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Optimizing Biocontrol Activity of *Paenibacillus xylanexedens* for Management of Hairy Root Disease in Tomato Grown in Hydroponic Greenhouses

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Abstract: Hairy root disease (HRD) caused by rhizogenic *Agrobacterium* biovar 1 strains affect tomato, cucumber, eggplant, and bell pepper grown in hydroponic greenhouses and can cause considerable yield losses worldwide. Recently, *Paenibacillus xylanexedens* strains (ST15.15/027 and AD117) with antagonistic activity against rhizogenic agrobacteria were identified. In this study, we present results of greenhouse trials of two consecutive growing seasons (2019 and 2020) to examine the potential of these two biocontrol organisms (BCOs) under practical conditions. BCO-treatment at a 10^7 colony forming units (CFU)/mL density resulted in a considerable reduction of the HRD infestation rate, confirming the biocontrol potential of the two *P. xylanexedens* strains. Results revealed that a single BCO strain (ST15.15/027) performed equally well as the mixed inoculum of both strains. The same level of biocontrol activity was even achieved when the BCO inoculum density was reduced to 10^5 CFU/mL. qPCR analysis further showed that *Paenibacillus* was still present in rockwool substrate near the end of both trials, indicating that they persist well in a rockwool environment and that application at the start of the trial is sufficient to protect tomato plants until the end of the trial. Altogether, these results are highly valuable for further optimization and exploitation of *P. xylanexedens* as a biocontrol product for the control of HRD in hydroponic greenhouses.

Keywords: biological control organism (BCO); greenhouse hydroponics; hairy root disease (HRD); *Paenibacillus*; rhizogenic *Agrobacterium*; rockwool

1. Introduction

Rhizogenic *Agrobacterium* biovar 1 is the causal agent of hairy root disease (HRD), which affects many hydroponically-grown crops, including tomato, cucumber, eggplant, and bell pepper [1–3]. Application of hydrogen peroxide is currently the most used management strategy to reduce HRD. However, rhizogenic *Agrobacterium* strains are becoming increasingly resistant towards hydrogen peroxide, with some strains reported to tolerate up to 600 ppm peroxide, a concentration that is not desirable in practice [4]. As a result, biological control was proposed as a more sustainable strategy in plant disease management to minimize the detrimental effects of chemical biocides for the environment [5].

The genus *Paenibacillus* consists of many soil-borne bacteria with plant growth-promoting or disease-protective traits [6]. Several studies showed antagonistic properties of *Paenibacillus* spp. or demonstrated their potential as biological control organisms (BCOs) for the management of several plant diseases caused by pathogenic bacteria, nematodes, fungi, and oomycetes [7–10]. Moreover, a number of *Paenibacillus*-based products were introduced as commercial biological control products [11], such as "Topsid (AC-1)" (Green-biotech, South Korea), which is used against fungal diseases in pear and apple [12,13]. Recently, a set of *Paenibacillus* strains was reported to display antagonistic activity towards rhizogenic *Agrobacterium* bv. 1 [14]. Moreover, a preliminary and short-term greenhouse trial demonstrated the effectiveness of the identified *Paenibacillus* strains in controlling HRD [14]. In that greenhouse trial, the two most promising BCOs (ST15.15/027 and AD117) were applied repeatedly, as a highly concentrated mixed inoculum and were evaluated for their biocontrol activity towards one particular rhizogenic *Agrobacterium* strain. This exciting proof-of-concept of the biocontrol potential of *Paenibacillus* for the management of HRD also raised a number of additional questions—(i) Do these *Paenibacillus* strains exhibit biocontrol activity towards other rhizogenic *Agrobacterium* strains, considering the high genetic and phenotypic diversity among rhizogenic *Agrobacterium* strains [4]? (ii) Can we further optimize inoculum density? (iii) Are the BCOs stably maintained in the rockwool environment towards the end of the trial and is repeated application throughout the trial necessary for effective HRD control? (iv) Is a single biocontrol strain sufficient to effectively control HRD? To confirm and extend the proof-of-concept established previously in the short-term greenhouse trial and to address these new questions, we performed two independent greenhouse trials, spanning two entire growing seasons in 2019 and 2020. In these greenhouse trials, we evaluated different BCO densities as well as the efficacy of a single strain to reduce HRD (ST15.15/027).

2. Materials and Methods

2.1. Bacterial Strains and Preparation of Bacterial Inocula

Rhizogenic *Agrobacterium* bv. 1 strain ST07.18/001, and *Paenibacillus xylanexedens* strains ST15.15/027 and AD117 were used in this study. *Agrobacterium* ST07.18/001 was previously isolated from the roots of a tomato plant showing HRD symptoms, as described before (Bosmans et al., 2015). Specific PCRs targeting the *virD2* and *rolB* gene, and the partial 16S ribosomal RNA (rRNA) gene sequence confirmed that strain ST07.18/001 is a rhizogenic *Agrobacterium* belonging to genomospecies G3. The *Paenibacillus* isolates used in this study were described previously and were shown to have antagonistic activity against rhizogenic *Agrobacterium* [14]. Both strains were classified as *P. xylanexedens* based on 16S rRNA sequences (99% identity with *P. xylanexedens* B22a^{T}), and the Average Nucleotide Identity (ANI) based on whole-genome sequence comparison using the BLAST algorithm (95.7% identity with *P. xylanexedens* PAMC 22703). Strains were stored at −80 °C and were grown on full-strength Tryptic Soy Agar (TSA; Oxoid) (rhizogenic *Agrobacterium*) or 0.1× TSA (*Paenibacillus*) and incubated at 25 °C for 48 h. BCO and rhizogenic *Agrobacterium* inocula were prepared by first transferring a single colony into 4 × 5 mL 0.1× tryptic soy broth (TSB) (*Paenibacillus*) or full-strength TSB (rhizogenic *Agrobacterium*) and incubating overnight at 25 °C and 120 rpm agitation. The next day, 20 mL overnight culture was added to 1 L full-strength TSB (*Paenibacillus* and rhizogenic *Agrobacterium*) and incubated at 25 °C and 120 rpm. After overnight incubation, the optical density (OD_{600}) was measured and suspensions were diluted to achieve the desired densities. Density of the inocula was confirmed by plate counting of a seven-step 10× serial dilution.

2.2. Greenhouse Experiments

The experiment was carried out in semi-commercial hydroponic greenhouse compartments equipped with a gutter growing system (FormFlex/Metazet, Wateringen, the Netherlands) at Research Station Hoogstraten (Hoogstraten, Belgium). Two-headed Beef tomato cv. Rebelski (De Ruiter, Bergschenhoek, the Netherlands) plants were grafted onto

rootstock Maxifort (De Ruiter, Bergschenhoek, the Netherlands) and subsequently grown in rockwool cubes (Grodan, Roermond, the Netherlands). Rockwool cubes, containing 6-week old seedlings, were placed on the rockwool slab (referred to as "planting date") with a spacing of 50 cm between the cubes, resulting in an overall stem density of approximately 2.5 stems m^{-2}. On the same day, the BCOs were applied by pouring 50 mL of the inoculum (densities ranging from 10^5 to 10^7 colony forming units (CFU)/mL for either a single or a mixed BCO inoculum) to each plant. This inoculation procedure was repeated for 10 consecutive days (DAP 0–9), and then once a week during the following four weeks (DAP 16, 23, 30, 37). After the 10-day application of the BCO, individual plants were artificially inoculated by pouring 50 mL of a 10^4 CFU/mL *Agrobacterium* ST07.18/001 solution once a week during four consecutive weeks (17, 24, 31, and 38 days after planting (DAP)). The untreated control plants were not treated with BCO, and were only artificially inoculated with rhizogenic *Agrobacterium*. In the greenhouse trial of 2019, the untreated control was compared with the application of a mixed inoculum (equal level of strains ST15.15/027 and AD117) in a total density of 10^7 CFU/mL (P_mix_10E07). In the 2020 greenhouse trial, the following treatments were included and compared with an untreated control—application of a mixed BCO inoculum (ST15.15/027 and AD117) in a total density of 10^7 CFU/mL (P_mix_10E07), and a single BCO strain (ST15.15/027) in a density of 10^5 or 10^7 CFU/mL (P_027_10E05, and P_027_10E07, respectively). For each BCO-treatment as well as the untreated control, 48 plants were monitored throughout the trial. Plants were visually inspected for HRD symptoms (excessive root formation in the substrate and emerging roots on top of the rockwool cube), approximately 10 weeks after planting (March 2019 and 2020) and near the end of the experiment (August 2019 and October 2020). Visual HRD symptoms were confirmed by a positive qPCR analysis targeting rhizogenic *Agrobacterium*, as described before [15]. At the same time, stem thickness was also measured right on top of the rockwool cube. In order to evaluate if stem thickness differed between healthy and infected plants, plants were divided into healthy and HRD-infected cohorts, based on observation of symptoms by the end of the experiment.

2.3. Quantification of Rhizogenic Agrobacterium and Paenibacillus in Root Samples

Quantification of rhizogenic *Agrobacterium* and *Paenibacillus* was done using a SYBR green-based qPCR (Thermo Fisher Scientific, Merelbeke, Belgium), targeting the open reading frame *orf13* gene and a specific motif on the *16S rRNA* gene, respectively, as described before [15,16]. Root samples of 12 plants for each treatment and the untreated control were taken at the beginning (March 2019 and 2020), and near the end of the trial (August 2019 and October 2020). More specifically, for each plant, two rockwool samples (in oppositely directed pairs; 10 cm deep) were taken using a sterile core borer (diameter 1.0 cm) at a distance of 2.5 cm from the plant stem, and were subsequently pooled to obtain one sample per plant. Next, the root fragments were sorted out, cut in small fragments (*c.* 0.5 cm) and used for DNA extraction. Genomic DNA was extracted from 250 mg root material using the DNeasy PowerSoil DNA extraction kit (QIAGEN, Venlo, NL), according to manufacturer's instructions, with one modification. In the third and fourth step of the protocol, the use of a vortex adapter was replaced by two cycles of 30 s in the Precellys 24 homogenizer (Bertin instruments, Montigny-le-Bretonneux, France) at 4000 rpm. qPCR amplifications were conducted in an ABI StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using MicroAmpTM optical 96-well reaction plates (Thermo Fisher Scientific, Merelbeke, Belgium). The following primer pairs were used: orf13-F1 (5′-TGGATTATTTTGTGGC-3′) and orf13-R2 (5′-CCTTGCCAATTGCCAGTA-3′) for quantification of rhizogenic *Agrobacterium* and 16S-F1 (5′-ACCCGTGAAAACGGTGATGAGC-3′) and 16S-R1 (5′-GTTGCTACGTGAATACCCAGT-3′) for quantification of *Paenibacillus*. Reaction conditions and cycling program were adopted from [16], with annealing temperatures of 53 °C and 60 °C, for the quantification of rhizogenic *Agrobacterium* and *Paenibacillus*, respectively. Quantification of samples yielding a Ct value greater than 30 or with a T_m different from 80 ± 1 °C (for rhizogenic *Agrobacterium*) and 83 ± 1 °C for *Paenibacillus*) were

considered to be negative. A negative control with nuclease-free water instead of template DNA and a positive control with 1 ng gDNA of rhizogenic *Agrobacterium* ST07.18/001 were included in every assay. Conversion of Ct values to log CFU/g root was conducted using previously established standard curves [17].

2.4. Statistical Analysis

Calculated infestation rates were analyzed by generating a generalized linear model (GLM) with a binomial distribution in R [18], converted into proportions and subjected to a Chi-square test in a pair-wise manner, all against all. Stem thickness and qPCR results were checked for normality by applying the Shapiro-Wilks test, and in both cases, the null hypothesis was rejected. Further analyses were conducted using ggstatsplot R packages and specifying the use of non-parametric statistics with the command *type* = *"np"* for the application of Kruskal-Wallis test for multiple or Mann-Whitney for two-treatment testing [19]. Pairwise comparison was conducted by applying Dunn test with Bonferroni correction. Values that were considered as outliers based on Tukey's method were taken out of the dataset. The ggplots2 package was used for creating the figures [20].

3. Results

3.1. Parameters Influencing the Biocontrol Activity of Paenibacillus xylanexedens

Different BCO treatment strategies were tested in two consecutive trials, and the infestation rate was determined at the beginning and towards the end of each trial. In both greenhouse trials, no HRD symptoms were observed 2.5 months (March) after the start of both trials. Application of the BCO mixture of the two *P. xylanexedens* strains ST15.15/027 and AD117 resulted in a considerable decrease of the HRD infestation rate, as compared to the untreated control by the end of the trials (Figure 1). In 2019, the infestation rate decreased significantly from 42% in the untreated plants to 6% for plants treated with the BCO mixture (χ^2 = 14.63, *p*-value < 0.001), while in 2020, a decrease in HRD infestation from 27% for untreated plants to 4% for plants treated with the BCO-mixture was observed (χ^2 = 4.57, *p*-value = 0.03; Table S1). When examining the effect of a single strain application (ST15.15/027), the results revealed a similar reduction of 4–6% in the HRD infestation rate, as compared to the mixed BCO inoculum (4%; χ^2 = 0.00, *p*-value = 1), indicating that the same level of HRD control could be achieved by applying only one BCO strain. Furthermore, application of strain ST15.15/027 at an initial density of 10^5 CFU/mL was as effective as an inoculum density of 10^7 CFU/mL (χ^2 = 0.00, *p*-value = 1).

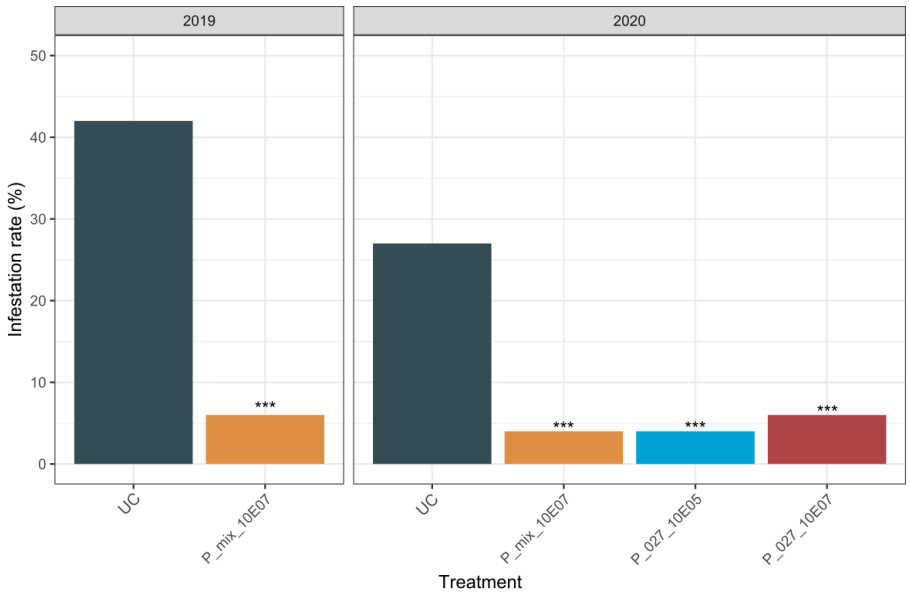

Figure 1. Comparison of HRD infestation rate between untreated plants and plants treated with *Paenibacillus xylanexedens* inocula at the end of the greenhouse trial in two consecutive growing seasons (2019 and 2020). The infestation rate was calculated as the ratio of tomato plants showing HRD symptoms (N = 48). All plants were artificially inoculated with rhizogenic *Agrobacterium* ST07.18/001. Either no BCO was applied (untreated control; UC) or plants were treated with different BCO inocula—a mixed inoculum of 10^7 CFU/mL *Paenibacillus xylanexedens* ST15.15/027 and AD117 (P_mix_10E07), or a single strain inoculum (ST15.15/027) in a density of 10^5 CFU/mL (P_027_10E05) or 10^7 CFU/mL (P_027_10E07). Significance is presented against the untreated control of the respective year: *** represents a p-value < 0.001.

3.2. Dynamics of Rhizogenic Agrobacterium and Paenibacillus Populations in Infested and Non-Infested Rockwool Substrate

Quantification of *Agrobacterium* and *Paenibacillus* was conducted approximately 10 weeks after planting and towards the end of the 2019 (August) and 2020 (October) trials, using qPCR (Figures 2 and 3). At the beginning of the 2019 trial, the rhizogenic *Agrobacterium* density in root samples of the untreated control and the BCO-treatment, did not differ significantly ($\log_e(W_{\text{Mann-Whitney}}) = 4.20$, p-value = 0.66) and showed a median value of 5.47 log CFU/g root material. Towards the end of the trial, a significant increase in *Agrobacterium* density was observed in the untreated control (M = 7.54 log CFU/g root; $\log_e(W_{\text{Mann-Whitney}}) = 3.33$, p-value = 0.038). In contrast, no significant increase in rhizogenic *Agrobacterium* density was observed during the trial for plants treated with the BCO mixture (M = 5.71 log CFU/g root; $\log_e(W_{\text{Mann-Whitney}}) = 4.09$, p-value = 0.734). Looking at the end of the trial, the rhizogenic *Agrobacterium* level in the BCO-treatment was significantly lower compared to the untreated control ($\log_e(W_{\text{Mann-Whitney}}) = 4.62$, p-value = 0.02).

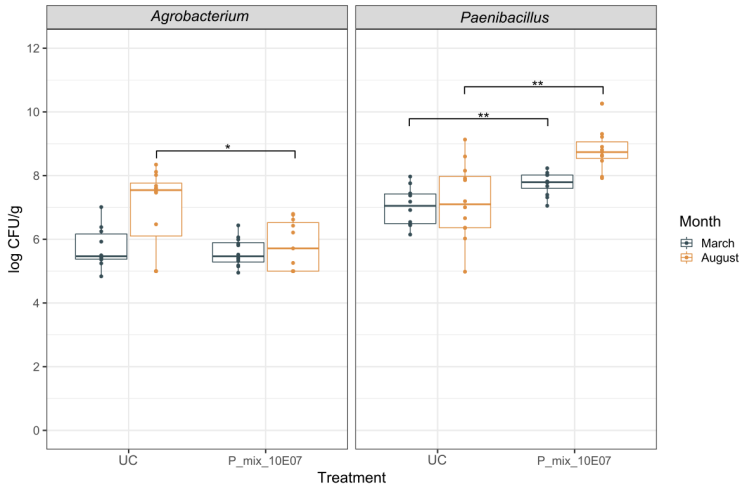

Figure 2. Population densities of rhizogenic *Agrobacterium* and *Paenibacillus* in tomato root samples at the beginning and towards the end of the 2019 greenhouse trial. The boxplots present the population density of rhizogenic *Agrobacterium* and *Paenibacillus* at the beginning (March) and towards the end of the trial (August) in root samples of untreated plants and plants treated with a BCO inoculum. All plants were artificially inoculated with rhizogenic *Agrobacterium* ST15.18/001. Either no BCO was applied (untreated control; UC) or plants were treated with a mixed inoculum of 10^7 CFU/mL *Paenibacillus xylanexedens* ST15.15/027 and AD117 (P_mix_10E07). Bacterial levels were quantified using a qPCR targeting either rhizogenic *Agrobacterium* or *Paenibacillus*, and are expressed in the log CFU/g root material. Significance levels of pairwise comparisons between untreated control and BCO-treatment are shown for both time points: * represents p-values < 0.05; ** represents p-values < 0.01.

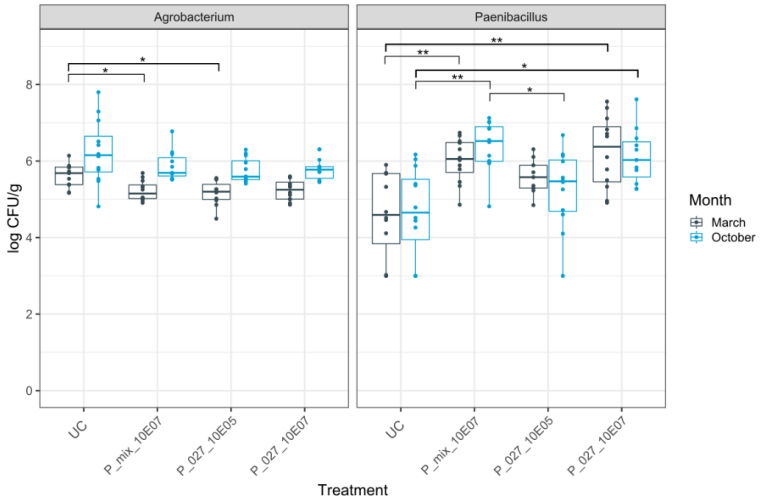

Figure 3. Population densities of rhizogenic *Agrobacterium* and *Paenibacillus* in tomato root samples at the beginning and towards the end of the 2020 greenhouse trial. The boxplots present the population density of rhizogenic *Agrobacterium* and *Paenibacillus* at the beginning (March) and towards the end of the trial (October) in root samples of untreated plants and plants treated with a BCO inoculum. All plants were artificially inoculated with rhizogenic *Agrobacterium* ST07.18/001. Either no BCO was applied (untreated control; UC) or the plants were treated with a mixed inoculum of 10^7 CFU/mL *Paenibacillus xylanexedens* ST15.15/027 and AD117 (P_mix_10E07), or a single strain inoculum (ST15.15/027) with a density of 10^5 CFU/mL (P_027_10E05) or 10^7 CFU/mL (P_027_10E07). Bacterial levels were quantified using a qPCR targeting either rhizogenic *Agrobacterium* or *Paenibacillus*, and are expressed in log CFU/g root material. Significance levels of pairwise comparisons between the untreated control and each BCO-treatment are shown for both time points: * represents p-values < 0.05; ** represents p-values < 0.01.

The *Paenibacillus* density at the beginning of the trial was significantly higher in root samples of plants treated with BCOs (M = 7.79 log CFU/g root), compared to the untreated control (M = 7.05 log CFU/g root) ($\log_e(W_{Mann\text{-}Whitney})$ = 2.89, *p*-value = 0.006). This difference was more pronounced near the end of the trial, with median *Paenibacillus* densities of 8.74 and 7.10 log CFU/g root for the BCO treatment and the untreated control, respectively. Moreover, the *Paenibacillus* density in the BCO-treatment significantly increased towards the end of the trial (M = 8.74 log CFU/g root; $\log_e(W_{Mann\text{-}Whitney})$ = 2.08, *p*-value = 0.0004), while no significant increase was observed in the untreated control ($\log_e(W_{Mann\text{-}Whitney})$ = 4.01, *p*-value = 0.76). Nevertheless, we did detect *Paenibacillus* in the untreated control (Figure 2).

In 2020, the increase in rhizogenic *Agrobacterium* density in the untreated control from the beginning (M = 5.68 log CFU/g root) towards the end of the trial (M = 6.15 log CFU/g root) was confirmed, although it was less pronounced than in 2019 ($\log_e(W_{Mann\text{-}Whitney})$ = 3.53, *p*-value = 0.053). However, a slight increase in rhizogenic *Agrobacterium* density of ~0.5 log CFU/g root was also observed in the BCO treatments, which was not observed in the 2019 greenhouse trial (Table S2). Higher rhizogenic *Agrobacterium* densities were observed in the untreated control compared to the treatments at the beginning of the season (χ^2 = 10.99, *p*-value = 0.01). However, towards the end of the trial, the differences in rhizogenic *Agrobacterium* densities between untreated control and treatments, seemed to have levelled off (χ^2 = 3.90, *p*-value = 0.27). With regards to *Paenibacillus* densities measured in plant root samples, most results obtained in 2019 were confirmed in the 2020 greenhouse trial. In March, the *Paenibacillus* density was significantly higher in plant root samples of BCO-treatments P_027_10E07 (M = 6.37 log CFU/g root) and P_mix_10E07 (M = 6.05 log CFU/g root) than in the untreated control (M = 4.59 log CFU/g root) (*p*-value = 0.003, and 0.008, respectively).

Likewise, towards the end of the trial, a significantly higher *Paenibacillus* density was observed in treatments P_027_10E7 (M = 6.03 log CFU/g root) and P_mix_10E07 (M = 6.52 log CFU/g root) compared to the untreated control (M = 4.65 log CFU/g root) (*p*-values = 0.03 and 0.001, respectively). However, the median *Paenibacillus* density in the low density BCO-treatment (P_027_10E5) was not significantly higher than in the untreated control at the beginning (M = 5.58 log CFU/g root; *p*-value = 0.56) and near the end of the trial (M = 5.47 log CFU/g root; *p*-value = 1.00).

Although the inoculum density did not result in significant differences in the HRD infestation rate, it did affect the *Paenibacillus* level at the end of the trial. The *Paenibacillus* density towards the end of the trial in treatment P_027_10E05 (M = 5.47 log CFU/g root) was lower than the BCO-treatment P_mix_10E07 (M = 6.52 log CFU/g root) (*p*-value = 0.03). Additionally, the density of *Paenibacillus* ST15.15/027 remained stable from March until October, with no significant differences in median values in neither the untreated control nor the treatments (Table S2) (Figure 3).

3.3. Evaluation of Stem Thickness as an Alternative Indicator for HRD

When stem thickness of healthy and infected plants near the end of the trial was compared, significantly higher median values were observed in infected plants both in 2019 and 2020 (Figure 4, Table S3). Near the end of the 2019 trial, stem thickness of infected plants displayed a median value 23.65 mm, which was significantly larger than that of healthy plants displaying a median value of 20.60 mm ($\log_e(W_{Mann\text{-}Whitney})$ = 6.17, *p*-value = 0.00019). Near the end of the 2020 trial, stem thickness of infected plants displayed a median value of 23.00 mm, which was significantly larger than that of healthy plants displaying a median of 21.75 mm ($\log_e(W_{Mann\text{-}Whitney})$ = 7.04, *p*-value = 0.014). However, in 2019, healthy and infected plants displayed a similar stem thickness (M = 17.50 mm and 17.80 mm, respectively) ($\log_e(W_{Mann\text{-}Whitney})$ = 6.83, *p*-value = 0.893), while a significant difference in stem thickness was observed between healthy and infected plants at the beginning of the 2020 greenhouse trial, although the difference was only marginal (M =

16.10 mm and M = 17.15 mm, respectively; $\log_e(W_{\text{Mann-Whitney}}) = 7.04$, p-value = 0.015) (Figure 4).

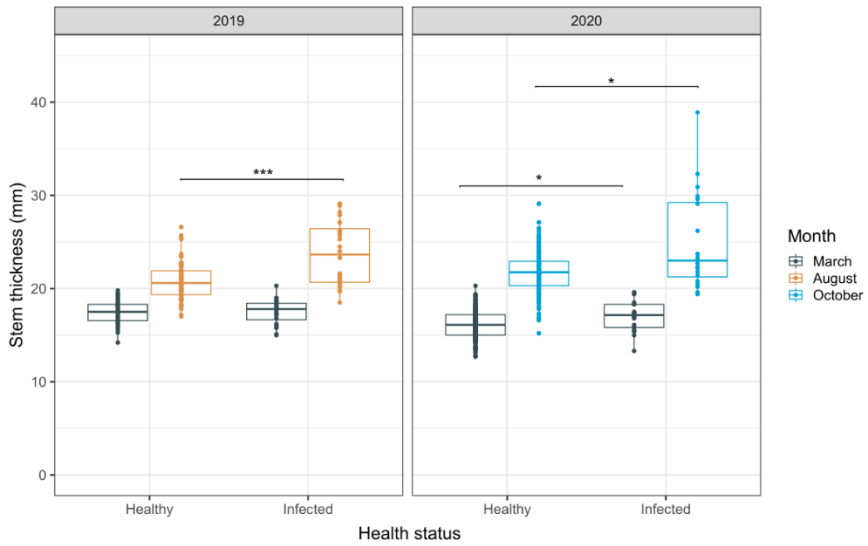

Figure 4. Comparison of stem thickness of healthy and infected plants at the beginning and towards the end of both greenhouse trials. Plants were classified as healthy if no HRD symptoms were observed at the end of the trial (N = 67 and N = 172 in greenhouse trial of 2019 and 2020, respectively), whereas plants showing HRD symptoms by the end of the experiment were classified as infected (N = 28 and N = 20 in greenhouse trial of 2019 and 2020, respectively). Stem thickness (expressed in mm) was monitored at the beginning (March) and towards the end of the trial (August and October for the 2019 and 2020 trial, respectively). Significance differences between healthy and diseased plants are indicated. p-values < 0.05 are represented by *, and those <0.001 are represented by ***.

4. Discussion

The genus *Paenibacillus* contains a considerable number of strains that present antagonistic activity against plant pathogens or act as plant growth-promoting bacteria [11]. Different *Paenibacillus* strains were examined as BCOs in soil-grown tomato greenhouses, including *Paenibacillus lentimorbus* 629 against root and crown rot caused by the pathogen complex consisting of *Fusarium oxysporum* f. sp. *lycopersici*, *Pyrenochaeta lycopersici*, and *Rhizoctonia solani* [21], *Paenibacillus elgii* HOA73 against gray mold caused by *Botrytis cinerea* [22], and *Paenibacillus ehimensis* RS820 against the root-knot nematode *Meloidogyne incognita* [23]. To date, *Paenibacillus* is not yet thoroughly tested for its biocontrol effectiveness in hydroponics tomato greenhouses, which present a totally different environment. In a previous study, *P. xylanexedens* strains showed biocontrol potential in a preliminary greenhouse trial [14]. However, this greenhouse trial was limited in duration, and only one inoculum (i.e., a mixed inoculum consisting of two *P. xylanexedens* strains (ST15.15/027 and AD117) was tested, meaning that the effects could not be attributed to a specific strain.

Hence, further research was needed to confirm the biocontrol potential of the *Paenibacillus* strains in greenhouse trials spanning an entire season and to investigate whether inoculum density and the use of a single BCO affects the biocontrol effectiveness. In this study, we unequivocally demonstrated the effectiveness of *P. xylanexedens* strains to reduce HRD in tomato hydroponics, in two consecutive greenhouse trials, spanning entire growing seasons. Additionally, in the greenhouse trial of [14], a genomospecies G9 strain was used, which is the most common genomospecies associated with HRD. Nevertheless, genomospecies G1, G3, G7, G8, and G9 were also associated with HRD in tomato crops [4]. This study demonstrated the effectiveness of *P. xylanexedens* strains to control a rhizogenic *Agrobacterium* genomospecies G3 strain, and indicates that the use of *P. xylanexedens* is not limited to genomospecies G9 strains.

It was demonstrated in this study that a single *Paenibacillus* strain performed equally well in reducing HRD, compared to a mixed inoculum. Compared to the use of a mixed inoculum, the use of a single strain had a number of advantages—(i) a single strain inoculum is easier to produce and apply; (ii) it is less complex to monitor a single BCO strain throughout the growing season; and (iii) there is no risk of incompatibility between strains once they are introduced in the rockwool environment [24]. On the other hand, some studies reported that improved biocontrol can be achieved by making use of mixed inocula [25–27]. When assessing the effect of the *Paenibacillus* density on biocontrol effectiveness, our results did not show a significant difference in the reduction of the HRD infestation rate when *Paenibacillus* was applied at 10^5 or 10^7 CFU/mL (single or mixed inoculum). These results suggest that a BCO density of 10^5 CFU/mL should be sufficient to obtain the desired HRD reduction. However, when looking at BCO densities in plant root samples, a slightly higher density was detected when higher inoculum densities (10^7 CFU/mL) were applied. This was observed at the beginning as well as towards the end of the greenhouse trial. Considering that the ratio between *Agrobacterium* and BCO was also an important factor influencing the biocontrol effectiveness [28], higher *Agrobacterium* densities could reduce the efficacy of the BCO when applied at a lower density.

Our results also clearly demonstrated that the *Paenibacillus* strains studied could persist in the rockwool environment. Approximately 7–9 months after application, *Paenibacillus* was still detected in plant root samples, and no decrease was observed in between the two time-points analyzed. This clearly points to *Paenibacillus*' ability to establish itself in a rockwool environment, and suggests that there is no need for repeated BCO-application throughout the season, which is a major advantage for its application in practice. This was in agreement with several studies that showed that members of the *Paenibacillus* genus belonged to the core microbiome of several important agricultural crops, reinforcing its close association with plants and its ability to thrive in the plant rhizosphere [29]. However, it should be noted that *Paenibacillus* was also detected in the untreated control in both the 2019 and 2020 greenhouse trial. This phenomenon could be partially attributed to the lack of specificity of the qPCR assay used in this study. Although the specificity of qPCR was confirmed for a number of related species and genera (unpublished results), the qPCR used in this study did not allow specific detection of *Paenibacillus* strains showing antagonistic activity. Therefore, it could not be excluded that *Paenibacillus* strains that are indigenously present in the root bacterial community of tomato grown on rockwool substrate, would also be detected. This is in agreement with a recent metagenetics study that showed that *Paenibacillus* is naturally abundantly present in rockwool samples of several hydroponics greenhouses [30].

There was also a large difference in *Paenibacillus* density in the untreated control treatments when the two subsequent trials were compared. For instance, in the untreated control of the 2019 greenhouse trial, a *Paenibacillus* density of 7.05 and 7.10 log CFU/g was detected at the beginning and towards the end of the trial, respectively, while in 2020 densities of 4.59 and 4.65 log CFU/g were detected in the untreated control. This could imply that a higher density of indigenous *Paenibacillus* was present in the 2019 trial compared to the 2020 greenhouse trial. This could be partially attributed to a thorough disinfection process that took place in between the two trials. Despite this difference in "basal level", the increase in *Paenibacillus* density in the treatments compared to the untreated control at the beginning of the trial (1.24 +/− 0.47 log CFU/g) and towards the end of the trial (1.49 +/− 0.45 log CFU/g) were in the same range for both greenhouse trials.

Finally, our results clearly showed that plants infected with HRD displayed a significantly larger stem thickness towards the end of the experiments in 2019 and 2020. This indicates that stem thickness could be considered a reliable marker of HRD-incidence besides the classic visual assessment of root proliferation in rockwool slabs. In the 2019 greenhouse trial, no significant difference was observed in stem thickness in healthy or HRD-affected plants at the beginning of the trial. At this time-point, however, no visual symptoms were yet observed. However, in 2020, already early in the trial, a significant

difference in stem thickness of healthy and infected plants was detected, despite the fact that no excessive root formation was observed. This suggests that the stem thickness might show the HRD-phenotype sooner than excessive root formation. Further research is needed to confirm this scenario.

In conclusion, in two consecutive growing seasons, it was clearly demonstrated that the *Paenibacillus xylanexedens* BCOs tested could effectively suppress HRD incidence to low levels. However, to further exploit the BCOs studied here in hydroponics greenhouses, more research is required to optimize the application strategy, and to elucidate the mode of action of the antagonistic activity of the BCO strains.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050817/s1. Table S1: Generalized Linear Model (GLM) results and pairwise comparisons of infestation rates for 2019 and 2020 trials. Table S2: Statistical analysis of densities of rhizogenic *Agrobacterium* and *Paenibacillus* in tomato roots based on qPCR for 2019 and 2020 trials. Table S3: Statistical analysis of stem thickness for healthy and HRD-infected plants for 2019 and 2020 trials.

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