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Biocontrol Endotherapy with *Trichoderma* spp. and *Bacillus amyloliquefaciens* against *Phytophthora* spp.: A Comparative Study with Phosphite Treatment on *Quercus robur* and *Fagus sylvatica*

Gilles Berger[1], Katarzyna Czarnocka[2], Bastien Cochard[1], Tomasz Oszako[2] and François Lefort[1]

1. Plants and Pathogens Group, Research Institute of Earth Nature and Environment, Hepia, HES-SO University of Applied Sciences and Arts Western Switzerland, Jussy, Geneva 1254, Switzerland

2. Department of Forest Pathology, Forest Research Institute, Sękocin Stary 05-090, Poland

Abstract: *Phytophthora* species are particularly aggressive plant pathogens and are often associated with the decline of many tree species, including oak and beech. Several fungi and bacteria species are known as potential antagonists usable as biological control agents. Phosphonate (H_3PO_3), commonly branded as phosphite, has also been used in the past years to protect trees against invasive *Phytophthora* spp.. This study aimed at comparing the effects of selected antagonist microorganisms and phosphonate, when applied by microinjection or leaf treatment. Antagonistic species were first selected for their high inhibitory activity against problematic *Phytophthora* species, such as *Phytophthora cactorum*, *P. quercina* and *P. plurivora* attacking *Quercus robur* and *Fagus sylvatica* in Polish forests. Three endophytic species *Trichoderma atroviride* (two strains), *T. harzianum* and *Bacillus amyloliquefaciens* showed a high control activity, and their efficacy was then assessed in comparison with a phosphonate treatment. Two application methods were experimented in this study: injection of a solution of spores or phosphonate into the sap vessels of beech or a foliar treatment on oak. Phosphonate and two strains of *Trichoderma* significantly reduced the necrotic area on oak leaves inoculated with *P. plurivora* and one strain of *T. atroviride* significantly reduced necrotic areas on beech branches. These results are therefore promising of a novel way to control *Phytophthora* spp. in forest stands and nurseries.

Key words: *Phytophthora* spp. endotherapy, *Trichoderma*, *Bacillus* sp., endophytes.

1. Introduction

The genus *Phytophthora* (Oomycetes: Peronosporaceae) contains some of the most aggressive plant pathogens, causing large economic and ecological damages worldwide in agriculture, horticulture and forestry. Most species have an extensive host spectrum, while a few are host specific [1]. *Phytophthora* diseases have been shown to be involved in the decline of many tree species across Europe [2]. The symptoms are very similar for most tree species, and include higher crown transparency, fine and lateral root rots, collar and trunk canker with tarry spots, wilting and branch dieback [1]. Owing to their complex and mutual interactions, numerous biotic and abiotic factors contribute to an aggravation of tree decline caused by *Phytophthora* species [3].

Among sensitive species, oak species have been subjected to serious and frequent dieback during the past century in Europe [4-7]. The most common species found in oak stands are *P. plurivora* and *P. quercina* [2, 6, 8], and the association with soil type and acidity would be responsible for oak decline [5].

In spite of early reports of the presence of *Phytophthora* species affecting beech (*F. sylvatica*) in the UK, this species was considered for a long time as non-problematic due to its wide adaptation capacity

Corresponding author: François Lefort, professor, research fields: plant pathology and biological control.

and competitive ability [9], however, with the climatic extremes of 2002 and 2003, typical *Phytophthora* symptoms have been increasingly reported on beech [10]. It is now a common view that climate changes could hasten the spreading of pathogens. Very few forecasts have been made available for *Phytophthora* species, with the exception of a study for *P. cinnamomi*, showing the likely spreading of the disease in Europe [11].

A wide choice of fungicides has been developed and made available to agriculture and horticulture. Except in forest nurseries, they are not adapted to forest trees treatments. Phosphite, also called phosphonate or phosphonic acid salt (H_3PO_3), did show a good control activity on *Phytophthora* species and very interesting direct and indirect protective effects on plants [12]. Better results in reducing *Phytophthora* infection were obtained by trunk injection [13-19] and foliar spray [20-24]. This chemical was chosen here for its known capacity to reduce the impact of *Phytophthora* infections and was then used as a positive control to be compared to the treatments with biological antagonists. The biocontrol capacity of some endophytic organisms, i.e., asymptomatic organisms with an internal colonisation, has mainly been studied through *in vitro* experiments [25-29]. *In situ* experiments were mainly carried out on herbaceous plants [30-33] or young tree seedlings [27, 34-37] and rarely on adult trees [38-40]. In this study, the authors proposed to compare the effects of selected antagonist microorganisms with phosphonate, when applied by microinjection or leaf treatment. The microorganisms retained for these works were fungi of the genus *Trichoderma* and bacteria from the *Bacillus* genus, which have been extensively studied for their properties of biological control and plant growth promotion [26, 41-45] and are potentially endophytes.

2. Material and Methods

2.1 Microorganism Isolates and Culture Conditions

P. cactorum, *P. plurivora* and *P. quercina* isolates were from the collection of the Forest Research Institute in Sękocin. The different strains of *B. amyloliquefaciens* and *Trichoderma* spp. were by the collection of the group plants and pathogens (Hepia) in Jussy. *T. atroviride* ITEC is from Lallemand Plant Care, France. *T. atroviride* UASWS0365 has been registered in Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession DSM 29610, and accessions are pending for the other *Trichoderma* spp. UASWS strains. The *B. amyloliquefaciens* strains P1 and C2 have been registered in DSMZ in patent deposit under the treaty of Budapest under the accessions DSM 32011 and DSM 32012, respectively. *Phytophthora* species were cultivated on V8 agar medium (V8 Campbel's soup, USA), fungi on potato dextrose agar (PDA) medium (Carl Roth, Switzerland) with 50 µg/mL ampicillin (Carl Roth, Switzerland) and bacteria on Luria Bertani agar (LBA) medium (Carl Roth). All cultures were maintained in the dark at room temperature.

2.2 In Vitro Dual Cultures

Confrontation between *Phytophthora* spp. and potential antagonists were performed as dual cultures in 9 cm diameter. Petri dishes contained 30 mL of a mixed sterile PDA + V8 medium (PDA 39 g/L, $CaCO_3$ 3.5 g/L and 200 mL/L of V8 Campbell's soup) for fungi species and LBA + V8 medium (LBA 35 g/L, $CaCO_3$ 3.5 g/L and 200 mL/L of V8 Campbell's soup) for bacteria. Each Petri dish was inoculated with a 0.5 cm diameter plug of a 7-day-old pure culture of one of the *Phytophthora* strains and a 0.5 cm diameter plug of one 7-day-old pure culture of an antagonistic organism, at a distance of 5 cm from each other. The experiment was repeated nine times per confrontation for statistical needs. The development of the pathogen was measured three times at the edge of the mycelium after 3, 5 and 7 d of confrontation. The statistical analyses were performed on Minitab using a one way ANOVA. The antagonistic effects were compared

using Tuckey's comparison test.

The antagonistic interaction between the different fungal strains and *Phytophthora* spp. was analysed with the scoring method described by Badalyan et al. [46, 47]. This method classified the interaction in three types—A, B and C and four subtypes—CA1, CA2, CB1 and CB2, where A = deadlock with mycelial contact, B = deadlock without mycelial contact, C = overgrowth of the pathogen without deadlock; CA1/CA2 = partial/complete replacement after the deadlock with mycelial contact, CB1/CB2 = partial/complete replacement after the deadlock without mycelial contact. The antagonist index (AI) was calculated by Eq. (1):

$$AI = A\ (N \times 1) + B\ (N \times 2) + C\ (N \times 3) + CA1\ (N \times 3.5) + CB1\ (N \times 4) + CA2\ (N \times 4.5) + CB2\ (N \times 5) \quad (1)$$

where, N is the amount of specific interaction recorded.

2.3 Foliar Treatments with Bacteria, Fungi and Phosphite on 2-Year-Old Oaks (Q. robur L.)

Leaves from 70 young oaks (2-year-old) cultivated in glasshouse, were treated with biological agents selected for their inhibitory activity on the three *Phytophthora* species used for inoculation. Foliar treatment was carried out with a paint brush, on 2 leaves/tree, prior to inoculation by *P. plurivora*. On each tree, a treated leaf and an untreated leaf were collected two weeks after treatment and kept in humid chambers at 20 °C in a climatic chamber in the dark.

Each of the following modalities of foliar treatment was repeated on 10 trees: (1) phosphite solution (1.2 g/L H_3PO_3) according to Gentile et al. [18]; (2) *T. atroviride* strain ITEC (1×10^8 conidia/mL); (3) *T. aureoviride* strain UASWS (1×10^8 conidia/mL); (4) *T. harzianum* strain B100 (1×10^8 conidia/mL); (5) *B. amyloliquefaciens* C2 (1×10^8 CFU/mL); (6) *B. amyloliquefaciens* P1 (1×10^8 CFU/ mL); (7) control consisted of ultrapure water application.

P. plurivora strain IBL254 was inoculated on control and treated leaves one week after leaves had been placed in humid chambers in the dark. Inoculation consisted in depositing a 25 mm^2 agar plug of a 7-day-old culture on V8 medium.

Leaves were scanned and necrotic surfaces were measured with Jen's Makroaufmass program[1], and statistic treatment of data was performed with the *R* program using the Kruskal-Wallis test.

2.4 Endotherapy through Trunk Injection on 30-Year-Old Beech Individuals Inoculated with P. plurivora

2.4.1 Inoculation of Beech Trees with *P. plurivora*

In a plot of beech (*F. sylvatica*), 80 trees of 30-year-old with similar diameter measured at a 1.3 m height from the ground, were selected for a randomised endotherapy experiment. All trees were inoculated with *P. plurivora* on branches 1 m away from the main trunk, in order to prevent any secondary infection from *P. plurivora*. Inoculations were performed as follows: on each branch, bark was removed on a surface of 3 cm × 2 cm with the help of a sterile razor blade, then a PDA plug (1 cm × 1 cm) from a 7-day-old culture of *P. plurivora* strain IBL254 was placed into contact with the exposed cambium. The inoculated area was covered with cotton moistened with sterile water, sealed with Parafilm™ wrap and finally covered with aluminium foil in order to prevent desiccation. The branches were kept in contact with the pathogen for three weeks.

2.4.2 Trunk Injection Modalities

Injections were performed with a direct injector system (Wedgle Direct-Inject, Arborsystems Inc., USA) and were carried out at a 1.5 m height from the ground. The following treatment modalities were applied to groups of 10 trees: (1) phosphite at the concentration of 250 g/L H_3PO_3. Injections were performed at a dose of 0.8 mL per 5 cm of trunk circumference yielding a dose of 50 g/L/cm of trunk, according to recommendations by Shearer et al. [15]

[1] http://ruedig.de/tmp/messprogramm.htm.

and Gentile et al. [18]; (2) *T. atroviride* strain ITEC (1×10^8 conidia/mL); (3) *T. aureoviride* strain UASWS strain (1×10^8 conidia/mL); (4) *T. harzianum* strain B100 (1×10^8 conidia/mL), conidia doses were of 0.8 mL per 10 cm of trunk; (5) *B. amyloliquefaciens* strain P1 at a concentration of 1×10^8 CFU/mL and at the final dose of 0.8 mL/10 cm of trunk; (6) *B. amyloliquefaciens* strain C2 at a concentration of 1×10^8 CFU/mL and at the final dose of 0.8 mL/10 cm of trunk.

The first injections took place three weeks after the first inoculation with *P. plurivora* and the branches were then harvested three weeks later.

2.4.3 Inoculation after Tree Injections on Non-inoculated Branches

Non-inoculated branches were harvested on the treated trees at the same time as inoculated branches. The latter were cut into 20 cm pieces, put in moisture chambers for three weeks at 20 °C in the dark and inoculated with *P. plurivora* following the same protocol as described above.

The size and surface of necrotic spots were then measured and compared to a positive control inoculated with *P. plurivora* without any injection and a negative control inoculated with sterile water only. Necrotic surfaces were drawn on transparent plastic sheets, which were then scanned to yield necrotic surface measurements by Jen's Makroaufmass programme. Statistic treatment of data was performed with the *R* programme using the Kruskal-Wallis and post-hoc tests after Nemenyi in the pairwise multiple comparison of mean ranks package (PMCMR) [48].

3. Results

3.1 Confrontation of P. plurivora, P. cactorum and P. quercina with Antagonistic Bacteria and Fungi

The dual cultures displayed different levels of mycelia growth inhibition as shown in Table 1. The ANOVA showed that all results were significantly different from the control at 3, 5 and 7 d for each *Trichoderma* strain in confrontation with *P. cactorum*. *T. harzianum* B100 had the strongest impact on the growth of *P. cactorum* with a reduction of 56.78% compared to the control. The isolates of *T. harzianum* B33 and *T. aureoviride* UASWS did not show any significant difference with *T. harzianum* B100, and were capable of reducing the mycelium growth in a

Table 1 Effect of *Trichoderma* spp. strains after 7 d of dual culture on the growth of *P. cactorum*, *P. plurivora* and *P. quercina*.

Fungal strains	Reduction of mycelium growth over control (%)		
	P. cactorum	*P. plurivora*	*P. quercina*
Control	0.00[a]	0.00[a]	0.00[a]
T. asperellum UASWS	36.75[bc]	41.29[c]	32.35[b]
T. atroviride UASWS0365	24.92[b]	19.84[b]	33.33[b]
T. atroviride ITEC	52.21[de]	54.56[d]	58.82[bc]
T. aureoviride UASWS	56.62[e]	51.10[cd]	54.09[bc]
T. hamatum UASWS	31.39[bc]	46.48[cd]	57.84[bc]
T. harzianum B05	39.75[cd]	51.21[cd]	56.86[bc]
T. harzianum B100	56.78[e]	50.63[cd]	63.73[c]
T. harzianum B33	55.84[e]	51.79[cd]	46.08[bc]
T. harzianum B97	53.94[de]	48.10[cd]	47.04[bc]
Standard error of the mean (SE)	0.0716	0.0932	0.0627
Degree of freedom	89	89	29
F-values	44.09	55.37	10.95
P-values	< 0.005	< 0.005	< 0.005

All values are mean of nine replicates, except for *P. quercina* where the mean is based on three replicates. Growth of the different *Phytophthora* species in control was without any antagonistic fungi.

Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to Tukey's comparison test.

range of 55.84%-56.62%. The other strains showed a smaller impact within a range of 24.92%-36.75% of mycelium growth reduction.

As shown by ANOVA, at all dates, all *Trichoderma* strains significantly reduced the growth over the control *P. plurivora*, except the *T. harzianum* B33 and *T. asperellum* at 3 d and *T. atroviride* at 3 d and 5 d.

T. atroviride ITEC had the biggest impact on the growth of *P. plurivora* with 54.56%, but did not show any differences with most of the other strains (*T. harzianum* B97, B33, B100, B05, *T. hamatum*), which reduced *P. plurivora* growth between 46.48% and 51.79%. All yielded results were significantly different from *T. atroviride* UASWS0365 (19.84%) and *T. asperellum* (41.29%).

Similarly, all *Trichoderma* strains significantly reduced the growth of the control *P. quercina* but only after 7 d. No results showed any significant difference over the control at 3 d or 5 d. *T. harzianum* B100 induced the most important growth reduction compared to the *P. quercina* control with 63.73%. The other strains showed growth reduction between 58.8% and 32.35%. The antagonistic interaction between *P. cactorum* and *P. plurivora* is shown in Table 2. Initial deadlock was observed as soon as 3 d for most of the strains. Two types (A and C) and three subtypes (CA1, CA2 and CB1) were identified. Based on the AI values, it appeared that *T. atroviride* was weakly active and all the other strains were strongly active against both *Phytophthora* species.

The two strains of *B. amyloliquefaciens* showed significant differences with all *Phytophthora* spp. after

Table 2 Interactions between antagonistic fungi and *P. cactorum* and *P. plurivora*.

Fungal strain	*P. cactorum*		*P. plurivora*	
	AI	Type/subtype	AI	Type/subtype
T. asperellum	30.00	C	30.50	CA1
T. atroviride UASWS0365	15.00	A	10.00	A
T. atroviride ITEC	38.50	CA2	34.50	CA1
T. aureoviride	30.50	CA1	33.50	CA1
T. hamatum	34.50	CA1	35.00	CA1/CA2
T. harzianum B05	34.00	CB1	37.50	CA2
T. harzianum B100	31.00	CA1	39.00	CA1/CA2
T. harzianum B33	38.00	CA2	36.50	CA1/CA2
T. harzianum B97	39.00	CA2	32.90	CA1

The AI was scored on Badalyan's scale with the interactions types and subtypes.
C = overgrowth of the pathogen without deadlock, A = deadlock with mycelial contact, CA1/CA2 = partial/complete replacement after the deadlock with mycelial contact, CB1 = partial replacement after the deadlock without mycelial contact.

Table 3 Effect of *B. amyloliquefaciens* strains after 7 d of dual culture on the growth of *P. cactorum*, *P. plurivora* and *P. quercina*.

Bacillus isolate	Reduction of mycelium growth over control (%)		
	P. cactorum	*P. plurivora*	*P. quercina*
Control	0.00[a]	0.00[a]	0.00[a]
B. amyloliquefaciens P1	33.58[b]	20.18[b]	34.31[b]
B. amyloliquefaciens C2	22.29b	22.46[b]	29.41[b]
Standard error of the mean (SE)	0.138	0.107	0.102
Degree of freedom	26	26	8
F-values	23.53	22.51	11.44
P-values	< 0.005	< 0.005	0.009

All values are mean of nine replicates, except for *P. quercina* where the mean is based on three replicates. Growth of the different *Phytophthora* species in control was without any antagonistic fungi.
Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to Tukey's comparison test.

7 d, but no difference between each other (Table 3). At 3 d and 5 d, no results were significantly different from the control except for *B. amyloliquefaciens* C2 at 5 d, showing a significant difference with *P. plurivora* control growth.

3.2 Foliar Treatments with Bacteria, Fungi and Phosphite on 2-Year-Old Oaks (Q. robur L.)

In this experiment, all treatments reduced the necrotic areas in comparison with the control. Four treatments were found significantly different *(P* ≤ 0.05) from the control after a Kruskal-Wallis comparison test. The treatments based on phosphite, *T. aureoviride* UASWS and *T. harzianum* B100 were able to significantly reduce the necrotic surfaces caused by *P. plurivora*. The treatment based on a direct application of phosphite gave the best results with an 85.77% necrosis reduction over the control, followed by *T. aureoviride* UASWS, and *T. harzianum* B100 with respectively 83.92% and 77% necrosis reduction, as shown in Table 4 and Fig. 1.

3.3 Endotherapy through Trunk Injection on 30-Year-Old Beech Individuals Inoculated with P. plurivora

3.3.1 Curative Results

All necroses that developed on the treated trees were smaller than the ones on the control trees, and necrosis surface reduction ranged from 62.33% to 9.65% (Table 5), but did not provide any significant results ($P < 0.05$) due to outliers (Fig. 2). It is therefore not possible to determine a curative efficiency for the different antagonists or the phosphite treatment. However, the necrotic surfaces on treated branches were significantly larger ($P < 0.05$) for each treatment than on the negative control branches, on which the wounds were already healing with the formation of a new bark, confirming that the inoculation methodology used was efficient. Additionally, a quantitative polymerase chain reaction (qPCR) was conducted in order to check the inoculation viability (data not shown) and clearly amplified *P. plurivora* DNA isolated from the necrosis margins, marking the

Table 4 Effect of the different preventive treatments on the reduction of necrotic surface due to *P. plurivora* on 2-year-old oak leaves.

Microorganisms isolates	Necrotic surface reduction over control (%)
Control	0.00[a]
B. amyloliquefaciens C2-	38.07[abcd]
B. amyloliquefaciens C2+	42.98[ab]
B. amyloliquefaciens P1-	51.56[abcd]
B. amyloliquefaciens P1+	69.71[abcd]
Phosphite -	77.56[bcd]
Phosphite +	85.77[d]
T. atroviride ITEC-	31.82[ab]
T. atroviride ITEC+	54.02[abcd]
*T. aureoviride*UASWS -	83.92[cd]
*T. aureoviride*UASWS+	51.63[abc]
T. harzianum B100-	77.00[bcd]
T. harzianum B100+	48.13[abc]
Standard error of the mean (SE)	0.0315
Degree of freedom	12
P-value	< 0.005

All values are mean of 8-10 replicates. Growth of *P. plurivora* in control was without any antagonistic fungi.

Treated leaves are labeled with the symbol "+", while untreated leaves are labeled with the symbol "-".

Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to Kruskal-Wallis post-hoc tests after Nemenyi in the pairwise multiple comparison of mean.

Table 5 Effect of the different curative treatments on the reduction of necrotic surfaces due to *P. plurivora* on 30-year-old beech branches.

Microorganisms isolates	Necrotic surface reduction over control (%)
Control	0.00[a]
B. amyloliquefaciens P1	62.33[a]
B. amyloliquefaciens C2	35.29[a]
Phosphite	46.07[a]
T. atroviride ITEC	9.65[a]
T. aureoviride UASWS	60.64[a]
T. harzianum B100	47.25[a]
Standard error of the mean (SE)	2.55
Degree of freedom	6
P-value	0.740

All values are mean of 10 replicates. Growth of *P. plurivora* in control was without any antagonistic fungi.

Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to the Kruskal-Wallis test.

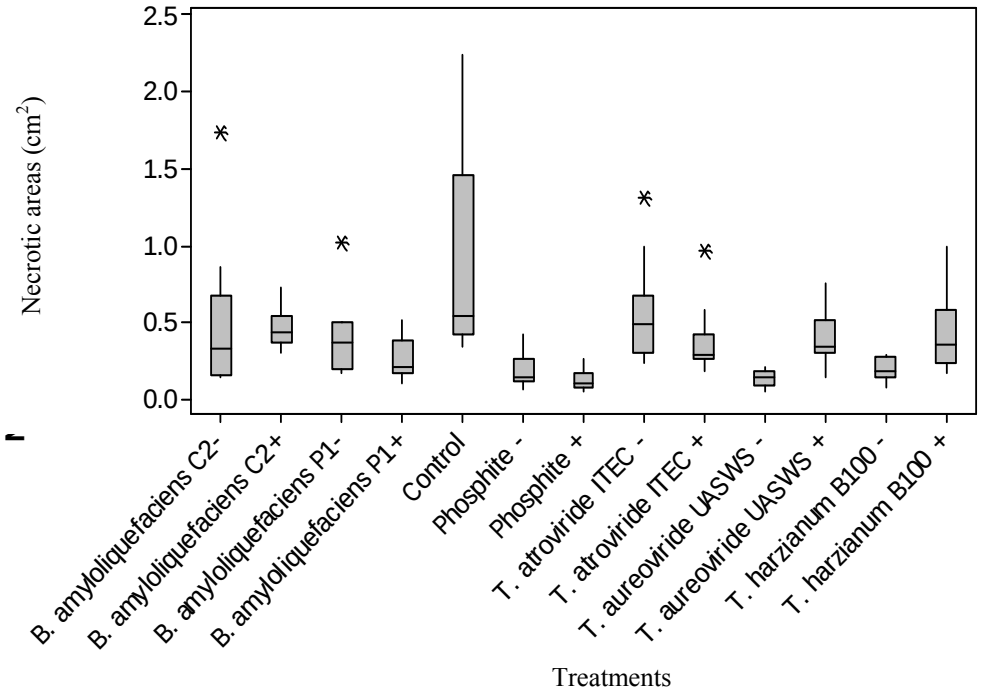

Fig. 1 Box plot of the necrotic areas developed on oak (*Q. robur*) leaves after 7 d in contact with *P. plurivora*.
The boxes represent the values between the first and the third quartile with the median in the middle. The line in the box represents the median, while the extremities of the vertical lines show the minimum and maximum values. * means the outliers.

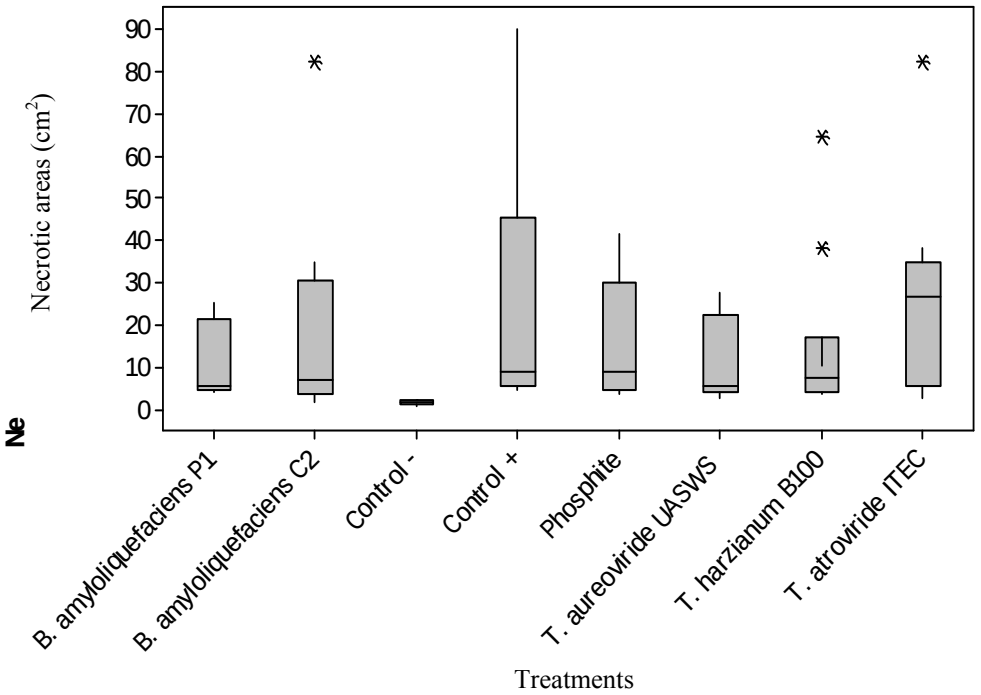

Fig. 2 Box plot of the necrotic areas on beech (*F. sylvatica*) branches for the curative treatment with positive and negative control.
The boxes represent the values between the first and the third quartile with the median in the middle. The line in the box represents the median, while the extremities of the vertical lines show the minimum and maximum values. * means the outliers.

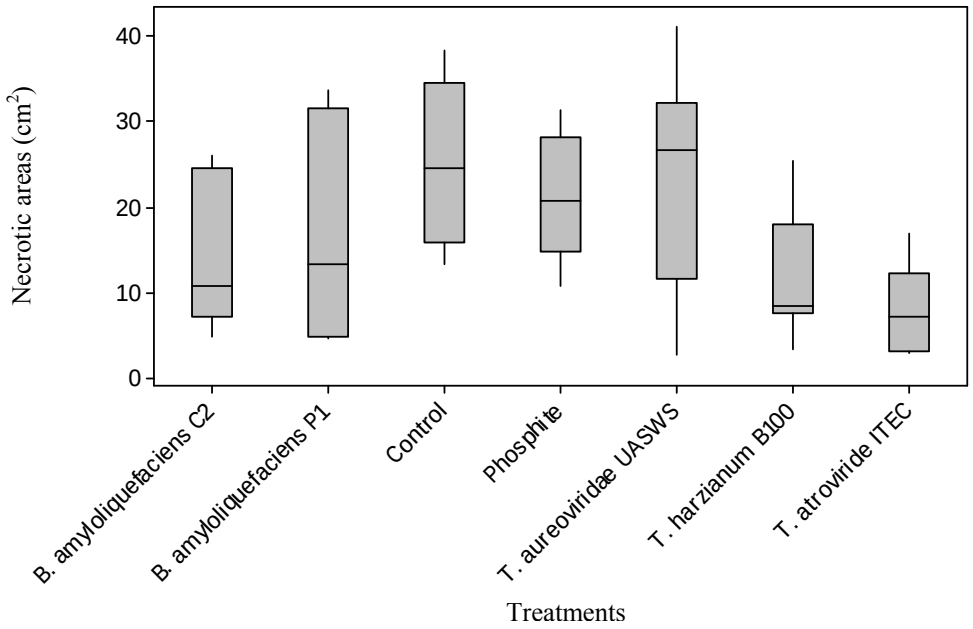

Fig. 3 Box plot of the necrotic areas on beech (*F. sylvatica*) branches for the preventive treatment with positive control.
The boxes represent the values between the first and the third quartile with the median in the middle. The line in the box represents the median, while the extremities of the vertical lines show the minimum and maximum values.

Table 6 Effect of the different preventive treatments on the reduction of necrosis surface due to *P. plurivora* on 30-year-old beech branches.

Microorganisms isolates	Necrosis surface reduction over control (%)
Control	0.00[a]
B. amyloliquefaciens P1	32.16[ab]
B. amyloliquefaciens C2	47.75[ab]
Phosphite	15.85[a]
T. atroviride ITEC	69.50[b]
T. aureoviride UASWS	10.10[ab]
T. harzianum B100	54.28[ab]
Standard error of the mean (SE)	1.42
Degree of freedom	6
P-value	0.004

All values are mean of 5-10 replicates. Growth of *P. plurivora* in control was without any treatment.
Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to the Kruskal-Wallis post-hoc tests after Nemenyi in the pairwise multiple comparison of mean.

presence of *P. plurivora* in the inoculated branches, whereas there was no amplification in the negative control.

3.3.2 Preventive Results

The preventively treated trees developed necrosis caused by *P. plurivora* that were smaller than the control ranging from a 10.10% to 69.5% reduction (Table 6). The treatment with *T. atroviride* ITEC and the phosphite treatment significantly reduced ($P < 0.05$) the necrotic surfaces when compared to the control as shown in Fig. 3.

4. Discussion

In Europe, *Phytophtora* invasive species are spreading and infecting new forest stands, and are commonly associated with oak and beech dieback [2]. As they are already present in the environment, new strategies should be developed in order to help the forests sustain these new threats. One strategy could be to use biological antagonists as natural barriers to *Phytopthora* spp.. endophytes, if antagonistic to *Phytophthora* spp., they could lead to a long term beneficial interaction, which would potentially increase the tolerance to new stress and therefore support the trees' capacity for adaptation. Besides, this alternative strategy could result in releasing time and resources to find new resistance genes and create

resistant cultivars. The first step of this work would involve identifying and selecting the most competent antagonist against the chosen *Phytophthora* spp.. All *Trichoderma* and *Bacillus* species tested here significantly reduced the growth of *P. cactorum*, *P. quercina* and *P. plurivora* in *in vitro* dual cultures. However, the different species of *Trichoderma* showed significant differences between each other, and the most effective species are *T. atroviride* ITEC, *T. aureoviride* UASWS and all strains of *T. harzianum*, except strain B05 in the dual culture with *P. cactorum*. These results are in adequacy with the common literature about *in vitro* biological control efficiency of strains of the same species [44, 49-54], but provide new information on the properties of these strains. The AI showed that all strains of *Trichoderma* spp. were strongly active against *P. cactorum* and *P. plurivora*, with the exception of *T. atroviride* UASWS0365. The slow growth of *P. quercina* did not give the opportunity to clearly determine an antagonist interaction with the different *Trichoderma* strains.

The other experiments used the organisms which performed best in the dual cultures experiment. This selection method through dual cultures is however influenced by the growth speed of the antagonistic organism, and could not represent a definitive judgment on the properties of any potential antagonist as already shown and discussed by different authors in Refs. [41, 55-57].

The two stains of *B. amyloliquefaciens* expressed a significant reduction of the three *Phytophthora* species, widening the antagonistic capacity already described for this species in Refs. [41, 55-57].

The experiment of foliar treatment on oak leaves showed that the phosphite treatment was capable of protecting treated and untreated leaves. This is due to its capacity of diffusing through the plant tissues as demonstrated by several studies [12, 20, 58, 59]. The significant result obtained by *T. aureoviride* UASWS and *T. harzianum* B100 showed that only the untreated leaves of treated plants were able to reduce the necrotic area, as compared to the control. This might be due to some secondary metabolites generated by the fungi, but further investigation would be needed to understand more about the interaction between these endophytic fungi in oaks and their production of specific secondary metabolites.

The endotherapy experiment confirmed that the inoculation method of *P. plurivora* was efficient as previous report with *P. citrophthora* [60]. The curative treatment did not show any significant results, but the preventive application did show promising results for *T. atroviride* ITEC, which was able to significantly reduce the necrosis size compared to the control and the phosphite treatment. The phosphite treatment was used here as a positive control, since it is usually known for reducing *Phytopthora* spp. activity.

However, pathogen penetration could be facilitated by the artificial creation of wounds, which is not the case in a natural infection. The artificial creation of wounds allowed to express the capacity to reduce lesion expanses, and did not integrate the possible pre-infection protective capacity of the treatments. This might also explain the surprisingly insignificant result of the phosphite treatment, which had been previously reported as efficient against *Phythophthora* spp. [18], as phosphite is known to play a role against *Phytophthora* spp. in the pre-invasive momentum [12].

5. Conclusions

In conclusion, this study showed that all tested strains had a potential in the biological control of the three tested species of *Phytophthora*. However, there were significant differences between the different *Trichoderma* strains in the dual culture experiment. The foliar treatment of oak leaves yielded positive results with the phosphite treatment and two *Trichoderma* stains, whereas the endotherapy experiment on beech against *P. plurivora* pointed the potential capacity of *T. atroviride* ITEC in reducing *P.*

plurivora infection. Further experiments would still be required in order to confirm the promising results of preventive treatments by foliar application or endotherapy and to improve a methodology able to reduce the impact of these two *Phytophthora* species. Such treatments could be part of an integrated control of the *Phytophthora* disease in forest stands and nurseries.

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