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# Evaluation of *Bacillus amyloliquefaciens* as a biocontrol agent against oak decline disease in *Quercus* trees

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## Abstract

*Aim of study:* This study aimed to investigate the biocontrol potential of *Bacillus amyloliquefaciens* against *Phytophthora cinnamomi* infection in *Quercus suber* (cork oak). Both *in vitro* and *in planta* experiments were conducted to assess the effectiveness of *B. amyloliquefaciens* as a biocontrol agent.

*Area of study:* The microorganism strains, *B. amyloliquefaciens* and *P. cinnamomi*, as well as the embryogenic lines of *Q. suber* used, have a Spanish origin.

*Materials and methods: In vitro* experiments involved evaluating the inhibitory effects of *B. amyloliquefaciens* on *P. cinnamomi* growth through dual-inoculated agar plates. *In planta*, dual inoculation tests were performed by co-inoculating plantlets with both *P. cinnamomi* and *B. amyloliquefaciens*. Physiological parameters, such as photosynthetic activity, chlorophyll content, and oxidative stress markers, were measured. All experiments were conducted under controlled conditions.

*Main results: In vitro* experiments revealed the inhibitory effects of *B. amyloliquefaciens* on *P. cinnamomi* growth. Infected plantlets displayed symptoms of root infection. Dual inoculation tests resulted in plant survival against *P. cinnamomi* infection. Analysis of physiological parameters indicated variations among treatments and clones, highlighting the distinct response of *Q. suber* plantlets to the pathogen and underscoring the importance of genetic variability for disease management.

*Research highlights:* This study provides insights into the potential of a strain of *B. amyloliquefaciens* as a biocontrol agent against *P. cinnamomi* infection in cork oak. Further investigations are warranted to elucidate the underlying mechanisms of susceptibility and resistance in different clones of *Q. suber*.

**Additional key words:** *Phytophthora cinnamomi*; cork oak; growth inhibition; dual inoculation; physiological parameters.

**Abbreviations used:** C (control); FAA (free amino acids), Fv/Fm (maximum quantum efficiency of PSII photochemistry), NBT (nitro blue tetrazolium chloride), PDA (potato dextrose agar), phy (*Phytophthora cinnamomi*), PIabs (photosynthetic performance index), PRGI (percentage of radial growth inhibition), ROS (reactive oxygen species), SPAD (soil plant analysis development), STA (total solubilized starch), TFL (total flavonoids), TPC (total phenolic compounds), TSS (total soluble sugars).

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# Introduction

Since the early 1980s, the oak decline disease, also known as Quercus decline and "Seca", has emerged as a significant concern in the Iberian Peninsula. This disease primarily affects trees belonging to the *Quercus* genus, including the cork oak (*Quercus suber* L), and its damaging effects resemble those caused by drought. The impact of oak decline disease has been substantial, leading to the decline of thousands of tree specimens and adversely affecting forest ecosystems (Tuset et al., 2001; García, 2016).

Extensive research has identified *Phytophthora cinnamomi* Rands, an oomycete pathogen, as the primary cause of oak decline disease in *Quercus* species (Tapias et al., 2006). However, chemical control methods for this disease present challenges due to the oomycetes' ability to develop resistance and the potential environmental impacts associated with such control measures (de Andrade Lourenço et al., 2022). Consequently, there is a pressing need to explore alternative approaches that effectively manage the disease while minimizing environmental harm.

By integrating genotype selection and harnessing the symbiotic interactions between microorganisms and *Quercus* species, sustainable strategies to combat oak decline disease can be implemented. In recent years, somatic embryogenesis has demonstrated successful outcomes in generating clones of *Q. suber*, as evidenced in the existing literature (García-Martín et al., 2001; Pintos et al., 2010; Gomez-Garay et al., 2014; Testillano et al., 2018). Thus, we have opted to employ this technique in our laboratory to endeavour the selection of the most resilient genotypes.

The use of plant tissue cultures, specifically through the application of somatic embryogenesis, provides a promising avenue for identifying and propagating disease-resistant genotypes of *Q. suber*. By subjecting plantlets cultivated *in vitro* from somatic embryos to pathogen inoculation in the rhizosphere, potential effects on the plants can be assessed. This technique allows for the study of various genotypic clones, enabling the determination of their susceptibility or tolerance to the pathogen. The results obtained from these tests can help identify genotypes that exhibit higher tolerance levels than others, thus opening doors for further research into these selected genotypes.

Genetic improvement of *Quercus* species for the resistance or tolerance to *P. cinnamomi* is a practical medium to long-term strategy in forestry management, especially in highly affected areas designated for reforestation. In addition to the use of antagonistic organisms such as mycorrhizae (Tapias et al., 2006) and other microorganisms, biological control of plant diseases involves the inhibition of plant pathogen populations through the utilization of living organisms such as bacteria, fungi, protozoa, or their metabolites. Currently, only a few biofungicides are available to inhibit the growth of *P. cinnamomi*, with the utilization of specific strains of the *Bacillus* spp. being a subject of study (de Andrade Lourenço et al., 2022).

Recent scientific evidence highlights the plant-protective properties of *Bacillus amyloliquefaciens* (González, 2019). This gram-positive aerobic bacterium, capable of surviving under certain anaerobic conditions, is widely distributed in soil and water. It is recognized as a prominent biological control agent, producing active compounds such as extracellular enzymes and lipopeptides (e.g., surfactin, fengycin, and iturin). These compounds, acting as biosurfactants, play a crucial role as antifungal agents by disrupting the cell walls of fungi and oomycetes such as *P. cinnamomi* (Silva et al., 2017). Furthermore, the genus *Bacillus* facilitates the solubilization of mineral plant nutrients in the soil, thereby increasing their availability to plants and promoting their growth (González, 2019).

Consequently, this study aims to evaluate the effectiveness of *B. amyloliquefaciens* as a biocontrol agent against *P. cinnamomi* in selected genotypes of *in vitro*-generated *Q. suber* plants using somatic embryogenesis. The objective is to contribute to the control of oak decline disease and gain a deeper understanding of the biocontrol potential of *B. amyloliquefaciens* in the context of oak decline disease management.

# Material and methods

## Maintenance and cultivation of *Phytophthora cinnamomi*

A strain obtained from Tragsa-Maceda (project TSA0069316) was used for the pathogen cultivation. The strain was grown on Petri dishes containing potato dextrose agar (PDA) at temperatures ranging from 25ºC to 30ºC under dark conditions. To assess the growth and morphology of the colonies, staining techniques were employed. Colonies grown for 5 to 10 days were stained with cotton blue (a few drops of 0.1% cotton blue solution, Sigma-Aldrich Mfcd00146574, were added to cover the samples and coverslips were gently placed over the samples) and observed under an optical microscope.

## Maintenance and cultivation of *Bacillus amyloliquefaciens*

A strain obtained from the FiVe-A research group at UCM (No. 970861) was used as the biocontrol organism. For its cultivation, the strain was grown on Petri dishes using a solid medium (PDA) at room temperature. To assess the morphology and characteristics of the potential biocontrol agent, staining techniques were employed. Staining with cotton blue was performed, and the samples were observed under an optical microscope.

## Dual plate confrontation between pathogen and biocontrol organism

To determine the effectiveness of the biocontrol agent in reducing pathogen growth, dual plate confrontations were conducted on PDA medium. To determine if there was antagonism between the organisms two assays were conducted.

The first experiment evaluated the inhibitory effects of volatile compounds using divided Petri dishes containing PDA medium, with two series, each comprising three replicates.

The second experiment focused on assessing soluble compounds' inhibitory effects on oomycete growth, utilizing undivided Petri dishes containing PDA medium. A circular disk measuring 1 cm in diameter and colonized by the potential antagonist, *B. amyloliquefaciens*, was placed in the middle of the plate, while a circular disk (1 cm in diameter) and colonized by the oomycete was placed on one side at a distance of 2 cm. As a control, plates containing only the pathogen disk were prepared in the same position. The pathogenic oomycete's radial growth towards the plate's center was evaluated daily for one week. The percentage of radial growth inhibition was calculated according to the formula (Ezziyyani et al., 2004):

$$PCIR = (R1 - R2) / R1 \times 100$$

where PCIR is the percentage of radial growth inhibition; R1 is the radial growth of the pathogen in the control plate; and R2 is the radial growth of the pathogen in the confrontation.

## Antibiosis assay with soluble compounds

A liquid culture (nutrient broth) of *B. amyloliquefaciens* was prepared, approximately 30 mL ($3*10^7$ CFU/mL) of which was centrifuged and subsequently filter-sterilized. The obtained extract was used to test whether *P. cinnamomi* could grow in the presence of the extract and the absence of the bacteria.

In the center of a Petri dish containing PDA medium, a 0.5×0.5 cm square of mycelium of *P. cinnamomi* was placed, and small wells were made on each side of the dish on the medium where the extract was dispensed. The same experiment was also conducted by placing extract-impregnated filter paper discs on the medium. Three experiments were conducted with three replicates each.

## Plant material

For the plant trial, embryos from two genotypes (ROP7B-763/02 and ADB1A-2/06) derived from somatic embryogenesis lines were selected. Mother tree ROP7B had been selected for cork production and quality by the Institute of Cork, Wood, and Charcoal (ICMC, Regional Government of Extremadura, Spain) and ADB1A for productivity, health status, and cork quality by the Agroforestry Department of the company Transformaciónn Agraria SA (TRAGSA). These clones were obtained from the project "Development of an *in vitro* test for the evaluation of tolerance to *P. cinnamomi* in holm oak and cork oak genotypes through Micropropagation" (Ref. TSA0069316. Entity: Ministry of Agriculture, Fisheries, and Food, Subdirectorate General of Forest Policy. National Program for Conservation and Improvement of Genetic Resources of holm oak and cork oak against the Oak Decline Syndrome. FEADES. Manager: TRAGSA).

The cork oak somatic embryos were grown in Sommer et al. (1975) medium with the addition of 500 mg/L glutamine, separated by genotype lines. Within each line, embryos that had clearly developed an embryonic axis and cotyledons were selected, specifically cotyledonary embryos ranging from 3 to 5 mm in size. These selected cotyledonary embryos were transferred to a maturation medium consisting of Sommer et al. (1975) basal medium supplemented with 10 g/L of activated charcoal. The pH was adjusted to between 5.5 and 5.7, followed by autoclaving at 121ºC and a pressure of 1 kg/cm² for 20 minutes. The selected somatic embryos were placed in groups of 5 or 6 per plate, with the axis always in contact with the medium. These embryos belonged to the same clone and were approximately the same size. They remained in this medium for a total of three months, spending the first month at 25ºC and the following two months in cold storage at 4ºC.

After the maturation process, the embryos were expected to be mature cotyledon embryos measuring between 1.5 and 2 cm. The embryos were kept in sterile distilled imbibition water for 24 hours before being sown in culture tubes with a germination medium. The germination medium consisted of Sommer et al. (1975) basal medium supplemented with 50 µL of 6-benzylaminopurine from a 1 mg/mL solution and 1 mL of indole-3-butyric acid from a 0.1 mg/mL solution as growth regulators. The pH was adjusted to between 5.5 and 5.7, followed by autoclaving at 121ºC and a pressure of 1 kg/cm² for 20 minutes.

## Inoculation of *Quercus suber* plantlets with *Phytophthora cinnamomi*

Six plantlet pairs of the same genotype, exhibiting similar morphological characteristics, were carefully selected to avoid potential phenotypic effects. Each plantlet pair was treated as a replicate, and the entire experiment was repeated twice for robustness and consistency. One plantlet from each pair was designated as the control, while the other was inoculated with the pathogen, ensuring subsequent comparison between the two groups. The

plantlets were removed from the germination medium and placed in DeWit® tubes.

The homogenate for inoculation was prepared from *P. cinnamomi* grown for a week on a PDA plate at 27±2ºC under dark conditions. A square piece of mycelium measuring 1.5 × 1.5 cm was cut from the plate and transferred to a sterile tube containing 25 mL of sterile distilled water. After thorough homogenization with water, the plantlets were inoculated. The inoculation process entailed placing 500 µL of the *P. cinnamomi* inoculum in direct contact with the plantlets' roots within the tubes to induce infection and 500 µL of sterile distilled water were added in the tubes containing control plantlets. The remaining homogenate was spread on PDA plates to assess the viability of the inoculum by observing its growth over the course of the plantlet infection.

After 5 days of growing in the plant culture chamber, the plantlets were assessed once the corresponding inoculations have been performed.

## Cross inoculation with *Bacillus amyloliquefaciens* and *Phytophthora cinnamomi*

Plantlets were grown in sterile glass jars on a hydroponic medium (vermiculite as support), with two groups of three plants for each genotype, and the experiment was replicated twice. On the day of transplantation, they were watered with sterile distilled water and sealed with parafilm. The plantlets were kept in a growth chamber under a photoperiod of 16 hours of light and 8 hours of darkness at a temperature of 20±2°C. Each genotype was subjected to one of the three following treatments: the treatment group involves the application of *B. amyloliquefaciens* before *Phytophthora* inoculation (*phy/bac*) while the other two groups act as controls, with one remaining untreated (*C*) and the other being exposed solely to the pathogen (*phy*).

Around 10-15 days after transplanting the plantlets into vermiculite, inoculation with *B. amyloliquefaciens* was performed. A total of 3 mL of *B. amyloliquefaciens* ($10^8$/mL CFUs) culture broth was directly poured onto the vermiculite, near the base of the plantlet. Fifteen days after the inoculation with *B. amyloliquefaciens*, infection with *P. cinnamomi* was initiated, and an infection control group was established using the homogenate of pure-cultured pathogen mycelium as described previously. The plantlets were kept in the growth chamber for one month, during which signs of infection began to appear.

## Evaluation of disease severity

The evaluation included comparing the inoculated plantlets with untreated control plantlets to determine the effects on vigor, growth, and infection symptoms, following the Diagnostic Protocols for Regulated Pests: *P. cinnamomi* (EPPO, 2004). Infected roots exhibited symptoms such as a darkened and necrotic appearance that was analyzed using the ImageJ® software to calculate the percentage of infected roots.

## Physiological measurements

*Measurement of chlorophyll content/greenness*

The chlorophyll content, measured as leaf greenness, was determined in one leaf from each plant using the SPAD index (Soil Plant Analysis Development; 502 PLUS KONICA MINOLTA). The index quantitatively assesses the leaf's green intensity based on measurements of light transmission at a wavelength of 650 nm (chlorophyll molecule absorption) and at a wavelength of 940 nm (where there is no absorption).

*Measurement of photosynthetic activity*

The fluorescence of one leaf from each plant was measured using HANDY PEA+. Data on the parameters of maximum quantum efficiency of PSII (Fv/Fm) and the photosynthetic performance index (PIabs) were recorded.

*Detection of superoxide anion by staining with nitroblue tetrazolium (NBT)*

A representative leaf (a leaf from each plant that possesses a phenotypic consistent with the majority of leaves on the same plant, chosen for the purpose of obtaining measurements or data that accurately reflect the typical physiological status of the plant) selected from each plant, where greenness and photosynthetic activity were measured, was finally used to detect the presence or absence of superoxide anion.

The protocol followed was described by Monteoliva et al. (2019). The selected leaf was incubated in a 0.25% NBT solution (0.2 g in 80 mL phosphate buffer) for 2 hours in darkness. After the incubation time, the NBT solution was removed and replaced with 96% ethanol to stop the reaction by incubating at 95°C for 5 minutes. Once the reaction was stopped, the next step was to remove chlorophyll from the leaves by immersing them in a solution of 96% ethanol/acetic acid (3:1). They should be kept in this solution until they lose their green color. The leaves were then washed and preserved in 70% ethanol. The leaves with formazan deposits were photographed, and the relative area, compared to the total area of each leaf, was quantified using ImageJ® software.

*Quantification of infection and metabolism markers*

The protocol described by López-Hidalgo et al. (2021) was used to obtain measurements of photosynthetic

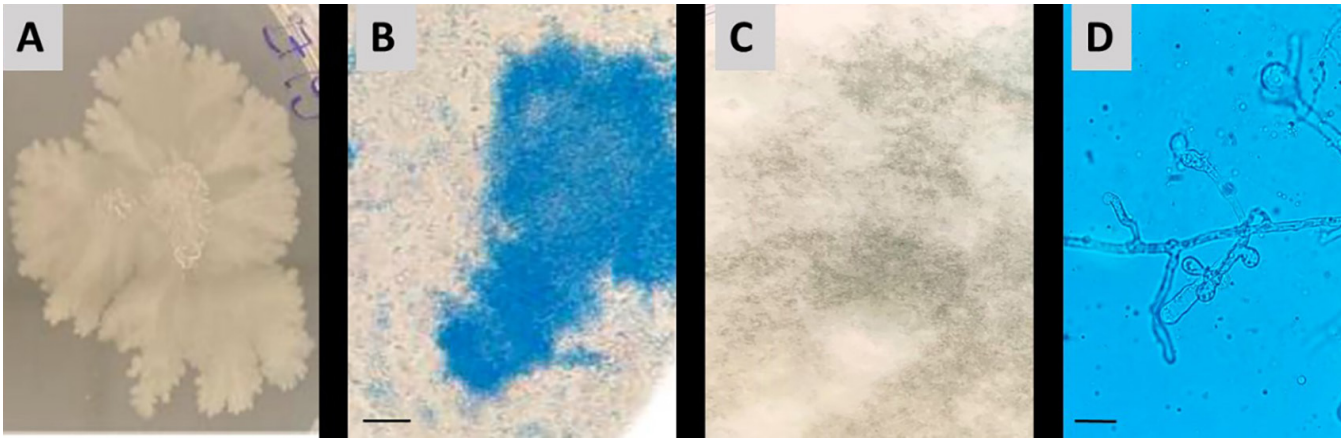


**Figure 1.** *Bacillus amyloliquefaciens* colony growing in PDA medium (A). Image of a culture of *B. amyloliquefaciens* in liquid medium stained with cotton blue, captured using an optical microscope at 100x magnification (B). *Phytophthora cinnamomi* exhibiting cottony mycelium growth in PDA medium (C). Images captured by optical microscopy showing the mycelium of *P. cinnamomi* grown on PDA medium stained with cotton blue at 100x magnification (D). Scale bar: 10 µm.

pigments, malondialdehyde, total phenolic compounds (TPC), total flavonoids (TFL), free amino acids (FAA), total soluble sugars (TSS), and total solubilized starch (STA) markers.

### Statistical analysis

A one-way analysis of variance (ANOVA) was employed to compare the means of the various treatment variables. The STATISTICA® software was utilized to conduct the statistical analysis. To demonstrate the grouping and differences among treatments, the Duncan test was applied.

## Results

### Dual confrontation assay between *Phytophthora cinnamomi* and *Bacillus amyloliquefaciens*

Both microorganisms, the pathogen *P. cinnamomi* and the potential biological control agent *B. amyloliquefaciens*, were easily identifiable due to their distinct growth patterns in the PDA medium, both visually and under the optical microscope (Fig. 1). The bacterial colony appears opaque with a rough, matted surface and has an irregular, lobed edge (Fig. 1A, B). The mycelium of *P. cinnamomi* is cottony in appearance (Fig. 1C) and under the microscope, it exhibits a siphonaceous or coenocytic structure. It appears torulose or verrucose, moderately branched, with abundant hyphal swellings (Fig. 1D).

During the dual confrontation of *P. cinnamomi* and *B. amyloliquefaciens* growing in divided Petri dishes, none of the replicates exhibited signs of inhibition caused by volatile compounds released by *B. amyloliquefaciens*. In all cases, the oomycete mycelium developed normally within the expected time frame (Fig. 2).

Our results demonstrated that *B. amyloliquefaciens* was capable of inhibiting (Fig. 3) or halting the growth of *P. cinnamomi* by attacking its hyphae upon contact. *P. cinnamomi* was unable to survive once it came into contact with *B. amyloliquefaciens*. The percentage of radial growth inhibition (PRGI) yielded a result of 64.68%.

The results of the antibiosis assay with *B. amyloliquefaciens* extract showed that after 5 days of growth, the mycelium reached the wells or the discs impregnated with the extract, growing around and above them (Fig. 4). Three days later, complete mycelial growth was observed throughout the plate. It was not possible to confirm whether the extract could inhibit mycelial growth.

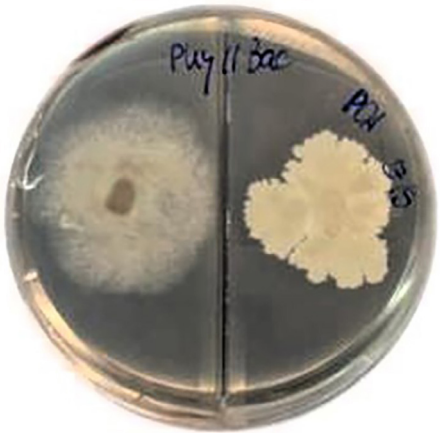

**Figure 2.** Dual plate confrontations on divided PDA medium. The left side of the plate shows the unaffected growth of the oomycete, while on the right side, colonies of *B. amyloliquefaciens* are observed. The image represents the growth after 5 days.

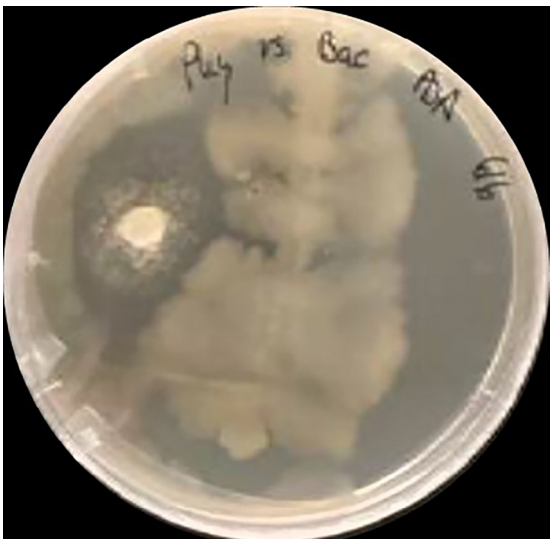


**Figure 3.** Plate confrontation on PDA medium between *P. cinnamomi* and *B. amyloliquefaciens*. Confrontation has grown for 5 days.

## *In vitro* tolerance test of *Q. suber* to *Phytophthora cinnamomi*

Five days after inoculation, the plantlets were removed from the DeWit tubes in a laminar flow cabinet, and the symptoms were recorded. Significant differences were observed between the inoculated plantlets and the controls. The most prominent difference was the occurrence of necrosis at the root tips of the infected plantlets, characterized by a black or dark brown coloration. In the control plantlets, root growth was observed, with nearly 100% of the roots displaying a healthy appearance and a white color. In contrast, the plantlets infected with *P. cinnamomi* showed symptoms of infection of their roots, which were black in color and necrotic in appearance (Fig. 5). After the inoculation experiment was completed, the remaining inoculum was plated on PDA plates to assess its viability and potential for infection.

The analysis of the proportion of infected roots was performed using the ImageJ® software, where the entire root was defined as 100%. This allowed to calculate the percentage of symptomatic root, which was 74.5% for the ROP7B-763/02 clone and 99.3% for the ADB1A-2/06 clone.

## Dual inoculation test with *P. cinnamomi* and *B. amyloliquefaciens*

None of the plants died due to *P. cinnamomi* infection during the experiment. The death of some older leaves was observed in several plants, which is normal for *in vitro* cultivated plants. The persistence of cotyledons for an extended period or early necrosis of cotyledons was also observed among both genotypes (Fig. 6).

The growth rate was used to evaluate the root growth, and overall, we observed that in the control plantlets, the roots grew an average of 18.6% ± 2.04%. In contrast, the infected roots grew an average of 8.89% ± 1.54%, and the plant with dual inoculation 26.3% ± 1.39%. There are significant differences between the two clones. While the infected plantlets of ROP7B-763/02 with *P. cinnamomi* experienced a 55% ± 4.08% decrease in the root growth rate, in the case of ADB1A-2/06, it was a 38.1% ± 1.04% decrease. On the other hand, the root growth rate in the plants subjected to dual inoculation was a 10.7 ± 0.33% increase for the ROP7B-763/02 plants, while the ADB1A-2/06 plants showed an 18.5% ± 2.45% increase.

## Photosynthetic pigments content and photosynthesis assessment

The chlorophyll measurements taken with the SPAD meter did not show significant differences for the ROP7B-763/02 clone between the treatments. However, in

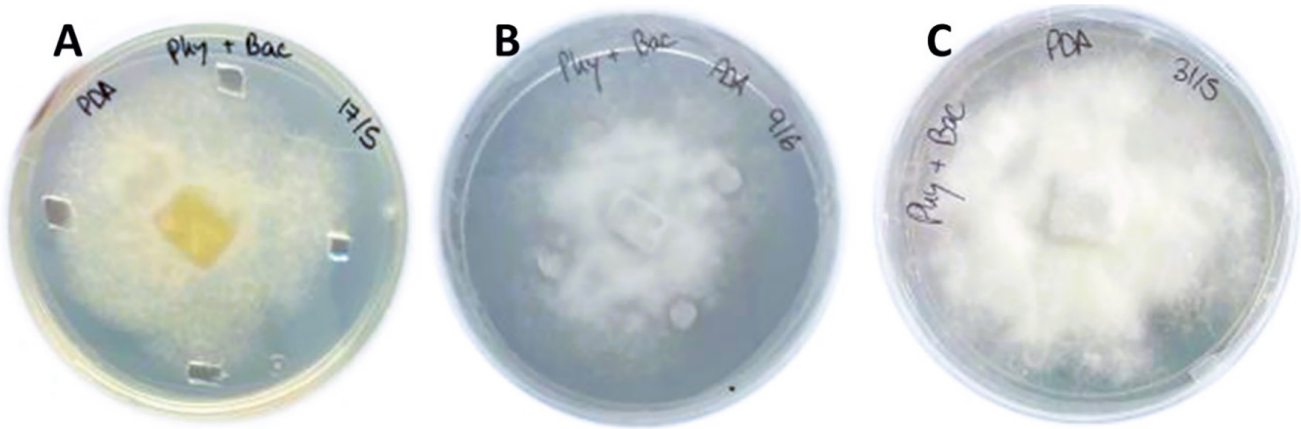


**Figure 4.** Antibiosis assay conducted with *P. cinnamomi* and filter-sterilized extract from *B. amyloliquefaciens* culture medium: Assay with wells containing extract grown for 5 days (A); assay with filter paper discs impregnated with the extract grown for 5 days (B); assay with filter paper discs impregnated with the extract grown for 7 days (C).

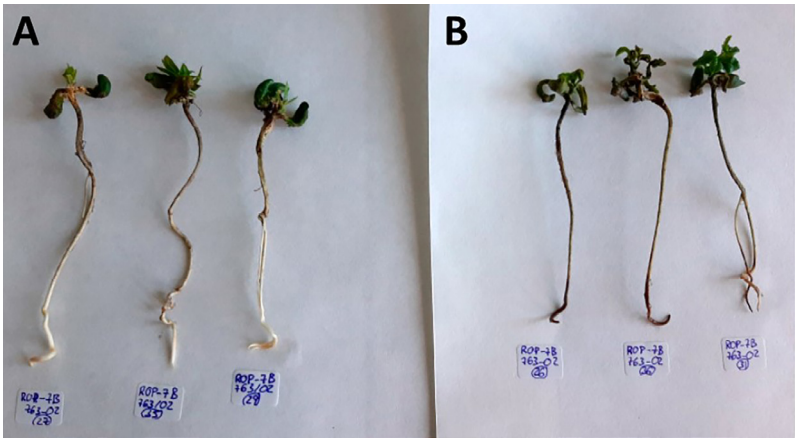


**Figure 5.** Control and *P. cinnamomi*-inoculated *Q. suber* plantlets: Control treatment of ROP7B-763/02 clone (A); ROP7B-763/02 inoculated plantlets showing infection symptoms (necrosis) in the roots (B).

the case of the ADB1A-2/06 clone, there was a significant reduction of approximately 50% in the treatment with *P. cinnamomi* and a slightly lower reduction of around 27% in the case of dual inoculation. The total chlorophyll content showed a reduction of approximately 25% for the plants inoculated with *P. cinnamomi*, regardless of the clone. However, there was a difference for the plants subjected to dual inoculation. The ROP7B-763/02 clone exhibited an increase of almost 30% compared to the control, while the ADB1A-2/06 clone showed a reduction of around 10%.

Regarding carotenoid content, ROP7B-763/02 plants showed a 10% increase in the treatment with *P. cinnamomi* and nearly a 40% increase in the double inoculation treatment. However, no significant differences in carotenoid content were observed for ADB1A-2/06 plants subjected to the different treatments.

In regards to the fluorescence results, two parameters were measured: the maximum quantum efficiency of PSII photochemistry (Fv/Fm) and the photosynthetic performance index (PIabs). No significant differences were detected between the treatments for Fv/Fm, and there was a slight decrease in PIabs in ROP7B-763/02 plants inoculated with *P. cinnamomi*.

## Measurement of oxidative stress

The percentage of blue-stained leaves represents the number of crystals formed due to the presence


**Figure 6.** Growth, infection, and analysis process of ADB1A-2/06 genotype plantlets: germinated embryo in a DeWit tube on agar medium showing the aerial part (A); plantlet transplanted into vermiculite medium inside a sterile glass jar (B); plantlet growing on vermiculite with newly inoculated *Phytophthora cinnamomi*, surrounded by an agar piece containing the mycelium (C); and plantlet treated with the biocontrol agent and the oomycete after one month of inoculation (D).

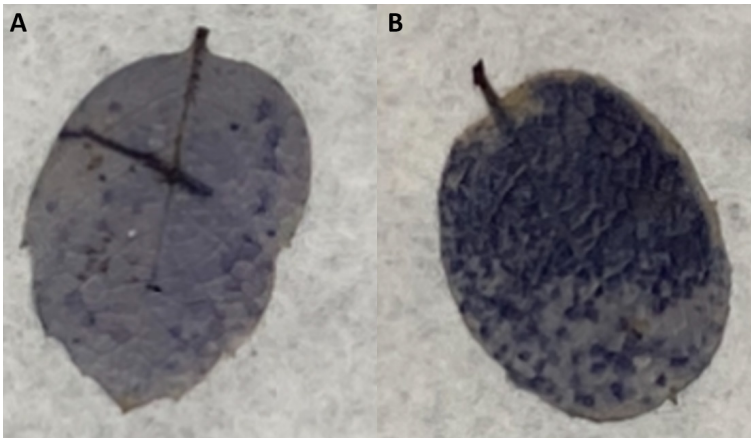


**Figure 7.** Images of cork oak leaves stained with NBT to assess oxidative stress: ROP7B-763/02 clone (A) and ADB1A-2/06 clone (B).

of the superoxide anion (Fig. 7). The value for the *P. cinnamomi* treatment was higher than for the control, and in ROP7B-763/02 genotype the treatment involving both organisms had a higher value compared to the treatment with the oomycete alone. Nevertheless, for the ADB1A-2/06 genotype, the control value was higher than in the other two treatments.

### Quantification of metabolism markers

Table 1 presents the values of various markers including FAA, TFL, TPC, TSS, and STA. The measurements were taken for each clone (ADB1A-2/06 and ROP7B-763/02) and under different treatments: *C*, *phy*, and *phy/bac*.

The variations observed provide insights into the potential metabolic responses of the plants to *P. cinnamomi* infection and the application of *B. amyloliquefaciens* as a biocontrol agent at free amino acids and flavonoids content but not at total phenolic compounds content and carbohydrates metabolism.

The combined treatment with *B. amyloliquefaciens* and *P. cinnamomi* (*phy/bac*) samples of ROP7B-763/02 clones showed higher levels of FAA compared to the control samples.

The TFL levels were relatively similar among the different clones and treatments, with only slight variations observed. Thus, no clear trend or significant differences can be inferred from these results. Interestingly, the *phy/bac* treatment showed the highest TPC levels in both clones, indicating a potential interaction between *P. cinnamomi* and *B. amyloliquefaciens* in enhancing flavonoids production. The TPC levels varied significantly among the clones. The ROP7B-763/02 clone exhibited higher TPC levels compared to the ADB1A-2/06 clone, regardless of the treatment.

The TSS levels varied across the clones and treatments, but no consistent pattern can be elucidated from the results. The STA levels were relatively consistent among the clones and treatments, with no significant differences observed.

## Discussion

Understanding the interactions between beneficial microorganisms and plant pathogens is crucial for the development of sustainable strategies for disease control in forestry. In this study, we aimed to investigate the potential of *B. amyloliquefaciens* as a biocontrol agent against *P. cinnamomi* infection in cork oak (*Quercus suber* L.). The objective was to evaluate whether *B. amyloliquefaciens* could help mitigate the spread of the disease known as *Quercus* decline or cork oak wilt, which is currently causing significant damage in Spain and Portugal, with devastating consequences. A series of experiments were conducted, both *in vitro* and *in planta*, to assess the biocontrol potential of *B. amyloliquefaciens*.

For the *in vitro* experiments, the distinct growth patterns of both microorganisms in the PDA medium facilitated their differentiation, *B. amyloliquefaciens* colonies exhibited opaque structures with rough, matted surfaces and irregular edges, while *P. cinnamomi* displayed cottony mycelium growth.

Several researchers have found evidence of inhibition by volatile compounds synthesized by *B. amyloliquefaciens* (Méndez-Bravo et al., 2018; González, 2019; Wang et al., 2022). However, in our evaluation of volatile compounds released by *B. amyloliquefaciens*, no inhibitory effects on the mycelial growth of *P. cinnamomi* were observed. The oomycete mycelium developed normally within the expected time frame, suggesting that volatile compounds could be not effective in inhibiting the growth of the pathogen. In contrast, soluble compounds produced by *B. amyloliquefaciens* demonstrated inhibitory effects on the growth of *P. cinnamomi*. *B. amyloliquefaciens*, along with

**Table 1.** Summary of the metabolism markers detected in different *Quercus suber* clones and treatments. The measurements are presented in micrograms per milligram of dry weight (µg/mg DW). Free amino acids (FAA), total flavonoids (TFL), total phenolics content (TPC), total soluble sugars (TSS), and starch (STA). *Phytophthora cinnamomi* (phy), and *Phytophthora cinnamomi* combined with *Bacillus amyloliquefaciens* (phy/bac).

| | **Control** | | ***phy*** | | ***phy/bac*** | |
|---|---|---|---|---|---|---|
| | **ADB1A-2/06** | **ROP7B-763/02** | **ADB1A-2/06** | **ROP7B-763/02** | **ADB1A-2/06** | **ROP7B-763/02** |
| FAA | 9.15±1.99 | 5.37±1.17 | 9.1±1.95 | 4.68±1.01 | 10.13±2.21 | 8.07±1.75 |
| TFL | 2.82±0.11 | 2.73±0.11 | 2.89±0.12 | 2.78±0.10 | 3.04±0.12 | 2.98±0.13 |
| TPC | 20.69±6.09 | 40.1±11.81 | 16.54±4.81 | 47.30±13.04 | 18.69±5.50 | 45.53±12.02 |
| TSS | 96.99±10.06 | 85.67±8.88 | 94.41±9.77 | 73.31±7.60 | 83.73±8.65 | 73.31±7.98 |
| STA | 85.48±2.12 | 91.38±4.83 | 93.65±3.55 | 80.49±4.25 | 92.29±3.07 | 88.22±3.63 |

other bacteria of this genus such as *B. subtilis*, has been used as a biocontrol method against various *Phytophthora* species and on different plant species affected by these pathogens, including soybean, avocado, and tomato (Méndez-Bravo et al., 2018; Liu et al., 2019; Ley-López et al., 2022). There are limited studies on the biocontrol of this pathogen in *Q. suber* using *B. amyloliquefaciens*. González (2019) tested several strains of *B. amyloliquefaciens in vitro* and in plants of *Quercus ilex*, *Q. suber*, and *Castanea sativa*, obtaining excellent *in vitro* results as these strains were able to reduce mycelial growth. The pathogen's mycelium was unable to survive upon contact with *B. amyloliquefaciens*, as the bacterium attacked the hyphae, ultimately halting its growth. The PRGI was calculated to be 64.68%, indicating a substantial inhibitory effect of the biocontrol agent on the pathogen. Other researchers, such as De Dios et al. (2020), also conducted *in vitro* antagonistic assays using various soil organisms, including *B. amyloliquefaciens*, against *P. cinnamomi*. They found that different strains of *B. amyloliquefaciens* exhibited inhibitory capacities ranging from 61.08% to 42.25%, which are lower than the results obtained in our study (64.68%). Additionally, an antibiosis assay was performed using filter-sterilized extracts from the culture medium of *B. amyloliquefaciens*. The mycelium of *P. cinnamomi* grew around and above the wells or discs impregnated with the extract, suggesting a limited and transient inhibitory activity of the extract over time.

*In planta* experiments were conducted to assess the response of the plantlets to *P. cinnamomi* infection. The infected plantlets exhibited symptoms of root infection, characterized by a distinct black coloration and a necrotic appearance. This visual observation was further validated by employing quantitative analysis using ImageJ® software. By defining the entire root length as 100%, it was possible to calculate the percentage of symptomatic roots, providing a more precise assessment of the extent of infection. The ROP7B-763/02 clone demonstrated a significant symptomatic root necrosis of 74.5%, indicating a considerable level of infection, while the ADB1A-2/06 clone exhibited an even higher symptomatic root proportion of 99.3%, suggesting a heightened susceptibility to *P. cinnamomi* infection. The results obtained in this study highlight the distinct response of *Q. suber* plantlets to the pathogen, emphasizing the importance of further investigations to elucidate the mechanisms underlying the varying levels of susceptibility observed among different clones. The significant genetic variability found within *Q. suber* genotypes plays a crucial role in combatting the rapid spread of this devastating disease. It offers the possibility of identifying individuals with diverse survival rates in severe "seca" outbreaks, thereby providing valuable tools for disease management and mitigation efforts (Tapias et al., 2006).

In the dual inoculation test with *P. cinnamomi* and *B. amyloliquefaciens*, none of the plants experienced mortality due to *P. cinnamomi* infection throughout the experiment. However, some older leaves displayed signs of death, which is commonly observed in *in vitro* cultivated plants. In other *in vitro* plant generation studies, such as with *Q. ilex*, it has been observed abundant phenol exudation and subsequent necrosis of tissues (Mauri & Manzanera, 2011). Additionally, it was observed that cotyledons either quickly necrosed or remained green throughout the process. This could be attributed to the fact that *in vitro* cotyledons are non-functional, which facilitates their atrophy, rapid degeneration, and necrosis (Mauri & Manzanera, 2011). The infected plantlets exhibited distinct symptoms in the roots, characterized by black coloration and a necrotic appearance.

To evaluate root growth, the growth rate was measured. The control plantlets exhibited an average root growth rate of 18.6%. In contrast, the infected roots showed a reduced average growth rate of 8.89%, while the plants subjected to dual inoculation displayed an increased growth rate of 26.3%. Notably, there were variations between the two clones. The infected ROP7B-763/02 plantlets experienced a 55% decrease in the root growth rate, whereas the ADB1A-2/06 plantlets exhibited a 38.1% decrease. Interestingly, in the case of dual inoculation, the ROP7B-763/02 plants showed a 10.7% increase in the root growth rate, while the ADB1A-2/06 plants displayed an 18.5% increase.

Undoubtedly, physical parameters are the most easily quantifiable as they can be visually assessed in the plant and, in many cases, may not necessarily result in plant destruction, thus providing potential benefits. However, generally, all infection processes are linked to changes in the plant's physiological parameters as a response to the presence of the pathogen (García, 2016).

Plant exposure to pathogen infection induces physiological and biochemical changes, including the impairment of photosynthesis, which directly damages the photosynthetic apparatus and reduces the photosynthetic rate (Elías, 2015). Based on the literature findings, we would expect a decrease in photosynthetic activity in the more susceptible genotypes to the infection, indicating higher stress levels. This would also lead to a reduction in chlorophyll content and leaf greenness (Navarro et al., 2009; San-Eufrasio et al., 2021). The assessment of photosynthetic pigment content and photosynthesis revealed notable differences among the treatments. In the ROP7B-763/02 clone, there were no significant variations in chlorophyll measurements between the treatments. However, the ADB1A-2/06 clone exhibited a significant reduction of approximately 50% in chlorophyll levels when treated with *P. cinnamomi*, and a slightly lower reduction of around 27% in the case of dual inoculation. Regardless of the clone, plants inoculated with *P. cinnamomi* showed a reduction of ~ 25% in total chlorophyll content. In contrast, the ROP7B-763/02 clone subjected to dual inoculation displayed an increase of almost 30% compared to the control, while the ADB1A-2/06 clone showed a reduction of ~ 10%. Carotenoid content showed variations among treatments and clones. The ROP7B-763/02 plants displayed a 10% increase in carotenoid content in the treatment with *P. cinnamomi* and a substantial 40% increase in the dual inoculation treatment. However, no significant differences in carotenoid content were observed for the ADB1A-2/06 plants under different treatments.

Fluorescence measurements assessed two parameters: the maximum quantum efficiency of PSII photochemistry (Fv/Fm) and the photosynthetic performance index (PIabs) which are commonly used as indicators of photochemical activity and stress response in plants (San-Eufrasio et al., 2021). No significant differences were detected between the treatments for Fv/Fm, and there was only a slight decrease in PIabs in ROP7B-763/02 plants inoculated with *P. cinnamomi*. The ROP7B-763/02 plants displayed a 10% increase in carotenoid content in the treatment with *P. cinnamomi* and a substantial 40% increase in the dual inoculation treatment. However, no significant differences in carotenoid content were observed for the ADB1A-2/06 plants under different treatments. Carotenoids play a crucial role in photoprotection, serving as a defense mechanism in plants against photooxidative damage. In response to diseases, plants enhance the production of carotenoids and other substances as a defensive measure (Elías, 2015).

Plants mount an oxidative burst as a response to biotic stresses. Upon recognition of pathogen-associated molecular patterns, plants undergo diverse physiological and biochemical changes to enhance their immune response. Reactive oxygen species (ROS) act as crucial secondary messengers that mediate downstream immune reactions (Wu et al., 2023). This process involves the transient generation of singlet oxygen species within various subcellular compartments, which are subsequently eliminated by antioxidant systems. The defense mechanisms regulated by ROS play a pivotal role and interact with other defense mechanisms (Tyagi et al., 2022).

To assess oxidative stress, the presence of the superoxide anion was evaluated by quantifying the percentage of blue-stained leaves. The superoxide anion represents one of the oxygen-derived free radicals (ROS) that impact plant physiology. It is catalyzed by superoxide dismutase within the chloroplasts, resulting in the formation of hydrogen peroxide, and subsequently undergoing further transformation through other antioxidant mechanisms (Morcillo, 2021). The percentage of blue-stained leaves was higher in the presence of *P. cinnamomi* compared to the control condition. The ADB 1A genotype may have a lower baseline defense response or may exhibit a weaker response to the pathogens compared to the ROP7B-763/02 genotype. In the case of the ROP7B-763/02 genotype, when subjected to the treatment involving both organisms, higher values were observed compared to the treatment with the oomycete alone. This suggests that the presence of both organisms triggers a more robust defense response.

Quantification of metabolism markers, including FAA, TPC, TFL, TSS, and STA, revealed interesting variations across the different clones and treatments.

There is a known positive correlation between proline accumulation and plant stress tolerance (Zeier, 2013). Therefore, based on this information, it would be expected that the more tolerant genotypes to the infection would exhibit increased levels of these amino acids, as they contribute to the plant's response to biotic and abiotic stress by preventing water loss and enhancing osmoprotection (San Eufrasio, 2021). Nevertheless, the control samples of both ADB1A-2/06 and ROP7B-763/02 clones exhibited higher levels of FAA compared to the *P. cinnamomi* control.

TFL levels remained relatively similar among the different clones and treatments, with only slight variations observed, indicating no clear trend or significant differences. TPC levels showed significant variation among the clones and treatments. The ROP7B-763/02 clone consistently exhibited higher TPC levels compared to the ADB1A-2/06 clone, regardless of the treatment. The synthesis of secondary metabolites, specifically phenolic compounds and flavonoids, is closely linked to the plant's defense response. Phenolic compounds, including simple phenolic acids and flavonoids, are a class of plant secondary metabolites that play a role in defense against herbivores and pathogens (Camisón et al., 2019). It is known that the

presence of *P. cinnamomi* triggers nonspecific defense responses, such as the accumulation of phenols, thickening of cell walls, and callose deposition (San-Eufrasio et al., 2021). It is expected that plants resistant to the disease would exhibit higher concentrations of these metabolites as part of their defensive response, similar to carotenoids. This hypothesis is based on the findings of Navarro et al. (2009), who observed subtle differences between susceptible and resistant individuals of *Q. ilex*, noting a slight increase in the resistant varieties, which could be attributed to biotic stress (*P. cinnamomi* infection) and abiotic stress (drought/water stress conditions). Flavonoids would act similarly, as they are phenolic compounds involved in antioxidant activity. A lower concentration of flavonoids would result in a reduction in the plant's antioxidant capacity, making it more susceptible to the disease (Camisón et al., 2019).

Severe alterations in carbohydrate metabolism and phloem transport observed in plants infected with pathogens (Elías, 2015) indicate the disruption of normal physiological processes. Soluble sugars play a crucial role in primary plant metabolism, serving as a source of energy and structural components, and participating in hormone-mediated signaling pathways. The increase in sugar concentration in the leaves of resistant plant varieties, known as "high-sugar resistance" (Morkunas & Ratajczak, 2014), suggests their involvement in inducible immunity mechanisms.

The observed variations in TSS levels among treatments and genotypes provide insights into the plant's response to pathogen infection. In our study, the control treatment exhibited the highest TSS levels in both genotypes, indicating the normal functioning of sugar metabolism in the absence of stress. However, when the plants were inoculated with the pathogen, slightly lower TSS values were observed, suggesting a disturbance in sugar accumulation. Surprisingly, the dual inoculation treatment resulted in even lower TSS levels, indicating a further reduction in sugar accumulation. This finding implies that the presence of the pathogen and the additional bacterial inoculation might have triggered physiological changes that hampered sugar accumulation. The "tug of war" between pathogens and plants for sugar resources (Liu et al., 2022) suggests that the microorganisms might utilize or redirect the available sugars for their survival and growth, thus reducing the sugar availability for the plant's own defense mechanisms.

Regarding starch metabolism, plants can reorganize it in response to stress, enabling them to release energy, sugars, and other metabolites crucial for their survival (Kalogeropoulou et al., 2022). Under stressful conditions, starch accumulation is often observed as a protective response. In our study, the control treatment showed variations in STA levels among genotypes. ROP7B-763/02 exhibited higher STA values compared to ADB1A-2/06, indicating genotype-specific differences in starch accumulation. However, when ADB1A-2/06 was subjected to *P. cinnamomi* infection, it showed higher STA levels compared to the control treatment, suggesting an active response to the stressor. Interestingly, the addition of *B. amyloliquefaciens* in the dual inoculation treatment led to a decrease in STA levels in both genotypes, indicating a disruption in starch metabolism. However, ROP7B-763/02 still maintained a higher level of starch compared to ADB1A-2/06. This suggests that ROP7B-763/02 might have a more efficient starch degradation mechanism or a different regulation of starch metabolism compared to ADB1A-2/06, allowing it to maintain a higher level of starch even under stressful conditions.

These results reflect the dynamic nature of starch metabolism and its regulation in response to stress. The observed variations in STA levels among treatments and genotypes highlight the complexity of plant responses to biotic stress and the need for further research to elucidate the underlying physiological mechanisms.

In conclusion, this study highlights two crucial findings: the variation in genotype tolerance and the effectiveness of the biocontrol agent *B. amyloliquefaciens* against *P. cinnamomi*. The experiments demonstrate contrasting levels of tolerance among different cork oak genotypes, while also revealing the varying degrees of inhibition exerted by the biocontrol agent. These results underscore the significance of genotype-specific responses and the potential of targeted biocontrol strategies to mitigate cork oak decline and its adverse consequences in affected areas.

# Authors' contributions

**Conceptualization:** A. Gómez-Garay, B. Pintos.
**Data curation:** Not applicable.
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