the case of *Pseudomonas* strain BI 7439, the development of a formulation with improved shelf life compared to the bacterial suspension is clearly a

prerequisite for any further development work. The finding that metabolites apparently contribute to the activity of the culture suspension indicates that the formulation development should not only concentrate on cell survival and shelf life but also make use of the metabolites produced by *Pseudomonas* strain BI 7439.

The experiments confirm the previously reported anti-*Fusarium* activity of *Trichoderma* strain BI 7376 and *Pseudomonas* strain BI 7439 and show that both are active also against *Fusarium* species other than *F. culmorum,* which was used in the previous experiments. A new finding is that *Trichoderma* strain BI 7376 has the potential to not only control soilborne but also seedborne *Fusarium* infection. The results also show that the concept of using a combination of a physical seed treatment, here exemplified by electron treatment, and microbial antagonists appears feasible.

Supplementary information

The online version contains supplementary material.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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