

Article

Dual Field Effects of *Trichoderma* Trunk Injections on Chestnut Trees: Suppression of Nut Rot and Enhanced Control of the Asian Chestnut Gall Wasp

Alessandra Benigno , Matteo Bracalini * , Viola Papini , Lorenzo Tagliaferri, Salvatore Moricca  and Tiziana Panzavolta 

Department of Agriculture, Food, Environment and Forestry, Plant Pathology and Entomology Section, University of Florence, Piazzale delle Cascine 28, 50144 Florence, Italy; alessandra.benigno@unifi.it (A.B.); viola.papini@unifi.it (V.P.); lorenzo.tagliaferri@unifi.it (L.T.); salvatore.moricca@unifi.it (S.M.); tiziana.panzavolta@unifi.it (T.P.)

* Correspondence: matteo.bracalini@unifi.it

Abstract

The European chestnut (*Castanea sativa* Mill.) is under increasing threat from the combined impact of the emerging fungal pathogen *Gnomoniopsis castaneae*, the causative agent of chestnut brown rot, and the Asian chestnut gall wasp (*Dryocosmus kuriphilus*), which is one of the most destructive invasive pests in chestnut orchards. This study evaluated the efficacy of endotherapy involving the injection of a multispecies *Trichoderma*-based biocontrol formulation (*T. harzianum*, *T. viride*, and *T. atroviride*) into the trunks of trees in two chestnut-growing areas in Tuscany, Italy. Independent trials were conducted using either a single spring application or two applications (autumn and spring) to assess the effects on brown rot, the gall wasp and its parasitoid, *Torymus sinensis*, as well as on major nut-feeding insects. Trunk injection significantly reduced the incidence of *G. castaneae* from 48.1% to 61.7% in untreated trees to 16.6%–28.1% in treated trees. The double application strategy showed promising results compared to untreated trees: increased larval mortality of *D. kuriphilus*, reduced bud infestation, and enhanced parasitisation by *Torymus sinensis*. These results demonstrate that trunk-injected *Trichoderma* is an effective, environmentally sustainable tool for the integrated protection of chestnut, suppressing brown rot, and supporting the biological control of the Asian gall wasp simultaneously.

Keywords: *Castanea sativa*; *Gnomoniopsis castaneae*; *Dryocosmus kuriphilus*; *Torymus sinensis*; tree endotherapy; biological control; chestnut brown rot



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1. Introduction

The European chestnut (*Castanea sativa* Mill.) is a highly valued tree species for fruit and timber production which contributes, especially in Mediterranean Europe, to essential ecosystem services, including biodiversity enhancement and forest ecosystem stability [1–4]. In Italy, chestnut groves represent an important component of rural and mountainous landscapes, fulfilling multifunctional roles of primary importance that bring economic, ecological, aesthetic, and social benefits [5]. The proliferation of invasive pests and emerging fungal pathogens severely challenges the long-term sustainability of these stands.

Among chestnut pests, the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu, stands out as a critical global threat driven by its rapid expansion [6]. This

invasive hymenopteran induces galls on vegetative organs, which restrict tissue development and impair the tree's photosynthetic capacity and nut production [7,8]. Although the classical biological control operated by *Torymus sinensis* Kamijo is widely considered a very successful case in forest entomology [9], severe localized outbreaks of *D. kuriphilus* still periodically arise due to microclimatic variations or transient mismatches in host–parasitoid synchronization [10]. Chestnut fruit production is also affected by several carpophagous insect pests, including tortricid moths of the genera *Cydia* and *Pammene* as well as weevils of the genus *Curculio*, whose larvae develop within the nuts and can cause infestation rates exceeding 50% and 90%, respectively [11].

Chestnut cultivation has also been challenged over the last fifteen years by brown or chalky nut rot, a major phytosanitary issue affecting chestnut reproductive organs caused by the ascomycete *Gnomoniopsis castaneae* (syn. *G. smithogilvyi*) [12,13]. The versatile lifestyle of *G. castaneae* as an endophyte, saprophyte, and opportunistic pathogen allows it to colonize branches, leaves, and galls induced by *D. kuriphilus*. The fungus can produce abundant inoculum, leading to severe internal fruit browning and high production losses [14–19].

Due to environmental concerns and operational constraints, the application of synthetic fungicides and insecticides to manage chestnut diseases and pests in forest ecosystems is subject to regulatory restrictions [2,5,20]. In the European Union, Regulation (EC) No. 1107/2009 establishes the framework for the approval of active substances and the authorisation of plant protection products, including biocontrol agents, based on their potential effects on human health and the environment [21,22]. These limitations suggest that the urgent development of low-impact, sustainable control alternatives, in line with current European regulatory frameworks, is necessary [21,22]. In this context, biological control based on antagonistic microorganisms, such as some members of the genus *Trichoderma*, represents one of the most promising management strategies due to a variety of biocontrol mechanisms displayed by the beneficial agents, which include direct and indirect mycoparasitism, competition for nutrients, and induced systemic resistance (ISR) [23–25]. In addition to their action against pathogenic fungi, *Trichoderma* species play a multifaceted role against phytophagous insect pests through the production of insecticidal or inhibitory secondary metabolites [26,27], as well as the induced release of host volatile organic compounds (VOCs) attractive to natural enemies of insect pests [28,29], thereby enhancing indirect plant defence.

Trunk injection (endotherapy) has emerged as a promising phytosanitary strategy that overcomes several limitations associated with conventional foliar and soil-applied treatments [30]. By delivering active compounds directly into the vascular system, endotherapy ensures targeted translocation throughout the canopy and root system, improving treatment efficiency while minimizing environmental dispersion and contamination [31,32]. This approach is consistent with the principles of sustainable agriculture, as it allows the use of lower quantities of active ingredients, reduces exposure of non-target organisms, and limits impacts on soil and water resources [5,33]. Beyond its precision of delivery, trunk injection may provide prolonged protection by allowing active compounds to persist within and move through the plant vascular system [5,32,34]. With regard to biological control agents, sustained efficacy may also depend on their ability to establish and persist within host tissues; however, this mechanism is highly dependent on the specific biological agent–host interaction.

In chestnut trees, fungicides, phosphites/phosphonates, and biological control agents, such as *Trichoderma* spp., have been successfully applied via endotherapy to manage major diseases, including ink disease caused by *Phytophthora* spp. and chestnut brown rot caused by *Gnomoniopsis* spp. [35–38]. Potassium phosphite/phosphonate formulations and especially selected *Trichoderma* strains have demonstrated significant reductions in disease

severity and yield losses while providing long-lasting protection, highlighting the potential of endotherapy as an effective and environmentally sustainable tool for integrated chestnut disease management [5,30]. *Trichoderma atroviride* P. Karst and *Bacillus amyloliquefaciens* (ex Fukumoto) Priest et al. have also shown potential in controlling the latent pathogen *G. castaneae* in European chestnut grafting scions soaked in propagules suspensions [34].

This study investigated, under field conditions, whether selected strains of *Trichoderma*, administered via endotherapy, could exert multiple beneficial effects in chestnut groves heavily infested by the fungal pathogen *G. castaneae* and by major insect pests. The specific objectives of the study were (i) to evaluate the capacity of the treatment to reduce the incidence and severity of chestnut nut rot caused by *G. castaneae*; (ii) to assess the cumulative effects of repeated applications of the biopesticide within a same growing season; (iii) to evaluate field-level interactions among the fungal treatment, *D. kuriphilus*, and its parasitoid *T. sinensis* by measuring parasitization rates; and (iv) to assess whether trunk injections of *Trichoderma* influence the incidence of damage caused by major nut-feeding insect pests, including species of *Cydia* Hübner, *Pammene* Hübner, and *Curculio* L.

2. Materials and Methods

The experiments were conducted in two chestnut-growing areas in northeastern Tuscany (central Italy), located about 45 km apart: two chestnut groves in Raggiolo (43°40'33" N, 11°42'33" E) in the province of Arezzo, and one in Marradi (44°04'11.7" N, 11°37'40.1" E) in the province of Florence. The sites exhibited structural and topographical variations typical of Apennine chestnut stands. Across all three groves, the average trunk diameter at breast height (DBH) was approximately 65 cm. Tree age within each grove ranged from 40 to approximately 150 years, reflecting the typical heterogeneous age structure of traditional open-grown chestnut orchards. The Marradi trial site consisted exclusively of the traditional cultivar 'Marrone di Marradi', with a tree density of 85 trees ha⁻¹ and a gentler slope of 25%. In contrast, the two Raggiolo plots represented traditional multi-cultivar stands composed predominantly of 'Raggiolana', 'Pistolese', 'Mondistollo', 'Marrone', and 'Tigolese', with tree densities of 85 and 95 trees ha⁻¹ and steep slopes of 35% and 50%, respectively. Field trials were carried out in Raggiolo during the 2021–2022 period and in Marradi during 2023–2024.

2.1. Trials Carried Out During the 2021–2022 Period at Raggiolo

2.1.1. Treatments

Two chestnut groves in Raggiolo, designated as chestnut grove 1 (CG1) and chestnut grove 2 (CG2), were selected for field trials. In each grove, a trunk-injection treatment was conducted on 17 May 2022. A total of 20 chestnut trees were randomly selected for the treatment. These constituted the experimental unit, whereas an additional 20 trees were selected as controls. To minimize potential environmental and microclimatic confounding factors across the sloped terrain, trees to be treated were assigned randomly across each site. This random selection ensured an even, unbiased distribution of treatment and control trees across the topographical gradient (lower, middle, and upper slope), across DBH size classes within the ~65 cm average range, and across the represented cultivars. Trunk injections utilized a freshly prepared conidial suspension comprising three *Trichoderma* species: *Trichoderma harzianum* Rifai, *T. viride* Persoon, and *T. atroviride* Karsten. *Trichoderma harzianum* and *T. viride* isolates were sourced from the mycological collection of the Plant Pathology and Entomology Division, DAGRI Department, University of Florence. The endotherapeutic solution was prepared by collecting conidia of *T. harzianum* and *T. viride* from pure cultures grown for 7 to 10 days on malt extract agar (MEA) in 9 cm-diameter Petri dishes. The fungal mixture was filtered through Whatman® Grade 1 filter paper

(Buckinghamshire, UK; 9 cm diameter, 11 µm pore size) to remove mycelial fragments. In contrast, *T. atroviride* was obtained from the commercial biological formulation Vintec® (Belchim Crop Protection, Londerzeel, Belgium), following the manufacturer's instructions. The three suspensions were then combined in equal volumes (1:1:1 ratio) to ensure that each species was present in equal amounts in the treatment mixture at a final concentration of 10^8 conidia/mL. Following the protocol of Berger et al. [39], a dose of 0.8 mL per 10 cm of trunk circumference was applied. Based on the trunk circumferences measured in the field, this resulted in an average injection volume of 16.1 ± 8.4 mL per tree (range: 7.3–48.8 mL). The total dose was divided and delivered through two injection ports per tree, positioned on opposite sides of the trunk. This approach allowed the treatment volume to be specifically tailored to the dimensions of each individual tree, ensuring optimal delivery of the biopesticide. Applications were performed at breast height (approximately 1.3 m above ground level) using the BITE® (Blade for Infusion in Trees) injection system (De Rebus Plantarum, Vicenza, Italy). Unlike traditional methods, this system is drill-free and non-pressurized; it employs a specialized tapered blade to create a single 5 mm vertical incision per port, reaching a depth of approximately 70–80 mm into the active xylem. This minimally invasive approach limits tissue damage and facilitates rapid wound closure, which typically occurs within a few weeks. The system utilizes specialized blades to create lenticular-shaped slots that exploit the plant's natural passive uptake; this process is further enhanced by a mechanically induced Venturi effect, ensuring efficient administration of the active ingredient [30].

2.1.2. Sampling for the Effects on ACGW

The year before the treatment, in June 2021, an initial baseline sample of 50 galls was randomly collected from all 40 trees in each chestnut grove. After the treatment, on 3 June 2022, an independent sample of 12 shoots was sampled from the four cardinal points of each selected tree (treated and untreated). In the laboratory, the number of galls per shoot was determined. Each gall was dissected with a scalpel under a Wild M3C stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland), recording the total number of cells, empty cells, parasitoids (separately for dead and alive individuals), ACGW larvae (separately for dead and alive individuals), and cells from which ACGW adults emerged.

2.1.3. Sampling for the Effects on Nut Parasites

During the autumn of 2022, when the fruit reached maturity, 20 nuts per tree were collected directly from the canopy of both treated and untreated chestnut trees in both chestnut groves, for a total of 800 nuts. The samples were taken to the laboratory for processing; half were used to assess the presence of *G. castaneae*, while the other half were examined for insect pests.

2.1.4. Isolation and Characterization of the Pathogen *G. castaneae*

Both apparently healthy and symptomatic nuts were examined in the laboratory under a Leica Wild M8 stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) to detect fungal reproductive structures on the pericarp surface. The nuts were then processed for fungal isolation on potato dextrose agar (PDA; Liofilchem, Roseto degli Abruzzi, Italy), as described below. The nuts were surface-sterilised by immersion in 3% NaOCl for one minute, after which they were rinsed with sterile distilled water. Five tissue fragments ($4\text{--}5\text{ mm}^2$) were aseptically excised from each nut and placed on 9 cm Petri dishes containing 2% PDA supplemented with 250 mg/L ampicillin and 10 mg/L rifampicin. Plates were incubated in the dark at 24 ± 1 °C for 7 days, and emerging colonies were subcultured onto 2% PDA. Isolates were morphologically characterized on malt extract agar (MEA, DIFCO, Detroit, MI, USA), assessing colony and margin shape, texture and mycelial pigmentation. For each morphotype, mycelial samples were mounted in lactophenol and examined using an optical

microscope (DM750; Leica Microsystems, Wetzlar, Germany) at 40× to describe conidial micromorphology; 100 structures per taxon were measured. Images were captured with an Optika 4083.B5 digital camera and processed with OptikaView 7 software. The identity of the pathogen was confirmed by sequence analysis of the internal transcribed spacer region, comprising the 5.8S rRNA gene and the adjacent ITS1 and ITS2 spacers. For DNA extraction, isolates were cultured on sterile cellophane overlays placed on 1% MEA in 90 mm Petri dishes and incubated in the dark at 22 °C. After ten days, approximately 50 mg of mycelium (fresh weight) was harvested from the cellophane surface and stored at −20 °C until processing. The genomic DNA was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol and subsequently stored at −20 °C. The ITS region was amplified using the pair of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The Polymerase Chain Reaction (PCR) was carried out in a reaction volume of 25 µL, comprising a 1× PCR buffer, a dNTP mixture at a concentration of 0.2 mM, MgCl₂ at a concentration of 1.5 mM, forward and reverse primers (each at a concentration of 0.5 µM), Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) at an activity of 1 U, and 100 ng of DNA template. The PCR cycling conditions comprised a denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The final extension stage was conducted at 72 °C for 10 min. The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) was utilized for the purification of the PCR products, in accordance with the manufacturer's instructions. The analysis of nucleotide sequences was conducted with the programme FinchTV version 1.4.0 (Geospiza, Inc., Seattle, WA, USA). The investigation was conducted on the basis of a comparative analysis of the consensus sequences under consideration with those which had previously been deposited in the GenBank database (National Center for Biotechnology Information—NCBI). This analysis, conducted by applying the Basic Local Alignment Search Tool (BLAST) (<https://www.ncbi.nlm.nih.gov/BLAST/>, accessed on 11 April 2025), web based interface, confirmed the identifications previously made through traditional morphological methods.

2.1.5. Analysis of Nut-Feeding Insect Pests

All sampled nuts were dissected under a Wild M3C stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) to check for the presence of insect pests. Pest damage was identified based on gallery features and larval morphology. Furthermore, when tortricid larvae were present, the species were identified using the keys of Brown & Komai [40].

2.2. Trial Carried Out During the 2023–2024 Period at Marradi

2.2.1. Treatments

To ensure that ACGW larvae of the same generation underwent two consecutive treatments, a first treatment was carried out in autumn (15 September 2023) and a second the following year in spring (24 May 2024). Trunk injections were carried out in a single chestnut grove on 10 trees following the same methodology as in the other chestnut groves (Raggiolo). Unlike the other site, the trees were previously sorted into two groups (treated/untreated) to have two units of 10 trees each, which would be homogeneous also in relation to (a) DBH size and (b) level of ACGW infestation.

2.2.2. Sampling for the Effects on ACGW

The first sampling was performed in 2023 (19 July 2023) before the treatments to determine the level of ACGW infestation and to select the trees for the treatments (as specified before) by collecting shoots from the 20 selected (treated/untreated) chestnut trees. Four shoots per tree were randomly collected from the four cardinal points of the

crown. Shoots were taken to the laboratory where the number of galls per shoot was determined. Each gall was dissected, recording the total number of cells, the number of empty cells, parasitized cells (separately for dead and alive parasitoids), ACGW larvae (separately for dead and alive individuals), and cells from which ACGW adults had emerged. In December 2023, after the first (autumn) treatment, 12 chestnut shoots were collected from each treated and untreated tree. The first three buds of each shoot, which are the preferred buds for oviposition [41], were dissected under a binocular microscope to verify the presence of wintering ACGW larvae. In June (10 June 2024), after the second (spring) treatment, 30 ACGW galls per tree were collected. Due to the particularly low levels of infestation observed in 2024 on all trees, the galls were collected directly rather than taking samples from the shoots, regardless of the treatment. Each gall was dissected, recording the total number of cells, the number of empty cells, parasitized cells (separately for dead and alive individuals), cells with ACGW larvae (separately for dead and alive individuals), and cells from which ACGW adults emerged.

2.2.3. Sampling for Nut Parasites

At fruit maturity (October 2024), 10 nuts were collected directly from the canopy of each chestnut tree (treated and untreated) selected for the study, for a total of 200 nuts. Following collection, samples were transported to the laboratory and subjected to the same processing, inspection, and evaluation procedures previously described for the Raggiolo trial. The same sample was screened for the presence of both *G. castaneae* and insect pests.

2.3. Statistical Analyses

Effects on ACGW: for Raggiolo, the percentages of dead ACGW (dead larvae inside cells and empty cells/total number of cells) and parasitized ACGW (cells with parasitoid/(total number of cells excluding empty cells)) were compared for each chestnut grove between the two years of the study (before and after the treatment) using the G test. In addition, differences in ACGW galls per shoot were analyzed using a Generalized Linear Mixed Model (GLMM) with a negative binomial distribution and a log-link function, including site, treatment, and their interaction as fixed factors, and tree as a random effect. To evaluate the effects of the treatment in both Raggiolo and Marradi, statistical analyses on ACGW parasitism and mortality were performed using a GLMM with a binomial distribution and logit link function. For the parasitism model, the count of parasitized cells was set as the target variable, with the total number of cells excluding empty cells specified as the binomial denominator. Conversely, for the mortality model, the count of dead ACGWs (dead larvae inside cells and empty cells) was set as the target variable, with the total number of cells specified as the binomial denominator. For Raggiolo, chestnut stand (site), treatment, and their interaction were included as fixed effects, whereas for Marradi, time (sampling before and after the treatment), treatment, and their interaction were considered. Gall size (considered as number of cells per gall) was included as a continuous covariate to account for its potential confounding effect on mortality and parasitism. Individual tree was specified as a random effect in all models. The number of infested buds at Marradi was analysed using a GLMM with a negative binomial distribution and a log-link function. Treatment was included as a fixed effect, while individual tree was specified as a random factor. Estimated marginal means were calculated on the original scale, with 95% confidence intervals.

Effects on nut parasites: regarding insect infestation, percentages of infested nuts from Raggiolo were analysed using the G test, comparing treated and untreated trees for both CG1 and CG2. The percentages of insect-infested nuts from treated and untreated trees in Marradi were analyzed using a chi-square (χ^2) test. For the fungal pathogen, the incidence

of *G. castaneae* was calculated as the percentage of infected nuts relative to the total number of nuts analysed. Differences in the incidence of *G. castaneae* between treated and untreated trees were assessed separately for each field experiment (Raggiolo and Marradi) using the chi-square (χ^2) test. Statistical significance for all analyses was set at $p < 0.05$. Assumptions underlying all statistical analyses were assessed using appropriate diagnostic procedures and were adequately met. Statistical analyses were conducted with SPSS v.28 (IBM Corp., Endicott, NY, USA).

3. Results

3.1. Trials Carried Out During the 2021–2022 Period at Raggiolo to Test the Effect of *Trichoderma* Treatment on the ACGW

In 2022, the ACGW infestation level was higher in CG1 than in CG2, as evidenced by a higher density of galls per shoot, 0.65 and 0.19, respectively (GLMM, $F_{1,1024} = 99.99$, $p < 0.001$). In contrast, the treatment did not yield a statistically significant effect on gall reduction ($F_{1,1024} = 2.56$, $p = 0.110$), and no significant site $\times$ treatment interaction was observed ($F_{1,1024} = 3.61$, $p = 0.058$). The binomial GLMM revealed a significant site $\times$ treatment interaction for ACGW mortality after the treatment ($F_{1,105} = 4.479$, $p < 0.05$), indicating that mortality in treated and untreated trees differed between sites (Figure 1). Overall ACGW mortality was higher in CG1 (21.7%) than in CG2 (13.5%; Figure 1). In CG1, mortality did not differ significantly between treated and untreated trees (22.1% vs. 21.2%, respectively; $p = 0.548$). In CG2, treated trees exhibited lower mortality (8.8%) compared to untreated ones (23.9%), although this within-site difference was not statistically significant ($p = 0.066$). Gall size did not affect mortality ($F_{1,105} = 3.585$, $p = 0.061$). This trend is further supported by the pre-treatment data; in 2021, mortality percentages in treatment-designated trees across both groves were higher than those observed in 2022 ($G = 31.527$, $df = 3$, $p < 0.001$). This temporal difference was significant only in CG1, which showed a 14.7% reduction in ACGW mortality in 2022 ($G = 10.451$, $df = 1$, $p < 0.01$), whereas the reduction in CG2 (5.5%) was not statistically significant ($G = 1.91$, $df = 1$, $p = 0.167$).

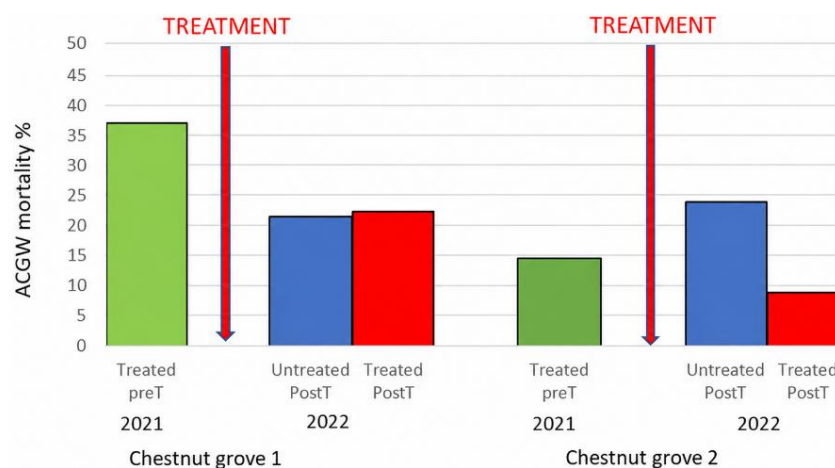


Figure 1. ACGW mortality percentages before (2021) and after (2022) *Trichoderma* treatment at Raggiolo (Tuscany, Italy). Chestnut grove 1: in 2021 $n = 227$, in 2022 $n = 1270$; Chestnut grove 2: in 2021 $n = 289$, in 2022 $n = 177$.

Regarding *T. sinensis*, a significant reduction in parasitization was recorded when comparing data from the year before the treatment with the following year ($G = 47.068$, $df = 3$, $p < 0.001$) in treatment-designated trees. However, the difference was statistically significant ($G = 31.121$, $df = 1$, $p < 0.001$) only in CG2 (98.7% and 80.0% respectively) but not in CG1 (88.1% and 81.6% respectively; $G = 2.805$, $df = 1$, $p = 0.094$) (Figure 2). Parasitism

was significantly affected by treatment ($F_{1,57} = 22.261, p < 0.001$), whereas neither chestnut stand ($p = 0.157$), gall size ($p = 0.502$), nor the site $\times$ treatment interaction ($p = 0.570$) was significant. Parasitism was consistently higher in treated than in control trees in both stands (CG1: 81.6% vs 63.5%, $p < 0.001$; CG2: 80.0% vs 57.9%, $p < 0.01$).

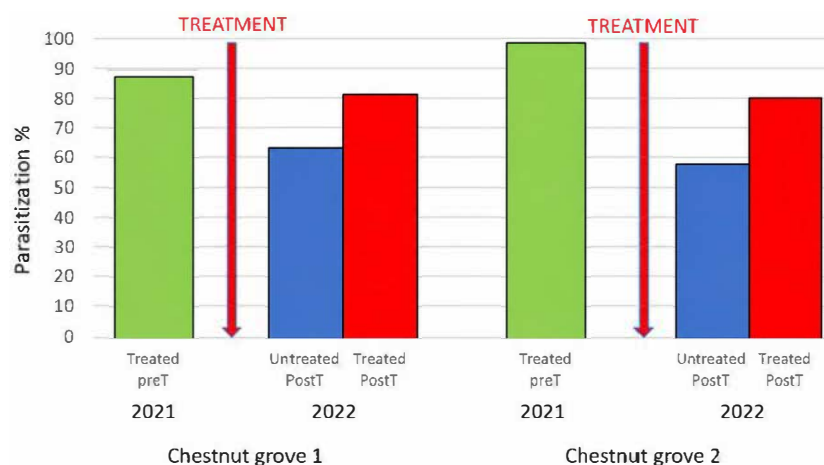


Figure 2. Percentage of ACGW parasitization before (2021) and after (2022) *Trichoderma* treatment at Raggiolo (Tuscany, Italy). Chestnut grove 1: in 2021, $n = 154$; in 2022, $n = 1025$; Chestnut grove 2: in 2021, $n = 216$; in 2022, $n = 157$.

3.2. Trials Carried Out During the 2023–2024 Period at Marradi to Test the Effect of *Trichoderma* Treatment on the ACGW

The GLMM analysis on ACGW mortality revealed a statistically significant interaction between treatment and time ($F_{1,971} = 5.513, p < 0.05$). Mortality increased slightly with increasing gall size ($\beta = 0.078$; OR = 1.082, 95% CI = 1.008–1.160). In contrast, the main effects of treatment alone ($F_{1,21} = 0.647, p = 0.435$) and time alone ($F_{1,971} = 1.596, p = 0.207$) were not statistically significant. Specifically, in 2023 before the treatment, ACGW larval mortality in the group of treatment-designated trees was not significantly different from that in control-designated trees (2.8% and 5.1%) (Figure 3). On the contrary, in 2024, after the treatment, ACGW mortality on treated trees was higher (14.8%) than that on untreated ones (2.8%) ($p < 0.05$) (Figure 3). In addition, the GLMM analysis revealed a significant effect of the treatment on the number of infested buds from samples collected during the winter after the treatment ($F_{1,536} = 6.368, p < 0.05$). Untreated trees showed a significantly higher mean number of infested buds (Mean $\pm$ SE: 0.135 ± 0.023) compared to the treated group (Mean $\pm$ SE: 0.065 ± 0.015) (Figure 4).

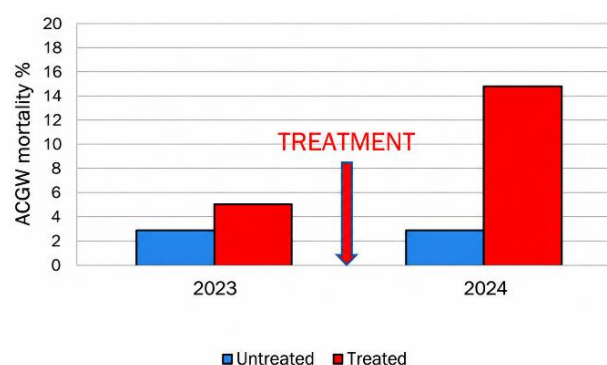


Figure 3. ACGW mortality percentages before (2023) and after (2024) *Trichoderma* treatment at Marradi (Tuscany, Italy). In 2023, $n = 1394$; in 2024, $n = 1321$.

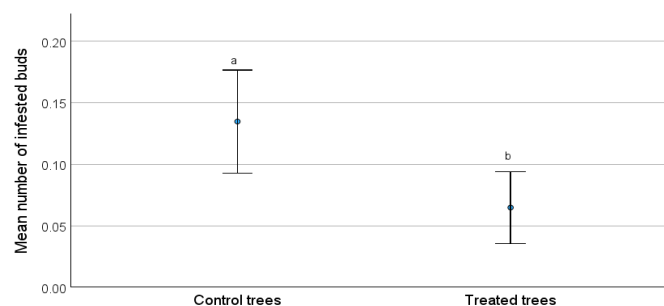


Figure 4. Mean number of infested buds per tree at Marradi (Tuscany, Italy) after treatment in December 2023. Error bars represent the standard error. Letters indicate statistically significant differences (GLMM). Control trees, $n = 260$; treated trees, $n = 278$.

Parasitism was significantly affected by the treatment $\times$ time interaction ($F_{1,878} = 30.684$, $p < 0.001$), indicating that parasitism in treated and untreated trees varied over time (Figure 5). Before treatment, parasitism was significantly lower in the treatment-designated trees than in the control (95.3% vs. 97.6%; $p = 0.001$), whereas after treatment (2024) the opposite pattern was observed, with significantly higher parasitism in the treated trees (98.0% vs. 92.8%; $p < 0.05$). Gall size also showed a small but significant negative effect on parasitism ($\beta = -0.073$, OR = 0.930, 95% CI = 0.866–0.999; $p < 0.05$). *Torymus sinensis* mortality was extremely low, with only nine dead individuals recorded across all trials (approximately 0.2%), including both treated and untreated chestnut trees. Given the very low number of mortality events, no inferential statistical analysis was performed.

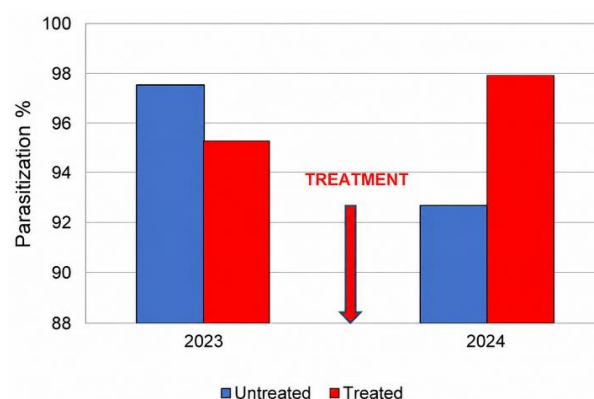


Figure 5. Percentage of ACGW parasitization before (2023) and after (2024) *Trichoderma* treatments at Marradi (Tuscany, Italy). In 2023, $n = 1341$; in 2024, $n = 1117$.

3.3. Effect of *Trichoderma* Treatment on Nut Pests of Chestnut

Nuts collected from treated and untreated trees were equally infested by spermo-carpophagus pests in all trials, i.e., CG1 and CG2, in 2022 at Raggiolo ($G = 6118$, $df = 3$, $p = 0.106$) as well as Marradi in 2024 ($\chi^2 = 2.15$, $df = 1$, $p = 0.142$) (Figure 6). Percentages of infested nuts varied from a minimum of about 23% to a maximum of about 48%, with the higher percentages at Marradi. Though there was no significant difference, the higher infestation percentages were on treated trees, thus confirming that the treatment did not affect nut pests.

In both chestnut groves at Raggiolo, a great part of the damage to the nuts was caused by *Cydia splendana* (Hübner) and *C. fagiglandana* (Zeller) (73% at CG1 and 71% at CG2 of the total pest damage), while *Pammene fasciana* (Linnaeus) was rarely recorded. The remaining part was due to *Curculio* spp. As regards Marradi, only *C. splendana* and *C. fagiglandana* were responsible for the damage; in fact, *Curculio* spp. were never recorded, and only a few larvae of *P. fasciana* were found inside nuts.

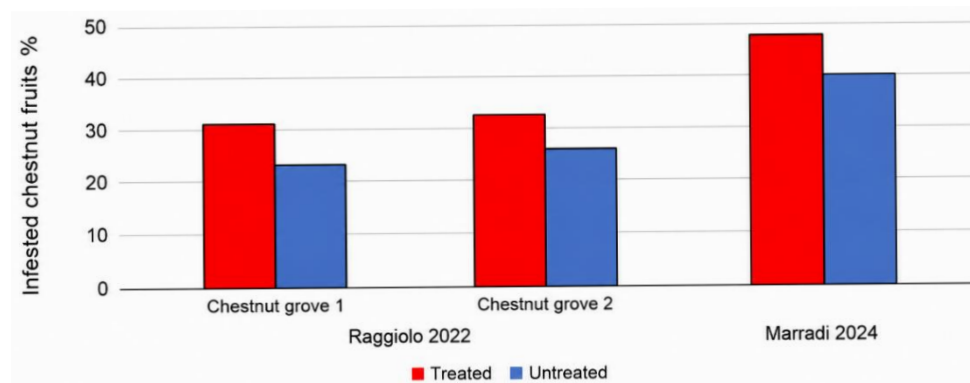


Figure 6. Percentage of infested chestnut fruits on treated and untreated trees in 2022 at Raggiolo (Tuscany, Italy) and in 2024 at Marradi (Tuscany, Italy) following the treatments. Raggiolo: Chestnut Grove 1, $n = 400$; Chestnut Grove 2, $n = 400$; Marradi: $n = 200$.

3.4. Effect of *Trichoderma* Treatment on *G. castaneae*

The identification of the pathogen was achieved through a combination of macromorphological, micromorphological, and molecular analysis. Sequence analysis confirmed the identity of *G. castaneae* isolates from Marradi (accession no. PZ622603) and Raggiolo (accession no. PZ622604). BLAST searches showed 100% sequence identity with the corresponding reference sequences deposited in the GenBank database. Field trials showed that treatments with *T. viride*, *T. harzianum*, and *T. atroviride* were associated with a reduction in *G. castaneae* incidence in treated trees compared to untreated ones. Lower incidence values were observed in treated trees in each of the three experimental chestnut groves. In the Marradi site, where two applications were performed within the same year, a markedly lower number of infected nuts ($n = 12$) was recorded in treated trees than in untreated trees.

The incidence of *G. castaneae* in treated trees was 28.1%, 24.2%, and 16.6%, whereas higher incidence values were recorded in the corresponding untreated trees (50.5%, 48.1%, and 61.7%) in the two Raggiolo sites and the Marradi site, respectively (Figure 7). Statistical analysis conducted using the χ^2 test revealed significant differences between treated and untreated trees ($p < 0.01$) in the respective field experiments. Overall, these findings indicate that the *Trichoderma*-based treatments were associated with a reduction in *G. castaneae* incidence under the experimental conditions, while no additional fungal complexes were detected in the analysed chestnut samples.

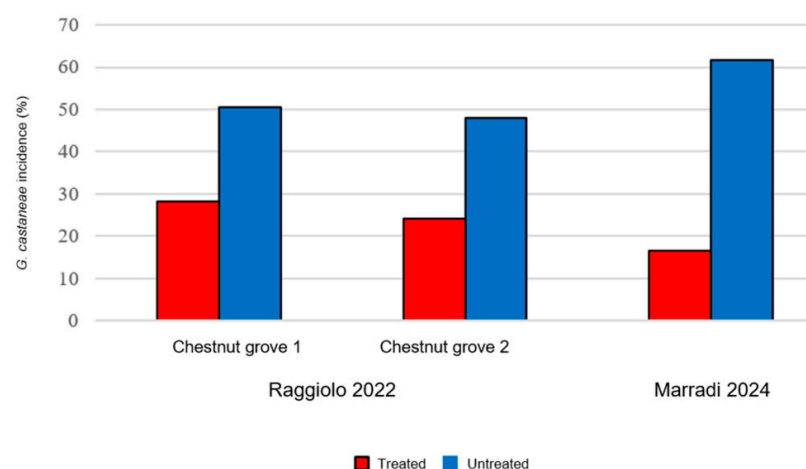


Figure 7. Percentage of nuts infected by *G. castaneae* on treated and untreated trees in 2022 at Raggiolo (Tuscany, Italy) and in 2024 at Marradi (Tuscany, Italy) following the biocontrol treatments. Raggiolo: Chestnut Grove, 1 $n = 400$; Chestnut Grove 2, $n = 400$; Marradi: $n = 200$.

4. Discussion

The development of sustainable and low-impact control strategies is an absolute priority for modern European plant protection, especially when it comes to multifunctional agro-forestry systems like chestnut groves [1,3]. While extensive data exist regarding the efficacy of biocontrol agents in enhancing plant defences against pathogens and pests in horticultural crops [25,42], less information is available for forest trees. In chestnut ecosystems, the co-occurrence of severe fungal diseases and destructive insect pests poses a major challenge to orchard management, as traditional agricultural strategies are often unfeasible or ecologically damaging [43]. In this context, *Trichoderma* trunk injection represents a promising strategy to overcome these limitations. Our findings demonstrated that endotherapy with *Trichoderma* spp. can affect chestnut parasites.

Trunk injections significantly suppressed the incidence of *G. castaneae* across all experimental sites. The highest protection was achieved at Marradi, where two applications in close succession (autumn and the following spring) lowered *G. castaneae* incidence to 16.6%, contrasted with 61.7% in the untreated control. These data strongly support the previous findings of Benigno et al. [5], who first documented that trunk injections with *Trichoderma* could successfully suppress chestnut nut rot under field conditions. The present study extends this previous work by evaluating the treatment across multiple field sites and application schedules. Importantly, the potential of trunk injection as a plant protection strategy has also been demonstrated in woody species other than chestnut. Trunk injection provides a targeted delivery route through the vascular system, potentially reducing spray drift and non-target exposure compared with conventional foliar applications [44]. Independent studies have shown that this approach can be effective against fungal pathogens in grapevine, where fungicide-loaded lignin-based nanocarriers delivered by trunk injection provided prolonged protection against pathogens associated with Esca disease [45]. Trunk-injected RNA molecules have also been shown to undergo systemic uptake and transport in apple trees and grapevines [46]. More recently, trunk injection has been investigated for the management of arthropod pests in other woody species, including *Gleditsia triacanthos* L. and *Quercus petraea* (Matt.) Liebl. [47]. These findings indicate that the applicability of endotherapy extends beyond chestnut and encompasses different categories of plant protection targets in diverse woody plants.

Several complementary mechanisms may account for the observed biocontrol efficacy. These include direct antagonism through mycoparasitism or the secretion of cell wall-degrading enzymes and antifungal metabolites across plant tissue micro-niches, as well as competition for nutrients and ecological niches. All of these are well-established modes of action for *Trichoderma* species [48,49]. The three-strain mixture was designed as a complementary *Trichoderma* consortium. This choice was based on the well-documented multifunctional nature of *Trichoderma* biocontrol, which depends on strain-specific combinations of mycoparasitism, antibiosis, competition, and host defence induction, and on evidence that compatible multi-strain consortia can provide broader and sometimes stronger protection than single-strain applications [50,51]. *T. atroviride* SC1 (Vintec®) was included as a commercial reference strain with established practical use, whereas the *T. harzianum* and *T. viride* isolates were selected as local antagonists after preliminary in vitro screening against *G. castaneae* [5]. However, because the strains were applied only as a mixture, the present study does not allow the contribution of each individual species to the observed biocontrol effect to be disentangled [51,52]. Similarly, the absence of a carrier-only injection control does not allow us to fully distinguish the effects of the injection procedure itself from those attributable to the applied *Trichoderma* species.

Beyond these mycoparasite-host fungus interactions, there is increasing evidence that *Trichoderma* can modulate plant immunity by producing bioactive compounds that can

trigger induced systemic resistance (ISR) and, in some cases, systemic acquired resistance (SAR). Such mechanisms may lead to long-lasting defence priming, enabling a more rapid and effective response upon pathogen challenge [25,53]. The sustained reduction in symptoms observed across both trials is consistent with this hypothesis, suggesting that host-mediated resistance substantially contributes to disease suppression alongside direct fungal antagonism [5]. More specifically, the vascular delivery provided by trunk injection may further favour the establishment and persistence of beneficial microorganisms within internal plant tissues. Nevertheless, the efficacy of endotherapy depends on tree anatomy, vascular connectivity, wound responses, and the formulation and mobility of the injected agent, which may explain differences in performance among sites and application schedules [30].

Regarding direct pest mortality, the double application strategy at Marradi led to a significantly higher winter mortality of *D. kuriphilus* larvae (14.8%) inside the galls compared to the control (2.8%), alongside a lower mean number of infested buds. This direct negative impact on the pest aligns with the findings of Poveda [54]. This author pointed out that *Trichoderma* species can exert direct entomopathogenic activity through host parasitism and the production of insecticidal or antifeedant secondary metabolites (e.g., peptaibols and chitinases), ultimately reducing the survival, longevity, and fitness of phytophagous insects. The first application (autumn) could be particularly crucial here, as it probably allowed *Trichoderma* to colonize the newly forming buds during the exact timeframe when early-stage ACGW larvae enter overwintering [7], exposing them to direct fungal metabolites or plant-induced defensive proteins.

Conversely, the treatment showed no significant effect on nut insects (*Cydia* spp., *P. fasciana*, and *Curculio* spp.). The lack of a significant effect on internal nut-feeding pests (*Cydia* spp., *Pammene fasciana*, and *Curculio* spp.) compared to the suppression observed for *D. kuriphilus* larvae highlights key nuances of trunk injection treatments, which often suffer from non-homogeneous spatial and temporal distribution within the tree canopy [32,55]. We hypothesize that this differential response is primarily driven by spatio-temporal tissue localization and feeding guild mechanics. The gall wasp attacks early-season vegetative tissues that are heavily vascularized and act as active, high-priority sinks during peak spring sap translocation [56,57], exposing gall tissues directly to systemic compounds moving acropetally via the transpiration stream [32]. In contrast, nut borers target reproductive structures later in the growing season, when systemic allocation to reproductive organs may be physiologically limited compared to earlier vegetative foliar sinks [56]. Furthermore, systemic resistance mechanisms (SAR/ISR) triggered by trunk-injected bio-elicitors have been shown to selectively reprogram foliar transcriptomes and elevate defence-related volatiles in the leaf canopy [58], which may specifically target foliar or gall-forming pests without affecting internal seed-boring larvae feeding on starch-rich endosperm. Future studies evaluating tissue-specific metabolite allocation across vegetative versus reproductive organs will be essential to test these mechanisms, utilizing established endotherapy tracking methodologies such as mass spectrometry (GC-MS or LC-MS/MS) [32,58], isotope-labelled tracing, or autoradiography to precisely map spatial residue partitioning [32,57].

The most striking and innovative result of this study concerns the potential multi-trophic interactions between trunk-injected *Trichoderma* strains, the pest *D. kuriphilus*, and its specific biological control agent *T. sinensis*. Across all experimental plots, *Trichoderma* treatments were associated with a significant increase in *T. sinensis* parasitization rates. Even during regional declines or fluctuations of the parasitoid population—as observed in Raggiolo—treated trees maintained significantly higher levels of parasitization compared to untreated trees. A potential, albeit working, hypothesis to explain this phenomenon lies

within the framework of indirect plant defences. It is well documented in the literature that root-associated or endophytic *Trichoderma* strains can trigger the production of volatile organic compounds (VOCs) by colonized plants [42,54]. For example, in a tri-trophic model system composed of tomato plants (*Solanum lycopersicum* L.), aphids (*Macrosiphum euphorbiae* Thomas), and their parasitoid (*Aphidius ervi* Haliday), Coppola et al. [26] demonstrated that *T. harzianum* colonization induces a defence priming state by upregulating genes involved in terpenoid biosynthesis and defence pathways, resulting in an altered blend of VOCs that significantly improves the foraging efficiency of the parasitoid wasp. A similar increase in field parasitism rates was reported by Contreras-Cornejo et al. [59], who found that *T. atroviride* inoculation in maize significantly increased the parasitism rate of *Spodoptera frugiperda* (Smith) larvae by its natural enemy *Campoletis sonorensis* (Cameron).

We hypothesize that *Trichoderma* injections may have similarly altered the volatile profile of *C. sativa*, preferentially attracting *T. sinensis* females. However, since measuring VOC emissions was outside the scope of this initial field-efficacy study, extending tri-trophic signalling mechanisms previously observed in herbaceous systems to a woody perennial such as *C. sativa* remains an unverified hypothesis. In fact, our primary objective was to evaluate field-level efficacy; hence, direct metabolomic, transcriptomic, and parasitoid behavioral analyses were not conducted. Furthermore, post-injection vascular colonization, tissue persistence, and viability of the injected *Trichoderma* strains were not verified via qPCR or re-isolation assays. Consequently, rigorous future investigations incorporating strain-specific molecular tracking, tissue viability protocols, and volatile profiling are necessary to confirm *Trichoderma* persistence and unravel the precise signalling pathways driving this tri-trophic interaction.

A major novelty of the present work lies in the double application strategy. The present study comprised two independent field trials conducted in Raggiolo, with a single injection, and in Marradi, with a double application. Across both trials, trunk injections resulted in a significant reduction in disease incidence and increased ACGW parasitization. In particular, results showed a strong effect associated with the double application in Marradi—which may also account for the significant increase in pest mortality observed in that single trial. However, environmental conditions and other site- and year-specific factors may also have contributed to the observed differences. Previous studies have demonstrated that *Trichoderma* spp. can colonize and persist in plant tissues for extended periods following inoculation [60]. Although the rate of endophytic colonization was not directly assessed in the present study, the first application (autumn) likely contributed to an initial establishment of the biocontrol agent within chestnut tissues, which was then reinforced by the second application (spring). This repeated exposure may have strengthened the biocontrol efficacy of *Trichoderma* spp. against *G. castaneae*, thereby contributing, via an additive biocontrol effect, to increased disease suppression under the consecutive treatment regime.

5. Conclusions

Overall, our findings suggest that trunk-injected *Trichoderma* formulations represent a promising, sustainable strategy for managing the brown nut rot pathogen *G. castaneae* and the gall wasp *D. kuriphilus*. However, the relative contribution of direct antagonism versus induced host resistance requires further elucidation. Future research should therefore focus on optimizing application timing and frequency to maximize efficacy under forest conditions. Furthermore, while the use of *Trichoderma* species in forest ecosystems shows considerable promise, their long-term field performance is not yet adequately documented. Multi-year studies integrating pest and pathogen suppression with investigations into the in planta viability and temporal colonization of these biocontrol agents are essential to understand their endurance and protective lifespan over time.

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Data Availability Statement: Data are available by e-mail on reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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