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Trunk injection of fosetyl-Al controls chestnut ink disease with dose-dependent phytotoxicity

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Abstract

Fosetyl-Al (aluminium tris-(ethyl phosphonate)) is a low-toxicity phosphonate commonly used to control oomycete-related diseases in crops. Trunk injection (endotherapy) delivers the fungicide directly into the vascular system, potentially improving efficacy while reducing environmental risks.

This two-year study evaluated the efficacy and phytotoxicity of trunk-injected fosetyl-Al to manage ink disease caused by *Phytophthora cinnamomi* in *Castanea sativa* saplings. Fifty saplings received fosetyl-Al injections at 0–4% using pressurized devices, followed by stem inoculation with the pathogen; a 2% soil irrigation treatment was included for comparison.

Trunk injections at 1%, 2% and 4% significantly suppressed stem necrosis and reduced root infections compared to irrigation and untreated control, with protection persisting for at least two years as reflected by reduced relative Area Under the Disease Progress Curve (rAUDPC) values. However, trunk injections also induced phytotoxic symptoms—including leaf burn, necrosis, and occasional plant death—with a clear dose-dependent relationship. While most plants recovered from moderate phytotoxicity, irreversible damage occurred at the highest doses, highlighting the critical need for precise dose adjustments, particularly in small-diameter trees. Logistic regression identified a Minimum Effective Dose (MED) range of 0.061–0.102 g/cm of stem circumference, corresponding to disease incidence targets of < 10% to < 1%, respectively, with phytotoxicity risks estimated between 10% and 25%.

These results demonstrate that trunk injection of fosetyl-Al provides effective, targeted, and environmentally friendly control of ink disease, while emphasizing the need for dose optimization to minimize adverse effects.

1. INTRODUCTION

The European chestnut (*Castanea sativa* Mill.), commonly known as sweet chestnut, is native to the Mediterranean region and represents the most important chestnut species in Europe, with its primary distribution in Western and Southern Europe (Seijo et al. 2017; Massantini et al. 2021). Its cultivation significantly expanded under the influence of Greek and Roman civilizations (Castellana et al. 2021) and became widespread throughout the Middle Ages (Conedera et al., 2004; Pereira-Lorenzo et al., 2011, 2019). Today, chestnut trees are found in both natural and managed environments, including traditional orchards and modern plantations (Pérez-Girón et al., 2020). Chestnuts hold considerable economic and nutritional importance. Their nuts can be consumed fresh or processed, and recent consumer interest has grown due to recognized nutritional qualities and potential health benefits. Chestnuts are low in fat and rich in vitamins, minerals, and beneficial compounds, such as polyunsaturated fatty acids (ω-3 and ω-6), tocopherol, and linoleic acid (Simopoulos, 1991; De Vasconcelos et al., 2010; Barreira et al., 2020; Rodrigues et al., 2020). Chestnut wood is also highly prized for construction, furniture making, and domestic applications, significantly contributing to rural economies. These varied uses have created strong cultural connections and a rich heritage associated with chestnut cultivation. In addition to economic and cultural value, chestnut orchards provide important environmental benefits. When well-

managed, these orchards are crucial for preserving biodiversity within rural landscapes (Diaz-Varela et al., 2018; Moretti et al., 2021). They offer diverse microhabitats—such as tree cavities, bark cracks, and small pockets of soil—that support various species, including fungi, plants, insects, birds, and mammals (Rubio, 2009; Zlatanov et al., 2013). Recognized under the European Directive 92/43/EEC (Habitat Directive Castanea sativa; code 9260), chestnut forests help conserve biodiversity, protect soils against erosion and wildfires, and function as carbon sinks, thereby contributing significantly to climate regulation, particularly in mountainous areas.

One of the most severe threats to European chestnut trees is ink disease, caused by soil-borne oomycetes of the genus *Phytophthora*, primarily *Phytophthora* × *cambivora* (Petri) Buism and *P. cinnamomi* Rand. First documented in southern Europe in the 18th century, the disease affects saplings in nurseries, young plantations, and mature trees, causing root and collar rot that can ultimately lead to plant death (Vannini et al., 2001), posing critical challenges for chestnut orchard management, forest conservation, and reforestation programs. After a decline, ink disease resurged in several European countries at the end of the twentieth century (Vettraino et al., 2001; Turchetti & Maresi, 2006; Prospero et al., 2023). Since *P. cinnamomi* is a thermophilic species (Zentmyer, 1981), its risk of proliferation may increase under climate change due to warmer winters (Bergot et al., 2004; Hernández-Lambraño et al., 2018). However, hotter and drier summers could limit its spread (Cidre-González et al., 2025), unless irrigation practices counteract these restrictive conditions and create favorable microenvironments for the pathogen.

This resurgence has renewed interest in phosphonate-based fungicides as a key component for managing chestnut ink disease within conventional agricultural systems (Dal Maso et al., 2015, 2017; González et al., 2017; Rosário et al., 2021; Brandano et al., 2023; Çakar et al., 2023; Morales-Rodríguez et al., 2025). The two most widely used phosphonate formulations in agriculture are potassium phosphonate (the mono- and di-potassium salt of phosphorous acid) and fosetyl-Al (aluminium tris-O-ethyl phosphonate), which are sometimes inaccurately referred to as phosphites (Manghi et al. 2021). Phosphonates are low molecular weight systemic products capable of translocating through the xylem and phloem to reach roots and leaves (Dann et al., 2021). Their activity as crop protectants is related to three complementary mechanisms of action that have been studied mostly against oomycetes plant pathogens (i) indirect stimulation of host plant defense responses; (ii) changes in the production of compounds produced by pathogens that affect the plant defense; and (iii) direct fungistatic effect towards pathogens (Dann et al. 2021; Manghi et al. 2021). Phosphonate treatments are widely reported to improve canopy health and vigor, while also restricting lesion development caused by *Phytophthora* spp. (Shearer et al., 2006; Gentile et al., 2009; Jung et al., 2010; Vettraino et al., 2010; Scott et al., 2015; Solla et al., 2021). More recently, their efficacy has also been demonstrated against fungal-driven diseases (Dann et al., 2021) and in protecting chestnut nuts from the ‘brown rot’ fungus *Gnomoniopsis castaneae* (= *G. smithogilvyi*) (Bastianelli et al., 2022).

Fosetyl-Al has been commercially used since the late 1970s and is registered for the control of a wide range of oomycete diseases. Its authorization for foliar application in chestnut is relatively recent in

some European countries, such as Spain, where it was granted as a minor use under Article 51 of Regulation (EU) No. 1107/2009 (Keyfol® WP, registration No. ES-00541; MAPA, 2025). Although foliar application is an established practice, it is frequently associated with substantial product losses, environmental contamination, and inefficient pesticide use (Berger and Laurent, 2019). These issues are amplified in tall trees, such as chestnut trees, treated with ground air-blast sprayers, as spray deposition declines with canopy height (Bock et al., 2013). In practice, many chestnut growers apply fosetyl-Al via irrigation when foliar spraying is not feasible. However, soil applications are prone to off-target losses through drainage, particularly under high rainfall or irrigation conditions, reducing plant uptake and increasing environmental risk (Lewis et al., 2016).

In this context, trunk injection (endotherapy) represents an alternative delivery strategy that allows direct introduction of fosetyl-Al into the vascular system, enabling rapid translocation through xylem and phloem (Archer et al., 2022). Trunk-injected fosetyl-Al has shown efficacy against *P. cinnamomi* in several woody hosts, including avocado and holm oak (Darvas et al., 1984; Romero et al., 2019), while reducing environmental exposure and eliminating spray drift (Wise et al., 2014). The present study focused on evaluating the efficacy of fosetyl-Al within the chestnut–*P. cinnamomi* pathosystem and on characterizing potential phytotoxic effects under controlled conditions, as a necessary step before field-scale implementation.

Nevertheless, trunk injection may induce phytotoxic responses, including leaf burn, necrosis, and, in some cases, plant death, as reported across a range of woody species and application scenarios (Pilbeam et al., 2000; Shearer et al., 2006; Hardy et al., 2001). Therefore, assessing both disease control and plant tolerance is essential before considering the application of trunk injection strategies in chestnut plantations.

This study evaluated the efficacy and phytotoxicity of trunk-injected fosetyl-Al in managing ink disease caused by *P. cinnamomi* in chestnut saplings. Specifically, the study aimed to: (i) compare the effectiveness of stem injection at different concentrations with soil irrigation, (ii) quantify the relationships between dose and response for disease suppression and phytotoxicity, and (iii) determine a minimum effective dose (MED) that optimizes efficacy while minimizing adverse effects.

2. MATERIAL AND METHODS

2.1. Experimental design

The experiment followed a completely randomized design comprising 5 treatment groups, each containing 10 asymptomatic *C. sativa* plants. Treatments included stem injection of fosetyl-Al solution at concentrations of 4%, 2%, and 1%, a control treatment receiving sterile water via stem injection, and an additional treatment where the intermediate fosetyl-Al concentration (2%) was applied through irrigation. Treatments were applied in June 2023. One month later, all plants were stem-inoculated with *P.*

cinnamomi, and then they were monitored for 2 years to evaluate both the preventive capacity and the phytotoxicity of fosetyl-Al treatments.

2.2. Plant material and *Phytophthora* spp. screening

In March 2023, 50 2-year-old *C. sativa* bare-root saplings obtained from a commercial nursery were transplanted into 60-L pots filled with a substrate mixture of peat, sand and horse manure (50:4:1, v/v/v). The plants were maintained outdoors at the facilities of the Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX, Plasencia, Spain), under a mean temperature of 18.28 ºC (mean minimum temperature of 7.39°C and mean maximum temperature of 31.06°C) according to records from the Spanish State Meteorological Agency for the period between March 2023 and July 2025. They received daily drip irrigation adjusted to their water requirements, and no fertilization was applied.

In May, all plants were tested for the presence of naturally occurring *Phytophthora* spp. detection before treatments. For this, 5 g of substrate from each pot, including rhizosphere roots, were flooded in a 9-cm Petri dish containing 10 mL of distilled water supplemented with 50 mg/L of hymexazol (Tachigaren 70WP®, Mitsui Chemicals Agro, Inc., Tokyo, Japan). Floating immature carnation petals were used as baits, following a technique adapted from Tello et al. (1991). The Petri dishes were kept at approximately 25°C in darkness, and the baits were subsequently examined under a microscope for the presence of sporangia. The potting substrate was also tested for the presence of *Phytophthora* spp. using susceptible plants as baits. For this, a pot was filled with 450 cm^3 of substrate from the corresponding plant, and 4 pre-germinated seeds of *Lupinus luteus* L. were sown. The pots were maintained at approximately 25°C under natural light for one week. The presence or absence of *Phytophthora* spp. was determined by *L. luteus* root analysis (Dunstan et al., 2016) on semi-selective NARPH medium (Hüberli et al., 2000). Two *Phytophthora* isolates obtained from these tests were molecularly identified by sequencing the ITS region (Grünwald et al., 2011).

2.2. Fosetyl-Al treatments

In June 2023, fosetyl-Al treatments were applied by injecting 45 mL of aqueous neutralized fosetyl-Al solution (Fosetyl-Al 80% WP) into the base of each plant stem at concentrations of 4%, 2%, and 1%. At the time of treatment, all plants were asymptomatic. Nevertheless, to ensure a balanced distribution of naturally-infested plants across groups, plants were assigned randomly, including 5–6 already infected but asymptomatic plants in each group. Injections were performed using pressurized injection devices (Ynject Go®, Fertinyect, Córdoba, Spain), following drilling with a 4 mm bit penetrating through the outer bark into the sapwood. The hole was not sealed after injection. Uptake times ranged from 30 minutes to 3 hours. The control plants received sterile water injections following identical procedures. Another group was treated by manually watering the pots once with 45 ml of a 2% solution of fosetyl-Al, instead of by injection.

Stem diameters were measured with a digital caliper to calculate the applied fosetyl-Al dose (g per cm of stem circumference) and assess its relationship with phytotoxicity and disease incidence. Treated plants

had a mean stem circumference of 5 ± 1.4 cm, and injected doses ranged from 0.04 to 0.40 g/cm of stem circumference, depending on treatment concentration.

2.3. Underbark inoculation test

In July 2023, an underbark inoculation test was performed to assess the ability of *P. cinnamomi* to colonize the tissue of a plant previously treated with fosetyl-Al.

The *P. cinnamomi* isolate used for the inoculation (CA01) was collected in 2017 from the rhizosphere soil of a symptomatic chestnut tree in Navaconcejo (Cáceres, Spain). Its identity was previously confirmed by sequence analysis of the ITS region (Grünwald et al., 2011). The isolate is preserved in the CICYTEX collection (Plasencia, Spain) and was selected for the assay based on preliminary aggressiveness tests using *L. luteus* seedlings.

The stem of each plant was inoculated twice, at 80 cm and 160 cm above the collar, according to the procedure followed by Milenkovic et al. (2018). After sterilizing the bark with 70% ethanol, a 7-mm-diameter hole was drilled through the bark into the wood using a steel cork borer. A plug of the same size was excised from the actively growing edge of the CA01 isolate, previously cultured on a 90-mm Petri dish with Potato Dextrose Agar (PDA) for seven days at 25°C in the dark. This fungal plug was inserted into the hole, replacing the removed bark plug. The inoculation site was then covered with moist cotton wool and sealed with Parafilm to maintain humidity and promote infection.

At 36 days after inoculation (DAI), the periderm at each inoculation site was carefully removed with a draw-knife to expose the phloem, and necrotic lesion length was measured using a digital caliper. For each plant, the reported value corresponds to the mean of the two inoculation points. To confirm the presence of *P. cinnamomi*, tissue samples taken from the lesion margins were re-isolated onto NARPH medium.

2.4. Ink disease progress and root isolation in naturally *P. cinnamomi*-infested plants

The typical aerial symptoms of *Phytophthora* infection, such as yellowing, leaf fall and collar lesions, were monitored for two years after treatment. Disease was assessed monthly using a visual symptom severity scale ranging from 0 to 4 (0 = healthy plant with no visible symptoms; 1 = foliage with light green coloration; 2 = approximately 50% of the leaves showing yellowing; 3 = extensive yellowing affecting nearly all (100%) of the foliage; 4 = complete plant collapse or death), simplified from Vettraino et al. (2001). The Area Under the Disease Progress Curve (AUDPC) was calculated using the method described by Shaner and Finney (1977) at 726 days after treatment (DAT). To account for differences in the time of disease development, the AUDPC values were normalized by dividing them by the maximum potential AUDPC value over the total time interval (from the first appearance of symptoms to the end of the evaluation period), following the approach proposed by Fry (1978). The resulting value was referred to as the relative AUDCP (rAUDPC). In addition, the presence or absence of disease symptoms was recorded for each plant to determine disease incidence.

Five months after treatment application, root infection was evaluated by carefully extracting approximately 100 g of root tissue from each plant. From each root sample, 40 fine root fragments (< 2 mm in diameter and approximately 10 mm long) were randomly selected and excised using a sterile scalpel.

The fragments were washed with tap water and plated on NARPH medium, which is selective for *Phytophthora* spp. After 2–5 days of incubation at 25°C in the dark, the Petri dishes were examined for the presence of *Phytophthora*, identified by the characteristic pattern of the colonies and hyphae. Subsequently, the isolates were subcultured on PDA medium at 25°C, where the identification was confirmed by the observation of hyphal swellings and the typical rosaceous colony pattern.

2.5. Phytotoxicity evaluation

Treated plants were monitored for signs of phytotoxicity on foliage and stems. Plants that exhibited leaf burn, leaf necrosis or sudden death within one week of treatment application were considered affected by phytotoxicity.

The phytotoxicity evaluation included measuring the quantum efficiency of photosystem II (ΦPSII) and stomatal conductance (gsw, mol H_2O $m^{-2}s^{-1}$) in light-adapted leaves using a Li-600 fluorometer (LI-COR®). Measurements were taken 19 DAT on three randomly selected, fully expanded leaves per plant, between 13:00 and 15:00 hours.

2.6. Statistical analysis

Data were analyzed in R (R Core Team, 2024) using RStudio software (RStudio, version 2025.05.0). The AUDPC was calculated using the *AUDPC()* function from the “agricolae” R package (de Mendiburu et al., 2023). Because rAUDPC, necrotic lesion length, and ΦPSII data did not meet the assumption of normality (Shapiro–Wilk test), but homoscedasticity was confirmed, treatment effects were analyzed using the Kruskal–Wallis test. When statistically significant differences were detected, pairwise comparisons were conducted using Dunn’s test with Bonferroni correction. Effect sizes were estimated using epsilon squared (ε^2) to quantify the magnitude of treatment effects. In contrast, stomatal conductance (gsw) was analyzed using Welch’s ANOVA to account for unequal variances among treatments. When significant differences were detected, pairwise comparisons were performed using the Games–Howell test with Bonferroni adjustment for multiple comparisons. These figures were done using the *ggbetweenstats()* function from the “ggstatsplot” R package (Patil, 2021).

Two logistic regression analyses were performed to examine the relationship between fosetyl-Al injected dose and the occurrence of phytotoxicity, as well as the occurrence of disease (incidence). The analyses were conducted using the *glm()* function with a binomial family and logit link, allowing the estimation of the probability of phytotoxicity (or disease) as a function of dose levels. Odds ratios (ORs) and their 95% confidence intervals (CIs) were computed by exponentiating the model coefficients using the *exp()* function. Model significance was evaluated through the likelihood ratio test. To assess the model's

discriminative ability, the area under the ROC curve (AUC) was calculated using the “pROC” R package (Robin et al., 2011).

Statistical analysis of root infection data was conducted using Fisher's exact probability test for an RxC matrix to evaluate the association between treatment and *P. cinnamomi* recovery. Pairwise comparisons were performed using the *pairwiseNominalIndependence()* function from the “rcompanion” R package (Mangiafico 2024), with Bonferroni correction applied to adjust for multiple testing.

3. RESULTS

3.1. *P. cinnamomi* growth following underbark inoculation test

Stem inoculation with *P. cinnamomi* isolate CA01 resulted in visible necrotic lesions only in the untreated control group and the soil irrigation treatment, indicating effective suppression by stem injections **(Figure S1)**. At 36 days after inoculation (DAI), lesion lengths differed significantly among treatments ($\chi^2 = 23.61$, $p < 0.001$), with a large effect size ($\varepsilon^2 = 0.51$, 95% CI [0.35, 1.00]) **(**Fig. 1**)**. Dunn’s post hoc test with Bonferroni correction showed that all injection concentrations (1%, 2%, and 4%) significantly reduced lesion sizes compared to the untreated control ($p < 0.001$). Median lesion size was 0 mm for all injection treatments, compared with 30.75 mm in the control. Lesion sizes in the 2% irrigation treatment did not differ significantly from either the control or the injection treatments. Its median value of 6.50 mm indicates that many plants developed no lesions, but high variability masked any detectable effect. Overall, only the stem injection provided consistent disease suppression. These results confirm that only stem injection provided consistent disease suppression. The zero median values observed for all injection treatments reflect a true biological response rather than a limitation of the analysis. Most injected plants showed no visible lesion development, while only a few individuals developed short necrotic lesions, leading to low mean values but a median of zero. The highly clustered distribution therefore suggests that the lesion has been arrested rather than partially suppressed.

3.2. Efficacy of fosetyl-Al on disease suppression and root recovery of *P. cinnamomi*

Disease progression was monitored over two years through visual symptom scoring, and the rAUDPC was calculated to quantify disease severity. All three injected treatments (1%, 2%, and 4%) and the 2% irrigated treatment exhibited a median rAUDPC of 0, indicating complete disease suppression in most cases **(**Fig. 2**)**. The Kruskal–Wallis test revealed statistically significant differences in rAUDPC values among treatments ($\chi^2 = 13.30$, $p = 0.009$). The effect size ($\varepsilon^2 = 0.44$, 95% CI [0.26, 1.00]) indicated a large treatment effect on rAUDPC. Dunn’s post hoc test with Bonferroni correction showed that the control group had statistically significantly higher disease severity than the 2% and 4% fosetyl-Al injection treatments ($p = 0.04$ and $p = 0.02$, respectively).

A total of 1,440 root fragments were analyzed to assess the presence of *P. cinnamomi* across the different treatment groups. No infections were detected in any of the stem-injected plants (1%, 2% and 4%), all of which showed an infection frequency of 0.00%. By contrast, *P. cinnamomi* was recovered from 8% of root fragments in the 2% irrigated treatment, suggesting partial efficacy. The untreated control exhibited the highest infection rate, with *P. cinnamomi* recovered from 15% of the root fragments. Fisher's exact test revealed statistically significant differences in the frequency of root infection across the treatments ($p < 2.2 \times 10^{-16}$). As shown in Table 1, pairwise comparisons confirmed that all injection treatments (1%, 2% and 4%) significantly reduced root infection rates compared to the untreated control (adjusted $p < 0.001$ in all cases). The 2% irrigated treatment also showed significant differences compared to the injected groups (1%, 2%, and 4%), with adjusted p values < 0.001, indicating lower efficacy of the soil application. However, no significant difference was observed between the 2% irrigated group and the untreated control after Bonferroni correction ($p = 0.317$), suggesting that irrigation treatment did not significantly reduce root infection. No significant differences were found between the different injection doses, indicating that all three concentrations achieved similar protective efficacy (Table 1).

Table 1
Pairwise Fisher's Exact Test results for *Phytophthora cinnamomi* root infection across treatment groups.

Group comparison	Fisher's p	Adjusted p (Bonferroni)
Control : 1%_injected	< .001	**< .001**
Control : 2%_injected	< .001	**< .001**
Control : 4%_injected	< .001	**< .001**
Control : 2%_irrigated	0.032	0.317
1%_injected : 2%_injected	1.000	1.000
1%_injected : 4%_injected	1.000	1.000
1%_injected : 2%_irrigated	< .001	**< .001**
2%_injected : 4%_injected	1.000	1.000
2%_injected : 2%_irrigated	< .001	**< .001**
4%_injected : 2%_irrigated	< .001	**< .001**

P-values were adjusted using the Bonferroni method. Statistically significant comparisons are highlighted in bold.

3.3. Dose-response relationship for disease incidence

The logistic regression analysis confirmed a significant association between the fosetyl-Al injected dose and disease probability (Fig. 3). The fitted equation, log(odds)disease = 1.397–58.699×dose. Dose had a

significant negative effect (β = − 58.70, SE = 24.75, z = − 2.37, p = 0.018), with a low odds ratio (OR = 2.5 × 10^{-26}, 95% CI [0.00, ∞]), indicating that higher doses decreased the likelihood of disease. The intercept was marginally significant (β = 1.397, SE = 0.78, z = 1.78, p = 0.075) and corresponded to OR = 4.04, suggesting moderate baseline odds of disease in the absence of treatment. The likelihood ratio test indicated a significant effect of dose on disease probability (χ^2 = 19.74, gl = 1, p < 0.001). The area under the curve (AUC = 0.93) confirms the model discrimination.

3.4. Dose-response relationship for phytotoxicity

No phytotoxic symptoms were observed in plants treated with fosetyl-Al via irrigation. However, trunk injection of the compound induced typical phytotoxic responses (such as leaf burn, necrosis, and sudden plant death) in some individuals within 48–72 hours post-application **(Figure S2)**. Irreversible damage leading to plant death occurred only in two individuals, both of which had received high doses (0.35 and 0.40 g/cm).

Logistic regression analysis confirmed a significant association between fosetyl-Al injected dose and phytotoxicity **(**Fig. 3**)**. The fitted equation, log(odds)phytotoxicity = -3.709 + 25.95 × dose, indicates a positive relationship between dose and phytotoxicity. The coefficient for dose was highly significant (β = 25.945, SE = 7.555, z = 3.43, p = 0.0006), showing that higher doses significantly increase the likelihood of phytotoxicity. Dose was a strong predictor of phytotoxicity, with an odds ratio (OR = 1.85 × 10^{11}, 95% CI [0.00, ∞], p = 0.001). The intercept had an OR = 0.02, 95% CI [0.00, 0.14], p = 0.001). The model demonstrated the predictive performance (AUC = 0.944), and the Chi-square test confirmed its robustness (χ^2 = 30.15, gl = 1, p < 0.001).

3.5. Minimum Effective Dose (MED) and associated phytotoxicity risk

Based on the fitted logistic regression models, the MED was estimated by applying the inverse logit transformation to selected disease risk levels. The MED required to achieve a 10% disease incidence was approximately 0.061 g/cm of stem circumference, corresponding to a predicted phytotoxicity risk of approximately 10.6% **(**Fig. 3**)**. To attain a more stringent disease threshold of 1%, the calculated MED increased to approximately 0.102 g/cm, with the associated phytotoxicity risk increased to around 25.7%.

3.6. Stomatal conductance and photosynthetic efficiency to assess phytotoxic effects

Figure 4 shows the effect of fosetyl-Al treatments on the quantum efficiency of photosystem II (ΦPSII) and stomatal conductance (gsw). The Kruskal–Wallis test revealed no statistically significant differences in ΦPSII among treatments (p = 0.07). In contrast, Welch's ANOVA indicated significant differences in stomatal conductance among treatments (p = 0.003). However, subsequent pairwise comparisons using the Games–Howell test with Bonferroni correction did not show statistically

significant differences between any specific pairs of treatments (Fig. 4). This discrepancy between the overall and pairwise tests may be explained by high within-group variability or limited statistical power to detect differences after correcting for multiple comparisons.

4. DISCUSSION

This study evaluated the efficacy and phytotoxicity of fosetyl-Al for managing ink disease caused by Phytophthora cinnamomi in Castanea sativa saplings under semi-controlled conditions. Treatments were applied either by trunk injection (1%, 2%, and 4%) or by soil irrigation (2%), using nursery-derived plants that included a proportion of asymptomatic but naturally infected individuals. The detection of *Phytophthora* spp. in nursery stock prior to treatment is consistent with previous reports describing widespread contamination in European nurseries (Jung et al., 2016; Moralejo et al., 2009; Prigigallo et al., 2015; Green et al., 2025), reinforcing the practical relevance of preventive approaches for newly established orchards and plantations.

The results confirmed the high efficacy of fosetyl-Al through trunk injection, which significantly reduced lesion development after underbark inoculation and completely prevented *P. cinnamomi* recovery from roots, whereas the soil irrigation treatment showed only partial protection. These results agree with earlier studies identifying trunk injection as an efficient delivery strategy for systemic compounds against soil-borne pathogens, including *P. cinnamomi* (Gentile et al., 2009; VanWoerkom et al., 2014; Scott et al., 2015; Dal Maso et al., 2017; Berguer and Laurent, 2019; Romero et al., 2019; Archer et al., 2022; Brandano et al., 2023). In addition, injection wounds showed visible external closure during the experimental period; however, internal wood integrity and long-term structural effects were not assessed and should be addressed in future field studies. As noted by Archer et al. (2022), a clear understanding of the anatomical and physiological responses of chestnut plants is essential before implementing injection technologies on a commercial scale.

Fosetyl-Al applied via soil irrigation demonstrated only partial effectiveness. The lack of significant differences between the 2% irrigated treatment and the untreated control indicates that soil irrigation is insufficient for reducing root infection. This limited efficacy is likely due to impaired phosphonate uptake in plants with compromised root systems, consistent with findings in almond trees (Wicks and Hall, 1988). Although nursery-infected saplings appeared healthy, latent infections likely reduced root function and hindered the uptake of treatment. Comparable studies in apple trees reported that foliar applications were more effective than soil drenches for phosphite translocation to roots (Nyoni et al., 2019). In avocados, trunk injection of fosetyl-Al significantly reduced root pathogen levels and improved tree health, although some foliar phytotoxicity occurred (Darvas et al., 1984). Trunk injection delivers the full dose of the active compound directly into the vascular system, eliminating reliance on functional root or foliar absorption surfaces. When roots or leaves are damaged, as in plants infected with *Phytophthora*, foliar or soil applications may not provide adequate uptake, increasing the risk of treatment failure and product loss.

The efficacy of soil-applied fosetyl-Al can be influenced by the physico-chemical properties of the soil and the interactions with soil microorganisms that affect its mobility and availability. The uptake of phosphonates by fungi and oomycetes, such as *Phytophthora* spp., is competitively inhibited by phosphate, making treatment success highly dependent on soil phosphate levels and pH (Barchietto et al., 1988; Fenn and Coffey, 1984; Manghi et al., 2021). Microbial oxidation of phosphonates to phosphate may further modify persistence and availability over time, depending on soil type and microbial activity (Casida 1960; Malacinski and Konetzka 1966; Guest and Grant 1991; Ohtake et al. 1996; Metcalf and Wolfe 1998; Schink and Friedrich, 2000; McDonald et al. 2001a, 2001b; White and Metcalf 2007; Gómez-Merino and Trejo-Téllez 2015; Nguyen 2025). Beyond efficacy, repeated soil applications may also alter soil chemistry and nutrient balance, as suggested by manganese toxicity reported after phosphorous acid drenches in chestnut seedlings (Rosário et al. 2021). In practice, soil-applied fosetyl-Al is susceptible to off-target losses via drain flow (Lewis et al., 2016), which reduces plant uptake and increases environmental exposure. Although soil impacts were not assessed here, these processes highlight the value of delivery methods that reduce off-target exposure.

A further practical advantage of trunk injection is durability. Disease suppression remained detectable for at least two years, supporting previous observations of prolonged protection in woody hosts (Romero et al. 2019). However, persistence may depend on dose and host physiology (Whiley et al., 1992; Hardy et al., 2001). Quantifying fosetyl-Al (sum of fosetyl, phosphonic acid and their salts) concentrations in different tissues over time would be valuable to define reapplication intervals and address concerns regarding potential residues in edible tissues.

Despite its strong efficacy, trunk injection also introduced a clear dose-dependent risk of phytotoxicity. Leaf burn, necrosis, and occasional plant death occurred within 48–72 h after treatment in a subset of injected plants, whereas no phytotoxic symptoms were observed in the irrigated treatment. Most affected saplings recovered and showed no visible damage after two years, suggesting that moderate injury can be compensated by subsequent growth, as reported previously (Pilbeam et al., 2000). Nonetheless, two plants exposed to the highest doses (0.35 and 0.40 g/cm of stem circumference) suffered irreversible damage, emphasizing the narrow margin between efficacy and intolerance in small-diameter trees.

Dose–response modelling provided a quantitative framework to balance protection and safety. The MED associated with a 10% disease incidence threshold was estimated at 0.061 g/cm, with an associated phytotoxicity probability of ~ 10.6%, whereas achieving a 1% disease threshold required 0.102 g/cm, increasing estimated phytotoxicity risk to ~ 25.7%. These results indicate that optimal disease protection may require accepting a moderate risk of phytotoxicity. Because phytotoxicity was recorded as presence/absence, these probabilities likely integrate predominantly mild to moderate symptoms rather than severity classes. Future research should quantify the severity of phytotoxicity to refine dosing strategies. These findings highlight the need to balance effective disease control with the risk of physiological damage and emphasize the importance of precise dose optimization in fosetyl-Al trunk injection protocols.

However, both the MED and the associated phytotoxicity risk were derived from young saplings under semi-controlled conditions and should not be directly extrapolated to mature trees. Field conditions introduce variability, and larger trees may show greater tolerance to phytotoxicity, due to their larger biomass and more developed physiological systems. In this context, a field study on *Quercus ilex* using 4% fosetyl-Al injections reported tree recovery without phytotoxic symptoms (Romero et al., 2019). Although no significant reduction of *P. cinnamomi* in roots was observed, a decreasing trend was noted. In contrast, our study demonstrated a statistically significant reduction in root infection, further supporting the effectiveness of stem injection under the tested conditions. Despite differences in species, environmental conditions and tree maturity between the two studies, both support the efficacy of fosetyl-Al trunk injection in controlling *P. cinnamomi*.

Previous studies have shown that phosphonate-induced phytotoxicity is dose-dependent, with symptoms becoming more severe as the application rate increases (Wicks and Hall, 1988; Pilbeam et al., 2000; Hardy et al., 2001; Barrett et al., 2003). This phenomenon occurs in a wide range of species from various genera and families. Phytotoxic effects have also been observed in fruits and reproductive tissues, for example, in avocados (Niekerk et al., 2018) and citrus fruits (Leroux, 2000), raising concerns about broader physiological impacts beyond foliage. Furthermore, it is difficult to make comparisons with previous studies, as most have evaluated phytotoxic responses to foliar applications or used potassium phosphonate instead of fosetyl-Al. Differences in the formulation of the compound, the method of application and the target tissues may influence the severity and nature of phytotoxic symptoms, which limits the direct comparability of results across studies.

Physiological indicators (ΦPSII and gsw) showed a decreasing trend in injected treatments, consistent with the observed phytotoxic symptoms. However, these differences were not statistically significant, likely due to limited statistical power and the fact that measurements were necessarily restricted to the least affected leaves, as severely damaged foliage could not be assessed.

Finally, integrated strategies could improve both efficacy and safety. Incorporating micronutrients or biostimulants in phosphonate formulations could mitigate phytotoxicity while enhancing efficacy, as demonstrated in studies using potassium phosphonate combined with micronutrients (Scott et al., 2015; Dal Maso et al., 2017). Similarly, Khdiar et al. (2022) reported that the foliar application of phosphite, combined with calcium chelate, reduced root lesion development. Stasikowski (2014) also found that both potassium phosphonate and calcium salts protect susceptible plants from *P. cinnamomi* infection, suggesting an additive protective effect when applied together. Given that *Trichoderma* spp. has demonstrated the ability to survive in environments containing phosphonates and that in vitro compatibility assays with potassium phosphonate and fosetyl-Al have shown promising results (Dhanya et al. 2016; Solis-Palacios et al. 2021; Sirikamonsathien et al. 2023), it would be worthwhile to explore the combined application of fosetyl-Al and *Trichoderma* through trunk injection as a potential integrated strategy for disease management.

Further research is needed to validate these findings under field conditions and in mature trees, where physiological responses, disease pressure, and environmental variability may differ from those in controlled environments. Long-term studies are necessary to evaluate the persistence of protection and the potential cumulative effects of repeated trunk injections. Evaluating alternative formulations, including combinations with micronutrients, biostimulants, or biocontrol agents, may enhance efficacy and reduce adverse effects. Investigating the translocation and degradation of phosphonates within plants and soil, particularly in relation to microbial communities and nutrient cycling, will inform the sustainability of treatment applications.

More broadly, integrating pathogen-free nursery stock, resistant rootstocks, and complementary biological control options alongside carefully dosed phosphonate use could strengthen long-term ink disease management in orchards and reforestation programs.

5. CONCLUSION

Trunk injection of fosetyl-Al provided effective control of *P. cinnamomi* in *C. sativa* saplings, consistently reducing stem lesion development and eliminating pathogen recovery from roots at all tested injection concentrations (1%, 2%, and 4%). Protection persisted for at least two years, as reflected by reduced disease progress (rAUDPC). Dose–response analyses identified a practical MED range of 0.061–0.102 g/cm of stem circumference, corresponding to predicted disease incidence thresholds of 10% and 1%, with associated phytotoxicity risks of approximately 10–25%. While most saplings recovered from moderate phytotoxic symptoms, occasional irreversible damage at the highest doses highlights the need for precise dose adjustment, particularly in small-diameter trees. Compared with soil irrigation, trunk injection provided superior and more reliable disease suppression and reduced off-target exposure. Future research should validate these findings under field conditions and in mature trees, quantify tissue residues and persistence to guide retreatment intervals, and evaluate optimized formulations and integrated strategies to maximize efficacy while minimizing adverse effects.

Abbreviations

rAUDPC	Area Under the Disease Progress Curve
PDA	Potato-Dextrose-Agar
NARPH	Natamycin-Ampicillin-Rifampicin-PCNB-Hymexazol
DAI	Days After Inoculation
DAT	Days After Treatment
MED	Minimum Effective Dose

Declarations

Supplementary Materials: Figure S1: Lesion development in chestnut saplings 36 days after stem inoculation with *Phytophthora cinnamomi* isolate CA01; Figure S2: Foliar phytotoxicity symptoms in chestnut leaves following trunk injection of fosetyl-Al.

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Figures

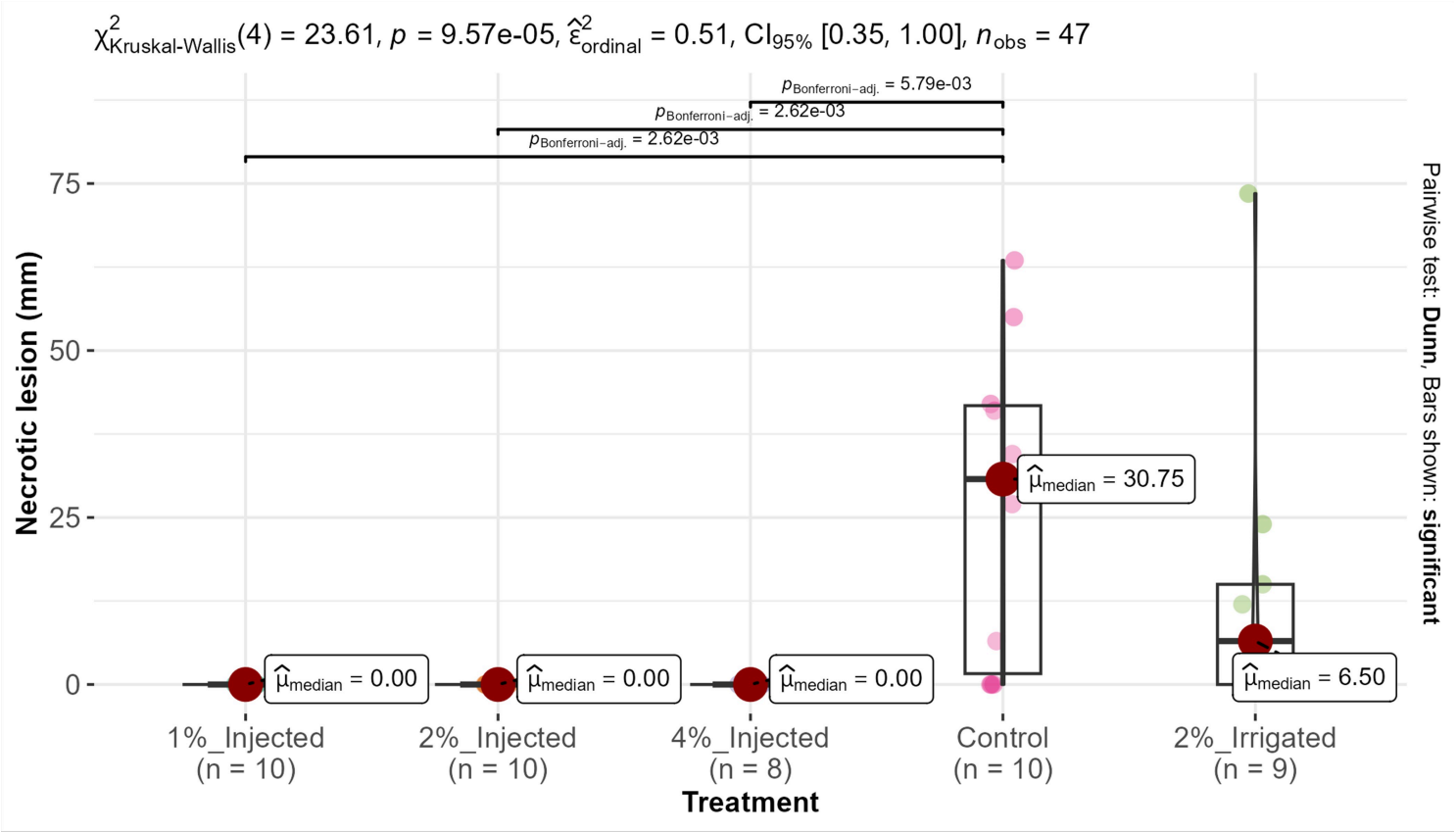


Figure 1

Length of stem necrotic lesions (mm) in *Castanea sativa* saplings 36 days after *Phytophthora cinnamomi* inoculation across the treatments. Treatments include three levels of fosetyl-Al trunk injection (1%, 2%, and 4%), a 2% irrigation treatment, and an untreated control group. The Kruskal–Wallis test indicated statistically significant differences among treatments ($p < 0.001$). Pairwise comparisons were conducted using Dunn's test with Bonferroni correction. Red dots represent group medians.

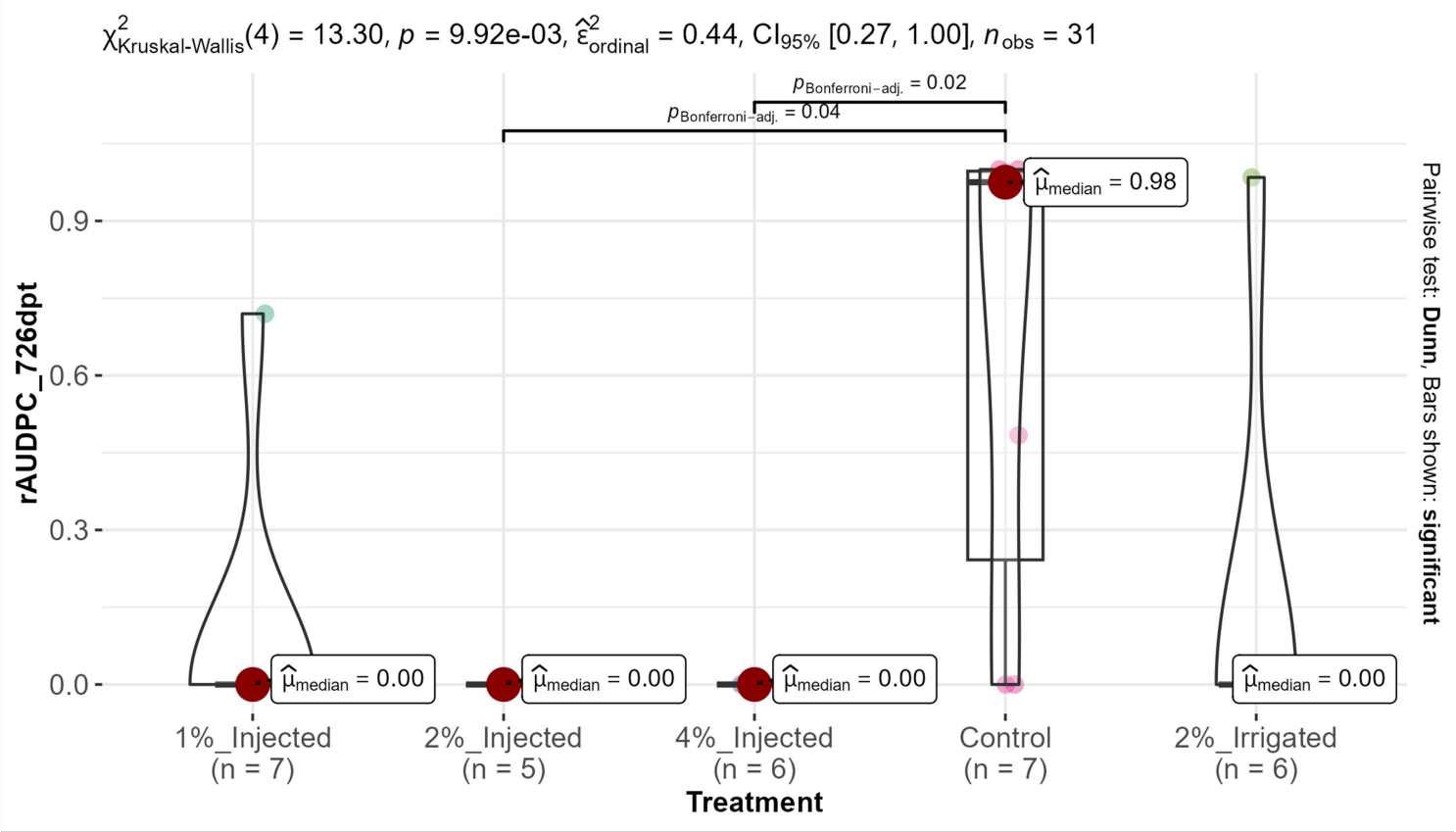


Figure 2

Violin plots showing the distribution of relative area under the disease progress curve (rAUDPC) at 726 days after treatment (DAT) across the treatments. Treatments include three levels of fosetyl-Al trunk injection (1%, 2%, and 4%), a 2% irrigation treatment, and an untreated control group. The Kruskal–Wallis test indicated statistically significant differences among treatments ($p < 0.001$). Pairwise comparisons were conducted using Dunn's test with Bonferroni correction. Red dots represent group medians.

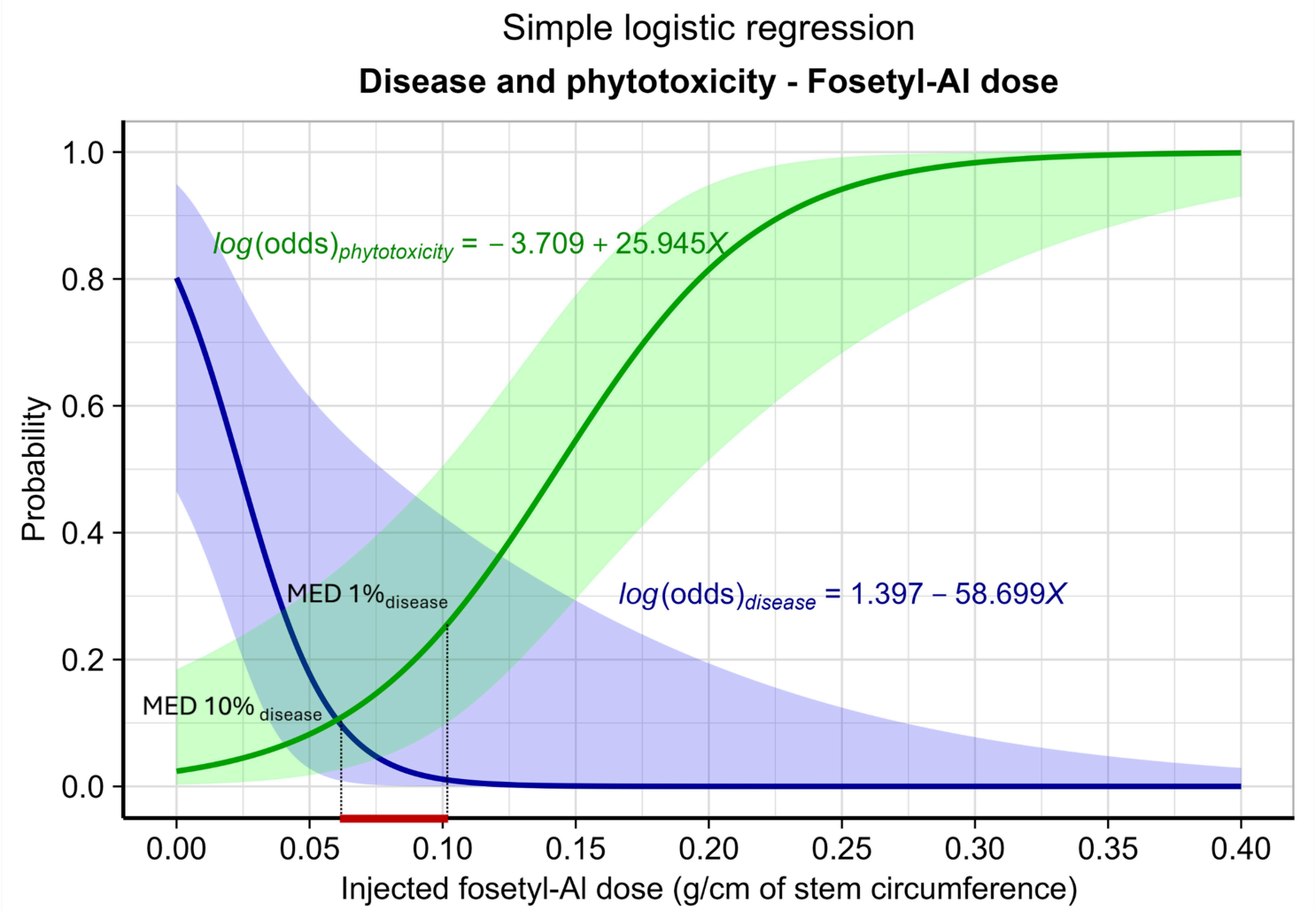


Figure 3

Logistic regression curves showing the relationship between injected fosetyl-Al dose (g/cm of stem circumference) and the probability of disease (blue line) and phytotoxicity (green line). Shaded areas represent 95% confidence intervals calculated from the logistic regression models. Equations correspond to the fitted logit (log(odds)) form for each response. Vertical dotted lines indicate the Minimum Effective Dose (MED) corresponding to 10% and 1% disease incidence (0.061 g/cm and 0.102 g/cm, respectively), along with their associated phytotoxicity risks. The red horizontal segment highlights the dose range between these two MED values.

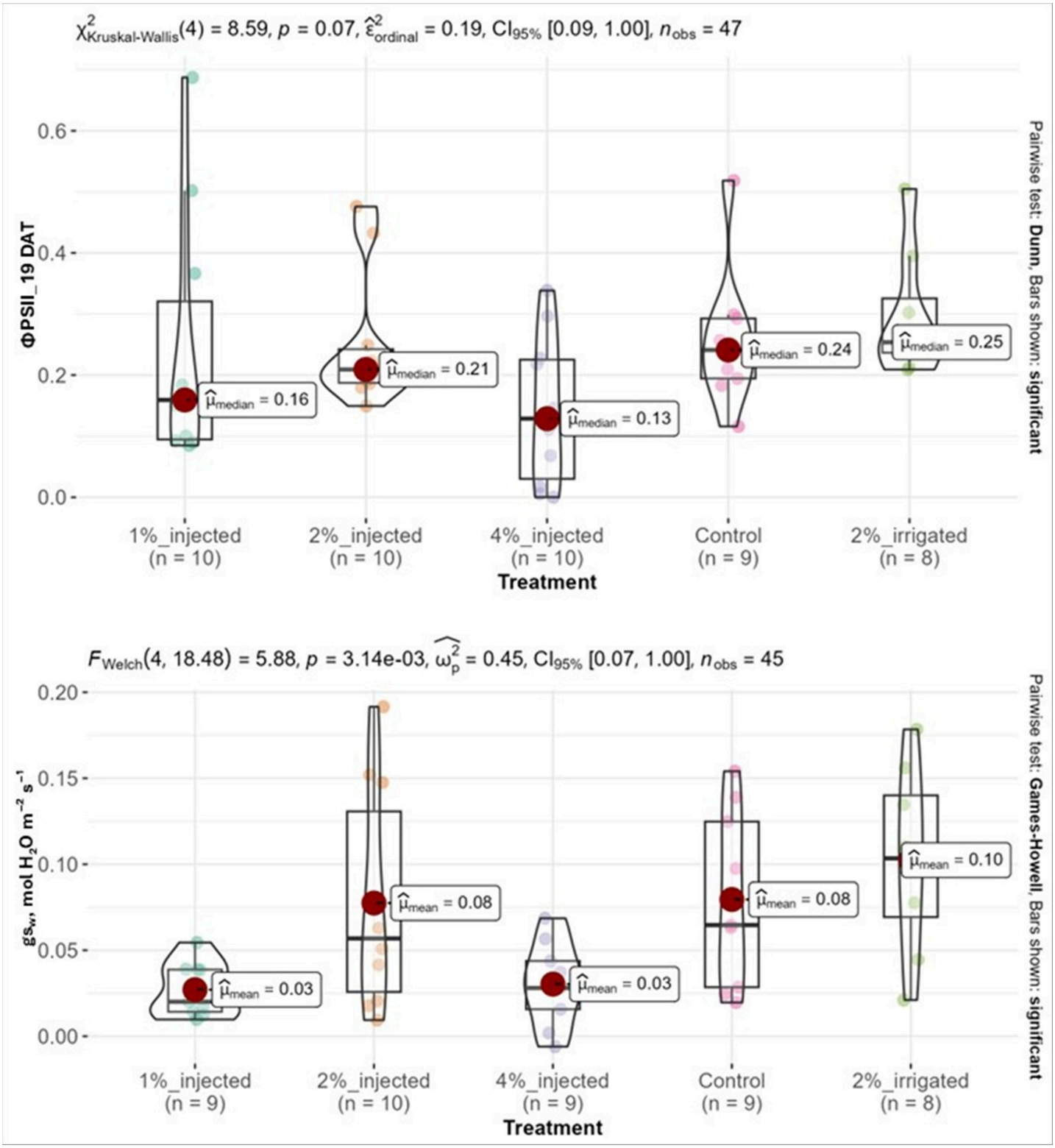


Figure 4

Effect of fosetyl-Al treatments on the quantum efficiency of photosystem II (ΦPSII, top) and stomatal conductance (gsw, bottom) of chestnut leaves, measured 19 days after treatment. Treatments include three levels of fosetyl-Al trunk injection (1%, 2%, and 4%), a 2% irrigation treatment, and an untreated control group. Data were analyzed using the Kruskal–Wallis test (ΦPSII) and Welch's ANOVA (gsw). Violin plots represent data distributions with embedded boxplots; red dots indicate group medians

(ΦPSII) or means (gsw). Pairwise comparisons were performed using Dunn's test (ΦPSII) or Games–Howell test (gsw), both with Bonferroni correction.

Supplementary Files

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