



## Report on results from mesocosm trials: Ideal product formulation and tailored IPM- protocols

### Deliverable 3.1

#### WP3 Development of new treatment and ground-based monitoring protocols to be used in integrated pest management

##### Summary:

*Phytophthora* species are a major threat to Fagaceae species in the Mediterranean basin. Potassium phosphonate is among the most efficient and ecofriendly molecule to mitigate disease impact caused by this group of pathogens. However, its use is currently restricted to sweet chestnut, while it is not registered as pesticide on oaks. The main aim of WP3 was to test the efficacy of several products, including biofumigants, antagonistic microorganisms, plant growth promoters and resistance inducers. Two mesocosm trials were implemented to test 13 different products both singularly and in combination on sweet chestnut, cork and holm oak seedlings. Based on the results, Biofence, Bactrium, Tricoten, Kalex (for chestnut) and Kalex EVO (for oaks) resulted the most effective products. Their combined application had positive effects on plant physiological parameters, root systems and defence mechanisms.

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## 1. Executive Summary

Species in the genus *Phytophthora* are among the main pathogens threatening Fagaceae, including cork and holm oak, and sweet chestnut. Among control strategies, treatments with potassium phosphonate (K-phosphonate), formerly named phosphite, are the most common and successful methods for controlling *Phytophthora* on woody trees in forest ecosystems. However, due to the European Directive EU 1009/2019, K-phosphonate is registered only on target crops/plants. Currently, K-phosphonate is registered on sweet chestnut, while it is not on oak species. Because of these new limitations on the use of phosphonates, valid alternative treatments should be considered, including biofumigants, antagonistic microorganisms, plant growth promoters and resistance inducers.

In order to identify alternative products to be used in IPM protocols for limiting the impact of *Phytophthora* species in Fagaceae forests, planned to be applied in a range of demonstration areas within WP4 of the Fagesos project, mesocosm trials were carried out to test the efficacy of fourteen products, with different mode of action, on cork oak, holm oak and sweet chestnut.

The products used were selected based on the literature and their availability in the market. Their efficacy was initially tested individually to select the most effective, among the different modes of action, in controlling *Phytophthora* infection. The selected products were then tested in combination with each other to verify the simultaneous beneficial action. Both trials were conducted on 6-months old seedlings of the three Fagaceae species. To identify the most promising products, analyses on the roots system, the health status of the plants, and physiological parameters were performed.

The first trials identified the products Biofence, Bactrium, Tricoten and Kalex (only for chestnut) and Kalex EVO (for cork and holm oak) as the most effective among those tested. The second trials also proved the effectiveness of the four products combined, showing positive effects on plant health, physiology and root system development, as well as reducing *Phytophthora* inoculum in the soil.

## 2. Introduction

Invasive *Phytophthora* species are among the most relevant biotic stressors in Fagaceae ecosystems, including evergreen Mediterranean oak and chestnut trees in Southern Europe (Jung et al., 2018). Once a *Phytophthora* species is introduced in a new environment its eradication is challenging, if not impossible. It produces resting structures, such as oospores, that can last in the soil for many years. Very few fungicides are active against *Phytophthora* spp. and their use in forest ecosystems is restricted or prohibited in most European countries.

Currently, the most common molecule used to mitigate the impact of *Phytophthora* spp. in several natural and semi-natural forest ecosystems worldwide is K-phosphonate, a compound used in agriculture as a soil fertilizer. K-phosphonate has significant fungicidal properties, suppressing mycelial growth, hyphal branch deformation, and lyses of chlamydospores, sporangia and zoospores (King et al., 2010). It works also as a resistance inducer, being able to activate defence mechanisms in the plant that prevent the development of infectious processes (Crane and Shearer, 2014). Phosphonates can be applied via foliar spray or trunk injection (endotherapy).

The main problem regarding the use of K-phosphonate is related to its registration. In several countries, such as Australia, South Africa and USA it is registered as systemic fungicide (Garbelotto and Schmidt, 2009; Hardy et al., 2001). In most European countries, including Italy, it was considered a fertilizer until 2023. However, since several studies highlighted that phosphonates have no nutritional role for plants, the European Directive EU

1009/2019 has restricted their use, and the new phosphonates-based products are usable only when homologated as pesticide on target crops/plants (Manghi et al., 2021). Currently K-phosphonate is not registered on evergreen oaks, but its use has been recently authorized in Italy and Spain (but not in Portugal) on chestnut. Because of these limitations on the use of phosphonates, valid alternative products must be introduced in IPM protocols.

Beneficial microorganisms, such as Proteobacteria (i.e. *Bacillus* spp.), colonize plant roots, providing benefits to their hosts, and regulating phytohormone synthesis (improving soil nutrient availability and disease resistance). They can also produce several bioactive metabolites that serve as antimicrobial compounds (Moon et al., 2021; Ritika and Uptal, 2021). As antagonist microorganisms, some *Trichoderma* strains are well known for being effective biocontrol agents that lessen the severity of plant diseases, typically affecting the roots (Sharma and Sharma 2020). The antagonistic effect of *Trichoderma* spp. over *Phytophthora* spp. development is mostly related to several mechanisms such as physical space limitation by root colonization, production of chemical substances suppressing *Phytophthora* growth and direct mycoparasitism (Vinale et al., 2008).

Among the other strategies for mitigating the impact of *Phytophthora* spp. that can be followed, biofumigation with Brassicaceae is one of the most effective in limiting the pathogen inoculum in the soil (Morales et al., 2016).

### 3. Mesocosm trials

In order to achieve the aim of identifying phosphonate's substitute, several products were tested in mesocosm trials, looking for 4 main different modes of action: 1) biofumigant biomolecules; 2) antagonist microorganisms; 3) Plant Growth Promoter Microorganism (PGPM); 4) resistance inducers.

#### 3.1 Experimental design

A complete randomized experiment was carried out, following the scheme listed in Figure 1 for the first mesocosm trial, and in Figure 2 for the second trial.

T01_PC	T01_PC	T01_TR	T01_TR
T01_PC	T01_PC	T01_TR	T01_TR
T01_PC	T01_PC	T01_TR	T01_TR
T01_PC	T01_PC	T01_TR	T01_TR
T01_PC	T01_PC	T01_TR	T01_TR
T02_PC	T02_PC	T02_TR	T02_TR
T02_PC	T02_PC	T02_TR	T02_TR
T02_PC	T02_PC	T02_TR	T02_TR
T02_PC	T02_PC	T02_TR	T02_TR
T02_PC	T02_PC	T02_TR	T02_TR
T03_PC	T03_PC	T03_TR	T03_TR
T03_PC	T03_PC	T03_TR	T03_TR
T03_PC	T03_PC	T03_TR	T03_TR
T03_PC	T03_PC	T03_TR	T03_TR
T03_PC	T03_PC	T03_TR	T03_TR
T04_PC	T04_PC	T04_TR	T04_TR
T04_PC	T04_PC	T04_TR	T04_TR
T04_PC	T04_PC	T04_TR	T04_TR
T04_PC	T04_PC	T04_TR	T04_TR
T04_PC	T04_PC	T04_TR	T04_TR
T05_PC	T05_PC	T05_TR	T05_TR
T05_PC	T05_PC	T05_TR	T05_TR
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T05_PC	T05_PC	T05_TR	T05_TR
T05_PC	T05_PC	T05_TR	T05_TR
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T06_PC	T06_PC	T06_TR	T06_TR
T06_PC	T06_PC	T06_TR	T06_TR
T06_PC	T06_PC	T06_TR	T06_TR
T07_PC	T07_PC	T07_TR	T07_TR
T07_PC	T07_PC	T07_TR	T07_TR
T07_PC	T07_PC	T07_TR	T07_TR
T07_PC	T07_PC	T07_TR	T07_TR
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T13_PC	T13_PC	T13_TR	T13_TR
T13_PC	T13_PC	T13_TR	T13_TR
T13_PC	T13_PC	T13_TR	T13_TR
T13_PC	T13_PC	T13_TR	T13_TR
PC	PC	NC	NC
PC	PC	NC	NC
PC	PC	NC	NC
PC	PC	NC	NC
PC	PC	NC	NC

**Figure 1.** Experimental design of the first mesocosm trials. The different colors represent the 13 different treatments (T01-T13); for each one, there were 10 seedlings inoculated with *Phytophthora cinnamomi* (PC) and 10 seedlings uninoculated (TR). Positive control consists in seedlings inoculated with *Phytophthora cinnamomi* untreated (PC) and negative control in seedlings uninoculated and untreated (NC).

PC-01	PC-21	PC-41	TR-01	TR-21	TR-41
PC-02	PC-22	PC-42	TR-02	TR-22	TR-42
PC-03	PC-23	PC-43	TR-03	TR-23	TR-43
PC-04	PC-24	PC-44	TR-04	TR-24	TR-44
PC-05	PC-25	PC-45	TR-05	TR-25	TR-45
PC-06	PC-26	PC-46	TR-06	TR-26	TR-46
PC-07	PC-27	PC-47	TR-07	TR-27	TR-47
PC-08	PC-28	PC-48	TR-08	TR-28	TR-48
PC-09	PC-29	PC-49	TR-09	TR-29	TR-49
PC-10	PC-30	PC-50	TR-10	TR-30	TR-50
PC-11	PC-31	PC-51	TR-11	TR-31	TR-51
PC-12	PC-32	PC-52	TR-12	TR-32	TR-52
PC-13	PC-33	PC-53	TR-13	TR-33	TR-53
PC-14	PC-34	PC-54	TR-14	TR-34	TR-54
PC-15	PC-35	PC-55	TR-15	TR-35	TR-55
PC-16	PC-36	PC-56	TR-16	TR-36	TR-56
PC-17	PC-37	PC-57	TR-17	TR-37	TR-57
PC-18	PC-38	PC-58	TR-18	TR-38	TR-58
PC-19	PC-39	PC-59	TR-19	TR-39	TR-59
PC-20	PC-40	PC-60	TR-20	TR-40	TR-60
PC+TR-01	PC+TR-21	PC+TR-41	NC-01	NC-21	NC-41
PC+TR-02	PC+TR-22	PC+TR-42	NC-02	NC-22	NC-42
PC+TR-03	PC+TR-23	PC+TR-43	NC-03	NC-23	NC-43
PC+TR-04	PC+TR-24	PC+TR-44	NC-04	NC-24	NC-44
PC+TR-05	PC+TR-25	PC+TR-45	NC-05	NC-25	NC-45
PC+TR-06	PC+TR-26	PC+TR-46	NC-06	NC-26	NC-46
PC+TR-07	PC+TR-27	PC+TR-47	NC-07	NC-27	NC-47
PC+TR-08	PC+TR-28	PC+TR-48	NC-08	NC-28	NC-48
PC+TR-09	PC+TR-29	PC+TR-49	NC-09	NC-29	NC-49
PC+TR-10	PC+TR-30	PC+TR-50	NC-10	NC-30	NC-50
PC+TR-11	PC+TR-31	PC+TR-51	NC-11	NC-31	NC-51
PC+TR-12	PC+TR-32	PC+TR-52	NC-12	NC-32	NC-52
PC+TR-13	PC+TR-33	PC+TR-53	NC-13	NC-33	NC-53
PC+TR-14	PC+TR-34	PC+TR-54	NC-14	NC-34	NC-54
PC+TR-15	PC+TR-35	PC+TR-55	NC-15	NC-35	NC-55
PC+TR-16	PC+TR-36	PC+TR-56	NC-16	NC-36	NC-56
PC+TR-17	PC+TR-37	PC+TR-57	NC-17	NC-37	NC-57
PC+TR-18	PC+TR-38	PC+TR-58	NC-18	NC-38	NC-58
PC+TR-19	PC+TR-39	PC+TR-59	NC-19	NC-39	NC-59
PC+TR-20	PC+TR-40	PC+TR-60	NC-20	NC-40	NC-60

**Figure 2.** Experimental design of the second mesocosm trial. The different colors represent the 4 different blocks: seedlings inoculated with *Phytophthora cinnamomi* (PC), seedlings treated and uninoculated (TR), seedlings inoculated with *Phytophthora cinnamomi* and treated (PC+TR) and seedlings uninoculated and untreated (NC).

### 3.1.1 Single products efficacy

The single product efficacy trials consisted in testing 13 different products. The trials were conducted on 6-month-old chestnut seedlings (*Castanea sativa*) at the Tuscia University (UNITUS), on cork oak (*Quercus suber*)

at the University of Sassari (UNISS) and on holm oak (*Quercus ilex*) at the Cordoba University (UCO). All three partners followed the same protocol for inoculation and treatments, as well as conditions, parameters and timing of the experiments.

Among the products selected for the trials there were:

- 1) Two biofumigant products, including the formulate **Biofence FL** (liquid and pellet form of *Brassica* spp. processing residues) and **Biofence dieci** (consisting in a flour of *Brassica* spp. processing residues).
- 2) One PGPM product called **Bactrium**, provided by the partner Atens, was tested. In addition, two different *Bacillus* strains (**BV** and **F35**) provided by UNISS were also included in the trials, both individually and applied together, due to their different effects on *Phytophthora* growth.
- 3) Three *Trichoderma*-based products: **Tricoten**, provided by Atens, a ***Trichoderma* complex** developed by UNITUS (Aleandri et al., 2015), and a strain of ***Trichoderma gamsii*** selected by the UCO team (Ruiz Gómez and de Miguel Rojas, 2021).
- 4) Five resistance inducers: **LL017**, a prototype bioproduct whose efficacy against oomycetes was proven to control *Phytophthora infestans* on potato-plants, and *Plasmopara viticola* on grapes; **Chitosan**, commercially available under different trading marks. For cork and holm oak trials the product Biorend, produced by Biogard, was used; for chestnut, Chitosano 5% SKL. Chitosan is one of the most prevalent biological inducers which increases plants tolerance to different biotic stresses; **Si-K basalt flour + effetto scudo** (Basalti Orvieto srl, Castel Viscardo (TR), Italy); **Kalex** (Alba Milagro, Parabiago, Italy), a K-phosphonate based product able to activate defence mechanisms in the plant that prevent the development of *Phytophthora* infectious processes; **Kalex EVO** (Alba Milagro, Parabiago, Italy), which is a copper and molybdenum-based product that reduces biotic stress and improves resistance through a complexed form of copper.

### 3.1.2 Combined products efficacy

Based on the results of the single products efficacy trials, the following products were selected for the second trials: **Biofence FL** as a biofumigant; **Tricoten** for the antagonist microorganism; **Bactrium** as PGPM; **Kalex** (for chestnut) and **Kalex EVO** (for cork and holm oak) as resistance inducers.

The trial consisted in a complete randomized experiment with 2 factors: *P. cinnamomi* inoculation (PC) and Treatments (TR), accounting for 4 blocks (PC, TR, PC+TR) and a negative control (NC). For each block, 60 repetitions were carried out, for a total of 200 plants. The four selected products were applied together, observing the timelines suggested by the manufacturers. A 60-days trial has been carried out, as the optimum timespan for assessment (Figure 2).

## 3.2 Inoculum preparation

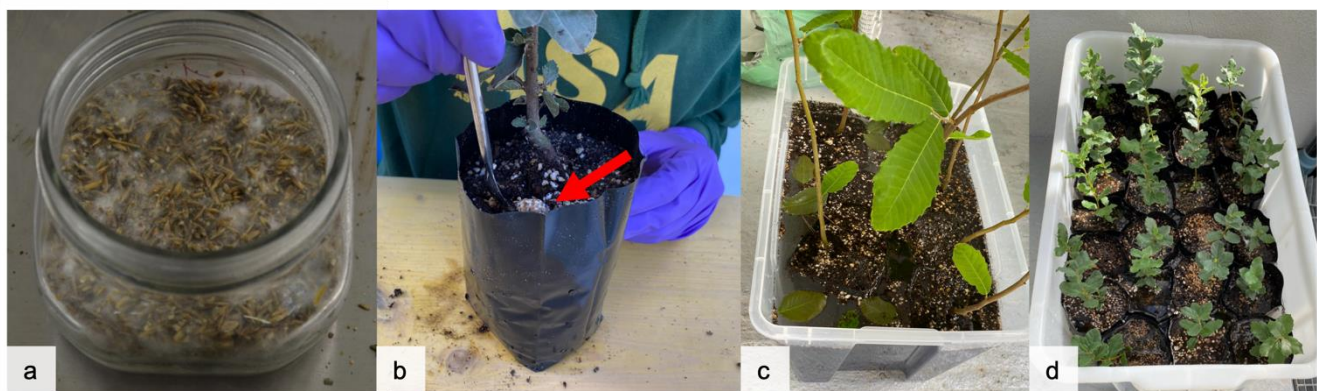
Inoculum of *P. cinnamomi* was prepared by growing individual isolates in 500-mL Erlenmeyer flasks (or glass jars) containing a mixture of 200 mL of decorticated organic millet seeds thoroughly moistened with 100 mL of filtered carrot juice (200 mL/L carrot, 3 g/L CaCO<sub>3</sub> and 800 mL/L distilled water), autoclaved twice for 20 min at 121°C before use. After cooling to room temperature, the mixture was inoculated with eight 5-mm-diameter plugs cut from the margins of an actively growing *P. cinnamomi* culture and incubated for 4-week at 20°C (Figure 3, a).

### 3.3 Inoculation

About 40 mL of *P. cinnamomi* inoculum was collected from the flasks and added to the soil of each pot (around the stem of the seedling). Negative control plants received 40 mL of the uninoculated mixture. For the inoculation, 4 holes were made around the plant with the help of a 10 mL pipette, and this is where the inoculum was distributed and subsequently covered with soil (Figure 3, b).

### 3.4 Flooding

To stimulate the production of sporangia, pots were flooded immediately after inoculation for 48 h by immersing pots in 10-litre buckets so with water level ca. 1 cm above the soil surface. To confirm viability of the inoculum and production of zoospores, *Q. suber* leaves (or other fresh leaves) were used as bait during flooding. Flooding was repeated after four weeks (Figure 3, c-d).



**Figure 3.** Different phases of the soil inoculation method: inoculum preparation (a), inoculation of *Phytophthora cinnamomi* mycelium in the soil (b), flooding of sweet chestnut (c) and cork oak seedlings (d).

### 3.5 Treatments

#### 3.5.1 First single product trials

Each treatment followed different preparation and distribution methods:

- **Biofence FL** was prepared following the manufacturer's instructions. It was diluted a 2% concentration with water and 2 mL/pot of the resulting solution were sprayed in the substrate;
- **Biofence dieci (pellet)** was used by adding in the substrate 2 g of product per pot and 40 mL of water for product activation;
- **Bactrium** was distributed in the soil by adding 5 mL/pot of the solution provided by the manufacturer;
- **Bacillus BV** and **F35** strains were tested by pouring 100 mL of a *Bacillus* cell suspension containing about  $1-2 \times 10^8$  cell/mL;
- **Tricoten** was applied by mixing in the first centimeters of the substrate 4 g/pot of the wettable powder;
- **Trichoderma complex** and **Trichoderma gamsii** were tested by washing with distilled water the plate of the 10 day-old actively growing colony, and applying 5 mL of the resulted suspension containing a concentration of  $10^9$  conidia/mL has been collected in the substrate;
- **Kalex** and **Kalex EVO** were uniformly sprayed over the leaves at 0.5% concentration;
- **LL017** and **Chitosan**: applied as it was provided by the manufacturer via foliar spray;

- **Si-k basalt flour + effetto scudo**: distributed in the soil at the dose of 6-20 mL/m<sup>2</sup> as suggested by the manufacturer.

### 3.5.2 Second combined product trials

In the second trial the four selected most effective products were used in combination. Their preparation was as described in the previous chapter. However, the treatments with the different products were made with the following timeline:

- 1) seedlings were first inoculated with *P. cinnamomi*;
- 2) after two days seedlings were treated with **Biofence FL**;
- 3) two weeks later a solution with **Bactrium** and **Tricoten** was prepared and distributed in the substrate;
- 4) one day later, seedlings were foliar sprayed with **Kalex** (chestnut) or **Kalex EVO** (holm oak and cork oak).

### 3.6 Disease Index and survival assessment

During the experiment inoculated and treated, and uninoculated and untreated seedlings were visually assessed, twice per week, by giving a disease class from 0 to 4, where:

Class 0: healthy plant with no symptoms

Class 1: symptoms on 1 to 30% of the plant

Class 2: symptoms on 31 to 60% of the plant

Class 3: symptoms on > 61% of the plant

Class 4: dead plant

At the end of the experiment, the seedlings were checked for their vitality, and mortality rate and survival analysis were calculated using the Kaplan-Meier curve.

### 3.7 Root assessment

At the end of the experiments, roots were extracted from pots and gently washed with tap water until removal of the substrate. Washing was made with the help of a sieve to capture fine roots excised from the root system due to manipulation. Then, the whole root was scanned with a densitometer, and images analysed with the software WinRHIZO™ (Regent Instruments Inc., Ottawa, ON, Canada). Single roots were cut off at the collar, analysed for the presence of root rot and necrotic lesions, and morphological traits of the roots, such as total root length (cm), were assessed. Then, the roots were divided into two fractions: coarse and fine roots, and each fraction weighed. Finally, each root fraction was weighed and air-dried in a conventional oven for 48 h at 85°C to calculate the dry weight.

### 3.8 Aboveground biomass

The aboveground biomass (stem and leaves) was excised from the root and weighed both together and separately. The stem length and the collar root diameter were recorded and then, the stem and all the leaves were air-dried to calculate dry weight.

### 3.9 Reisolation

In order to determine whether the pathogen was still viable in the soil at the end of the experiment, the soil was assessed using the baiting methods. It consisted in putting a rhizosphere soil sample in a container, flooding it with distilled water and placing non-wounded young leaves floated over the clean water surface acting as baits for zoospores. After 3 days the leaves that show the typical necrotic lesions of *Phytophthora* were plated on selective media (SMA or PARPH) for the isolation of the colonies (Jung et al., 1996; Scanu et al., 2014). Re-isolations was also made from necrotic roots and collar tissues using SMA (or PARPH) selective medium.

### 3.10 Physiology assessments

Photosynthetic and stomatal conductance rates were measured weekly through the experiments. Five plants from each block/treatment were assessed for photosynthesis and conductance, with a Li6400XT IRGA, with a minimum of 3 minutes for stabilization. Another 10 plants for each block were only assessed for conductance, which is a quicker measurement. Fluorescence (Quantum Yield of the PSII) was also weekly assessed from the 5 plants used for physiology assessment, with the FluorPen FP100.

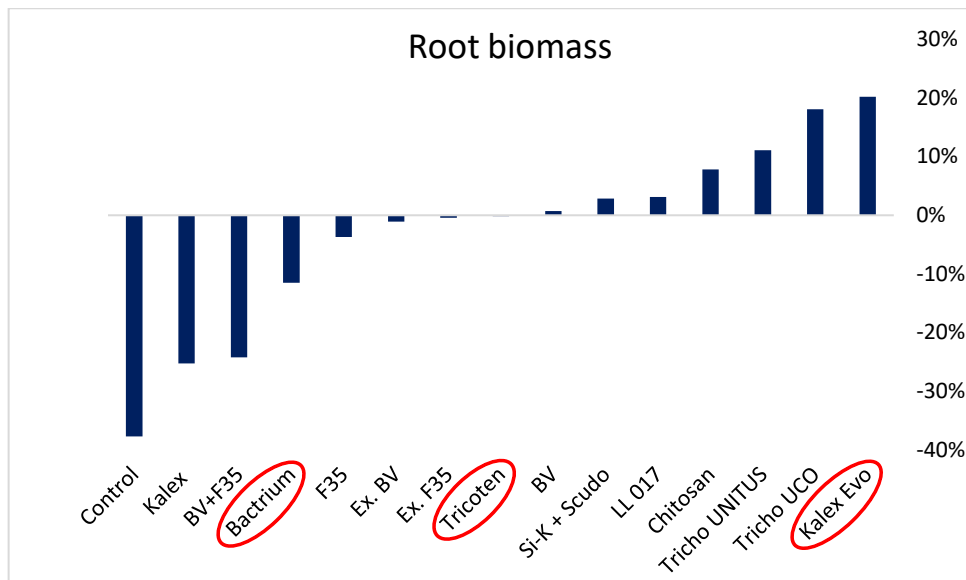
## 4. Results

### 4.1 First experiment - single products efficacy

#### 4.1.1 Cork oak

##### 4.1.1.1 Root assessment

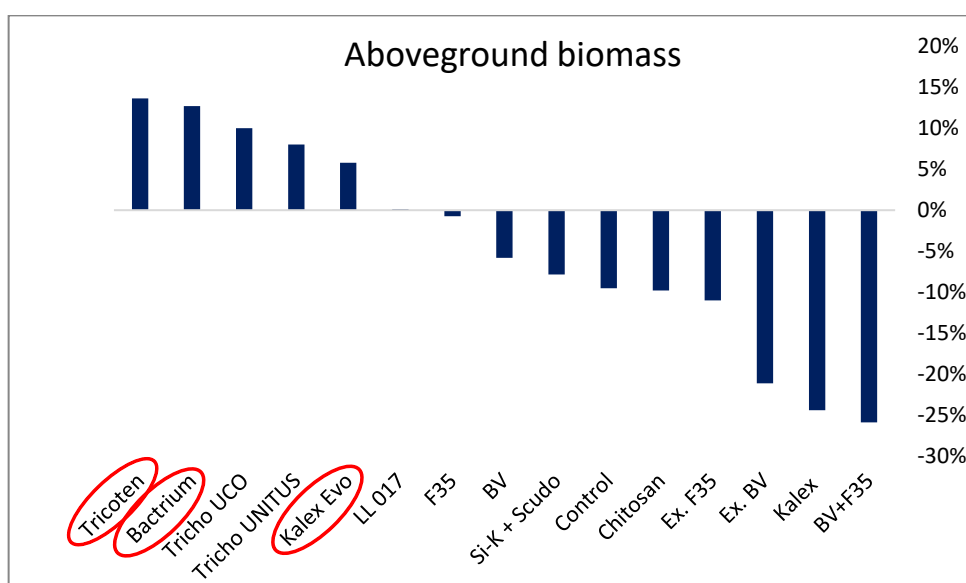
In the first mesocosm trial, the results showed that in the cork oak seedling inoculated with *P. cinnamomi* and without treatments (positive control), the root systems had an average weight reduction of 37%. In contrast, the weight of the root system of the seedlings treated with Tricoten, the *Bacillus* strain BV, and also the extract of the two *Bacillus* strains was nearly the same of those not inoculated and not treated (baseline). In the seedlings treated with Kalex, the two *Bacillus* strains applied together (F35 and BV), and the F35 strain a reduction between 3% and 25% was detected, respectively. The highest increase in the root biomass was observed in the seedlings treated with Kalex Evo (+ 20%) (Figure 4). Seedlings inoculated with *Trichoderma* complex (UNITUS) and *T. gamsii* (UCO) had a root weight higher than the uninoculated and untreated seedlings of 18% and 8%, respectively. Lastly, the treatments with Chitosan, LL017 and Si-k basalt flour + effetto scudo had a slight increase in root weight(8% and 3%) (Figure 4).



**Figure 4.** Root biomass variation (%) of the 6-months old cork oak seedlings inoculated with *Phytophthora cinnamomi* and treated with the different products compared with the uninoculated and untreated seedlings (baseline).

#### 4.1.1.2 Aboveground biomass

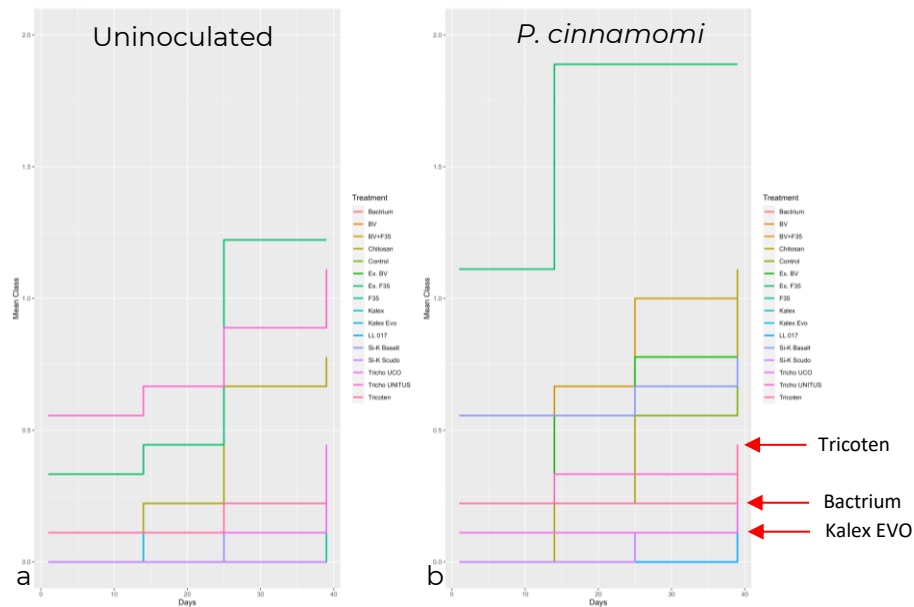
The analysis at the end of the experiment showed that the seedlings inoculated with *P. cinnamomi* and treated with Tricoten and Bactrium had the highest increase of the aboveground biomass weight, 14 and 13%, respectively, comparing to the uninoculated and not treated seedlings (baseline). The two *Trichoderma* treatments (*Trichoderma* complex and *T. gamsii*) led an increase of 10% and 8%, respectively. The seedlings treated with Kalex EVO increased the biomass of 6%. In the remaining seedlings inoculated and treated a reduction of the aboveground biomass compared with the untreated and uninoculated seedlings was detected, ranging from 1% (*Bacillus* F35 strain) to 26% of the two *Bacillus* strains applied together (F35 and BV). In the plants untreated and inoculated with *P. cinnamomi* (control) a reduction of 9% was detected (Figure 5).



**Figure 5.** Aboveground biomass variation (%) of the 6-months old cork oak seedlings inoculated with *Phytophthora cinnamomi* and treated with the different products compared to the uninoculated and untreated (baseline).

#### 4.1.1.3 Disease index

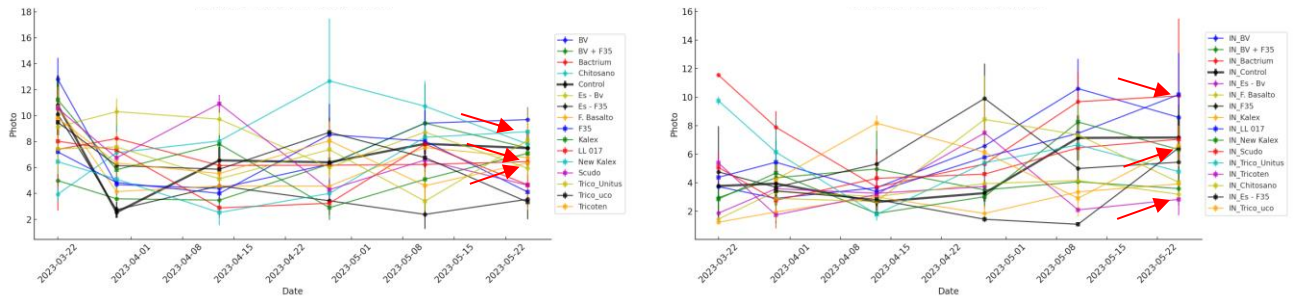
The disease index assessments observed in the cork oak seedlings showed that some treatments were effective. The lowest mean class of disease severity was detected in the seedlings treated with Kallex EVO, Si-k basalt flour + effetto scudo, *Trichoderma* complex (UNITUS), *T. gamsii* (UCO) and Tricoten respectively. In contrast, the treatments with *Bacillus* strain BV and Chitosan were shown to be less effective. The highest mean class was detected in the control (untreated and inoculated) seedlings (Figure 6).



**Figure 6.** Average disease index class for the different treatments in the uninoculated (a) and inoculated seedlings with *Phytophthora cinnamomi* (b). The red arrow represents the three different products selected for the combined trial.

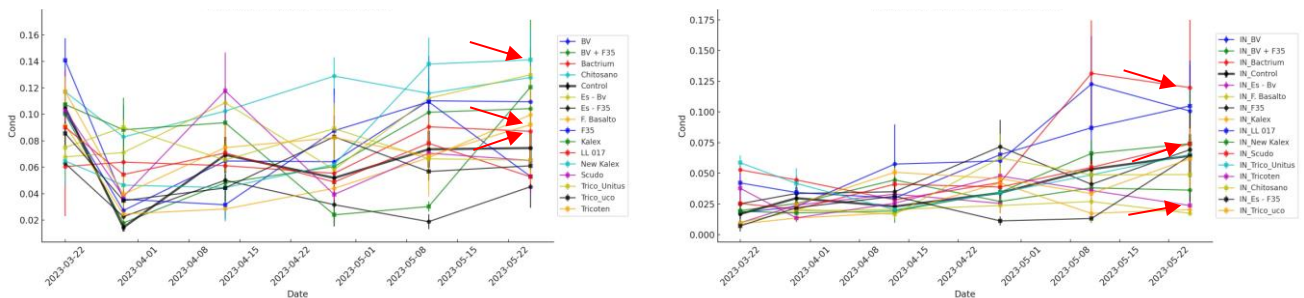
#### 4.1.1.4 Physiological parameters

The analyses of physiological parameters showed that most of treated seedlings did not present differences in net photosynthesis rate compared with the controls (both inoculated with *P. cinnamomi* and uninoculated). In particular, in the uninoculated seedlings, few treatments (*T. gamsii*, Basalt flour + effetto scudo and *Bacillus* F35) showed a significant reduction of the photosynthesis rate, while the other treatments did not differ significantly from the controls (Figure 7, left). In the seedlings inoculated and treated with Bactrium, LL017 and *Bacillus* BV, the photosynthesis rates were higher than the controls, while in all the other treatments they were lower. However, no significant statistical differences were detected (Figure 7, right).



**Figure 7.** Net photosynthesis rate observed for all the treatments in the seedlings uninoculated (left) and inoculated with *Phytophthora cinnamomi* (right). Red arrows represent the treatments selected for the second trial.

The conductance flow of the uninoculated seedlings showed that several treatments performed better than controls, including Bactrium, Tricoten and Kalex EVO (Figure 8, left). In the inoculated seedlings a rate higher than controls was detected for the treatments with Bactrium, the two *Bacillus* strains (BV and F35), LL017 and Kalex EVO, while the other showed a lower rate than the controls. However, lower value for the inoculated seedlings were detected compared with those uninoculated (Figure 8, right).

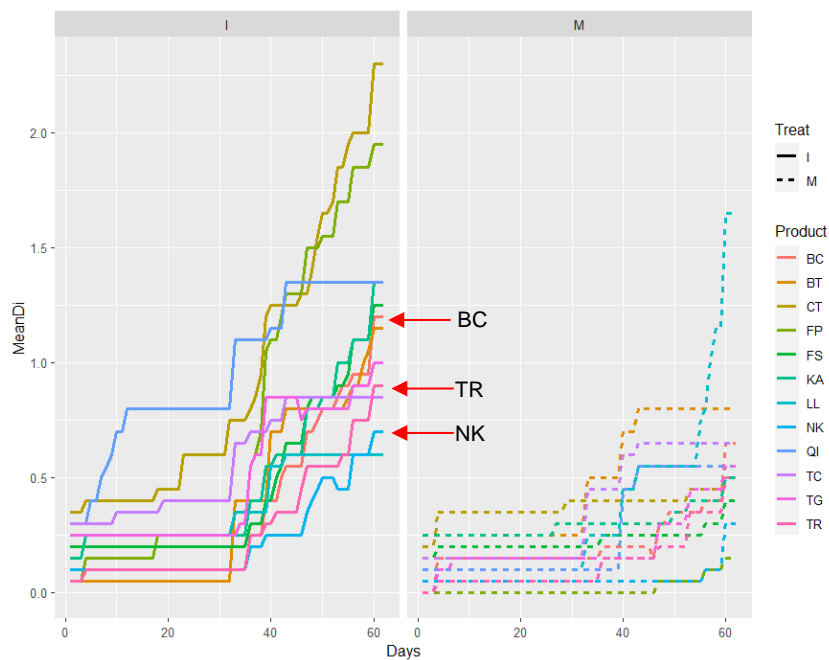


**Figure 8.** Conductance flow observed in the seedlings uninoculated (left) and inoculated with *Phytophthora cinnamomi* (right). Red arrows represent the treatments selected for the second trial.

## 4.1.2 Holm oak

### 4.1.2.1 Disease index

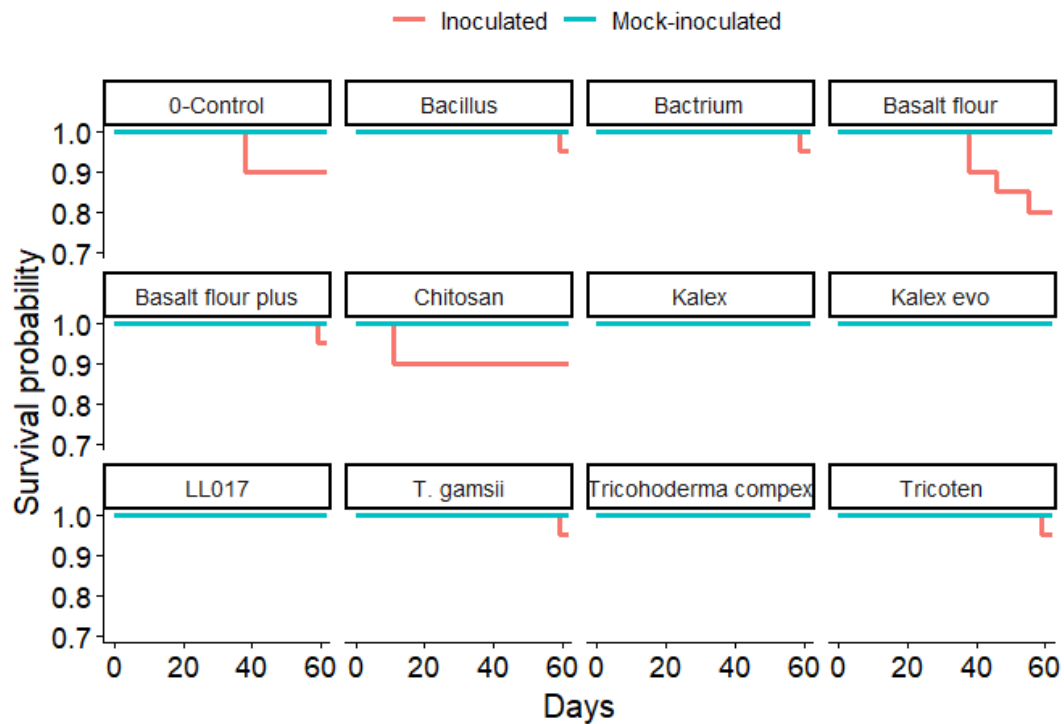
At the end of the experiment the lowest mean Disease Index was detected on holm oak seedlings treated with Si-k basalt flour + effetto scudo. However, when comparing with the uninoculated seedlings the treatment Kalex EVO showed significant differences compared to negative controls in *Phytophthora*-inoculated seedlings ( $p < 0.001$ ), followed by Tricoten. Treatments with FP (Si-k basalt flour) alone and *Bacillus* F35, Chitosan and LL017 were the less effective (Figure 9).



**Figure 9.** Average disease index (DI) class in the inoculated with *Phytophthora cinnamomi* (left) and uninoculated (right) holm oak seedlings for the different treatments: BC= Bactrium, BT= *Bacillus* UNISS, CT= control, FS= basalt flour+effetto scudo, FP= basalt flour, KA= Kalex, LL= LL017, NK= Kalex Evo, QI= chitosan, TC = *Trichoderma* UNITUS, TG= *Trichoderma* UCO, TR= Tricoten. The red arrow represents the three different products selected for the combined trial.

#### 4.1.2.2 Mortality

Most of the treatments did not show differences between *Phytophthora*-inoculated and uninoculated seedlings at the end of the experiment (Figure 10). Plants treated with *Trichoderma* complex, Kalex, Kalex EVO and LL017 showed a survival rate of 100%, and only control treatment and those treated with basalt flour and chitosan showed significant differences in mortality between uninoculated and *Phytophthora*-inoculated seedlings (Table 1).



**Figure 10.** Kaplan-Meier survival analysis of the inoculated with *Phytophthora cinnamomi* (orange lines) and uninoculated holm oak seedlings (light blue lines).

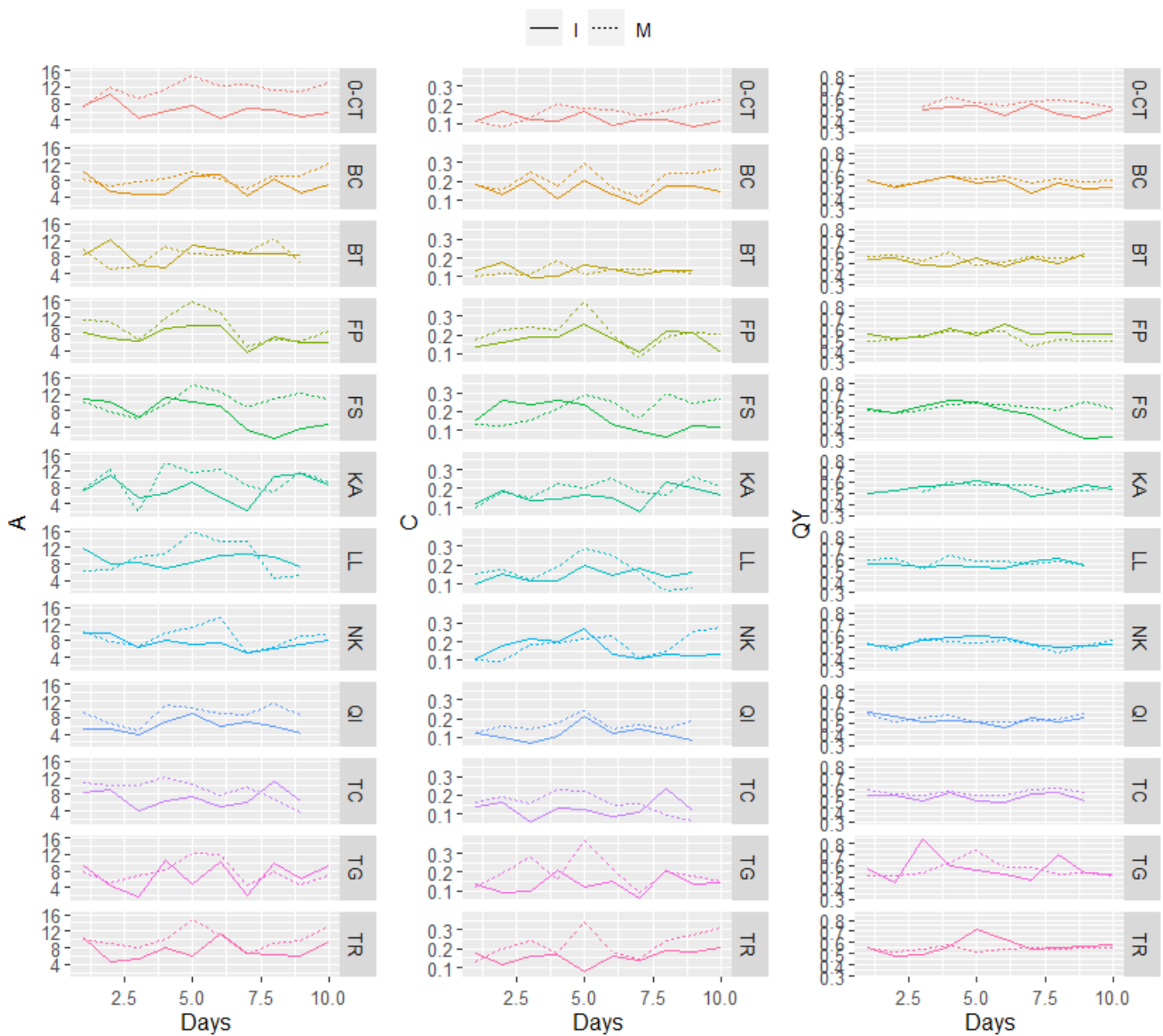
#### 4.1.2.3 Physiological parameters

The analyses of physiological parameters showed that most of treated holm oak seedlings did not present differences in net photosynthetic carbon assimilation ( $A$ ) rate, stomatal conductance rate ( $g_s$ ) and fluorescence (PSII Photosynthetic efficiency -  $\Phi$ PSII) compared with the inoculated control (Figure 11). Control seedlings showed significant differences between uninoculated and *Phytophthora*-inoculated seedlings at the end of the experiment in both  $A$  and  $C$ , with higher values for uninoculated seedlings. Interestingly, plants treated with basalt flour and “effetto scudo” (FS), showed significant differences in  $A$  at the end of the experiment, but the values of plants inoculated with *P. cinnamomi* did not show differences regarding negative control (seedlings mock-inoculated and without treatment). Inoculated seedlings of Chitosan treatment showed the minimum values of  $A$  and  $C$  overall.

**Table 1.** Results of the Kaplan-Meier survival analysis of holm oak seedlings in the first mesocosm trial.

Treatment	Thesis	n°	Observed	Expected	(O-E) <sup>2</sup> /E	(O-E) <sup>2</sup> /V
Control	<i>P. cinnamomi</i>	20	2	0.517	<b>4.248*</b>	4.449
Control	Uninoculated	20	0	0.547	0.547	0.574
<i>Bacillus</i>	<i>P. cinnamomi</i>	20	1	0.547	0.375	0.394
<i>Bacillus</i>	Uninoculated	20	0	0.547	0.547	0.574
Bactrium	<i>P. cinnamomi</i>	20	1	0.547	0.375	0.394
Bactrium	Uninoculated	20	0	0.547	0.547	0.574
Basalt flour	<i>P. cinnamomi</i>	20	4	0.494	<b>24.876**</b>	<b>26.9**</b>
Basalt flour	Uninoculated	20	0	0.547	0.547	0.574
Basalt flour plus	<i>P. cinnamomi</i>	20	1	0.547	0.375	0.394
Basalt flour plus	Uninoculated	20	0	0.547	0.547	0.574
Chitosan	<i>P. cinnamomi</i>	20	2	0.501	<b>4.49*</b>	4.696
Chitosan	Uninoculated	20	0	0.547	0.547	0.574
Kalex	<i>P. cinnamomi</i>	20	0	0.547	0.547	0.574
Kalex	Uninoculated	20	0	0.547	0.547	0.574
Kalex EVO	<i>P. cinnamomi</i>	20	0	0.547	0.547	0.574
Kalex EVO	Uninoculated	20	0	0.547	0.547	0.574
LL017	<i>P. cinnamomi</i>	20	0	0.547	0.547	0.574
LL017	Uninoculated	20	0	0.547	0.547	0.574
<i>T. gamsii</i>	<i>P. cinnamomi</i>	20	1	0.547	0.375	0.394
<i>T. gamsii</i>	Uninoculated	20	0	0.547	0.547	0.574
T. complex	<i>P. cinnamomi</i>	20	0	0.547	0.547	0.574
T. complex	Uninoculated	20	0	0.547	0.547	0.574
Tricoten	<i>P. cinnamomi</i>	20	1	0.547	0.375	0.394
Tricoten	Uninoculated	20	0	0.547	0.547	0.574

$\chi^2 = 44.5$ ; df = 23; p < 0.01

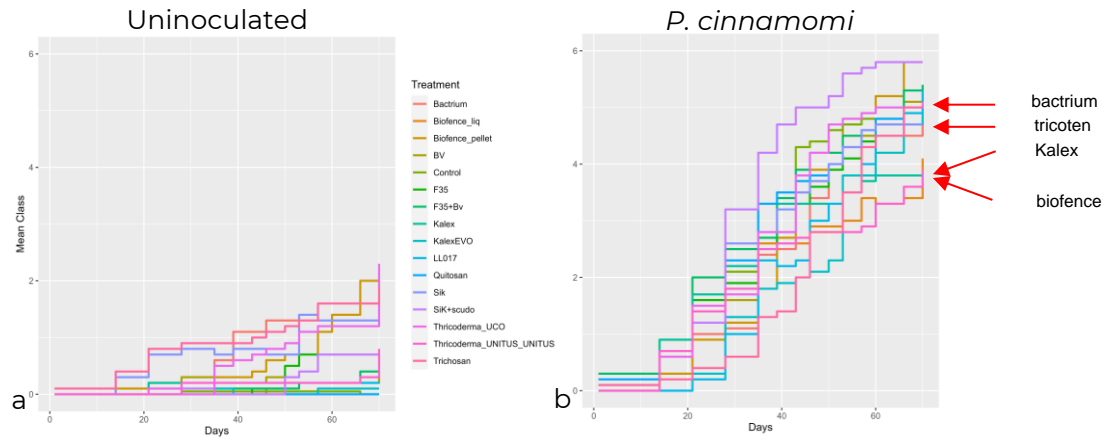


**Figure 11.** Physiological parameters (photosynthetic carbon assimilation (A),  $g_s$  stomatal conductance rate (C),  $\Phi$ PSII - fluorescence (QY)) of the plants for the first mesocosm experiment. I= plants inoculated with *Phytophthora cinnamomi* (continuous lines); M= uninoculated holm oak seedlings (dashed lines).

### 4.1.3 Sweet chestnut

#### 4.1.3.1 Disease index

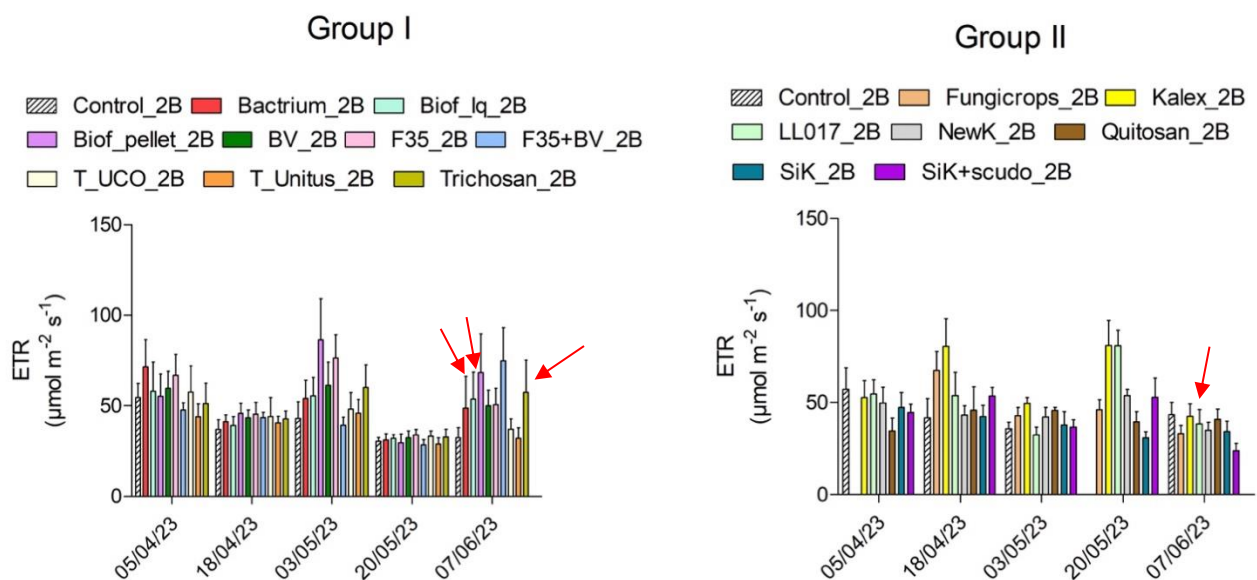
The results of this trial showed that the means of the disease class index of the seedlings inoculated with *P. cinnamomi* were significantly higher than those uninoculated (Figure 12). In particular, the highest class was detected in the seedlings treated with Si-k basalt flour + *effetto scudo*, followed by Biofence (pellet) and the two *Bacillus* strains. The lowest mean classes were detected in the treatments Biofence (liquid), followed by Tricoten and Kalex.



**Figure 12.** Average disease index class for the different treatments in the uninoculated (a) and inoculated with *Phytophthora cinnamomi* (b) chestnut seedlings. The red arrow represents the four different products selected for the combined trial.

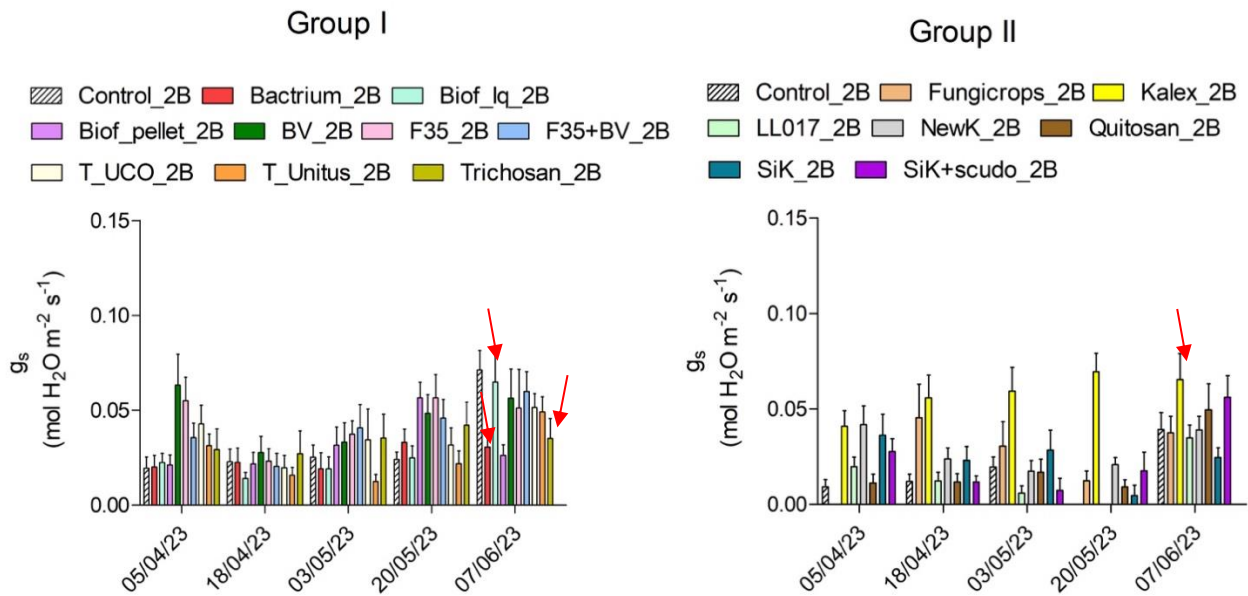
#### 4.1.3.2 Physiological parameters

In the 60 days experiment, in order to have as small a survey window as possible, the plants to be measured for electronic transport, stomatal conductance and photosynthesis rate were divided into two groups. The results of the electronic transport (ETR) showed that, at the end of trials, most of the treatments included in the first group, showed rates higher than control, except for *Trichoderma* complex and *T. gamsii* (Figure 13, left). Plants inoculated and treated with Bactrium, LL017 and *Bacillus* BV showed a photosynthesis rate higher than the control, while in plants that had received any of the other treatments a lower value was detected (Figure 13, right).



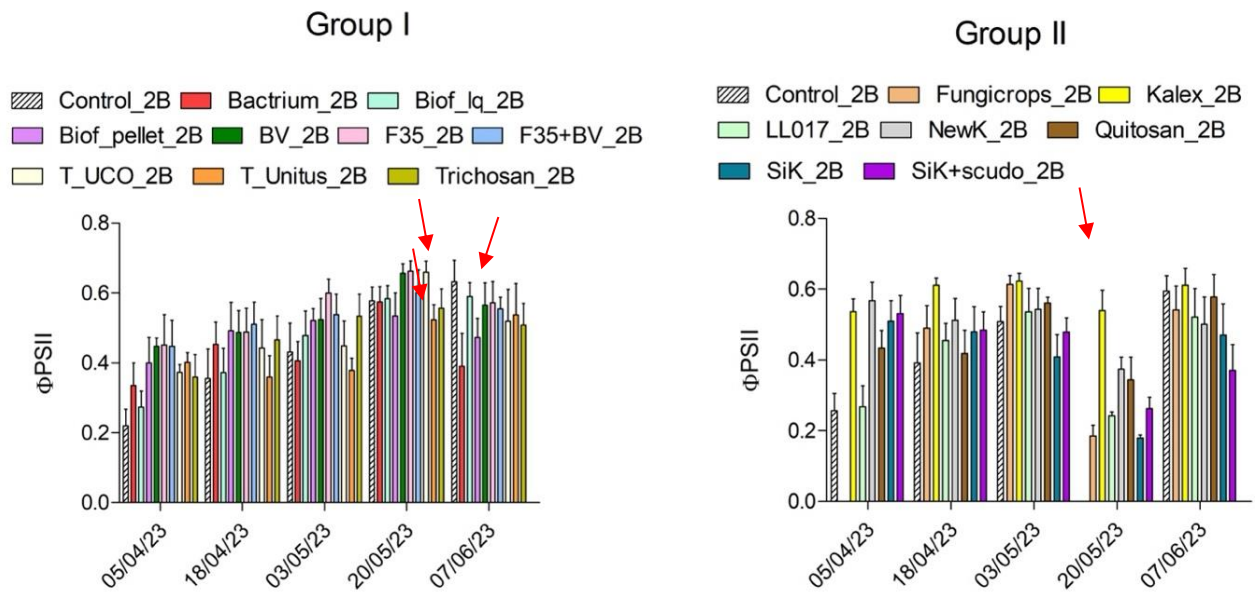
**Figure 13.** Electronic transport in the seedlings inoculated with *Phytophthora cinnamomi* for the two groups of treatments. The red arrows indicate the four treatments selected for the second mesocosm experiment.

In the stomatal conductance ( $g_s$ ), in group I, the treatments *T. gamsii* (UCO), *Trichoderma* complex (UNITUS), Bactrium, biofence (pellet), and Tricoten showed values lower than the control (Figure 14, left), while for other treatments the detected rates were equal or higher than control. In group II, only the treatment basalt flour showed a value significantly lower than control, while for the other treatments higher values were detected. Overall, Kalex treatment was detected as the most effective (Figure 14, right).



**Figure 14.** Stomatal conductance ( $g_s$ ) observed in the seedlings inoculated with *Phytophthora cinnamomi* for the two groups of treatments. The red arrows indicate the four treatments selected for the second experiment.

Concerning the quantum yield of photosystem II ( $\Phi_{PSII}$ ), in group I values lower than control were detected for all treatments (Figure 15, left). However, among treatments, Biofence showed the highest rate. In group II, the treatments Kalex, LL017 and chitosan showed values comparable with those of the control, while for the other treatments values lower than control were detected (Figure 15, right).



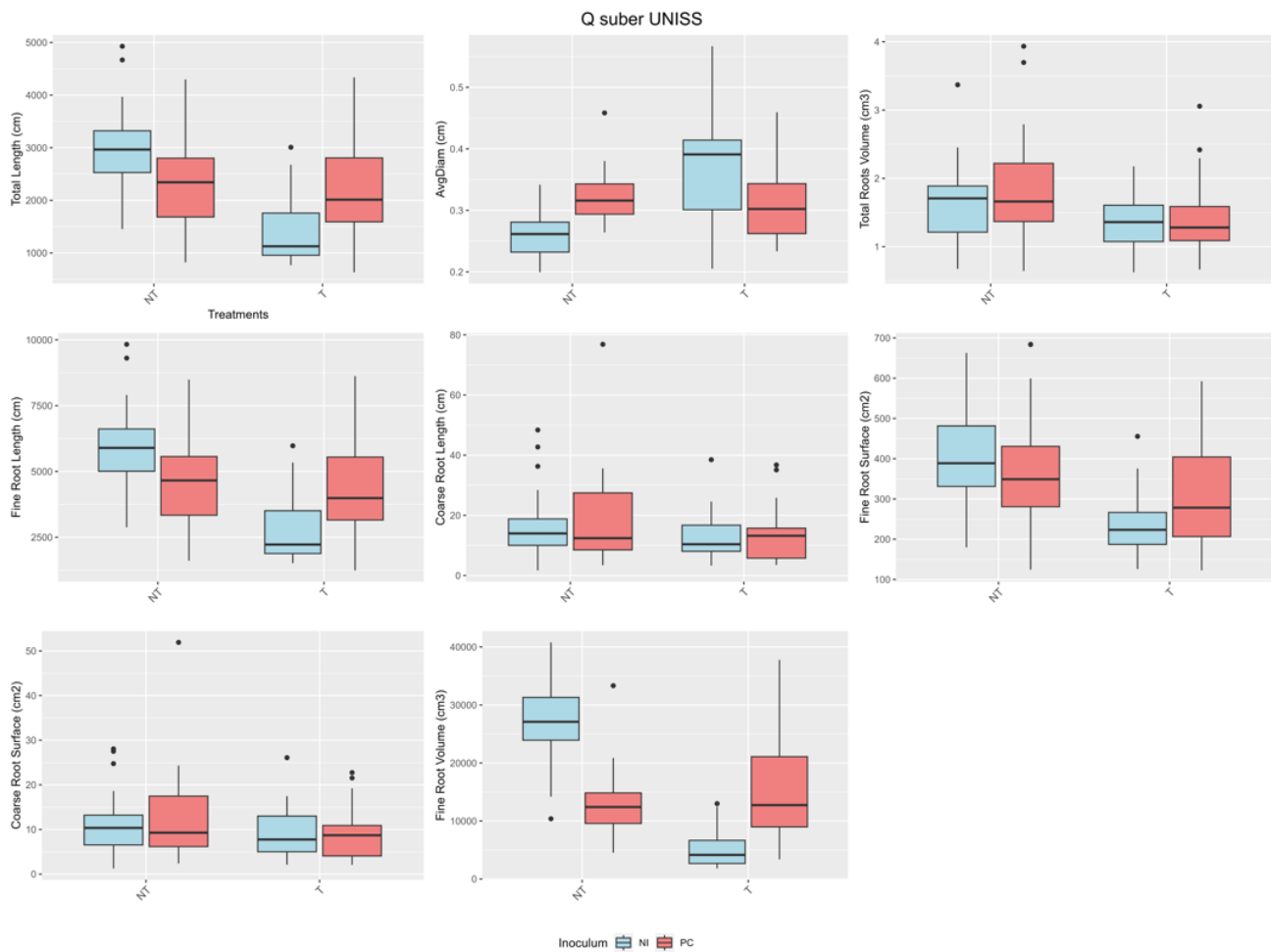
**Figure 15.** Quantum yield of photosystem II ( $\Phi_{PSII}$ ) observed in the seedlings inoculated with *Phytophthora cinnamomi* for the two groups of treatments. The red arrows indicate the four treatments selected for the second experiment.

## 4.2 Second experiment - combined product efficacy

### 4.2.1 Cork oak

#### 4.2.1.1 Root assessment

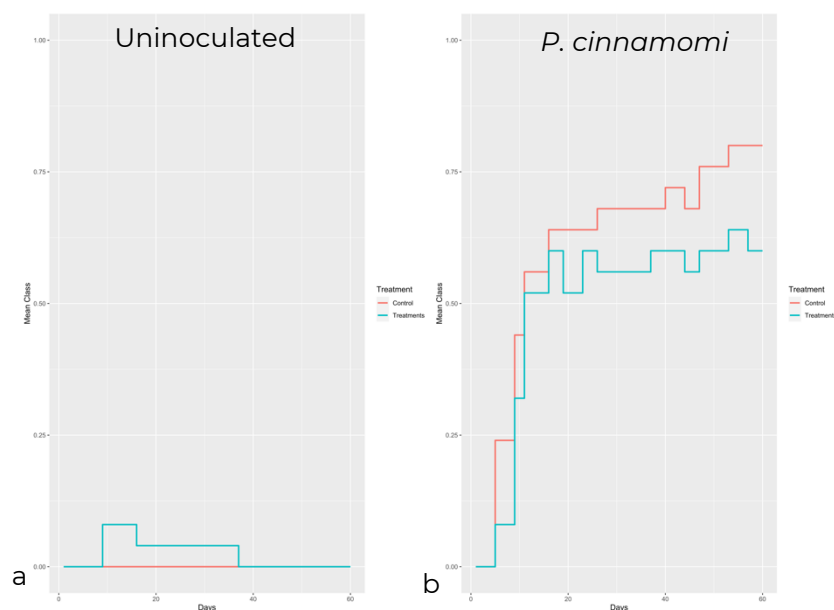
Root analyses showed that the values of average total length, fine root length, fine root surface and fine root volume in cork oak seedlings subjected to treatments were slightly higher in those inoculated with *P. cinnamomi* than in the uninoculated ones. Overall, the average diameter of the roots was higher in the uninoculated plants compared with those inoculated with *P. cinnamomi*. The coarse root surface and length were about the same both in inoculated and uninoculated, and treated and untreated seedlings. However, in all the analysed parameters, no significant statistical differences were detected among neither the inoculated and uninoculated, nor the treated and untreated seedlings (Figure 16).



**Figure 16.** Average value of the cork oak root parameters analysed (from left to right total length, average diameter, total roots volume, fine root length, coarse root length, fine root surface, coarse root surface and fine root volume) in the untreated (NT) and treated (T) seedlings. The red boxplot represents the seedlings inoculated with *Phytophthora cinnamomi* (PC) and the blue represent the uninoculated (NI).

#### 4.2.1.2 Disease index

In the combined products trial, the results showed that the mean disease index detected in the treated seedlings was lower than in those not treated, albeit slightly (Figure 17). In the uninoculated blocks, treated seedlings did not show disease symptoms.



**Figure 17.** Average disease index class for the untreated (red line) and treated (light blue line), uninoculated (a) and inoculated with *Phytophthora cinnamomi* (b) cork oak seedlings.

#### 4.2.2 Holm oak

##### 4.2.2.1 Root assessment

The results of the holm oak roots assessment showed significant differences between inoculated and uninoculated seedlings in all parameters, except for the dry weight, the diameter and the coarse root volume (Figure 18). Root dry matter, average root diameter and percentage of fine roots were significantly influenced by treatment compared with the positive control (Table 2).

**Table 2.** Results of the Dunn's z-test analysis of root biomass from the experiment with holm oak. NC: Negative control. NCIT: Plants treated and mock-inoculated. PC: Positive control. PCIT: Plants treated and inoculated with *P. cinnamomi*.

	$\chi^2$	p-value	NC - NCIT		NC - PC		NC - PCIT		NCIT - PC		NCIT - PCIT		PC - PCIT	
			z	p	z	p	z	p	z	p	z	p	z	p
Dry weight	10.3	0.02**	-0.7	0.239	2.4	0.009**	0.6	0.283	3.1	0.001**	1.3	0.1	-1.8	0.03 <sup>†</sup>
Dry matter %	22.2	<0.001***	2	0.022*	4.2	0.001**	0.3	0.371	2.18	0.015*	-1.7	0.044	-3.9	<0.001***
Average Diam	10.2	0.02**	0.4	0.337	-2.5	0.006**	-0.5	0.309	-2.9	0.002**	-0.9	0.179	2	0.021*
Fine root %	6.2	0.01	-1.1	0.129	-0.03	0.487	-2.1	0.016*	1.1	0.137	-0.9	0.16	2.1	0.018*

<sup>†</sup>: marginally significant; \*, \*\*, \*\*\*: Statistically significant at 0.05, 0.01 and 0.001 probability levels.

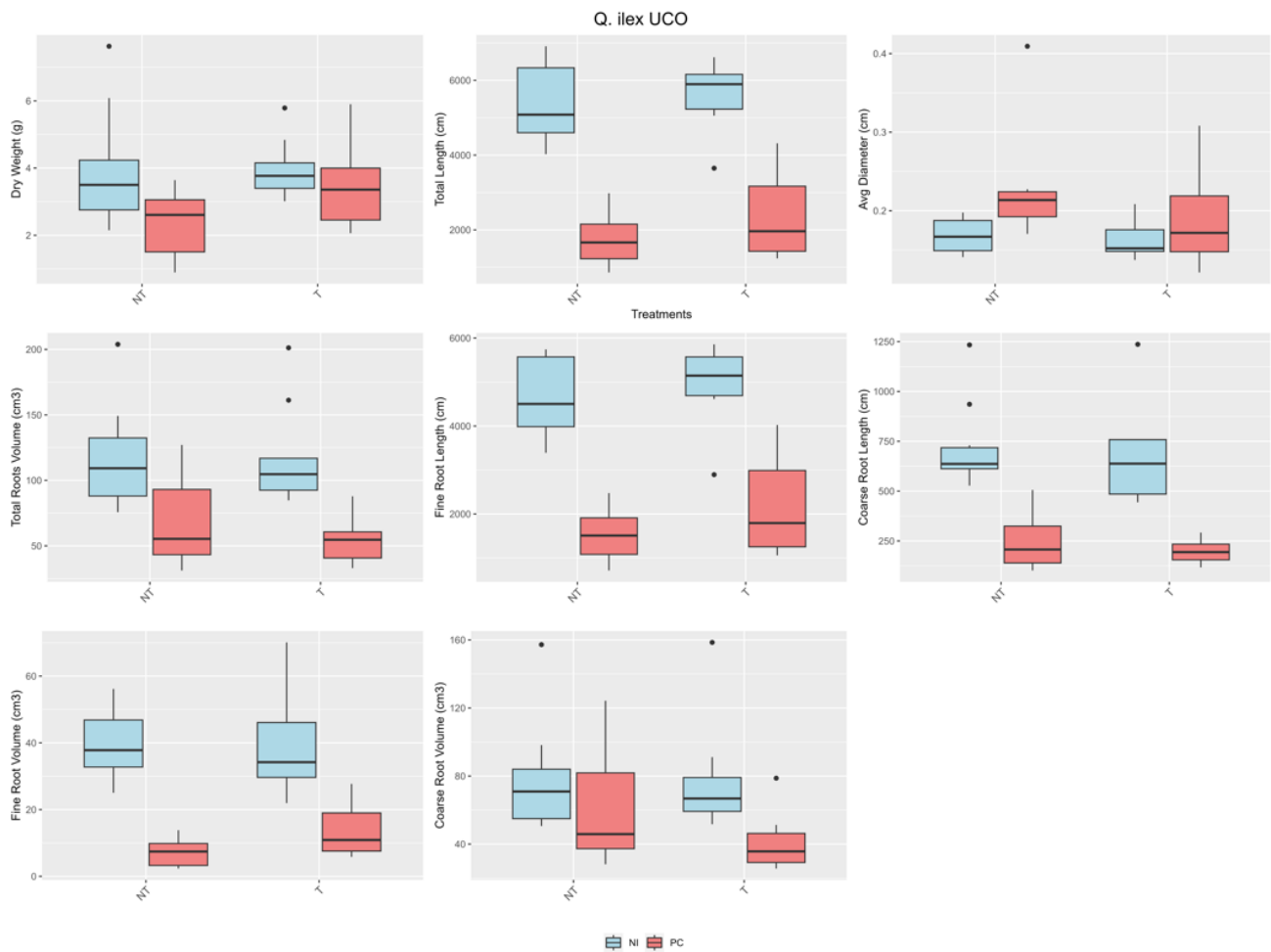
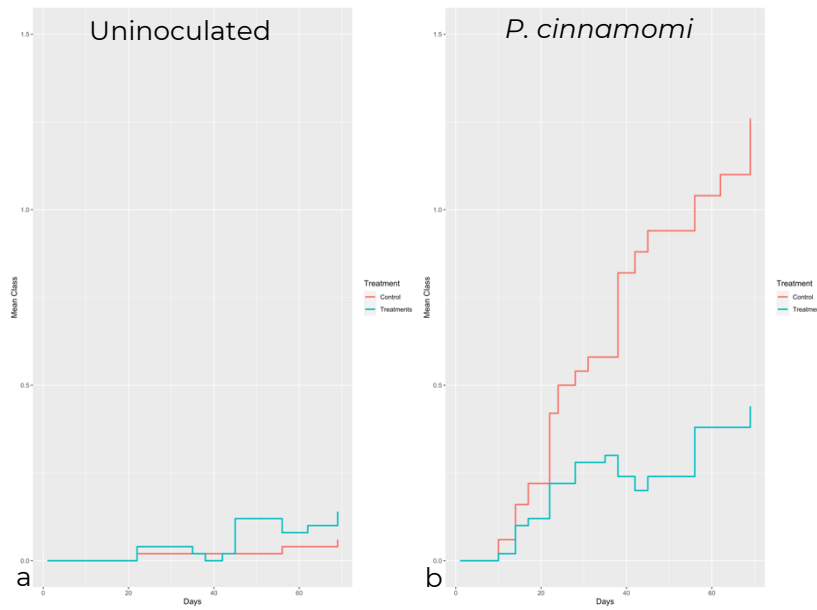


Figure 18. Average value of the holm oak root parameters analysed (from left to right: dry weight, total length, average diameter, total roots volume, fine roots length, coarse root length, fine root volume and coarse root volume) in the untreated (NT) and treated (T) seedlings. With red boxplot are represented seedlings inoculated with *Phytophthora cinnamomi*, and the blue boxplot represents the uninoculated.

#### 4.2.2.2 Disease index

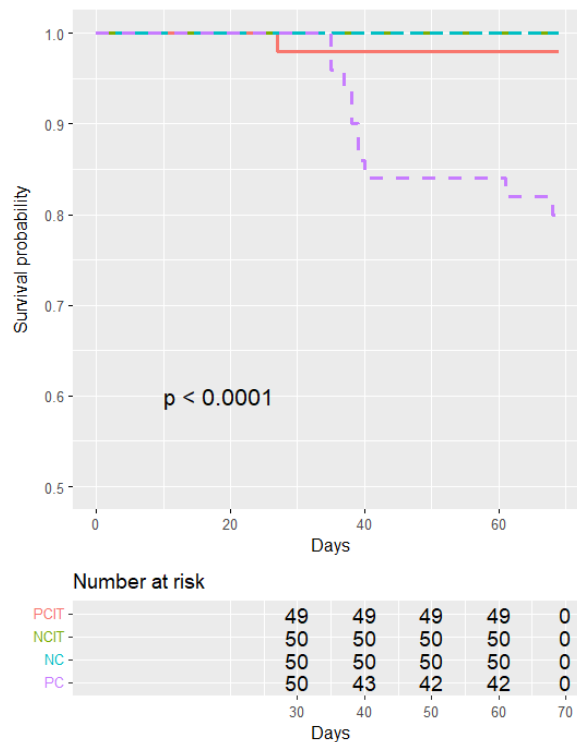
The disease index assessment carried out during the trial showed that the mean classes of the untreated seedlings inoculated with *P. cinnamomi* were significantly higher than those of treated seedlings (Figure 19, b). In contrast, in the uninoculated seedlings (Figure 19, a) the mean disease classes were slightly above zero.



**Figure 19.** Average disease index class for the untreated (red line) and treated (light blue line) and uninoculated (a) and inoculated with *Phytophthora cinnamomi* (b) holm oak seedlings.

#### 4.2.2.3 Mortality

Control plants showed highly significant differences between inoculation treatments, and plants treated with all the products showed a significant lower mortality than control plants (Chisq= 27.9 on 3 degrees of freedom,  $p < 0.001$ ) (Figure 20). Only one seedling was dead at the end of the experiment.



**Figure 20.** Kaplan-Meier survival analysis of the plants treated and inoculated with *Phytophthora cinnamomi* (orange), inoculated with *Phytophthora cinnamomi* (purple), uninoculated (light blue) and uninoculated and untreated seedlings (green).

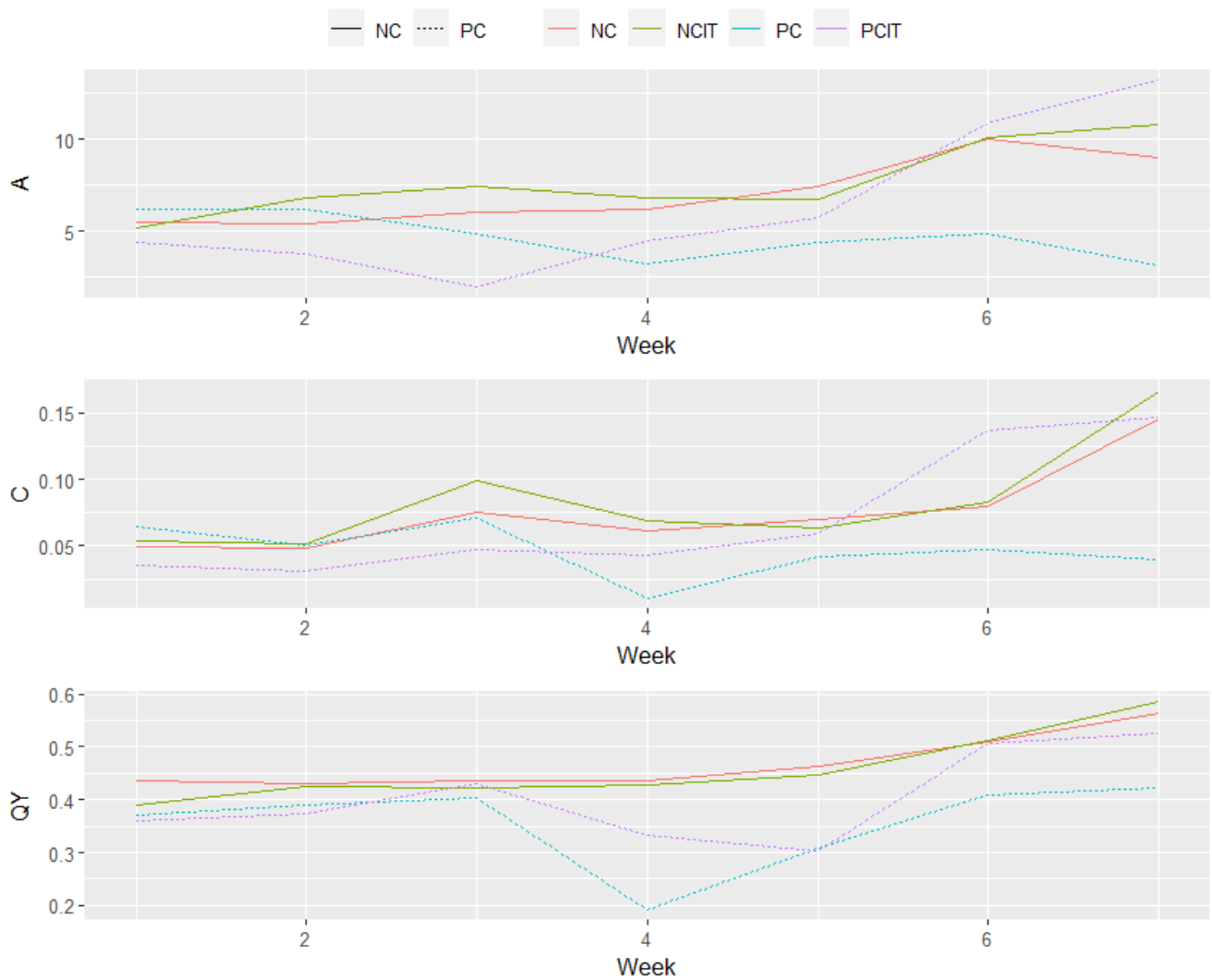
#### 4.2.2.4 Physiological parameters

The second mesocosm experiment showed strong differences in gas exchanges due to both inoculation and treatment parameters, without interaction between them (Table 3). The treatment with the selected products increased gas exchange rates of plants, while inoculation of *P. cinnamomi* decreased it.

**Table 3.** Two-way ANOVA results of photosynthesis rate (*A*) for the second mesocosm experiment.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
<i>Phytophthora</i>	1	123	122.96	13.694	0.000311	***
TREATMENT	1	44.8	44.77	4.986	0.02719	*
<i>Phytophthora</i> :TREATMENT	1	9.6	9.56	1.065	0.303945	
Residuals	136	1221.1	8.98			

Seedlings inoculated with *P. cinnamomi*, and seedlings inoculated with the pathogen and treated with the selected products showed a clear trend during the experiment increasing *A* and  $g_s$ , meanwhile positive control (seedlings inoculated with *P. cinnamomi* but not treated) decreased its gas exchange parameters during the experiment. The increase of *A*,  $g_s$  and  $\Phi_{PSII}$  during the experiment is related with the change in environmental conditions. The experiment was carried out in a greenhouse, where the control of light and temperature was limited. As spring advanced, temperature and daylight hours increased and therefore, also the physiological activity of the seedlings. Therefore, the different trends of treated and untreated plants inoculated with *P. cinnamomi* was highly significant (Figure 21).

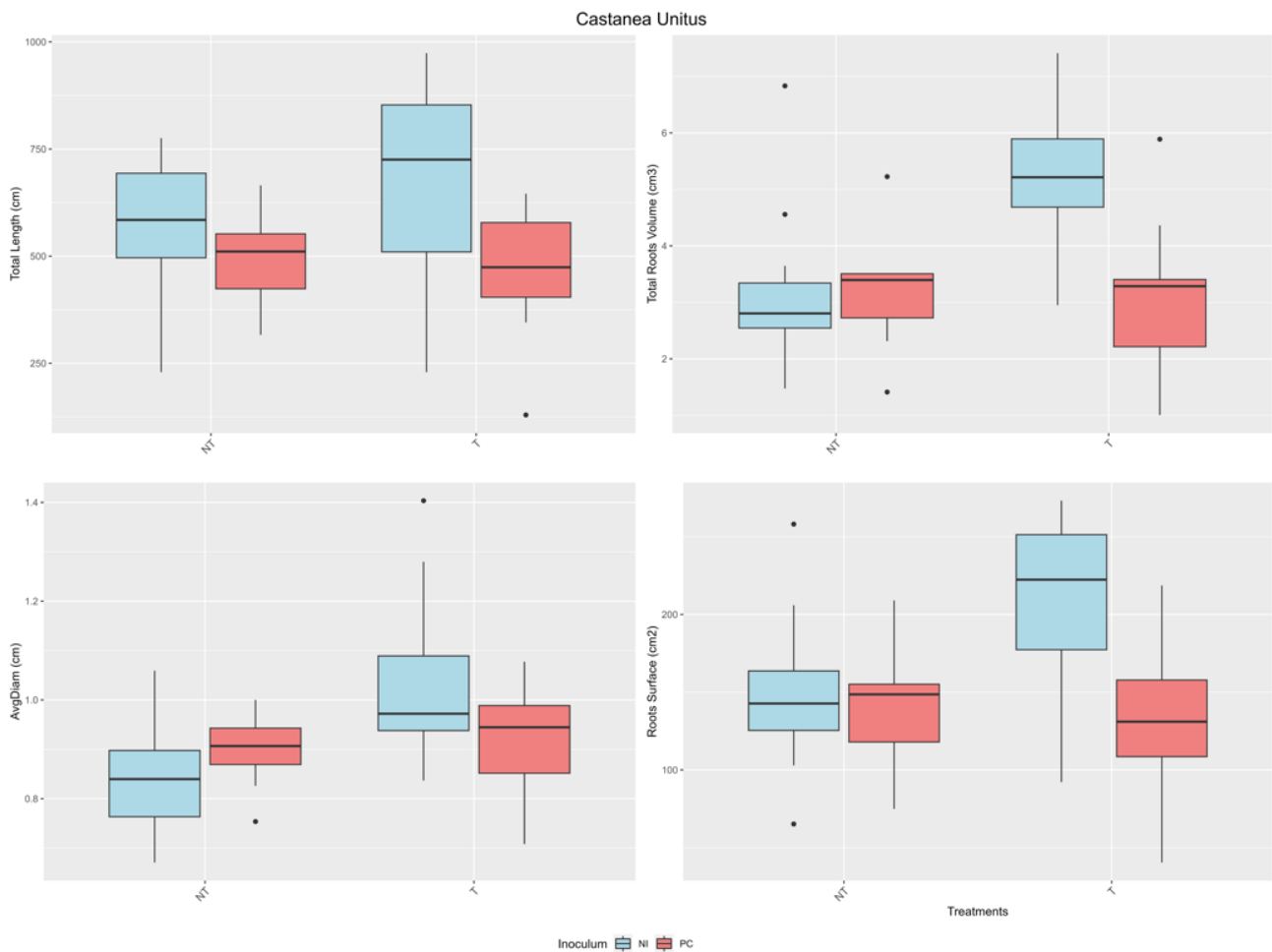


**Figure 21:** Physiological parameters of the second mesocosm experiment: photosynthetic carbon assimilation (A),  $g_s$  stomatal conductance rate (C),  $\Phi_{PSII}$  - fluorescence (QY) for the not treated and not inoculated (NC), only treatments but uninoculated (NCIT), treated and inoculated with *Phytophthora cinnamomi* (PCIT) and inoculated with *Phytophthora cinnamomi* but untreated (PC). Continuous lines represent the uninoculated seedlings, while dashed lines the inoculated with *Phytophthora cinnamomi*.

#### 4.2.3 Sweet chestnut

##### 4.2.3.1 Root assessment

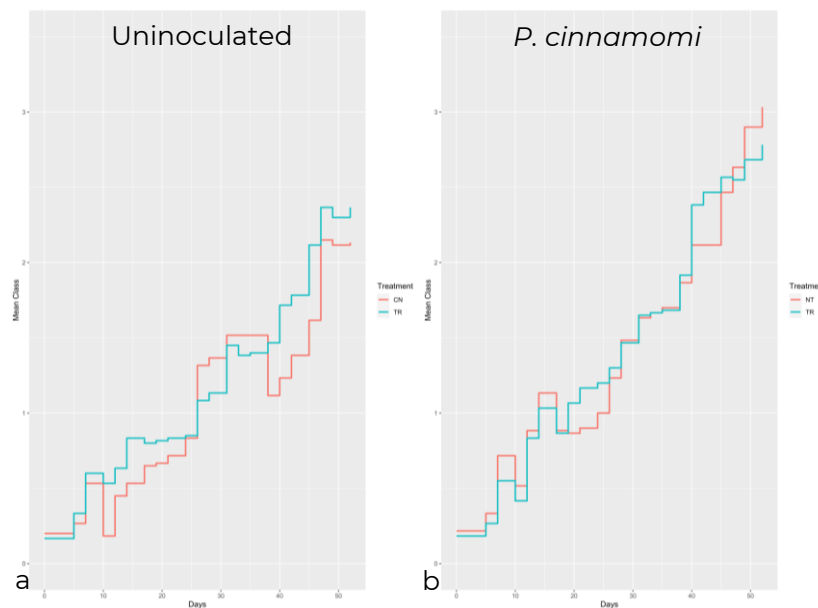
All root parameters assessed on treated sweet chestnut seedlings inoculated with *P. cinnamomi* showed values lower than those uninoculated and treated (Figure 22).



**Figure 22.** Average value of the root parameter analysed (total length, total roots volume, average diameter and roots surface) in the untreated (NT) and treated (T) chestnut seedlings. With red boxplot are represented those inoculated with *Phythophthora cinnamomi*, and the blue boxplot represents the uninoculated.

#### 4.2.3.2 Disease index

The analysis of the mean classes value of the disease index assessment showed that the treated and inoculated with *P. cinnamomi* seedlings were slightly higher than those untreated (Figure 23, b). However, since some symptoms were detected also in the uninoculated plants, both treated and not treated, seedlings may have some imbalance during the trial (Figure 23, a).



**Figure 23.** Average disease index class for the sweet chestnut seedlings untreated (red line) and treated (light blue line), uninoculated (a) and inoculated with *Phytophthora cinnamomi* (b).

## 5. Conclusions

Managing *Phytophthora* diseases in forestry is a challenge due to a lack of available products to be used in forest ecosystems. Therefore, to develop new treatments and ground-based monitoring protocols to be used in integrated pest management two mesocosm trials were implemented. The first experimental trial was carried out on chestnut, cork and holm oak seedlings and included single product treatments to identify the most promising ones to control *Phytophthora* disease. Products were selected based on their known efficacy against *Phytophthora*, but also considering their availability on the market.

On **cork oak** the results suggest that treatments can effectively protect and even enhance cork oak root and aboveground biomass under pathogen attack. Disease index assessments revealed that the treatments Kalex, Kalex EVO and Tricoten were the most effective in reducing symptom severity in cork oak seedlings. The results of the physiological parameters measurement showed that most treatments affect positively their rates, whether seedlings were inoculated with *P. cinnamomi* or not. Notably, Bactrium was among the few products where the photosynthesis rates increased. Stomatal conductance rate was higher in several treatments, such as Bactrium, Tricoten, and Kalex EVO, compared to controls.

In the **holm oak** experiment, Kalex EVO showed the most significant reduction in disease severity compared to uninoculated seedlings and the negative control (inoculated with *P. cinnamomi* and untreated), followed by Tricoten. The analysis on the physiological parameters showed that, in most of the treatments, seedlings had higher rates than those detected on *Phytophthora*-inoculated and untreated seedlings, showing an efficacy in limiting the impact of *P. cinnamomi* infection.

Finally, the disease index assessment on **sweet chestnut** indicated that the lowest mean disease classes were observed on seedling treated with Biofence (liquid), Tricoten and Kalex. Regarding the physiological parameters, the electronic transport (ETR) results showed that most treatments had higher rates than the control at the end of the trials.

Based on the results of the first mesocosm trials, the 4 most effective products for each category (PGPM, antagonist microorganisms, biofumigant and biostimulant) were identified. Then they were used in combination in the second mesocosm trial. Apart from the effectiveness in the mesocosm tests, other factors were considered for the selection of the products to be used in field application of the IPM protocols. These included their availability in the market; their registration for use in the indicated species; and their eco-friendly character. In the case of the biofumigant, the liquid Biofence was chosen because it worked better than the pellet formulation and it is an eco-friendly product that can be used safely. Tricoten and Bactrium were chosen as antagonists and plant growth inducers. Thus, during the project, ATENS will use the WP4 data for their registration. In the case of the resistance inducer, Kalex EVO was chosen as it is registered as fertilizer and therefore can be used on cork and holm oak stands. In the case of sweet chestnut, K-phosphonate was effective, and it is currently registered as Century (BASF) in Italy and Spain.

The results of the second combined products experiments showed that the root system of the treated cork and holm oak seedlings showed a better development compared with the untreated seedlings. In particular, holm oak had on average a higher root length, especially fine roots, compared with the inoculated and untreated seedlings, demonstrating the treatments were very effective on this host.

The same result was detected for the Disease Index, with a significant reduction in symptom development on treated and inoculated seedlings compared to the untreated and inoculated ones. This was remarkable on both cork and holm oak seedlings, confirming the efficacy of the four different products selected and applied together. Positive trends of the physiological parameters were detected on treated cork and holm oak seedlings, which reflect the increase of root biomass and the health of treated seedlings. Hence, the second mesocosm experiment showed that treatments effectively mitigated the negative impacts of *P. cinnamomi* on seedlings.

Overall, treated sweet chestnut seedlings had a higher root biomass than the inoculated with *P. cinnamomi* seedlings. The analysis of the mean class values from the disease index assessment showed that chestnut seedlings treated and inoculated with *P. cinnamomi* had slightly higher values than those untreated. However, some symptoms were also detected in the uninoculated seedlings, both treated and untreated, suggesting a possible imbalance due to environmental greenhouse conditions during the trial.

Despite some minor exceptions, mainly due to environmental and physiological factors that slightly influenced the course of the trials, the results of all parameters analysed showed the effectiveness of the combined treatments of the 4 products identified in the preliminary trials. This made it possible to identify the most promising products that will be used in the implementation of the IPM protocols in chestnut, holm and cork oak stands identified in the project for the field treatments.

## 6. References

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