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Biocontrol of *Stemphylium vesicarium* and *Pleospora allii* on Pear by *Bacillus subtilis* and *Trichoderma* spp.: Preventative and Curative Effects on Inoculum Production

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Abstract: Trials under controlled and field conditions were conducted to establish the effect of strategies of application of biological control agents (BCAs) in the reduction of *Stemphylium vesicarium* and *Pleospora allii* inoculum production on pear leaf debris. Six BCAs based on different strains of *Trichoderma* spp. (Tr1, Tr2) and *Bacillus subtilis* (Bs1, Bs2, Bs3 and Bs4) were evaluated. Two strategies were tested in controlled experiments: application before (preventative strategy) or after (curative strategy) pear leaf debris colonization by *S. vesicarium*, evaluating the growth inhibition and sporulation of *S. vesicarium* and the pseudothecia production of *P. allii*. When the BCAs were applied preventatively, the efficacy of treatments based on *B. subtilis* was higher than those based on *Trichoderma* spp. in controlling the pathogen colonization, but that of controlling the inoculum production of *S. vesicarium* and *P. allii* was similar. However, when the BCAs were applied curatively, *Trichoderma* based products were more effective. In field trials, *Trichoderma* spp. Tr1 and *B. subtlilis* Bs1 produced a consistent 45–50% decrease in the number of *S. vesicarium* conidia trapped compared to the non-treated control. We conclude that *Bacillus subtilis* Bs1 and *Trichoderma* spp. Tr1 and Tr2 can be expected to reduce fungal inoculum during the pear vegetative period by at least 45–50%. Additionally, *Trichoderma* spp. Tr1 and Tr2 have the potential to reduce the fungal overwintering inoculum by 80% to 90%.

Keywords: brown spot of pear; *Stemphylium vesicarium*; *Bacillus subtilis*; *Trichoderma* spp. biological control; colonization; inoculum reduction

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1. Introduction

Brown spot of pear (*Pyrus communis* L.), caused by the fungus *Stemphylium vesicarium* (Wall.) E. Simmons, is of major economic relevance in certain pear-producing areas in Europe [1] due to the high yield loss.

The pathogen has both sexual and asexual reproduction phases in its biological cycle, producing two kinds of inoculum; the sexual inoculum corresponds to ascospores produced by the Ascomycete *Pleospora allii* (Rabenh.) Ces. & De Not [2], and the asexual inoculum corresponds to conidia produced by the Deuteromycete *S. vesicarium* [3]. Ascospores are generated into pseudothecia that develop on pear leaf litter and other plant debris. The pseudothecia develop mainly from late summer to early autumn, with the highest levels of ascospore release between February and June, but ascospores have also been observed from July to October [1]. Although ascospores are pathogenic on pear [4] they play an important role in the colonization of plant debris on the orchard ground. Asexual inoculum, corresponding to the conidia formed in erect conidiophores, is mainly produced on plant debris on the orchard ground rather than on the trees. Conidia are produced from April to November with a maximal release from July to September [5,6]. The *S. vesicarium* life cycle is characterized by two different stages [1]; a pathogenic phase on the aerial pear

tree organs during the pear-growing period and a saprophytic phase corresponding to permanent colonization of the plant debris over the year.

Management of the disease is based on protectant fungicides being applied several times during the pear growing period, either at a fixed schedule or according to the BSPcast forecasting system [7,8]. However, the strategy based solely on the application of fungicides is not enough to control the disease, and additional management measures are necessary. Actions aimed at reducing inoculum production, such as leaf litter removal and the application of biological control agents based on commercial formulates of *Trichoderma* spp. have been tested [4,9,10]. *Trichoderma*-based products have been reported to be partially effective in reducing the number of *P. allii* ascospores [4] and *S. vesicarium* conidia [10]. These sanitation methods and the application of *Trichoderma* spp. based products reduced the incidence of pear brown spot by more than 60% [9]. However, more effective biological control agents and application strategies are needed to achieve the objective of decreasing the inoculum production throughout the year, so increasing the efficacy of disease control. In our previous studies, different *Bacillus subtilis* strains were evaluated in vitro and ex vivo for reducing *S. vesicarium* inoculum production [11]. Three of them were selected based on their in vitro and ex vivo activity and on their ability to reduce the inoculum production from 40% to 70%. The potential of these biological control agents for controlling brown spot of pear must be further investigated and screened under field conditions prior to its practical use.

The objective of this work was to evaluate different strategies of application of Biological Control Agents, based on different strains of *Trichoderma* spp. and *Bacillus subtilis*, in the reduction of *S. vesicarium* and *P. allii* inoculum production under controlled and field conditions.

2. Materials and Methods

The efficacy of selected biological control agents and the application strategy (preventative or curative) in reducing the growth and inoculum production of the pathogen was evaluated under controlled environmental conditions and in field trials.

2.1. Biological Control Agents

Six biological control agents (BCAs) based on *Bacillus subtilis* and *Trichoderma* spp. were tested. The *Trichoderma* based biofungicides evaluated were Tusal (Certis Europe), composed of a mixture of *T. atroviride* and *T. asperellum* strains (1×10^8 cfu/g), and Trianum-P (Koppert Biological Systems), composed of *T. harzianum* strain T-22 (1×10^9 spores/g), both products are water-dispersible granules, coded here as Tr1 and Tr2 respectively. The amount of commercial product applied in the experiments was 2 g/L for Tr1 and 0.3 g/L forTr2.

The *Bacillus subtilis* based products were Serenade Max (Bayer Crop Science) (Bs1 in this work), a wettable powder composed of *B. subtilis* strain QST 713 (5.13×10^{10} cfu/g), and Serenade ASO (Bayer Crop Science) (Bs2 in this work), a suspension concentrate composed of *B. subtilis* strain QST 713 (1×10^{12} cfu/mL). The dosage of commercial product applied was 4 g/L for Bs1and 9.3 mL/L for Bs2. Two experimental strains of *B. subtilis* B1 and B3 (Culture Collection of the Institute of Food and Agricultural Technology, University of Girona, Girona, Spain) were included. Strains B1 and B3 (Bs3 and Bs4 respectively in this work) were selected for their potential in vitro antagonism to *S. vesicarium*, control of brown spot of pear under ex vivo conditions, and efficacy to decrease the inoculum production, according to previous research [11]. Suspensions of 10^8 cfu/mL *B. subtilis* strains were obtained from 24 h culture grown in 20 mL LB broth at 24 °C on an orbital shaker at 70 rpm. *B. subtilis* strains Bs3 and Bs4 were tested separately with a dose of application of 10^8 cfu/mL.

2.2. Pathogen Strain and Plant Material

Stemphylium vesicarium strain EPS26 isolated from infected *P. communis* fruit, obtained from the Culture Collection of the Institute of Food and Agricultural Technology (University of Girona, Girona, Spain) was used. Conidial suspensions of 10^6 conidia/mL were prepared from cultures grown on tomato agar plates [12].

Pear leaves cv Passe Crassane were collected after leaf fall in autumn in a commercial organic pear orchard. Collected leaves were maintained in the greenhouse until use. In experiments under controlled conditions, disks of 15 mm diameter were cut from pear leaves and sterilized in an autoclave for 20 min at 120 °C and 1 atm. In trials under natural conditions, the leaves were used directly.

2.3. Evaluation of Preventative and Curative Application Strategies of Biological Control Agents in Controlling Fungal Growth and Inoculum Production under Controlled Environment Conditions

2.3.1. Preventative Strategy

The objective of these experiments was to determine the capacity of BCAs to control the growth and inoculum production of *S. vesicarium* when applied before *S. vesicarium* colonization of dead pear leaves. Seven treatments were evaluated, the six BCAs (Tr1, Tr2, Bs1, Bs2, Bs3, and Bs4) plus a non-treated control. The effect of the BCAs was evaluated under darkness and under 16 h-light photoperiod in two separate experiments. In both cases, the same protocol and experimental design were used. An *S. vesicarium* EPS26 mycelial plug was inoculated in the center of tomato agar Petri plates (9 cm diameter) and incubated at 22.5 °C with a 16-h-light photoperiod until the fungal mycelium colonized approximately half of the plate surface, about six days after inoculation. Autoclaved pear leaf disks were immersed in the corresponding BCA suspension, at previously defined doses, and transferred to the *S. vesicarium* colonized tomato agar plates. Six leaf disks treated with the same BCA were placed on each plate, equidistant from the center, avoiding contact with the fungal colony. For the non-treated control, pear leaf disks were immersed in sterile distilled water. The *S. vesicarium*-BCA inoculated plates were incubated at 22.5 °C for 16 days in darkness or under 16 h-light photoperiod, depending on the experiment. A randomized block design was used in each experiment, with three replicates of one plate per treatment. Each experiment (under darkness or photoperiod) was conducted twice. A total of 21 Petri dishes and 126 leaf disks were used per experiment repetition. At 3, 6, and 9 days after BCAs application, *S. vesicarium* growth inhibition was determined by measuring the distance from the periphery of the fungal colony to the margin of the BCA-inoculated leaf disk, and 16 days after the application, *S. vesicarium* sporulation was quantified. Three of the six-leaf disks in each Petri plate were taken out, placed individually in a tube with 2 mL of sterile distilled water, and crushed at 5000 rpm for 30 s with a homogenizer (PT MR300, Kinematica AG, Littau, Switzerland. To avoid germination of the conidia, 20 µL of blue lactophenol (5%) were added to each tube, aliquots of 10 µL were collected and the total conidia of *S. vesicarium* were counted in each sample under an optical microscope at 100× (BX50, Olympus Optical CO., LTD, Tokyo, Japan).

Sixteen days after BCA application, the *S. vesicarium*-BCAs inoculated plates with the three remaining leaf discs per plate were incubated over 29 days at 14 °C under continuous darkness, the optimal conditions to induce the development of *Pleospora allii* pseudothecia. At the end of the incubation period, the total pseudothecia of *P. allii* formed per leaf disk were determined. Three leaf disks were evaluated per treatment replicate (corresponding to the three remaining leaf disks in each plate). Leaf disks were transferred to a tube with KOH (10%) and heated for 45 min to clarify the plant tissue. Then the leaf disks were washed with sterile distilled water and analyzed individually. The number of pseudothecia per leaf disk was counted at 100× under an optical microscope (SMZ1500, Nikon Corporation, Tokyo, Japan) and processed through image analysis software (Optika Tm Vision Pro v2.7, Ponteranica, Italy).

2.3.2. Curative Strategy

The capacity of BCAs to inhibit the growth and inoculum production of *S. vesicarium* when applied after fungal colonization of dead pear leaves was determined. Six BCA treatments were evaluated (Tr1, Tr2, Bs1, Bs2, Bs3, Bs4) and two controls. One was with sterile distilled water, and additionally, a non-treated control with LB broth was included because a significant increase in the growth and sporulation of *S. vesicarium* had been observed in previous studies, which was attributed to the LB content of Bs3 and Bs4 suspensions (unpublished data). The values obtained in these Bs3 and Bs4 treatments were relativized from the relationship between the untreated control and the control with LB. The efficacy of control of the BCAs applied one and three days after the inoculation of *S. vesicarium* was evaluated. Six autoclaved pear leaf disks were distributed on the surface of moistened sterile filter paper placed inside a sterile petri plate (9 cm). Then a 20 µL-drop of 10^5 conidia/mL suspension of *S. vesicarium* EPS26 was deposited on each leaf disk, and the plates were incubated at 22.5 °C with a 16-h light photoperiod. The BCAs were applied to the leaf disks 24 h (1 DPI) or 72 h (3 DPI) after *S. vesicarium* inoculation as a 50 µL-drop of the corresponding BCA suspension at the previously described dose. Non-treated control disks were inoculated with either sterile distilled water or LB broth. A randomized block design was used in each experiment, with three replicates of one plate per treatment. Each replicate consisted of a plate with six-leaf disks. Plates with the inoculated leaf disks were incubated at 22.5 °C and 16-h light photoperiod. Seven days after pathogen inoculation, *S. vesicarium* aerial mycelium was assessed on three out of six-leaf disks per plate according to the following scale: 0: no aerial mycelium; 1: aerial mycelium covering 1 to 50% leaf disk surface; 2: aerial mycelium covering 51 to 90% leaf disk surface; 3: aerial mycelium covering more than 90% leaf disk surface. Individual observations of the surface of each leaf disk were at 100× under an optical microscope (BX50, Olympus Optical CO., LTD, Tokyo, Japan). Additionally, *S. vesicarium* sporulation was determined on the same leaf disks as described previously. The plates with the remaining three-leaf disks were incubated at 14 °C under continuous darkness to stimulate the formation of pseudothecia of *P. allii*. Forty-five days after *S. vesicarium* inoculation, the number of pseudothecia per leaf disk was quantified as previously described. The experiment was performed in duplicate.

2.3.3. Data Analysis

Data were analyzed using R statistical software [13]. The effects of BCAs on *S. vesicarium* growth, conidia, and pseudothecia production were determined by analysis of variance. The effect of light was also determined in preventative experiments. In all cases, the means were compared with Fisher's protected least significant difference test at $p = 0.05$. Since the effect of the experiment was significant, the data for each experiment were analyzed separately. The datasets were tested for equality of variances (Levene) and normality of residuals (Shapiro–Wilk). The values of different parameters for treatments with BCA relative to the values in non-treated controls were used to evaluate the efficacy of BCA in each experiment or trial. The control efficacy (*C*) of BCAs was calculated as the percent control in Equation (1). *C* is commonly reported [14] which:

$$C = \left(1 - \frac{\overline{X}_{treated}}{\overline{X}_{non\ treated}}\right) \times 100 \quad (1)$$

where $\overline{X}_{treated}$ is the mean value in different BCAs treatments and $\overline{X}_{non\ treated}$ the mean value in non-treated control. The control efficacy (*C*) were obtained for each experiment and repetition.

2.3.4. Image Analysis Using Scanning Electron Microscopy

Additional samples of pear leaf disks from curative experiments, previously described, were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.1–7.4) for 4 h at 4 °C, and dehydrated in a graded ethanol series. Samples were critical point dried

with CO_2 in a pressure bomb (K850, Emitech, UK). The specimens were mounted on metal stubs with colloidal silver (Electrodag 1415, Acheson Colloids Company, Port Huron, MI, USA) followed by a gold sputter coat depending on thickness (ca. 30 nm) in a sputter system (K550, Emitech, England). The samples were observed with a scanning electronic microscope (SEM) with 3.5 nm resolution and 15 kV of acceleration voltage (DSM 960A, Zeiss, Oberkochen, Germany). Image capture and measurements were using a digital image acquisition system by Quartz PCI program (Version 5.1, Quartz Imaging Co., Vancouver, BC, Canada).

2.4. Evaluation of Application of Biological Control Agents to Control Inoculum Production under Field Conditions

Trials were performed in a 0.5 ha experimental pear orchard located in Girona (Catalonia, Northeastern Spain, 41°57′37.16″ N, 2°49′51.84″ E). The trials were carried out between 23 March 2018 and 18 September 2018. Environmental parameters were monitored with a CR10X datalogger (Campbell Scientific Ltd., Loughborough, UK) connected to temperature and relative humidity (RH) (model HC2AS3), leaf wetness (model 237L), and rainfall (model ARG100) sensors. Temperature and RH were measured every 10 min, and leaf wetness and rainfall every 20 s. Mean temperature, mean relative humidity, total wetness duration, and total rainfall were determined hourly.

Pear leaves cv Passe Crassane collected after leaf fall in autumn in a commercial organic orchard, as described above, were used. The pear leaves were inoculated in the laboratory with a 1.8×10^5 conidia/mL suspension of *S. vesicarium* EPS26 by pulverization until run off with a hand pressure sprayer, then incubated in the dark for 48 h at 22.5 °C in a plant growth chamber (PGR15; Conviron, Winnipeg, MB, Canada) to ensure fungal colonization and inoculum production. The inoculated leaves were used in two different trials carried out in the experimental orchard, based on the spore traps used: (i) mechanical spore traps and (ii) rain catch-type spore traps.

The first application of all the BCAs tested was on 23 March, treatments with Bs1, Bs2, Tr1, and Tr2 were repeated on 5 April, 11 May, and 6 June. BCAs were applied using a hand-compression sprayer. Volumes of 350 mL (trial A) or 75mL (trial B) per treatment were used.

2.4.1. Mechanical Spore Trap Trial

For trial A, bottomless square devices (30 cm × 30 cm) with wooden lateral walls were placed on the orchard ground, filled with 350 g of *S. vesicarium* inoculated leaf debris, and covered with a fiberglass net to allow direct contact between leaves and soil [4]. The treatments consisted of the six BCAs (Tr1, Tr2, Bs1, Bs2, Bs3, Bs4) and a non-treated control. The treatments were arranged along three rows in a completely randomized block design, with three replicates of one device per treatment. The spore traps had 76 mm × 26 mm glass slides with the underside painted with a silicon solution (Lanzoni S.R.L., Bologna, Italy). Two slides were positioned 1 to 2 cm above the leaf debris on each device (replicate). The glass slides were removed and replaced with new ones every 15–20 days, depending on the climatic conditions (especially rainfall). The samples on the slides were fixed using jelly solution (Lanzoni S.R.L., Bologna, Italy). *Stemphylium vesicarium* conidia were counted in a longitudinal traverse using an optical microscope (BX50, Olympus Optical CO., LTD, Tokyo, Japan) and the number of spores per square centimeter was calculated.

2.4.2. Rain Catch-Type Spore Trap Trial

In trial B, rain catch-type spore traps were constructed from a modification of the original description [15], using a 15 cm diameter plastic funnel connected to a 750 mL plastic bottle filled with 2 mL of 20% $CuSO_4$ solution to prevent the germination of conidia and as a general preservative. The spore traps were placed in a pipeline of 15 cm diameter and positioned approximately 30 cm above the ground. *Stemphylium vesicarium* inoculated leaf debris (4–7 g) in a fiberglass net bag were deposited in the plastic funnel. The same treatments described in the previous trial were evaluated. Treatments were arranged in

three rows in a completely randomized block design with three replicates of one spore trap per replicate. Plastic bottles with spore samples were removed and replaced with newly prepared ones every 20 days or earlier, depending on the rainfall. The total amount of rainwater was recorded for each spore trap and a subsample (50–90 mL) was processed for inoculum quantification. The subsample solution was filtered through a gridded sterile cellulose nitrate filter (47 mm diameter and 0.45 µ pore size; MF-Millipore, Ireland). Filters were examined under an optical microscope (B × 50, Olympus, Center Valley, PA, USA), the number of conidia in a four traverse was counted, and the number of conidia per square centimeter and conidia concentration (conidia/mL) was calculated. Finally, the total number of conidia in the total amount of rainwater recorded was obtained.

2.4.3. Data Analysis

Data were analyzed separately for each trial using R statistical software [13]. The effect of treatments in the cumulated *S. vesicarium* conidia captured, conidia/cm^2 in trial A or total conidia in trial B, during the whole trial period was determined by analysis of variance. The means were compared with the Fisher's protected least significant difference test at $p = 0.05$. The datasets were tested for equality of variances (Levene) and normality of residuals (Shapiro–Wilk). The control efficacy of the BCA was calculated as the percent control (*C*) previously described.

2.4.4. Quantitative Synthesis of Bcas Effect Across Experiments and Field Trials

Control efficacy (C) obtained in the experiments performed under controlled conditions and field trials was analyzed globally with the purpose to summarize the efficacy of different BCAs. The average control efficacy (C) of the BCAs calculated as the percent control, previously described, was used. Principal component analysis (PCA) was performed to evaluate the relationship between the efficacy of control using preventative or curative strategies of BCA application under controlled conditions and control efficacy obtained in field trials. Analysis was performed using the *princomp* procedure of R statistical software [13]. Eleven variables were included in the analysis; three of which related to the preventative strategies of BCA application ($C_{leaf\ disk\ colonized\ by\ S.\ vesicarium}$, $C_{conidia\ production}$, and $C_{pseudothecia\ production}$), six variables related to the curative strategy ($C_{mycelium\ index\ 1\ dpi}$, $C_{mycelium\ index\ 3\ dpi}$, $C_{conidia\ production\ 1\ dpi}$, $C_{conidia\ production\ 3\ dpi}$, $C_{pseudothecia\ production\ 1\ dpi}$, $C_{pseudothecia\ production\ 3\ dpi}$), and two variables related to field trials ($C_{conidia\ trapped\ mechanical\ spore\ trap}$, $C_{conidia\ rain\ catch\text{-}type\ spore\ trap}$). Average data from each experiment were used in PCA analysis. Additionally, to summarize and compare the control efficacy of BCAs, the average of the efficacy (*C*) obtained when the BCAs were applied preventative, curatively, and in field trials was represented for each BCA.

3. Results

3.1. Preventative Strategy

Four experiments were performed under controlled conditions. The biological control agents (BCAs) were applied in a preventative strategy, before colonization of dead pear leaves by the pathogen *S. vesicarium*.

3.1.1. Growth of *S. vesicarium*

While all the leaf disks in the non-treated control (NT) were colonized by *S. vesicarium*, its growth was controlled by the application of BCAs based on *Bacillus subtilis*. In petri dish assay, the growth of *S. vesicarium* decreased significantly ($p < 0.001$) on pear leaf disks treated with Bs1, Bs2, Bs3, or Bs4 where the development of the *S. vesicarium* colony was inhibited (Table S1). The inhibition zones of *S. vesicarium* colony growth around the BCAs treated pear leaf disks ranged from 12.5 mm (experiment 4) to 23.0 mm (experiment 1), so the hyphae of *S. vesicarium* could not reach the plant tissue. In the four experiments, the BCA Bs4 was the most effective, whereas the BCAs Bs1, Bs2, and Bs3 showed some differences in efficacy depending on the trial. However, some of the BCAs treated pear leaf

disks were colonized by *S. vesicarium*. In all four experiments, the overall mean percentage of disks colonized by *S. vesicarium* nine days after inoculation was 39% (Bs1 and Bs2), 21% (Bs3), and 19% (Bs4). In contrast, the *Trichoderma* spp. based products (Tr1 and Tr2) did not inhibit the growth of *S. vesicarium*. In the four experiments, *S. vesicarium* colonized 95% of leaf disks treated with Tr1 and 64% of those treated with Tr2, after nine days of incubation. For all BCAs evaluated, the efficacy in reducing the *S. vesicarium* growth and pear leaf colonization was consistent at three, six, and nine days after BCAs application.

3.1.2. Conidia of *S. vesicarium*

The effect of light on *S. vesicarium* conidia production was significant ($p < 0.0001$). No sporulation was observed on leaf disks incubated in darkness (experiments 1 and 2), while abundant conidia were observed on pear leaf disks incubated under 16 h light/8 h dark photoperiod (experiments 3 and 4), with a mean of 1139 conidia/cm^2 ($\pm$154).

With a photoperiod incubation, a significant effect of BCAs treatment was observed on conidia of *S. vesicarium* production in comparison to the non-treated control (Table 1). In both experiments, some BCA treatments significantly reduced the number of conidia produced ($p = 0.0034$ and $p = 0.0057$ for experiments 3 and 4 respectively) compared to the non-treated control. In experiment 3, fungal sporulation on leaf disks treated with Tr2, Bs1, Bs4, and Bs3 was significantly lower ($p = 0.0034$) than in the non-treated control with a reduction in sporulation of 43% (Tr2), 46% (Bs1), 83% (Bs4), and 97% (Bs3). In experiment 4, sporulation was significantly lower in all BCA treated leaf disks compared to non-treated control disks ($p = 0.0057$). The reduction of conidia production in comparison to the non-treated control was 60–64% (Bs1, Bs3, and Bs4), 95% (Bs2), and 83–92% (Tr1 and Tr2).

Table 1. Effect of preventative applications of biological control agents (BCAs) on conidia production of *Stemphylium vesicarium*. BCAs used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2). NT: non-treated control. Means with the same letter in each experiment are not significantly different according to Fisher's protected least significant difference test ($p < 0.01$).

Experiment	Treatment	Conidia/cm^2	SE [z]
3	NT	2238.4 [a]	±33.3
	Bs1	1198.8 [b,c]	±225.4
	Bs2	1446.1 [a,b]	±731.8
	Bs3	75.4 [d]	±66.2
	Bs4	385.6 [c,d]	±185.7
	Tr1	1485.9 [a,b]	±234.7
	Tr2	1274.3 [b]	±65.4
4	NT	3225.5 [a]	±245.5
	Bs1	1288.9 [b]	±539.7
	Bs2	150.9 [b]	±66.2
	Bs3	1144.3 [b]	±610.1
	Bs4	1226.1 [b]	±758.6
	Tr1	547.0 [b]	±225.0
	Tr2	257.8 [b]	±92.6

[z] Mean Standard Error.

3.1.3. Pseudothecia of *P. allii*

The effect of light was not significant ($p = 0.7103$) in the production of *P. allii* pseudothecia. In non-treated controls, the production ranged from 141 to 166 pseudothecia/cm^2 in the four experiments. The pseudothecia of *P. allii* produced on leaf disks treated with the different BCAs was significantly lower ($p < 0.0001$) than that observed in the non-treated control (Figure 1). The production in BCAs treated leaf disks was 13–60 (Bs1), 9–55 (Bs2), 4–45 (Bs3), 2–17 (Bs4), 10–31 (Tr1), and 12–62 (Tr2) pseudothecia/cm^2. On leaf disks treated with Bs4, the significantly lowest number of pseudothecia (Figure 1) was observed, con-

sistently through the four experiments. The efficacy in reducing pseudothecia production observed for all BCAs in comparison to non-treated control was, as an average of the four experiments, 76% (Bs1), 77% (Bs2), 80% (Bs3 and Tr2), 85% (Tr1), and 92% (Bs4).

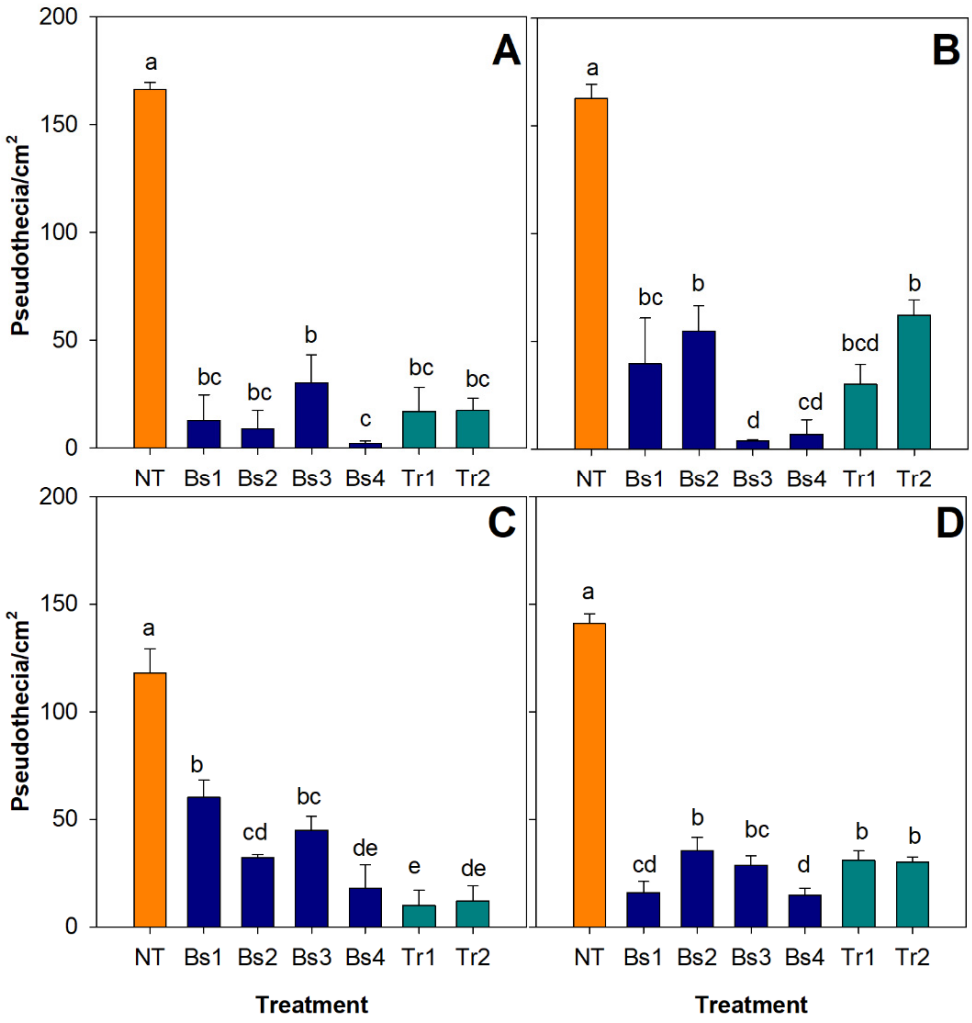

Figure 1. Effect of preventative applications of biological control agents (BCAs) in the pseudothecia of *Pleospora allii* production. BCAs used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) or *Trichoderma* spp. strains (Tr1, Tr2). NT: non-treated control. Results are given as the mean and standard error of four experiments ((**A**): experiment 1; (**B**): experiment 2; (**C**): experiment 3, and (**D**): experiment 4). Bars with the same letter are not significantly different according to Fisher's protected least significant test ($p < 0.01$).

3.2. Curative Strategy

3.2.1. Colonization of *S. vesicarium*

For the majority of leaf disks in the non-treated control, 90% of their surface was covered by the mycelium of *S. vesicarium*. A significant reduction ($p < 0.0001$) of mycelium colonization was observed at 1 DPI and 3 DPI on leaf disks treated with BCAs compared to the non-treated control in two experiments (Table 2). Consistently, the lowest colonization indices corresponded to leaf disks treated with *Trichoderma* spp. based products (Tr1 and Tr2), but the differences with the other treatments were not significant.

Table 2. Effect of curative applications of biological control agents (BCAs) on *Stemphylium vesicarium* mycelial growth. BCAs used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2) applied 1 or 3 days post-inoculation. NT: non-treated control. Means with the same letter in each experiment are not significantly different according to Fisher's protected least significant difference test ($p < 0.01$).

		1 DPI [x]		3 DPI	
Experiment	**Treatment**	**MI [y]**	**SE [z]**	**MI**	**SE**
1	NT	2.55 [a]	±0.29	2.67 [a]	±0.33
	Bs1	2.33 [a]	±0.19	2.00 [b]	±0.33
	Bs2	1.89 [a,b]	±0.48	1.44 [b,c]	±0.44
	Bs3	1.23 [b,c]	±0.19	1.28 [c]	±0.17
	Bs4	1.23 [b,c]	±0.19	1.22 [c]	±0.52
	Tr1	1.22 [b,c]	±0.29	1.11 [c]	±0.11
	Tr2	0.78 [c]	±0.40	1.22 [c]	±0.22
2	NT	2.77 [a]	±0.11	3.00 [a]	±0.00
	Bs1	1.11 [c,d]	±0.40	2.00 [b,c]	±0.19
	Bs2	1.55 [b,c]	±0.11	1.44 [d,e]	±0.11
	Bs3	1.96 [b]	±0.21	2.44 [b]	±0.11
	Bs4	2.06 [a,b]	±0.27	1.78 [c,d]	±0.11
	Tr1	0.89 [c,d]	±0.29	1.33 [d,e]	±0.19
	Tr2	0.44 [d]	±0.11	1.11 [e]	±0.29

[x] Days Post Inoculation. [y] Mycelium Index (from 0 to 3). [z] Mean Standard Error.

3.2.2. Sporulation of *S. vesicarium*

Although mycelia of *S. vesicarium* was observed on leaf disks in the two experiments, conidia formation was observed only in the second trial. For this reason, only results corresponding to the second experiment are presented. In the non-treated control, the mean for conidia/cm^2 was between 2377 and 2459 at 1 and 3 DPI respectively. The number of *S. vesicarium* conidia/cm^2 was significantly lower ($p < 0.0001$) on leaf disks treated with BCAs applied 1 DPI than in the non-treated control (Table 3), with Bs4 and Tr1 being the BCAs with the highest efficacy.

Table 3. Effect of curative applications of biological control agents (BCAs) on *Stemphylium vesicarium* conidia production. BCAs used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2), applied 1 or 3 days post-inoculation. NT: non-treated control. Means with the same letter in each experiment are not significantly different according to Fisher's protected least significant difference test ($p < 0.01$).

	1 DPI [y]		3 DPI	
Treatment	**Conidia/cm^2**	**SE [z]**	**Conidia/cm^2**	**SE**
NT	2376.9 [a]	±136.5	2458.7 [a]	±384.2
Bs1	1565.7 [b]	±93.1	710.6 [c]	±56.0
Bs2	729.4 [d]	±76.5	1358.2 [b]	±123.7
Bs3	1196.4 [c]	±52.7	289.9 [c,d]	±140.8
Bs4	402.5 [e]	±88.7	641.8 [c,d]	±32.1
Tr1	163.4 [f]	±72.5	132.0 [d]	±28.8
Tr2	1465.1 [b]	±16.6	370.9 [c,d]	±59.9

[y] Days post-inoculation. [z] Mean standard error.

The mean reduction of conidia production in comparison with the non-treated control was 93.1% for Tr1, 83.1% for Bs4, 69.3 for Bs2, and from 34.1 to 49.7% for Bs1, Bs3, and Tr2. Similar results were observed when the BCAs were applied 3 days after fungal inoculation (3 DPI). All BCAs significantly reduced *S. vesicarium* sporulation ($p < 0.0001$), the lowest levels of fungal sporulation being with treatments Bs3, Bs4, Tr1, and Tr2. This reduction

compared to the non-treated control was 91.6 and 92.5% for Bs4 and Tr1, respectively, 85.4% for Bs1, 84.4% for Tr2, and 63.4% and 67.8% for Bs3 and Bs2, respectively. Consistently, whether applied at 1 DPI or 3 DPI, conidia production was lowest on the leaf disks treated with *B. subtilis* Bs4 or *Trichoderma* spp. Tr1. No treatment resulted in a total absence of conidia production.

3.2.3. Pseudothecia of *P. allii*

Pseudothecia of *P. allii* were observed in pear leaf disks in all experiments. Applications of BCAs 1 day after the inoculation of *S. vesicarium* significantly reduced ($p < 0.0001$) the number of pseudothecia/cm^2 compared to the non-treated control, except for Bs4 in experiment 1 (Figure 2). In the first experiment, the leaf disks treated with BCAs based on *B. subtilis* were less effective in controlling pseudothecia production compared to the *Trichoderma* spp. applications. The number of pseudothecia/cm^2 on leaves treated with different strains of *B. subtilis* (Bs1, Bs2, Bs3, and Bs4) ranged from 42 to 61 (experiment 1) and from 43 to 68 (experiment 2). On leaves treated with *Trichoderma* spp. strains (Tr1 or Tr2), the number of pseudothecia/cm^2 was between 7 and 12 (experiment 1) and from 14 to 29 (experiment 2). The reduction of pseudothecia production compared to the non-treated control was from 38 to 51% (experiment 1) or from 54 to 62% (experiment 2) for Bs1, Bs2 and Bs3. Using Tr1 or Tr2 the reduction ranged from 86 to 92% (experiment 1) and from 74 to 87% (experiment 2).

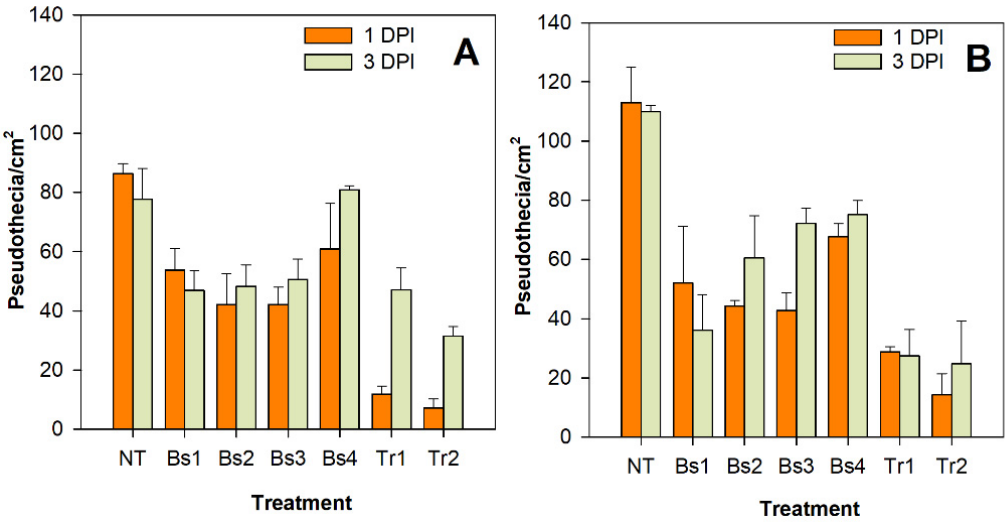

Figure 2. Effect of biological control agents (BCAs) applied 1 or 3 days after *Stemphylium vesicarium* inoculation (DPI) in the pseudothecia of *Pleospora allii* production. BCAs used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) or *Trichoderma* spp. strains (Tr1, Tr2). NT: non-treated control. Results are given as the means and standard error ((**A**): experiment 1, (**B**): experiment 2). Bars with the same letter are not significantly different according to Fisher's protected least significant test ($p < 0.01$). Lower case letters correspond to 1 DPI and upper case letters to 3 DPI treatments.

Pseudothecia production was also significantly reduced ($p < 0.0001$) compared to the non-treated control, except for Bs4 in experiment 1, when BCAs were applied 3 days after the inoculation of *S. vesicarium* (Figure 2). In addition, production in non-treated control leaf disks (78–110 pseudothecia/cm^2) was similar to that for the 1 DPI non-treated control. The pseudothecia production on leaves treated with different BCAs at 3 DPI was similar or slightly higher than that observed on the 1 DPI treated leaves, mainly in treatments with *Trichoderma* spp. (Tr1 or Tr2) in experiment 1. On leaves treated with different strains of *B. subtilis* (Bs1, Bs2, Bs3), the number of pseudothecia/cm^2 ranged from 43 to 51 in experiment 1, and 36 to 72 in experiment 2. On leaves treated with *Trichoderma* spp. (Tr1 or Tr2), the number was from 31 to 47 pseudothecia/cm^2 in experiment 1) and from 24 to

27 pseudothecia/cm^2 in experiment 2. The reduction of pseudothecia production with Bs1, Bs2, and Bs3 treatments compared to non-treated control was from 35 to 40% in experiment 1 and from 34% to 67% in experiment 2. Using Tr1 or Tr2, the reduction was between 39 and 60% in experiment 1 and from 75% to 77% in experiment 2.

3.2.4. Image Analysis of the Interaction between *S. vesicarium* and BCAs

Images obtained by scanning electron microscopy are presented in Figure 3. Normal growth of mycelia of *S. vesicarium* and conidia formation was observed in the non-treated control (Figure 3A,B). *B. subtilis* based BCA applications resulted in the colonization of leaf disks by high populations of the bacteria (Figure 3C–F). Bacterial cells of Bs1 had a high affinity for the fungal hyphae (C). The *B. subtilis* Bs2 treatment produced abnormal alterations on hyphae and conidia of *S. vesicarium* (D), and a reduction of *S. vesicarium* mycelium development (magnified in D). *B. subtilis* strains Bs3 (E) and Bs4 (F) produced alterations in conidia morphology (indicated with arrows). Regarding the *Trichoderma* spp. based BCAs, there was high colonization by spores and mycelia of the Tr1 strain (Figure 3G). The mycelium of *Trichoderma* spp. Tr1, which was thinner than the *S. vesicarium* mycelium, grew around the *S. vesicarium* hyphae. Additionally, broken and empty (H) and altered (I) conidia of *S. vesicarium* were observed. On leaf disks treated with *Trichoderma* spp. Tr2, the BCA conidia were not abundant, but the density of *Trichoderma* spp. mycelium was high (Figure 3J–L). Normal growth and development of *S. vesicarium* hyphae and conidia were altered by the *Trichoderma* spp. Tr2 hyphae interaction (J,K), which grew over and around them (L).

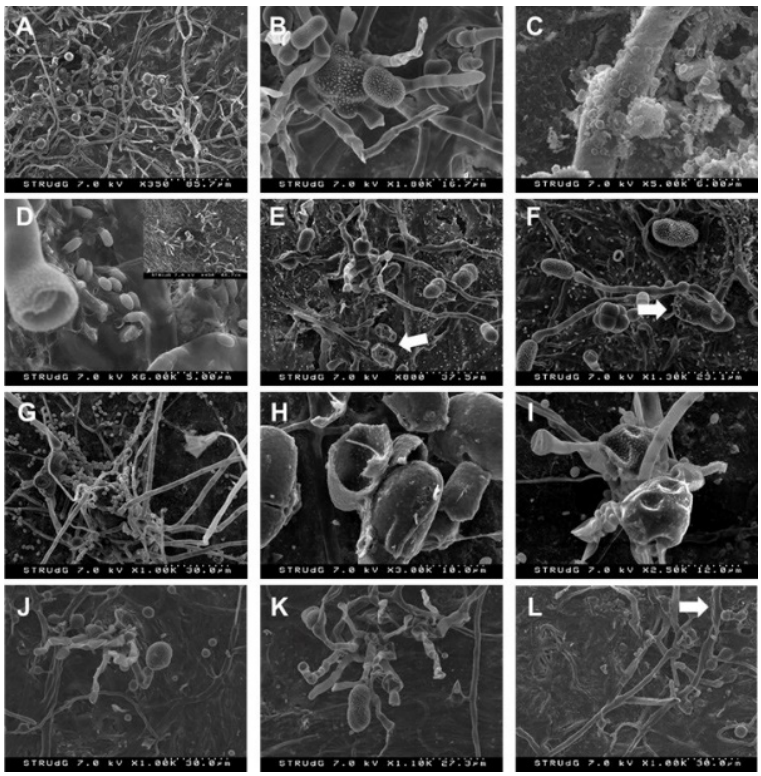

Figure 3. Colonies of *Stemphylium vesicarium* in non-treated control (**A**,**B**). Colonies of *S. vesicarium* and different *Bacillus subtilis* strains of Bs1 (**C**), Bs2 (**D**), Bs3 (**E**), and Bs4 (**F**). Colonies of *S. vesicarium* and *Trichoderma* spp. Tr1 (**G**,**H**,**I**) and Tr2 (**J**,**K**,**L**). Different biological control agents were applied 3 days after the inoculation of the *S. vesicarium.* Images were obtained using a scanning electron microscope.

3.3. Evaluation of Biological Control Agents to Control Inoculum Production under Field Conditions

Frequent and abundant rainfall and long wet periods with temperatures favorable for growth and sporulation of *S. vesicarium* characterized the climatic conditions during the trials (Figure 4C).

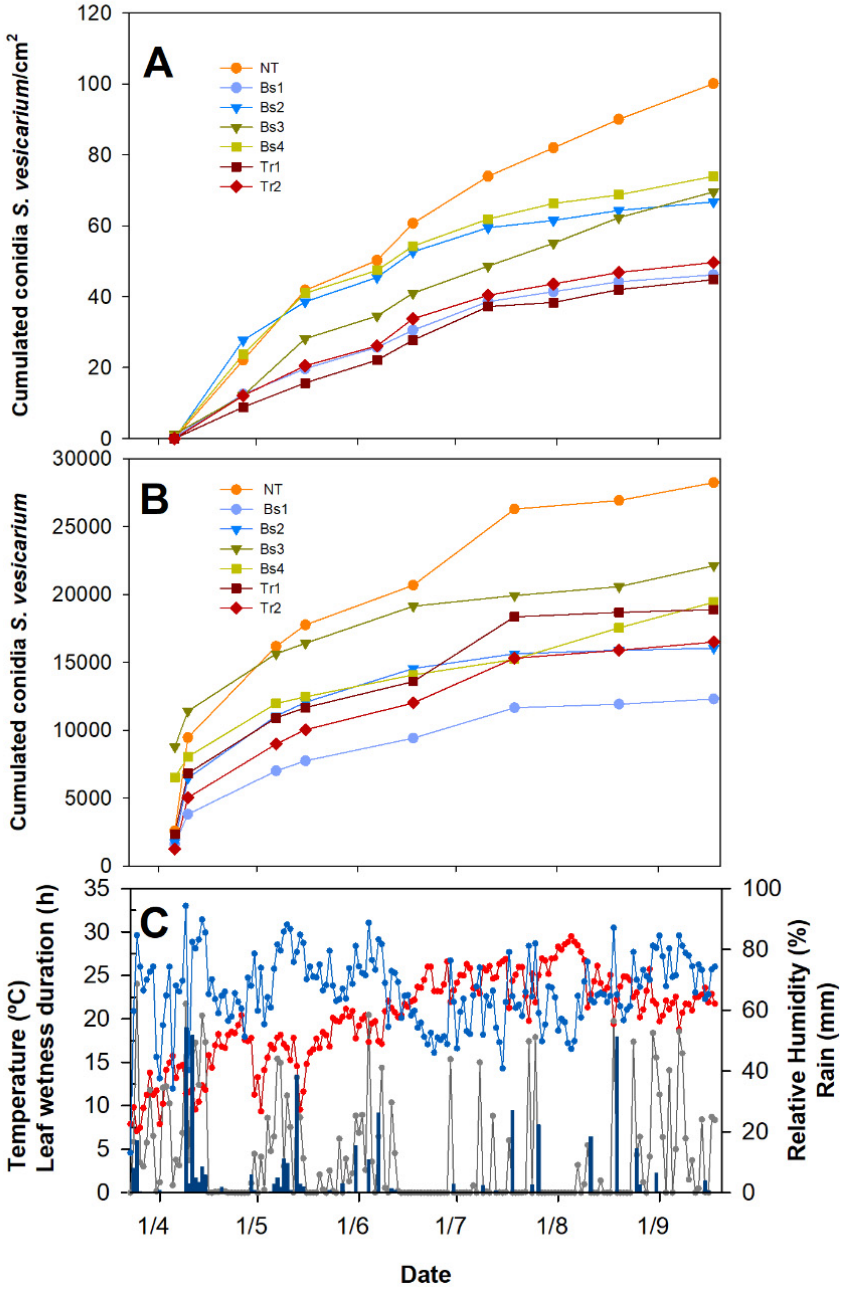

Figure 4. Dynamics of *Stemphylium vesicarium* observed in trials 1 (**A**) and 2 (**B**) under orchard conditions; (**C**): dynamics of Relative Humidity (•), Mean daily temperature (•), daily wetness duration (▬) and rain (bars). Conidia were trapped with mechanical spore-traps (**A**) or rain-catch traps (**B**). The results presented are the accumulated captured conidia. In trial 1 (**A**) conidia of *S. vesicarium*/cm^2 is shown whereas in trial 2 (**B**) the total of conidia caught of each period is shown. Biological control agents used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2). NT: non-treated control.

The dynamics over time of *S. vesicarium* conidia trapped using mechanical spore traps showed that, throughout the trials, conidia production was higher in the non-treated control than in the BCAs treatments (Figure 4A). At the beginning of the trial, two groups could be differentiated regarding conidia production. The highest amount of conidia were trapped in the non-treated control, whereas less were trapped from leaves treated with BCAs. Two weeks later, three groups could be differentiated in relation to the number of conidia trapped. The non-treated control had the highest amount of trapped conidia, the second group included the treatments with Bs2, Bs3, and Bs4, and the third group, with treatments Bs1, Tr1, and Tr2 had the lowest amount of trapped conidia. When the total amount of conidia captured throughout the trial was analyzed, the number of *S. vesicarium* conidia trapped was significantly lower ($p < 0.0001$) on leaves treated with BCAs (Figure 5A). The amounts trapped with Bs2, Bs3, and Bs4 treatments were significantly different from Bs1, Tr1, and in some cases Tr2 applications. The lowest amount of conidia trapped was with the latter treatments (Bs1, Tr1, and Tr2), with the total amount of *S. vesicarium* trapped reduced by 50 to 54% compared to the non-treated control, with 45 to 50 conidia/cm^2. The reduction in conidia trapped was from 26 to 33% for the other treatments in comparison to the non-treated control.

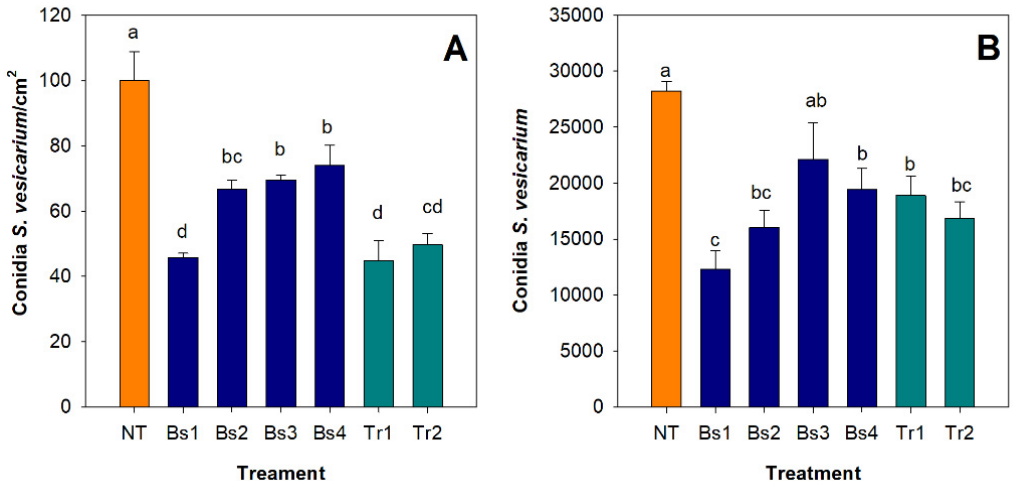

Figure 5. Total conidia of *Stemphylium vesicarium* trapped in trial 1 (**A**) and 2 (**B**), under orchard conditions. Conidia were trapped with mechanical spore-traps (**A**) or rain-catch traps (**B**). In trial 1 (**A**) total conidia *S. vesicarium*/cm^2 is shown whereas in trial 2 (**B**) the total of conidia caught throughout the trial period is shown. Biological control agents used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2). NT: non-treated control. Results shown are the accumulated conidia captured. Bars marked with the same letter are not significantly different according to Fisher's protected least significant test ($p < 0.01$).

The number of conidia trapped in the non-treated control using the rain-catch traps was higher than in the BCA treatments throughout the trial (Figure 4B). The dynamics of the conidia trapped from leaves treated with BCAs was similar to that observed using mechanical spore-traps, but without clear differences between treatments. Throughout the trial, the lowest values of conidia trapped were observed on leaves treated with Bs1. The total amount of *S. vesicarium* conidia trapped was significantly lower ($p < 0.0001$) from leaves treated with BCAs, compared to non-treated controls, except for Bs3 (Figure 5B). Applications of Bs2, Bs3, Bs4, Tr1, and Tr2 reduced the total amount of trapped *S. vesicarium* conidia from 33 to 43% compared to the non-treated control. The amount of conidia trapped from leaves treated with Bs1was reduced by 57% on average compared to the non-treated control.

3.4. Quantitative Synthesis of BCAs Effect Across Trials

The eigenvalues for the principal components analysis showed that the first two components explained 74.4% of the total variance. The first component had higher eigenvectors (0.13927 to 0.4008) related to variables associated with curative treatments and field trials. The second component had positive eigenvectors (0.1640 to 0.6300) for most of the variables related to experiments performed under controlled conditions (preventative and curative strategies) and large negative eigenvectors (−0.16892 to −0.4706) for variables related to field trials. Thus, the variation among BCAs could be explained by these variables (Figure 6A). In the scatter diagram (Figure 6A), the analysis distributes the BCAs into three groups. Group I, in the first quadrant, includes *Trichoderma* spp. Tr1 and Tr2 strains, featured by high efficacy of control when applied with a curative strategy and in controlling inoculum production in field trials. Group II, in between quadrant II and III, comprises *B. subtilis* Bs2, B3, and B24 strains, characterized by high efficacy of control in preventative applications under controlled conditions but low efficacy in field trials. Finally, group III, on the bottom of quadrant IV is composed of *B. subtilis* Bs1 strain, with low positive values of Component 1, which means low efficacy of control when applied in curative strategy but some efficacy in field trials. Similar results are shown in Figure 6B, where *Trichoderma* spp. BCAs, Tr1, and Tr2, showed higher efficacy of control (69.5% to 73.7%) when applied curatively than *B. subtilis* based BCAs, Bs1, Bs2, Bs3, or Bs4, (44.6% to 50.8%). Contrarily, when applied preventatively, globally *B. subtilis* strains showed higher efficacy (63.6% to 81.7%) than *Trichoderma* spp. strains (50.6% to 58.4%). The BCAs that presented high efficacy when applied curatively (Tr1 and Tr) also showed the highest efficacy in controlling the inoculum production in field trials (44.2 and 45.4). However, strain Bs1, with low efficacy when applied curatively showed high efficacy in controlling the inoculum in field trials (55.3%). The efficacy in field trials of the other *B. subtilis* strains (Bs2, Bs3, and Bs4) was lower (26.1% to 38.3%).

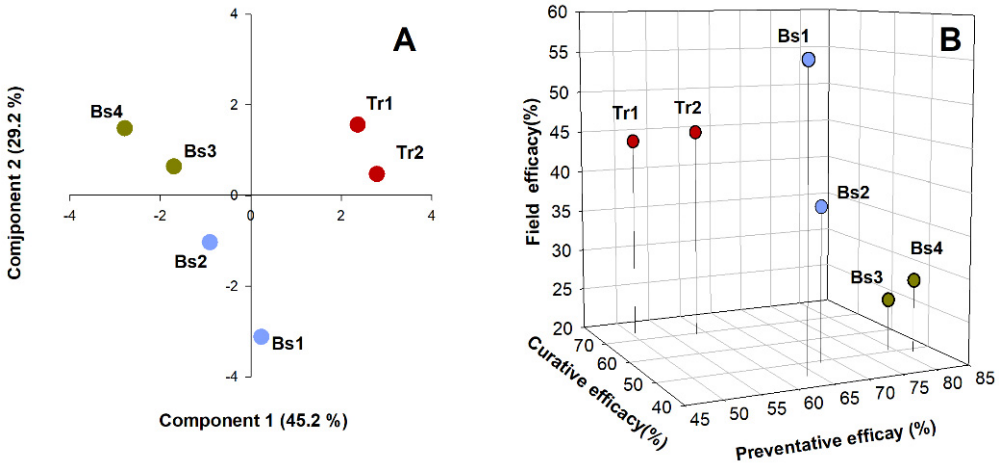

Figure 6. (**A**) Principal component analysis score plot (component 1 versus 2) of six biological control agents. Eleven variables were included in the analysis. Biological control agents used were *Bacillus subtilis* strains (Bs1, Bs2, Bs3, Bs4) and *Trichoderma* spp. strains (Tr1, Tr2). (**B**) Global efficacy obtained when the BCAs were applied preventatively, curatively, and in field trials were represented for each one. Average data of 17 independent experiments were used.

4. Discussion

Stemphylium vesicarium colonizes the plant debris on the soil of pear orchards and, when the climatic conditions are favorable, sporulation occurs and conidia of *S. vesicarium* are formed. These conidia, mainly released during spring and summer, can reach the aerial organs of pear trees and, if conditions are appropriate, produce infections. Under unfavorable conditions for *S. vesicarium* development, mainly during autumn, pseudothecia of *P. allii* develop. These pseudothecia mature during autumn and winter, producing

asci with ascospores which are released during rainy events, mainly from late winter to spring [1]. This means that the leaf debris in pear orchards is permanently colonized by *S. vesicarium* or *P. allii* [10] but with variations in the quantitative inoculum produced over the year [1,16]. Different studies have demonstrated that sanitation methods, including *Trichoderma* spp. applications on leaf litter, significantly reduce the inoculum amount and the disease levels of brown spot of pear [4,9,10]. Additionally, *Bacillus subtilis* has also been found to reduce *S. vesicarium* inoculum [11]. BCAs should be evaluated for their effect on inoculum production (conidia, pseudothecia, ascospores), and the strategy for their application in relation to the growth of *S. vesicarium* and colonization of leaf debris, should be defined before being included in an integrated disease management program. Timing of application may be critical for biological fungicides [17]. For these reasons, the efficacy of BCAs based on *B. subtilis* and *Trichoderma* spp. against *S. vesicarium* and *P. allii* growth and sporulation were evaluated in the present work. Two strategies of BCAs application were tested: preventative (before the plant debris was colonized) and curative (when the plant debris was already colonized). Experiments were performed under controlled and field conditions because the efficacy may vary in different situations. Pear leaf debris was used under controlled conditions. The use of pear leaf disks to test the efficacy of biocontrol agents rather than only artificial growth medium was to emulate the natural conditions (plant material) and had the advantage of avoiding the interference of artificial media on the mechanisms of the biocontrol. In fact, the composition of the culture medium may affect the products produced by the BCA.

The results show that preventative applications of BCAs based on *B. subtilis* were more effective in the inhibition of *S. vesicarium* growth than products based on *Trichoderma* spp. In the four experiments, only 19 to 39% of pear leaf disks were colonized by *S. vesicarium*, in which the pathogen colony growth stopped permanently. The differences in the efficacy of the two BCAs species tested may be due to the mechanism of action, probably related to the compounds produced by the strains of *B. subtilis* which diffused through the medium. It has been widely described that *B. subtilis* can produce lipopeptides and other metabolites with antifungal activity as surfactins, fengycins, bacisubins, and polyketides [18–21]. For example, the metabolite 3-hydroxypropionaldehyde, produced by *B. subtilis* strain CU12, has been shown to efficiently inhibit the growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum* [19,22]; and the strain SG6 could effectively inhibit the growth of *F. graminearum*, producing morphological changes in the hyphae [23]. In the work presented here, *B. subtilis* was grown on pear leaf debris to avoid the effect that the culture medium might have on the production of antimicrobial products but not on their diffusion; no bacterial colonies were observed outside the leaf disks. In contrast, the two BCAs based on *Trichoderma* spp. did not prevent the growth of *S. vesicarium* in 64% to 95% of disks. This indicates that no substances capable of affecting the growth of *S. vesicarium* were produced or diffused through the culture medium. However, when the BCAs were applied when the leaf debris were already colonized by the pathogen, 1 or 3 days post-inoculation, all reduced the growth of the *S. vesicarium*, the products based on *Trichoderma* spp. being more effective. Based on these results, the mechanism of action of BCAs based on *Trichoderma* spp. may be a direct interaction of the antagonists with the pathogen.

Light (photoperiod) was critical for the in vitro production of *S. vesicarium* conidia but not for the formation of pseudothecia of *P. allii*. No sporulation was produced in darkness, but pseudothecia formation was not affected by the absence of light. The effect of light on sporulation has been described in other species of *Stemphylium* [24], but this is the first time that the effect of light on *S. vesicarium* and *P. allii* sporulation on pear has been demonstrated.

The BCAs Bs1, Bs3, and Bs4 based on *B. subtilis* were capable of consistently decreasing the amount of *S. vesicarium* conidia produced when applied preventatively, with significant reductions, from 46% to 97%, compared to the non-treated control. The decrease in sporulation can be partially explained by the fact that *B. subtilis* strains inhibited the colonization

of leaf disks by *S. vesicarium*. However, even in the leaf disks colonized by *S. vesicarium*, a decrease in sporulation was observed. The capacity of *B. subtilis* to inhibit sporulation has been described in other fungi, such as *F. graminearum* in which *B. subtilis* SG6 was able to effectively inhibit growth and sporulation [23]. *Trichoderma* spp. based BCAs reduced the levels of sporulation when applied preventatively. It is remarkable that *Trichoderma* spp. Tr2 was capable of reducing the *S. vesicarium* conidia production to the levels of *B. subtilis* strains, between 43% and 92%. Consequently, despite *S. vesicarium* colonizing the majority of leaf disks treated with *Trichoderma* spp., the inhibition of sporulation was very high. The successful use of fungal antagonists against pathogens to reduce sporulation on necrotic tissue has been reported for several plant-pathogen pathosystems. *Pyrenophora tritici-repensis* on wheat straw using *Limonomyces resipellis; Botrytis cinerea* on dead leaves of strawberries using *Glicocladium sativus*, and *Cochliobolus sativus* on wheat using *Trichoderma* sp. [25] are some examples. Saprophytic yeasts are also reported to be capable of reducing sporulation of *Botrytis cinerea* on tomato and bean [26]. When the BCAs were applied following a curative strategy, one or three days after *S. vesicarium* inoculation, the reduction in conidia production was maintained for those based on both *B. subtilis* and *Trichoderma* spp. compounds.

Regarding the production of *P. allii* pseudothecia, all BCAs, both *B. subtilis* and *Trichoderma* spp. based biocontrol agents consistently reduced the number of pseudothecia produced when applied preventively in the four experiments, with reductions from 76% to 92% compared to the non-treated control. Application following a curative strategy at 1 or 3 DPI also resulted in a reduction of pseudothecia production. The reduction was lower for BCAs based on *B. subtilis* (29% to 67%) than for *Trichoderma* spp. BCAs (86% to 92%). These results agree with reports based on similar studies; *V. inaequalis* ascospore production on leaf disks incubated under controlled conditions was inhibited 83.7% to 90.4% by using *Microsphaeropsis* sp., *Athelia bombacina*, *Chaetomium globosum*, or *Trichoderma* sp. on apple leaf litter [17,27–30]. Carisse et al. [17] suggested that, under laboratory conditions, *M. ochracea* was more efficient at reducing the production of ascospores by *V. inaequalis* when applied before the formation of pseudothecia. Although the mechanism of action is not known exactly, the authors suggested that the BCA parasitizes *V. inaequalis* mycelium, preventing mating and, later, the formation of pseudothecia. Results obtained here using *Trichoderma* spp. showed that the efficacy of inhibition of pseudothecia formation was very high when applied both preventively and curatively in relation to pathogen colonization, but always before the formation of the pseudothecia. The effect of BCAs on pseudothecia formed and ascospore production was not determined in our study. If the number of pseudothecia decrease, it is clear that the number of ascospores released is expected to decrease, but it is unknown whether the formed pseudothecia would remain viable. The effect of BCAs on the viability of the pseudothecia once formed has not been determined. The *Trichoderma* spp. mechanisms of action were not a part of this study, but the results obtained and electron microscopy suggest that *Trichoderma* spp. based BCAs were able to interact directly with *S. vesicarium* mycelium, conidia, and pseudothecia in a hyperparasitism relationship, whether applied preventively or curatively. In general, the biocontrol mechanisms of *Trichoderma* spp. include competition and mycoparasitism, and stimulation of plant resistance [19,31]. In the saprophytic phase of *S. vesicarium*, stimulation of plant resistance is not possible. Mycoparasitism includes the secretion of cell wall-degrading enzymes such as chitinases, glucanases, and proteases to dissolve the cell wall of the fungal hosts and penetrate the cells [32]. The effect of *Trichoderma* on fungal germination has also been described, for example, *Trichoderma atroviride* produces endochitinases encoded by gene *ech42* that inhibit spore germination and hyphal elongation [33]. On the other hand, competition for nutrients is probably a less important mechanism in experiments performed under controlled conditions. Under these conditions, it can be concluded that, in general, when the BCAs were applied preventatively, those based on *B. subtilis* controlled pathogen colonization more effectively than those based on *Trichoderma* spp., but the efficacy was similar in controlling the inoculum production of *S. vesicarium* and *P. allii*.

However, when the BCAs were applied after *S. vesicarium* had colonized the leaf debris, *Trichoderma*-based products were more effective.

To determine the efficacy of controlling *S. vesicarium* inoculum production under natural conditions, two trials were performed. In each trial, different methods were used to determine the quantity of ascospores and conidia released: mechanical and rain-catch traps. The mechanical method has been widely used in epidemiological studies of airborne plant pathogenic fungi [34]. This method is aimed at trapping the ascopores or conidia released mainly due to rainfall events and transported aerially, as occurs under natural conditions. The environmental conditions and the soil contact to leaf debris, BCAs, and saprophytic fungi were similar to natural conditions. The rain-catch trap method has also been used in some airborne fungal studies [15], trapping a large number of conidia and ascospores, but it does not simulate the real conditions of inoculum release and aerial transportation. Using mechanical spore traps, *Trichoderma* spp. based BCAs Tr1 and Tr2 and *B. subtilis* Bs1 were effective in decreasing the number of conidia of *S. vesicarium* trapped by 50% compared to the non-treated control. This control of inoculum production was observed consistently during all trials. Similar results, mainly for Bs1 and Tr2, were obtained with the rain-catch traps. As expected, the efficacy of control by BCAs in field trials was in general lower than under controlled conditions. BCAs applications on leaves under natural conditions are exposed to external factors that may alter their activity and efficacy. This has been observed with fungal antagonists used to control *V. inaequalis* [27]. In our trials, the leaf debris was inoculated with *S. vesicarium* and incubated to ensure high levels of pathogen colonization, established within leaves and occupying the ecological niche, before the BCAs were applied. Apart from mycoparasitism and antibiosis as described previously, the mechanisms of biocontrol for the BCAs applied under natural conditions had not been studied before. Here, the competition for nutrients and space may play an important role in the mechanism of action for both *Trichoderma* spp. and *B. subtilis*. Competition for nutrients in biological control has been described in other pathosystems. One example is *V. inaequalis*, which increases the permeability of host plant cell membranes, resulting in an increased nutrient supply from the host, and it has been suggested that the BCA *Cladosporium cladosporioides* H39 may interfere in this process [29,35]. Competition for space or nutrients has long been considered one of the classical mechanisms of biocontrol by *Trichoderma* spp. but it is extremely difficult to demonstrate [31,36]. It may be assumed that multiple mechanisms are involved in biocontrol systems, but in most cases, only some of the possible mechanisms have been elucidated [32].

In vitro activity tests are not considered good tools to predict in vivo activity of biocontrol agents such as *Trichoderma* spp. [32,37]. However, the relationship observed in our work between the results from experiments performed under controlled conditions and in field trials indicates that the methodology used under controlled conditions is appropriate for BCAs evaluation and to determine if the biocontrol mechanisms would be effective under field conditions. This is partially explained by the type of plant material used in our experiments, pear leaf debris, similar to that in natural field conditions. However, it should be taken into account that environmental conditions in the field are not always favorable and that much more complex relationships with other populations of microorganisms occur.

Four applications of BCAs Bs1, Bs2, Tr1, and Tr2 were done at the beginning of the trials, from the end of March to June, but there were no applications between July and September. However, there was a reduction in inoculum levels throughout the trials, indicating that the BCAs maintained viable populations. Some differences in efficacy observed between trials for the same BCA, for example, Tr1, may be due to the microenvironmental conditions in the different spore-traps and their effect on the viability of BCA.

To design a sanitation strategy in pear orchards affected by brown spot, focused on reducing the inoculum of *S. vesicarium* or *P. allii* by using applications of BCA, two aspects should be considered: the efficacy of biocontrol depends on the application timing, and the number of applications is limited due to economic aspects. The application of BCAs to prevent pseudothecia of *P. allii* formation is critical because this application should be before

the pseudothecia are produced. Considering that *P. allii* pseudothecia are mainly produced during autumn, one application of BCAs should be at the beginning of leaf fall, the objective being to decrease the rate of pseudothecia produced. The other treatments may be done in spring, especially when the amount of released ascospores is high. The model PAMcast (*Pleospora allii* Maturation forecast) predicts the percentage of pseudothecia that are mature and with ascospores disposable to be released with rain events [38]. PAMcast may be used as a reference for timing the first BCAs application in spring. A third treatment might be necessary for June to ensure the viability of the BCAs. A good colonization capacity and viability over time are characteristics necessary in an inoculative strategy of BCAs application [39]. The objective should be that applications of BCAs in spring and June are effective almost throughout the growing season. The viability of the BCA populations in autumn is not so critical, since the autumn applications are aimed at avoiding pseudothecia formation in this period.

The combined use of *B. subtilis* and *Trichoderma* spp. strains, for example, Bs1 and Tr1 or Tr2 should also be considered, because a wider range of biocontrol mechanisms may operate in mixed BCA populations [40]. Combinations of *B. subtilis* and *Trichoderma* spp. have been tested against *Rhizoctonia solani* on cucumber seed and dry bean [41,42].

These and previous results [9] open up the possibility of using a brown spot disease control strategy in IPM programs and also in organic pear production, since sanitation methods may be an alternative to the application of chemical fungicides. Brown spots of pear can be partially managed using sulfur or copper in organic farming. However, the heavy usage of copper can lead to significant environmental problems (European Food Safety Authority EFSA, 2013), so there is an increasing need for new safe and environmentally-friendly alternatives, such as biopesticides [18]. Moreover, BCAs based on *Trichoderma* spp. and *B. subtilis* have the advantage of being compatible with biofumigation [39]. The effective use of biological control agents (BCAs) is a potentially major component of sustainable agriculture [40].

From the results presented here, it can be expected that Bs1 based on *Bacillus subtilis* and Tr1 or Tr2 based on *Trichoderma* spp. could reduce fungal inoculum during the pear vegetative period by at least 50% under natural conditions. Additionally, Tr1 and Tr2 may reduce the fungal overwintering inoculum under controlled conditions by 80%. These results are encouraging if a similar effect could be obtained in commercial pear orchards. More trials in pear orchards through the entire season are needed to evaluate these, and other, biological control agents and their effect on *S. vesicarium/P. allii* inoculum and the disease progress.

5. Conclusions

It can be concluded that, overall, with the preventative application of BCAs under controlled conditions, the efficacy for controlling pathogen colonization was higher for those based on *B. subtilis* than those based on *Trichoderma* spp., but similar for controlling the inoculum production of *S. vesicarium* and *P. allii*. However, when the BCAs were applied curatively, *Trichoderma*-based products were more effective. In field trials, *Trichoderma* spp. based BCAs Tr1 and B. subtilis Bs1 applied curatively proved to be consistently effective in decreasing the number of conidia of *S. vesicarium* trapped by 50% compared to the non-treated control. As a general conclusion, it can be expected that Bs1 is based on Bacillus subtilis and Tr1 or Tr2 is based on *Trichoderma* spp. could reduce fungal inoculum during the pear vegetative period by at least 50% and Tr1 and Tr2 may reduce the fungal overwintering inoculum by 80% to 90%.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081455/s1, Table S1: Effect of preventative applications of biological control agents (BCAs) on growth inhibition of *Stemphylium vesicarium*.

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