Received: 9 September 2020 | Revised: 13 July 2021 | Accepted: 13 July 2021

DOI: 10.1111/efp.12711

SHORT COMMUNICATION

Forest Pathology WILEY-BLACKWELL WILEY

Antagonistic effect in vitro of three commercial strains of *Bacillus* sp. against the forest pathogen *Diplodia corticola*

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Funding information
Diputació de Barcelona, Àrea de Territori i Sostenibilitat; Generalitat de Catalunya, Departament d'Agricultura, Ramaderia, Pesca i Alimentació

Editor: Alberto Santini

Abstract

Botryosphaeria canker of *Quercus suber* (causal agent: *Diplodia corticola*) poses a major threat for cork production along the western Mediterranean Basin. Infection by *D. corticola* induces cankers, which reduce tree vigour and compromise phellem regeneration. European policies in forest health advise the use of environmentally friendly methods, such as biocontrol, rather than the use of chemicals in natural stands. In this study, we assessed the antagonistic potential of three commercial products involving bacteria (*Bacillus amyloliquefaciens* and *Bacillus mojavensis*) using two culture media [potato-dextrose-agar medium (PDA), and PDA amended with copper oxychloride, an inorganic fungicide] against *D. corticola*. Five indices based on colony dimensions showed significant antagonistic effects of bacteria on fungal growth in vitro. The copper salt showed high toxicity against *D. corticola*. Preliminary results suggest that the three commercial products might be used as biocontrol agents against *D. corticola*, whose control capacities in the field deserve further investigation.

KEYWORDS
Bacillus amyloliquefaciens, *Bacillus mojavensis*, biocontrol, Botryosphaeria canker, cork oak disease

1 | INTRODUCTION

Botryosphaeria canker is a major threat for Mediterranean forests since it causes severe economic and ecological impacts in productive cork oak (*Quercus suber* L.) stands in western Europe. The disease is caused by the fungus *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque [Ascomycota, Dothideomycetes, Botryosphaeriaceae; formerly misidentified as *Diplodia mutila* (Fr.) Mont. (Alves et al., 2004)], causing severe dieback in mature *Quercus* spp., with necrosis in the stem and branches, and mortality of seedlings. The host range of the pathogen includes several Mediterranean oak species such as *Quercus canariensis* Willd., *Quercus coccifera* L., *Quercus ilex* L. and *Q. suber*. In cork oak, the pathogen causes branch necrosis and canker in the phelloderm exposed by debarking, which severely limits cork production, reduces the value of the extracted cork and severely reduces the vigour of the infected trees (Luque et al., 2008).

The disease has been traditionally treated using chemicals such as benomyl ($C_{14}H_{18}N_4O_3$; Benlate/WP, Du Pont Ibérica, S.A.) and thiophanate-methyl ($C_{12}H_{14}N_4O_4S_2$; Pelt/SC, Aventis CropScience España). Nevertheless, European policies for forest management advise against the use of fungicides; the use of benomyl is banned whereas thiophanate-methyl use was permitted until October 2020. As a consequence of these policies, the development of new control methods that reduce or even replace the use of chemicals is urgently

needed (Serrano et al., 2015). Biological control based on the use of antagonistic organisms represents one of the most promising alternatives against Botryosphaeria canker. Moreover, the Gram-positive genus *Bacillus* spp. has been suggested as a suitable biocontrol agent against phytopathogens also in combination with copper oxychloride [$Cu_2(OH)_3Cl$; Jeyaraman & Robert, 2018].

The aims of this study, therefore, were (i) to investigate the possible antagonistic effect of three commercial strains of *Bacillus* sp. against *D. corticola* in vitro and (ii) to evaluate the synergistic effects of possible biocontrol agents and copper salt for reducing vegetative growth of *D. corticola*.

2 | MATERIALS AND METHODS

2.1 | Inoculation and in vitro assessment

Potential antagonism between *D. corticola* and *Bacillus* spp. was analysed in dual-culture assays. A wild-type fungal strain isolated from *Q. suber* in Portugal (CAA007-1) was supplied by Dr. Artur Alves (Universidade de Aveiro). The fungal strain was cultured in 3.90% w/v potato-dextrose-agar medium (PDA; Biokar) amended with 0.03% w/v streptomycin sulphate (PDAs; $C_{21}H_{39}N_7O_{12} \bullet 1.50\ H_2SO_4$, Sigma-Aldrich) for one week at 21 ± 2°C before subculture. In total, 56 Petri dishes containing 20 ml PDA were prepared and a paper disc (30 mm in diameter), previously autoclaved twice, was placed aseptically on the cool medium in each dish (Figure 1a). In parallel, the same number of dishes were prepared containing 20 ml of PDA amended with 0.50% w/v of copper oxychloride (PDAc; Industrial Química Key S.A.) and sterile paper discs placed on the medium, as described above. Each dish was inoculated with a 5 × 5 mm plug of fresh mycelium taken from the edge of 21-day-old fungal colonies growing on PDA. Fungal plugs were placed on the opposite side of the plate with respect to the paper discs, as shown in Figure 1a.

Three bacterial treatments were assayed in the study with fourteen cultures per treatment (i.e. PDA and PDAc). Each treatment consisted of bacterial inoculation of the paper disc immediately after fungal subculture, by applying 100 µl of aqueous bacterial suspension (i.e. living microorganism and their metabolites). Bacteria assayed were as follows: (Bt1) aqueous suspension [28.53% w/v, that is 1.42×10^9 CFU/ml (Jeyaraman & Robert, 2018)] of *Bacillus amyloliquefaciens* Wang et al. (i.e. *B. amyloliquefaciens* subsp. *plantarum* strain D 747 AMYLO-X® WG, CERTIS Europe BV); (Bt2) liquid formulation from fermentation cultures of *B. amyloliquefaciens* (1×10^8 CFU/ml; Probelte S.A.U.) and (Bt3) liquid formulation of the fermentation cultures of *B. amyloliquefaciens* and *Bacillus mojavensis* Roberts et al. (1×10^8 CFU/ml; Probelte S.A.U.) in mixed suspension (1:1; concentration of each species was determined before broth mixing). Bacterial concentration in treatment Bt1 was calculated according to manufacturer's specifications (i.e. resuspension at desired concentration from lyophilized powder). Concentrations of viable cells in the remaining two treatments (i.e. Bt2 and Bt3) were quantified by counting colony-forming units per millilitre after growth in Nutrient Broth (NB; Scharlau Microbiology) amended with 1.50% w/v agarose (Nutrient Agar medium; NA) (Figure S1). Fourteen additional plates of PDA and PDAc were used as negative controls by treating the filter paper discs with 100 µl double-sterilized deionized water.

Each dish was marked with a diametric axis and two perpendicular axes from the fungal culture (Figure 1a; Figure S2). Dual-cultures were incubated under room conditions (21 ± 2°C; ~8 h of natural light per day) for 22 days. The length of the colony along four perpendicular radii (a, b, c, d; c being the axis towards the paper disc. Figure 1a; Figure S2) as well as the 45° left and right axis (L and R, respectively) was measured when fungal colonies reached the paper

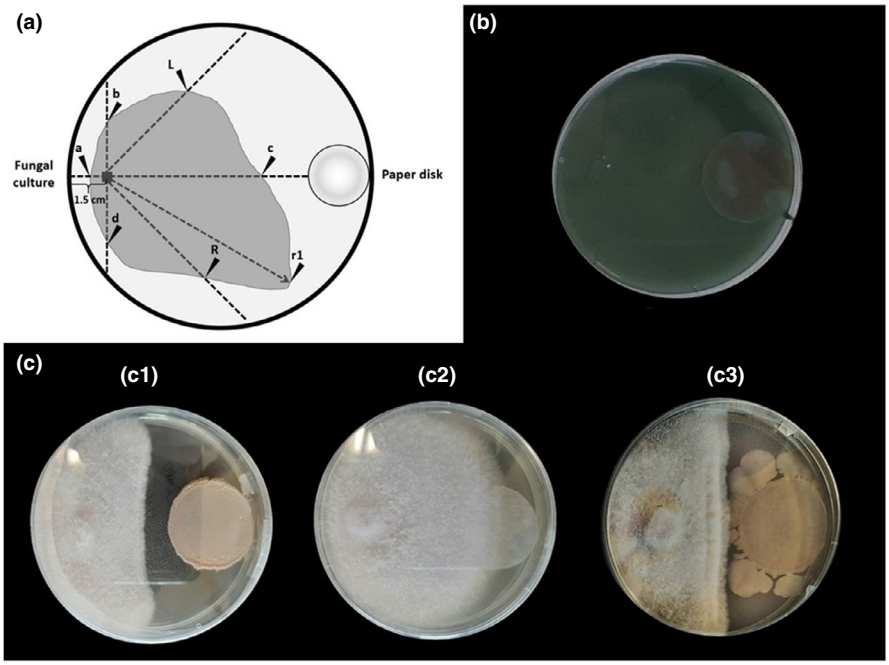

FIGURE 1 Images of the in vitro assay. (a) Scheme for dual-culture for antagonism assessment (modified from Martínez-Álvarez et al., 2016). a–d: perpendicular colony radii; L and R: 45° left and right axis in respect of c, respectively; r1: longest radius of fungal colony in any direction. (b) PDA amended with copper oxychloride (0.50% w/v; PDAc) showing absence of growth either for fungus or for bacteria 14 days after the beginning of the experiment (days post-culture; dpc). (c) (c1) dual-cultures of *D. corticola* and *B. amyloliquefaciens* (treatment Bt1) 14 dpc; (c2) control culture 14 dpc, and (c3) dual-culture of *D. corticola* and *B. amyloliquefaciens* (treatment Bt1) 22 dpc with formation of a clear mycelial barrier

discs in 80% of the PDA control plates. In addition, the longest radius of the fungal colonies was measured in any direction (r1) as shown in Figure 1a; Figure S2.

The shape coefficient (Sc = [L+R]/2 − c) and the percentage of inhibition of radial growth (I = 100 × [(r1 − c)/r1]; Martínez-Álvarez et al., 2016) were calculated as indicators of antagonism. The antagonistic index developed by Campanile et al. (2007) was calculated as:

$$AI = 100 \times [((a + b + d)/3) - c]/(a + b + d).$$

In addition, a variant of AI index, which we named the dual antagonism index (DAI), was also computed as:

$$DAI = 100 \times [((L + R + r1)/3) - c]/(L + R + r1).$$

2.2 | Statistical analysis

Differences in Sc between control and treatments were analysed using ANOVA and Fisher's least significant difference (LSD) test as post hoc analysis in the R environment (https://www.r-project.org/). The 'Desctools' package (https://cran.r-project.org/web/packages/DescTools/index.html) in R was used to analyse variations in c, I, AI and DAI values between control and treatments through Kruskal–Wallis rank-sum tests followed by Dunn's tests as post hoc analysis.

3 | RESULTS AND DISCUSSION

It took 14 days for 80% of *D. corticola* colonies to reach the sterile papers discs in PDA control cultures. At that time, the five indices calculated were significantly different between control and dual-culture treatments (Table 1; Figure 1c) demonstrating a clear antagonistic effect between the three strains of *Bacillus* sp. and *D. corticola*. Treatments Bt1 and Bt2 differed in bacterial densities (1.42×10^9 and 1×10^8 CFU/ml, respectively); a different strain of *B. amyloliquefaciens* was used in these two treatments; nevertheless, the values obtained in the indices I, AI and DAI were not significantly different (Table 1). In contrast, the length of the axis towards the antagonist (c) was significantly reduced in the more concentrated treatment Bt1 (p-value <.01), the Sc being significantly lower when compared with Bt2 (p-value <.01; Table 1). These results suggest a similar effect of the bacteria against *D. corticola*. In this regard, the results indicated that a lower density of *B. amyloliquefaciens* (i.e. 1×10^8 CFU/ml) may reduce fungal growth, potentially lowering the costs of an biocontrol product. However, this hypothesis requires validation through in planta assays to confirm the effects of each product under field conditions. In contrast, the mixed inoculum of *B. amyloliquefaciens* and *B. mojavensis* (Bt3) did not cause significant differences in any pairwise comparisons among treatments, except for the Sc when compared with Bt2 (p-value = .03; Table 1). This result was probably caused by the fast growth of *B. amyloliquefaciens*, since *B. mojavensis* colonies were not detected in Bt3 cultures. The combined effect of both organisms should be further investigated in future work by determining culture conditions that reduce the competition between bacterial species.

Biological control of phytopathogens can be developed using different organisms, including bacteria and fungi. In an early study, Przybył (2003) evaluated antagonism between *D. mutila* and three species of *Pseudomonas* that infect *Fraxinus excelsior* L. Longer stem lesions formed on positive control seedlings (inoculated with *D. mutila*) than those forming on plants treated with bacteria (1×10^8 CFU/ml) and *D. mutila*, suggesting a promising use for bacteria in the biocontrol of this fungal species. Campanile et al. (2007) showed that some fungi as *Trichoderma viride* Pers. and *Fusarium tricinctum* (Corda) Sacc in dual-cultures against *D. corticola* produced an AI index ≥20%. Those authors also found AI values of 16.60% when the pathogen was cultured in a medium containing metabolites of *F. tricinctum*. However, this fungus has been reported as a pathogen in herbaceous plants (e.g. *Hordeum vulgare* L.) reducing its potential as a suitable biocontroller in natural stands. In contrast, growth of *D. corticola* was not inhibited in the presence of culture filtrates of *T. viride* (Campanile et al., 2007). These results suggested that the mechanism of antagonism was through a combination of both physical (surface colonization) and chemical (production of antifungal compounds) factors. In this study, the experiments conducted using

TABLE 1 Antagonism indicators between *D. corticola* and *Bacillus* spp. in PDA after 14 days in culture

	Index				
Treatment	**c (cm)[1]**	**Sc (Ø)[2]**	**I (%)[1]**	**AI (%)[1]**	**DAI (%)[1]**
Control: *D. corticola* vs. dsdw	5.27 ± 0.22 a	−0.09 ± 0.08 a	3.48 ± 1.48 a	−86.46 ± 7.44 a	0.16 ± 1.54 a
Bt1: *D. corticola* vs. *B. amyloliquefaciens* 1.42×10^9 CFU/ml	2.21 ± 0.11 b	0.65 ± 0.13 b	42.57 ± 2.34 b	11.94 ± 3.72 b	29.86 ± 2.41 b
Bt2: *D. corticola* vs. *B. amyloliquefaciens* 1×10^8 CFU/ml	2.72 ± 0.06 c	1.27 ± 0.11 c	37.96 ± 1.88 b	0.94 ± 3.41 b	33.69 ± 1.95 b
Bt3: *D. corticola* vs. *B. amyloliquefaciens* + *B. mojavensis* 1×10^8 CFU/ml	2.54 ± 0.04 bc	0.95 ± 0.05 b	39.15 ± 1.74 b	7.00 ± 2.04 b	31.66 ± 1.26 b

Note: Small letters (a–c) denote significant differences (p-value <.05). Post hoc analysis: [1]Dunn's test; [2]LSD. Mean values and standard error are shown.

Abbreviation: dsdw, double-sterilized deionized water.

Bacillus spp. resulted in lower values of AI than those reported by Campanile et al. (2007) (Table 1), which could mean that the fungal antagonists tested by Campanile et al. (2007) may be more effective in competition for space or nutrients than the bacteria tested in the present work.

Monitoring of dual fungus-bacteria cultures until the 22nd day after initiating the experiment indicated that the fungus and bacteria did not make physical contact, whereas control dishes were completely covered by dense mycelium (Figure 1c). Moreover, fungal colonies sometimes exhibited the formation of a thick mycelial barrier adjacent to the antagonist (Figure 1c.c3). These observations indicate the possible production of antifungal compounds (antibiosis) by the bacteria, rather than direct competition for space. The antibiosis hypothesis deserves more study in order to identify the compounds that reduce vegetative growth of *D. corticola* in presence of the studied bacteria. This knowledge, together with in vivo assays, would clarify the potential of commercial *Bacillus* spp. as suitable agents in the biocontrol of Botryosphaeria canker.

In this bioassay neither fungi nor bacteria showed any growth in PDAc, demonstrating the toxic effect of the copper salt at the assayed dose. Jeyaraman and Robert (2018) reported that a mixed treatment of *B. amyloliquefaciens* and copper oxychloride resulted in a moderate reduction of cankers caused by *Macrophoma theicola* Petch (percentage of lesion length reduction in *Camellia* sp. >16%). In that study, mixed applications provided similar lesion reduction when compared with the use of copper salt and bacteria separately. Consequently, synergistic effects between copper salt and bacteria seem barely noticeable, although future studies in vitro are required to identify whether lower doses of copper salt could reduce fungal growth allowing bacteria proliferation. On the other hand, Serrano et al. (2015) assayed copper-calcium sulphate against *D. corticola* and reported that doses below 10 mg/L stimulated mycelial development in vitro in contrast to the highest assayed dose (i.e. 1 g/L) that yielded >90% inhibition. This result agrees with our observations since we found complete inhibition of fungal and bacterial growth with the higher concentration of copper oxychloride (i.e. 5 g/L). These results suggest that tests on this copper-based compound against *D. corticola* should be extended to the field in order to characterize its actual efficacy under different disease pressures.

This study reveals the promising role as biocontrol agents of the three analysed bacillus-based treatments. Future research should be focused on clarifying the mechanisms driving the observed antagonisms (antifungal compounds, competition by nutrients, etc.) as well as supporting current results with in planta bioassays. Once the efficacy of treatment was validated, specificity of product (since other mutualistic or commensal fungi—e.g. endophytes—could be potentially affected), optimal dose and most suitable application method (foliar or trunk lesion application, rhizosphere treatment, etc.) should be evaluated.

ACKNOWLEDGEMENTS

This work was partially funded by the Generalitat de Catalunya, Departament d'Acció Climàtica, Alimentació i Agenda Rural (formerly Departament d'Agricultura, Ramaderia, Pesca i Alimentació), and the Diputació de Barcelona, Àrea de Territori i Sostenibilitat. Reagents for treatments Bt2 and Bt3 were generously supplied by the company Probelte S.A.U. Authors specially thank Dr. Artur Alves (Universidade de Aveiro, Portugal) for his expert advice and for providing the fungal strain used in the study.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

Alves, A., Correia, A., Luque, J., & Phillips, A. (2004). *Botryosphaeria corticola*, sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia*, *96*(3), 598–613. https://doi.org/10.2307/3762177

Campanile, G., Ruscelli, A., & Luisi, N. (2007). Antagonistic activity of endophytic fungi towards *Diplodia corticola* assessed by in vitro and in planta tests. *European Journal of Plant Pathology*, *117*(3), 237–246. https://doi.org/10.1007/s10658-006-9089-1

Jeyaraman, M., & Robert, P. S. A. (2018). Bio efficacy of indigenous biological agents and selected fungicides against branch canker disease of (*Macrophoma theicola*) tea under field level. *BMC Plant Biology*, *18*(1). https://doi.org/10.1186/s12870-018-1445-8

Luque, J., Pera, J., & Parladé, J. (2008). Evaluation of fungicides for the control of *Botryosphaeria corticola* on cork oak in Catalonia (NE Spain). *Forest Pathology*, *38*(3), 147–155. https://doi.org/10.1111/j.1439-0329.2007.00526.x

Martínez-Álvarez, P., Fernández-González, R. A., Sanz-Ros, A. V., Pando, V., & Diez, J. J. (2016). Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings. *Biological Control*, *94*, 1–10. https://doi.org/10.1016/j.biocontrol.2015.11.011

Przybył, K. (2003). Effect of *Pseudomonas* spp. on inoculation of young plants of *Fraxinus excelsior* stem with *Diplodia mutila*. *Dendrobiology*, *50*, 29–32.

Serrano, M. S., Romero, M. A., Jiménez, J. J., De Vita, P., Ávila, A., Trapero, A., & Sánchez, M. E. (2015). Preventive control of Botryosphaeria canker affecting *Quercus suber* in southern Spain. *Forestry*, *88*(4), 500–507. https://doi.org/10.1093/forestry/cpv016

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Muñoz-Adalia, E. J., Meijer, A., Campillo-Brocal, J. C., & Colinas, C. (2021). Antagonistic effect in vitro of three commercial strains of *Bacillus* sp. against the forest pathogen *Diplodia corticola*. *Forest Pathology*, 51, :e12711. https://doi.org/10.1111/efp.12711