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Evaluation of *Trichoderma asperellum* ICC012 and *T. gamsii* ICC080 to protect almond pruning wounds from infections caused by fungal trunk pathogens

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Summary. This study evaluated the potential of *Trichoderma asperellum* ICC012 and *T. gamsii* ICC080 to protect almond pruning wounds from infections caused by three major almond trunk pathogens: *Diplodia seriata*, *Eutypa lata*, and *Neofusicoccum parvum*. Dual-culture antagonism assays and two *in planta* wound protection trials were conducted to assess their efficacy. In the first trial, treatments with *T. asperellum* ICC012 + *T. gamsii* ICC080 were applied one or seven days before or after pathogen inoculation to test the impact of application timing, while the second trial focused on preventive strategies, comparing single versus double applications prior to inoculation. Both *Trichoderma* strains alone and mixed were able to inhibit pathogen growth *in vitro*. Experiments *in planta* showed that only pre-infection applications significantly protected pruning wounds, though their efficacy differed by pathogen and treatment strategy. Protection was highest against *E. lata* and *D. seriata*, in which a single treatment prevented infection, whereas *N. parvum* proved more challenging; only a double pre-inoculation application markedly improved its control. Our results demonstrate that preventive wound protection by *T. asperellum* ICC012 + *T. gamsii* ICC080 is essential for effective control. Incorporating these biocontrol agents into almond orchard management can substantially reduce trunk disease infections and limit reliance on synthetic fungicides in Mediterranean production systems.

Keywords. *Diplodia seriata*, *Eutypa lata*, *Neofusicoccum parvum*, nut crops, *Prunus dulcis*.

INTRODUCTION

Almond (*Prunus dulcis* [Mill.] D.A.Webb) is a widely grown nut crop in many Mediterranean countries, as well as in California (USA), South Africa, and some countries in South America and Australasia. Almond trees are well adapted to Spanish semiarid Mediterranean conditions mainly found in the southeastern regions and parts of the Ebro Valley (Gradziel, 2017). Accord-

ing to Food and Agriculture Organization (FAO, 2024), Spain is one of the top producers of almonds after California (USA).

Fungal trunk diseases on fruit and nut crops affecting different cultivars and caused by many diverse pathogens are currently becoming one of the main concerns worldwide, due to important production losses and their negative economic impact (Guarnaccia *et al*., 2022; Martino *et al*., 2025; Luque-Cruz et al., 2026). Fungal trunk diseases of almond trees have been associated with pathogenic species belonging to several distinct taxonomic groups, including members of the families *Botryosphaeriaceae*, *Calosphaeriaceae*, *Cytosporaceae*, *Diaporthaceae*, *Diatrypaceae*, *Togniniaceae*, *Hymenochaetaceae*, *Pleosporaceae*, and *Tympanidaceae* (Slippers *et al*., 2007; Inderbitzin *et al*., 2010; Gramaje *et al*., 2012; Olmo *et al*., 2016; Markakis *et al*., 2017; Lawrence *et al*., 2018; Nouri *et al*., 2018; León *et al*., 2020; Holland *et al*., 2021b; Goura *et al*., 2023).

The role of wounds in the epidemiology and management of fungal trunk diseases in fruit and nut crops is crucial (Guarnaccia *et al*., 2022). In almond cultivation, pruning wounds serve as important infection courts for various fungal pathogens that cause trunk diseases, leading to sunken cankers, vascular tissue girdling, branch dieback, and, in severe cases, tree death (Inderbitzin *et al*., 2010; Gramaje *et al*., 2012; Holland *et al*., 2021b).

Researchers both in California and Spain have explored a variety of management strategies to protect pruning wounds in almonds through chemical and biological control. In Spain, Olmo *et al*. (2017) demonstrated the effectiveness of the fungicide thiophanate-methyl for pruning wound protection against infections caused by Botryosphaeriaceae species. Subsequently, Holland *et al*. (2021a) confirmed the efficacy of the same product in California against five almond trunk pathogens *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Cytospora sorbicola* Norph., Bulgakov, T.C. Wen & K.D. Hyde, *Ceratocystis destructans* L.A. Holland, D.P. Lawr. & Trouillas and *Eutypa lata* (Pers.) Tul. & C. Tul.

However, biological control has gained a strong interest in managing pruning wound diseases due to the progressive banning of chemical fungicides together with the high restrictions that they currently face worldwide due to their harmful effects on human health and the environment (Lahlali *et al*., 2022). Biological control agents (BCAs) such as *Trichoderma atroviride* P. Karst. SC1, *T. paratroviride* Jaklitsch & Voglmayr RTFT014, and *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams J1446 had proven their efficacy to protect almond wounds against fungal trunk pathogens in California (Holland *et al*., 2021a; Travadon *et al*., 2023a, b). While in Spain, the BCA *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 AC17 has recently been suggested as the best candidate for the biological control of Botryosphaeriaceae fungi infecting almond pruning wounds (Romero-Cuadrado *et al*., 2024).

Apart from the application of BCAs based on bacterial strains in Spain, all other biological control studies have been performed in California investigating BCAs products authorised or developed there. Nevertheless, in Mediterranean European almond producing countries, there is a lack of information about management strategies that may be useful for wound protection against trunk pathogens using fungal based BCAs. Thus, the objective of our work was to determine the potential biocontrol activity and wound protection of *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg (isolate ICC012) and *T. gamsii* Samuels & Druzhin. (isolate ICC080) against three frequently found almond trunk disease pathogens in Mediterranean conditions: *Diplodia seriata* De Not., *E. lata*, and *N. parvum*.

MATERIALS AND METHODS

Fungal isolates

In this study, we used three isolates of the following species of almond trunk pathogens: *D. seriata* (BAL-45, Binissalem, Balearic Islands), *E. lata* (GIHF-311, Caravaca de la Cruz, Murcia), and *N. parvum* (BAL-42, Sant Llorenç des Cardassar, Balearic Islands). These isolates were obtained from almond trees showing symptoms of cankers and internal wood necrosis. They were hyphal tipped prior to storage in 15% glycerol solution at -80°C into 1.5 mL cryovials at the fungal collection of the Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Spain. Moreover, *Trichoderma asperellum* (isolate ICC012) and *T. gamsii* (isolate ICC080) cultures, which are the components of the commercial product Blindar®, were provided by Gowan Crop Protection Limited, and they were also stored as described before.

Dual culture antagonism assay

Antagonistic effect of the *Trichoderma* isolates against the selected almond trunk pathogens was determined using dual culture assays on Potato Dextrose Agar (PDA, Biokar-Diagnostics, Zac de Ther, France) plates.

Pathogens inoculum was obtained from the margins of seven-day-old colonies actively growing on PDA. From these edges, mycelial plugs (6 mm in diam-

eter) were extracted for use in the confrontation assays. *Trichoderma* isolates were cultured on PDA and incubated for ten days at 25°C in darkness. To harvest conidia, the agar surface was flooded with 10 mL of sterile distilled water (SDW) and gently scraped with a sterile spatula. The suspension was filtered through a double layer of cheesecloth and adjusted using a hemocytometer to a final concentration of 10^6 conidia mL^{-1}. Moreover, these conidial suspensions were used to prepare an additional *Trichoderma* suspension containing a mixture of both isolates (*T. asperellum* + *T. gamsii,* 50% each), which was also adjusted to 10^6 conidia mL^{-1}. Drops (20 µL) of the three different *Trichoderma* conidial suspensions were used for the dual confrontation experiments.

The assay was performed in 90 mm Petri dishes containing PDA. A six mm pathogen plug was placed on one side of the dish, while a 20 µL drop of the corresponding *Trichoderma* suspension was placed at the opposite edge. All plates were incubated at 25°C in darkness for seven days. Control treatments consisted of PDA plates inoculated solely with the pathogen plug under the same incubation conditions to provide a baseline for radial growth comparison.

The experiment was conducted twice with four replicate plates per pathogen/*Trichoderma* suspension combination, and the area occupied by each microorganism was measured using ImageJ software (Rueden *et al*., 2017), based on a reference distance common to all images. The percent of mycelium growth inhibition was calculated using the formula: Percent Growth Inhibition (PGI) = [(B – A) / B] × 100, where A is the area of pathogen mycelium growth coinoculated with *Trichoderma* and B is the area of the pathogen mycelium growth alone in the control plate (Úrbez-Torres *et al*., 2020).

Pruning wound protection trials

Two different pruning wound protection trials against selected trunk pathogens were established using 1-y-old almond plants 'Avijor' grafted onto GF-677 rootstock.

Pathogens inoculum preparation

Inoculum of *D. seriata* and *N. parvum* were prepared as conidial suspensions according to Elena and Luque (2016). A mycelial plug of each pathogen, previously grown on PDA for seven days at 25°C, was put on a center of a water agar plate (WA) (Bacto Agar; BD Biosciences, NJ). Several sterile fragments (approximately n = 20) of pine needles (three cm long) were put on the media surface at about 1.5 cm surrounding the mycelial plug. Plates were incubated for four weeks at 25°C under combined near-UV and white fluorescent light (Philips TL-D 18W BLB and Sylvania Standard F18W/33-640-TS cool white, respectively) at a photoperiod of 12 and 12 h, until pycnidia formation. One day before inoculation, pine needles (approximately n = 30) with pycnidia were placed in a 250 mL Erlenmeyer flask containing 50 mL of sterile distilled water. The solution was kept overnight (approximately 16 h) at 4°C to prevent conidia germination, and in constant agitation with the help of a magnetic stirrer to induce conidia release from the pycnidia. On the next day, the suspension was vacuum-filtered through a 60-µm Steriflip filter (Millipore Corporation, Billerica, MA) to get a cleaner suspension. The conidial suspension was adjusted to 2×10^4 conidia mL^{-1} using a hemocytometer. Inoculum of *E. lata* consisted of a suspension of mycelial fragments from liquid cultures, which was prepared as described by Travadon *et al*., (2013). To produce the starter culture, ten 2 × 2-mm plugs from a seven-day culture on PDA were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of potato dextrose broth (PDB; Condalab, Spain) and incubated five days at 25°C and 150 rpm. A blender cup (Oster 4655ESP) was used to homogenize 40 mL of the PDB culture (one min, speed three), and two mL of this homogenized starter culture was then inoculated to a 125 mL Erlenmeyer flask containing 40 mL of PDB. After incubation five days at 25°C and 150 rpm, the entire 40 mL liquid culture was homogenized and the concentration of mycelial fragments, which were primarily <0.5 mm in length, was adjusted with SDW to 2×10^4 fragments mL^{-1} using a hemocytometer.

Trichoderma treatments preparation

A water suspension of formulated *T. asperellum* ICC012 + *T. gamsii* ICC080 (Blindar®; 3×10^7 CFU per gram of formulated product) at ten g L^{-1} was used. The viability of the conidia in the commercial product was checked to be at a minimum of 90% before each trial. For this purpose, a serial dilution of the conidia suspension was plated on PDA and the colony forming units were counted after 24–48 h incubation at room temperature.

First pruning wound protection trial

Main stems (0.5 cm diameter) of one-year-old potted almond plants 'Avijor' grafted onto GF-677 rootstock were pruned to 30 cm above the grafting point to simulate a fresh pruning wound. For each pathogen four dif-

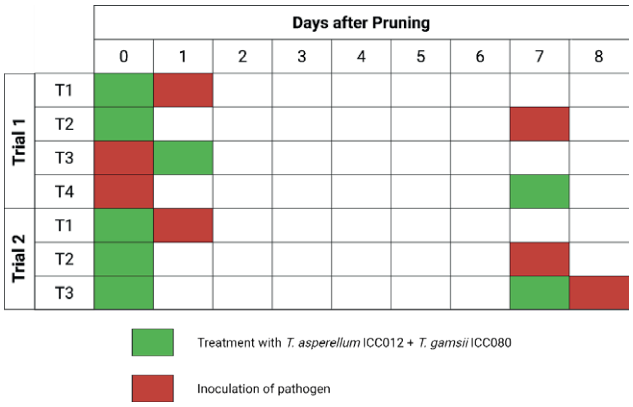

Figure 1. Schematic diagram illustrating the experimental design and treatment timing across the first and second pruning trials. In the first trial, for each pathogen (*Diplodia seriata*, *Eutypa lata* or *Neofusicoccum parvum*) four different treatments with *Trichoderma asperellum* ICC012 + *T. gamsii* ICC080 were evaluated: two pre-infection strategies (T1 and T2) and two post-infection strategies (T3 and T4). In the second trial, three pre-infection strategies with one (T1 and T2) or two (T3) *T. asperellum* ICC012 + *T. gamsii* ICC080 applications were evaluated.

ferent treatments with *T. asperellum* ICC012 + *T. gamsii* ICC080 were evaluated: two pre-infection strategies (T1-Plants treated immediately after pruning and inoculated 24 h after the treatment; and T2-Plants treated immediately after pruning and inoculated 7 d after the treatment) and two post-infection strategies (T3-Plants inoculated immediately after pruning and treated 24 h after infection; and T4-Plants inoculated immediately after pruning and treated seven days after infection) (Figure 1). Positive controls included non-treated but inoculated wounds with each pathogen at 24 h or seven days after pruning. Negative controls included pruned non-treated and non-inoculated plants to determine if natural infections occurred. *Trichoderma* treatments and the inoculation with each of the pathogens were performed by pipetting a drop of 20 µL onto the pruning wound, respectively. For each combination of treatment and pathogen, nine replicates (plants) were used. Plants were maintained in a temperature-controlled greenhouse and arranged in a completely randomized design. The experiment was repeated.

The plants were maintained in the greenhouse for two months. After this time, a three cm fragment of the main stem of each plant was cut below the wound and collected. In the laboratory, these three cm fragments were flame sterilized with 70% ethanol. Then, the bark around the fragments was removed. In each fragment, a tissue piece (≈ one mm) from the surface of the pruning wound was discarded, and five small pieces of necrotic tissue were plated onto one plate of two% malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS), in an attempt to recover the inoculated fungi and evaluate fungal colonization. Plates were incubated for up to 10 d at 25°C in the dark. If a piece of necrotic tissue yielded either *D. seriata*, *E. lata* or *N. parvum*, it was rated as colonized by the respective pathogen. From the data of these isolations, the mean percent recovery (MPR) (number of pieces colonized by each pathogen relative to the total number of pieces plated) was determined from all positive controls and treatments. Treatment effectiveness was calculated as the mean percent disease control (MPDC) using the formula: MPDC = 100 × [1 - (MPR treated plants / MPR non-treated control plants)] (Úrbez-Torres *et al*., 2020).

Second pruning wound protection trial

In this trial, inoculum preparation of the pathogens and *Trichoderma*, pathogens inoculation, and *Trichoderma* treatments were conducted as described previously.

Three pre-infection strategies with one or two *T. asperellum* ICC012 + *T. gamsii* ICC080 applications were studied: T1-Plants treated immediately after pruning and inoculated 24 h after treatment; T2-Plants treated immediately after pruning and inoculated seven days after treatment; and T3-Plants treated immediately after pruning and seven days later, and inoculated 24 h after the second treatment (Figure 1). Positive controls included non-treated but inoculated wounds with each pathogen at 24 h or seven days after pruning. Negative controls included non-treated and non-inoculated plants to determine if natural infections occurred. For each combination of treatment and pathogen, eight replicates (plants) were used. Plants were maintained in a temperature-controlled greenhouse for two months and arranged in a completely randomized design. The experiment was repeated. Fungal isolation, MPR and MPDC calculation were performed as described before.

Statistical analyses

For the dual culture antagonism assay the antagonistic activity of *Trichoderma* spp. against the fungal trunk pathogens was evaluated as the mean percent inhibition (MPI) of mycelial growth measured after seven days, calculated from the corresponding replicates (four per experiment and repetition). Regarding the pruning wounds protection experiments, the MPR by each pathogen was determined from all positive con-

trols and treatments calculated from the corresponding replicates (nine and eight plants per experiment in the first and second trials, respectively). Statistical analyses were conducted using R version 4.2.0 (R Core Team, 2024). For the experiment and treatment effect, values were analyzed using the Kruskal–Wallis multiple comparison test ($P < 0.05$). When differences were significant, Dunn's post hoc test was applied using the packages "agricolae" and "dunn.test" (De Mendiburu, 2023; Dinno, 2024).

RESULTS

Dual culture antagonism assay

The antagonistic activity of *T. asperellum* ICC012, *T. gamsii* ICC080, and a 50% mixture of both isolates was evaluated against three fungal trunk pathogens*: E. lata*, *D. seriata*, and *N. parvum*, as shown in Figure 2. The mean percent inhibition (MPI) of mycelial growth after seven days of incubation is presented in Figure 3.

The percentage of inhibition of mycelial growth of *D. seriata*, *E. lata* and *N. parvum* by *Trichoderma* strains used in this experiment ranged from 59.35% (*T. gamsii* ICC080 with *D. seriata*) to 80.89% (*T. asperellum* ICC012 + *T. gamsii* ICC080 with *N. parvum*).

For *E. lata*, the *T. asperellum* ICC012 strain resulted in an MPI of 80.57%, the *T. gamsii* ICC080 treatment in 61.06%, and the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture in 80%. The *T. asperellum* ICC012 alone and the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture significantly inhibited the growth of *E. lata* compared to *T. gamsii* ICC080 alone. In the case of *D. seriata*, the *T. asperellum* confrontation had an MPI of 73.85%, *T. gamsii* resulted in 59.35%, and the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture showed 65.48% inhibition. Again, *T. asperellum* ICC012 alone resulted in the highest inhibition, followed by the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture and *T. gamsii*

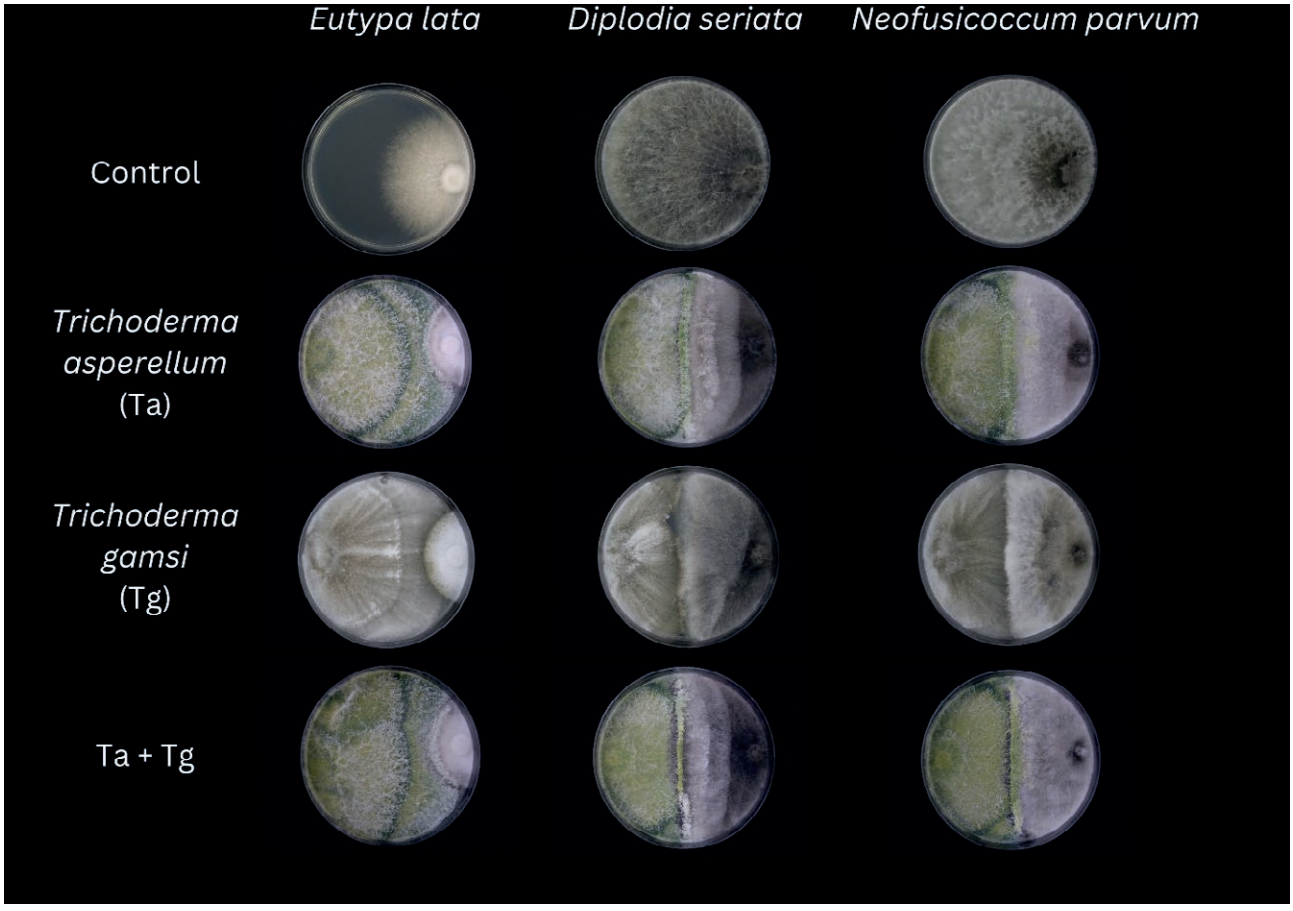

Figure 2. Dual culture antagonism experiment. Petri plates show antagonistic activity of each *Trichoderma* strain used in this study and the mixture of both strains (left side of the plate) against the fungal trunk pathogens: *Eutypa lata*, *Diplodia seriata*, and *Neofusicoccum parvum* (right side of Petri plate) seven days after incubation on PDA at 25°C.

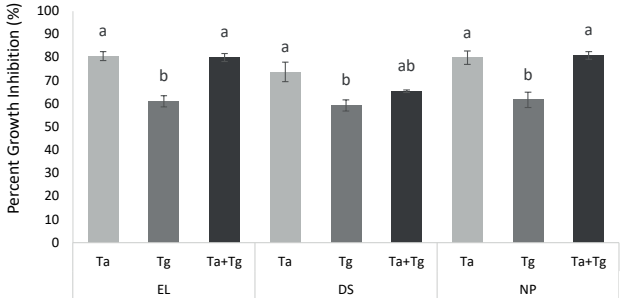

Figure 3. Antagonistic activity of *Trichoderma asperellum* isolate ICC012 (Ta), *T. gamsii* isolate ICC080 (Tg) and *T. asperellum* + *T. gamsii* 50% each isolate (Ta+Tg) against fungal trunk pathogens *Eutypa lata* (EL), *Diplodia seriata* (DS), and *Neofusicoccum parvum* (NP). Values represent the mean percent inhibition of mycelial growth measured after seven days calculated from eight replicates and bars represent standard errors of the means. For each pathogen, columns with the same letter were not statistically different according to Dunn's post hoc test ($P < 0.05$).

ICC080. For *N. parvum*, *T. asperellum* ICC012 showed an MPI of 79.96%, *T. gamsii* ICC080 had 61.77%, and the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture resulted in 80.89%. As occurred with *E. lata*, *T. asperellum* ICC012 and the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture exhibited significant inhibition compared to *T. gamsii* ICC080 alone.

Results analysis showed that the inhibition effects of *T. asperellum* ICC012 and *T. asperellum* ICC012 + *T. gamsii* ICC080 were not significantly different for any of the pathogens. For *E. lata* and *N. parvum* both *T. asperellum* ICC012 and *T. asperellum* ICC012 + *T. gamsii* ICC080 were significantly more effective for mycelial growth inhibition than *T. gamsii* ICC080 alone. But, for *D. seriata* there were statistically significant differences only between *T. asperellum* ICC012 and *T. gamsii* ICC080.

Pruning wound protection trials

First pruning wound protection trial

The efficacy of *T. asperellum* ICC012 + *T. gamsii* ICC080 was evaluated using four different treatment strategies: two pre-infection treatments and two post-infection treatments. Statistical analysis showed no significant differences between the two experimental replicates (*E. lata* $P = 0.727$; *D. seriata* $P = 0.262$; *N. parvum* $P = 0.056$), so data were pooled. The MPR and MPDC values are presented in Table 1. Fungal pathogens were not isolated from the non-inoculated negative controls.

For *E. lata*, the pre-infection treatment T1 presented a MPR of 16.6% and a MPDC of 79.9%. Pre-infection treatment T2 led to a MPR of 7.7% and a MPDC of 90.6%, while the post-infection treatments T3 and T4 exhibited MPR values of 38.8% and 54.4%, respectively, with corresponding MPDC values of 53.1% and 34.4%. For *D. seriata,* the treatment T1 had a MPR value of 37.7% and a MPDC of 57.5%, and treatment T2 had a MPR of 35.5% and a MPDC of 60.1%. Post-infection treatments T3 and T4 had MPR values of 75.5% and 66.6%, with MPDC values of 15.1% and 25.1%, respectively. For *N. parvum*, the pre-infection treatments T1 and T2 resulted in MPRs of 64.4% and 84.4%, with

Table 1. First trial. Mean percent recovery (MPR) and mean percent disease control (MPDC) of *Eutypa lata*, *Diplodia seriata* and *Neofusicoccum parvum* from pruning wounds treated with *Trichoderma asperellum* ICC012 + *T. gamsii* ICC080 in preventive (T1-Plants treated immediately after pruning and inoculated 24 h after the treatment; and T2-Plants treated immediately after pruning and inoculated seven days after the treatment) or curative strategies (T3-Plants inoculated immediately after pruning and treated 24 h after; and T4-Plants inoculated immediately after pruning and treated seven days after).

Treatment	Inoculation day	Value	*Eutypa lata*		*Diplodia seriata*		*Neofusicoccum parvum*	
Control			83.3±5.6	A	88.8±6.1	A	93.3±5.6	A
T1	1 after treatment	MPR	16.6±7.2	B	37.7±9.2	CD	64.4±9.9	B
		MPDC	79.9		57.5		30.7	
T2	7 after treatment	MPR	7.7±4.8	B	35.5±10.1	D	84.4±6.3	A
		MPDC	90.6		60.1		9.2	
T3	1 before treatment	MPR	38.8±10.9	A	75.5±9.9	B	91.1±5.9	A
		MPDC	53.1		15.1		2.03	
T4	7 before treatment	MPR	54.4±9.4	A	66.6±9.2	BC	90±6.9	A
		MPDC	34.4		25.1		3.2	

MPR, mean percent recovery.
MPDC, mean percent disease control was calculated as 100 x [1-(MPR treatment/MPR control)].
Values followed by the same letter(s) in each column were not statistically different using the Kruskal-Wallis multiple comparison test. When differences were significant, Dunn's post hoc test was applied.

Table 2. Second trial. Mean percent recovery (MPR) and mean percent disease control (MPDC) of *Eutypa lata*, *Diplodia seriata* and *Neofusicoccum parvum* from pruning wounds treated with *Trichoderma asperellum* ICC012 + *T. gamsii* ICC080 in three preventative strategies (T1-Plants treated immediately after pruning and inoculated 24 h after treatment; T2-Plants treated immediately after pruning and inoculated seven days after treatment; and T3-Plants treated immediately after pruning and seven days later, and inoculated 24 h after the second treatment).

Treatment	Inoculation day/ number of applications	Value	*Eutypa lata*		*Diplodia seriata*		*Neofusicoccum parvum*	
Control 1			73.3±12.3	A	53.3±18.4	A	66.6±16.05	A
T1	1 day/1	MPR	12.5±5.1	B	7.5±3.5	B	57.5±10.6	A
		MPDC	82.95		85.9		13.7	
Control 2			20±13.6	A	0	-	40±20	A
T2	7 days/1	MPR	0	B	1.2±1.2	-	13.7±5.9	AB
		MPDC	100		nc		65.6	
T3	7 days/2	MPR	1.2±1.2	B	0	-	5±5	B
		MPDC	93.7		nc		87.5	

MPR, mean percent recovery.
MPDC, mean percent disease control was calculated as 100 x [1-(MPR treatment/MPR control)].
Values followed by the same letter(s) in each column were not statistically different regarding its respective control using the Kruskal–Wallis multiple comparison test. When differences were significant, Dunn's post hoc test was applied.

MPDC values of 30.7% and 9.2%, respectively. The post-infection treatments T3 and T4 yielded MPR values of 91.1% and 90.0%, with MPDC values of 2.03% and 3.2%, respectively.

These results indicated that the application of *T. asperellum* ICC012 + *T. gamsii* ICC080 was most effective when applied in preventive strategies than curative strategies. This was very clear in the case of inoculation with *E. lata*, in which only preventive treatments T1 and T2 showed statistically significant differences with the untreated control ($P = 0.0002$). For *D. seriata* all treatments were significantly different from the untreated control ($P = 1.7\ 10^{-7}$), and for *N. parvum* only the preventive treatment T1 was significantly different from the untreated control ($P = 0.008$).

Second pruning wound protection trial

The pruning wound protection efficacy of *T. asperellum* ICC012 + *T. gamsii* ICC080 was further evaluated using only three pre-infection strategies with one or two applications of the product. Statistical analysis showed no significant differences between the two experimental replicates (*E. lata* $P = 0.896$; *D. seriata* $P = 0.223$; *N. parvum* $P = 0.226$), so data were pooled. The MPR and MPDC values are presented in Table 2. Fungal pathogens were not isolated from the non-inoculated negative controls.

For *E. lata*, the treatment T1 resulted in a MPR of 12.5% and an MPDC of 82.9%, treatment T2 had a MPR of 0% and a MPDC of 100%, while treatment T3 showed a MPR of 1.2% and a MPDC of 93.7%. For *D. seriata*, the treatment T1 resulted in a MPR of 7.5% and a MPDC of 85.9%, but this fungus could not be reisolated from the control plants infected seven days after pruning, thus invalidating the evaluation of the efficacy of the treatments T2 and T3, although their MPR values were very low (1.2% and 0%, respectively). For *N. parvum,* the treatment T1 resulted in a MPR of 57.5% and a MPDC of 13.7%, while treatments T2 and T3 had a MPR of 13.7% and a MPDC of 65.6%, and a MPR of 5% and a MPDC of 87.5%, respectively.

For *E. lata*, all preventive treatments with *T. asperellum* ICC012 + *T. gamsii* ICC080 were significantly different from their respective untreated controls ($P = 0.0007$ for T1 and $P = 0.031$ for T2 and T3). In *D. seriata* the treatment T1 was also significantly different from the untreated control ($P = 0.008$). But, for *N. parvum* only the treatment T3, with two applications of *T. asperellum* ICC012 + *T. gamsii* ICC080, was significantly different from its control ($P = 0.047$).

DISCUSSION

The dual culture and pruning wound assays collectively demonstrate that *T. asperellum* ICC012 + *T. gamsii* ICC080 can protect pruning wounds from infections caused by some of the major almond trunk pathogens in both *in vitro* and *in planta* experiments.

Our *in vitro* data showed *T. asperellum* is a potent antagonist against *D. seriata*, *E. lata*, and *N. parvum*. The combination of the strains *T. asperellum* ICC012

+ *T. gamsii* ICC080 showed inhibition levels statistically comparable to *T. asperellum* alone for all three pathogens, whereas *T. gamsii* alone was less effective. This is similar to the findings by Holland *et al*. (2021a), who observed that a mixed *T. harzianum* Rifai + *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx product (RootShield Plus®) was considerably less effective (only ~63% protection) than a single-strain *T. atroviride* treatment (93% protection) in almond trials. According to our results, the composition and compatibility of strains in a bioproduct are thus crucial for ensuring its effectiveness. In our case, combining *T. asperellum* ICC012 and *T. gamisii* ICC080 did not compromised antagonism performance relative to the one showing the best results alone, suggesting these two are complementary. Indeed, the species *T. asperellum* and *T. gamsii* have distinct but synergistic traits: *T. asperellum* produces a rich arsenal of antibiotics, competes aggressively for nutrients/space, and mycoparasitizes pathogens (Verma *et al*., 2007; Wu *et al*., 2017), while *T. gamsii* is noted for prolific volatile antifungal metabolites and an ability to tolerate cooler temperatures (Rinu *et al.,* 2014). By combining biological control agents, the product can exploit multiple modes of action and remain active under a wider range of environmental conditions. In fact, Di Marco *et al*. (2022) already showed that the *T. asperellum* ICC012 + *T. gamsii* ICC080 mixture was effective against diverse fungal grapevine trunk pathogens such as *Fomitiporia mediterranea* M. Fisch., *N. parvum* and *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams.

In planta, preventive treatments of pruning wounds with the mixture of *T. asperellum* ICC012 + *T. gamsii* ICC080 provided substantial protection, whereas post-infection (curative) applications were far less effective. These results underscore that the *Trichoderma* biocontrol is most effective as a protectant applied to fresh wounds, and notably less so as a curative applied after pathogen infection has occurred (Guzmán-Guzmán *et al*., 2023).

Neofusicoccum parvum proved to be the most challenging pathogen as it is an aggressive, fast-colonizing fungus known to cause extensive almond dieback (Holland *et al.,* 2021b). Our curative treatments were not effective for *N. parvum* (recovery of the fungus from inoculated wounds remained high), and even a single pre-infection application sometimes showed suboptimal control. Recovery of *N. parvum* from inoculated wounds was significantly reduced only when two consecutive pre-infection applications were used, probably allowing *Trichoderma* strains to firmly establish in the wounded area. Travadon *et al*. (2023a) reported that wound protection against *N. parvum* significantly improved when the pathogen was inoculated seven days after *Trichoderma* application, instead of 24 hours. They attributed this result to *N. parvum* high virulence and rapid wood colonization, whereas *E. lata* (a less virulent and more slowly invading pathogen) infections could be reduced by *Trichoderma* even with a shorter establishment period. Consistently, in our study *E. lata* was effectively controlled by a single pre-infection treatment, highlighting that preventive wound colonization by *Trichoderma* can almost completely supress infections by this pathogen. However, when *E. lata* was allowed to infect wounds before treatment, *Trichoderma* was much less effective. On the other hand, *D. seriata* exhibited intermediate aggressiveness between *N. parvum* and *E. lata*. Its generally less aggressive behaviour when compared with *N. parvum* can made wounds easier to protect. Our results showed that even one preventive treatment of *T. asperellum* ICC012 + *T. gamsii* ICC080 provided effective control of *D. seriata*.

These patterns reinforce that the timing of BCAs application is critical, and that pathogen aggressiveness based on how fast it is able to colonize the wound niche influence how effective the BCA wound protectant effect will be. Holland *et al*. (2021a) reported that a single application of *T. atroviride* SC1 (a commercial strain in product Vintec®) achieved 81–100% protection of almond pruning wounds against various pathogens, like the results obtained with the best fungicide (thiophanate-methyl). Similarly, Travadon *et al*. (2023a) conducted almond field trials in California and demonstrated that *T. atroviride* SC1 and an experimental *T. paratroviride* strain could prevent *E. lata* and *N. parvum* infections as effectively as thiophanate-methyl, when applied after pruning. *Trichoderma* treatments provided optimal protection when wounds were treated immediately post-pruning, before rainfall, as it can contribute to spore dispersal (Úrbez-Torres *et al*., 2010; Fujiyoshi *et al*., 2021; Jiménez Luna *et al*., 2022; Travadon *et al.,* 2023a).

Finally, it is interesting to note that our results using *T. asperellum* ICC012 + *T. gamsii* ICC080 extend the successful use of *Trichoderma*-based biocontrol in Mediterranean conditions, because these strains (originally formulated as "Remedier®"/"Blindar®") had already been used in Europe for grapevine fungal trunk diseases management and are known to colonize woody tissues effectively (Di Marco *et al*., 2022).

In Spain, Olmo *et al*. (2017) demonstrated that pruning wound protection in almonds orchards could be achieved with chemical fungicides; thiophanate-methyl was shown to be the most effective fungicide reducing *Botryosphaeriaceae* infections and lesion lengths, and it

was recommended for inclusion in integrated pest management (IPM) strategies. The BCAs used in our experimental treatments provide an alternative to such chemicals. As regulatory trends in the Europe, EU is limiting fungicide use. Thus, many chemical pesticides (including thiophanate-methyl) have been phased out due to environmental and health concerns. Consequently, interest in BCAs for fungal trunk disease management has increased. In California, a range of BCAs has already been explored in addition to *Trichoderma*-based products. A *C. rosea* strain J1446 showed excellent wound protection on almond and cherry, even matching fungicide control for *E. lata* and *N. parvum* in those hosts (Travadon *et al.,* 2023b). Until recently, comparable biocontrol research in Mediterranean almonds was scarce. Romero-Cuadrado *et al*. (2024) reported one of the first such Spanish studies, focusing on bacterial antagonist *P. aeruginosa* strain (AC17), which suppressed *N. parvum*, *B. dothidea*, and *D. seriata* cankers on almond, with efficacy equivalent to *T. atroviride* and conventional fungicide treatments. Their work confirms that multiple biocontrol agents (fungal and bacterial) can deliver high levels of protection. Together, these findings show that biological wound protectants can effectively complement or replace chemical fungicides in almond trunk diseases management. An IPM approach to trunk diseases should combine cultural measures (sanitation, optimal pruning timing) with wound protection using fungicides or BCAs across the nursery and orchard stages (Guarnaccia *et al*., 2022). Our findings strongly support this approach, offering a practical biological tool for the wound protection component.

In conclusion, *T. asperellum* ICC012 + *T. gamsii* ICC080, especially when applied as a preventive biocontrol treatment (e.g. at pruning), showed high efficacy in protecting almond pruning wounds from infection by *D. seriata*, *E. lata*, and *N. parvum*. This efficacy is comparable to that reported for other successful BCAs and fungicides in California and Spain. The success of *T. asperellum* ICC012 + *T. gamsii* ICC080 in our study contributes to the progressive adoption of BCAs for the management of fruit and nut crops diseases.

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