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# Use of Phosphonate Salts to Control Chestnut 'Brown Rot' by *Gnomoniopsis castaneae* in Fruit Orchards of *Castanea sativa*

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**Abstract:** The fungus *Gnomoniopsis castaneae* is the causal agent of the "brown rot" of sweet chestnut fruits. These days, this pathogen represents one of the main limiting factors for the sustainability of fruit production worldwide. Although heat treatment post-harvest is efficient in completely inactivating the pathogen, the application of appropriate protocols to control "brown rot" in chestnut orchards is required to help in reducing the latent population of the fungus in fruit tissues, and the consequent development of "brown rot" symptoms in the field before the post-harvest handling process. The present study aims to evaluate and compare the efficiency of products at a minimum environmental impact in experimental trials conducted in chestnut orchards in Central Italy for two consecutive years in 2019 and 2020. Phosphonate-based salts and, specifically, Zn-phosphonate were efficient in reducing the impact of the disease and the pathogen inoculum in fruits with an efficacy comparable to the fungicide Tebuconazole. A unique treatment at the blooming time produced the best results for both Zn-phosphonate and Tebuconazole, also giving indirect evidence of female flowers as a main site of infection. Phosphonate salts, and at first Zn-phosphonate, are highly effective to protect chestnut fruits from the 'brown rot' fungus *G. castaneae*. Its use in orchard management may complement the post-harvest heat treatment during the processing of fruits. Although a still ongoing debate on phosphonate salts use and efficacy in agriculture, they can be considered an optimal fungicide in chestnut orchards because of the low environmental impact when used at the recommended doses, the high translocability and stability, and the multiple mechanisms of action.

**Keywords:** *Gnomoniopsis smithogilvyi*; fungicide; low-impact commercial molecules; agroforestry; chestnut orchards; control disease

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## 1. Introduction

The fungus *Gnomoniopsis castaneae* G. Tamietti (syn. *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest) [1–3] is the causal agent of the "brown rot" of sweet chestnut fruits. These days, this pathogen represents one of the main limiting factors for the sustainability of fruit production. The disease outbreak can be dated to the first decade of the 21st century, when European and Australian chestnut growers first noticed a severe increase in internal rot in the fruits, locally affecting up to 80% of production in particular seasons [2,4,5]. The name "brown rot" refers to the typical symptomatology, which includes a progressive rot and browning of the endosperm and embryo [1]. The symptoms are mainly expressed postharvest, although rotting fruits can be found in burrs on the tree before harvest [4]. Notably, fruits that appear healthy could have latent infections of the pathogen [6] that rapidly colonize the endosperm during the postharvest period in case of conditions of optimal temperature and the presence of water. Indirect evidence supports the hypothesis that female flowers are the main site of infection of *G. castaneae* [7]. The

severe impact of the pathogen in the last decade was associated with a massive presence of inoculum in the environment boosted by climate change [8] and in synergy with infestation by the Chinese gall wasp *Dryocosmus kuriphilus* Yasumatsu [9–11]. Quantifying the impact suggests that incidence may be as high as 93.5% in northwest Italy, [2,8], 72% in Australia [5], and 21% in Switzerland [12]; the disease was more recently recorded in North America [13] and Chile [14]. Recent studies demonstrated that the pathogen can be easily controlled in post-harvest fruit handling by physical methods, indicating that the water-bath phase is the critical step. A strict parametrization of this phase was required to effectively inactivate *G. castaneae* in fruits. Specifically, treatment of fruits at 50 °C for a maximum of 45 min provided optimal conditions to completely inactivate *G. castaneae* inoculum during postharvest handling [6]. However, the control of "brown rot" in post-harvest fruit handling only partially solves the problem since, in particular seasons, the fruit stocks enter the post-harvest handling process at a rather high proportion of fruits with "brown rot" symptoms developed when they were still in the burrs on the trees, and during the harvest phase. Thus, the application of appropriate protocols to control "brown rot" in chestnut orchards is required to help reduce the fungus in fruit tissues, and the consequent development of "brown rot" symptoms in the field. Besides the homologation of some fungicide molecules for their use on chestnut, Tebuconazole, Boscalid, and Pyraclostrobin, a specific protocol to control the disease does not exist. Recently, in field trials, Silva-Campos et al. [15] reported that the use of pyraclostrobin and difenoconazole-based fungicides combined were the most effective in suppressing the level of nut infection caused by *G. castanea*. Moreover, a modern approach to control this disease in a peculiar agroforestry ecosystem such as chestnut groves, cannot rely on the use of molecules with relevant environmental impact. The present study aims to evaluate and compare the efficiency of products at minimum environmental impact between them and a currently homologated fungicide as a positive control. The results presented are the results of experimental trials conducted in chestnut orchards in Central Italy for 2 consecutive years in 2019 and 2020.

## 2. Materials and Methods

### *2.1. Evaluated Products*

Three different products were evaluated for their efficacy in mitigating the impact of brown rot: treatment Kalex® (AlbaMilagro International Ltd., Parabiago, Italy) containing 50% *w*/*w* potassium-phosphite ($KH_2PO_3$), is a liquid fertilizer and enhancer of the natural resistance of plants against external pathogenic agents; Kalex Zn® (AlbaMilagro International Ltd., Parabiago, Italy) containing 4% *w*/*w* Ureic nitrogen, 36% *w*/*w* zinc-phosphonate ($O_6P_2Zn_3$), is an innovative mineral fluid fertilizer containing, in the form of zinc phosphonates, high quantities of phosphorus and zinc, with its known fungicidal activity; Mystic® 430 SC (Nufarm Italia Ltd., Milano, Italy) containing 40.18% (*w*/*v*) Tebuconazole ($C_{16}H_{22}ClN_3O$), is used as conventional chemical treatment against fungal contamination (Table 1). Doses used were those recommended by producers: Kalex®, 200 mL/L, endotherapy; Kalex Zn®, 300 mL $hL^{-1}$, crown spray; Mystic® 430 SC, 35 mL $hL^{-1}$, crown spray.

### *2.2. In Vitro Tests*

Before application in open field conditions, the products were tested for their fungistatic and fungicide efficacy "in vitro" against mycelium and conidia of *G. castaneae*. The inhibition tests were carried out on a potato dextrose agar (PDA) medium amended with each product separately. For each product, eight concentrations (0.1 and 0.5, 1, 2, 5, 7.5, 10 and 20 mL $L^{-1}$) for mycelial radial growth assessment and fourteen concentrations (0.001, 0.01, 0.1, 1, 10 and 100 µL $L^{-1}$, 1, 2, 5, 7.5, 10, 20, 50 and 100 mL $L^{-1}$) for conidia germination assessment were compared. Plates with PDA only were used as control. For each of the tests, there were five replicate plates per treatment dose. All the in vitro experiments were repeated twice.

**Table 1.** Details of treatments, rate, application date, and phenological stage for field trials conducted at the chestnut orchard.

| Year | Product | Application | Rate | Date | Phenological Stage |
|---|---|---|---|---|---|
| 2019 | Kalex® | endotherapy | 0.8 mL/tree | 13 June | After bud burst |
| 2019 | Kalex Zn® | crown spray | 3 L/ha | 25 June and 6 July | Blooming and burr formation |
| 2019 | Mystic® 430 SC | crown spray | 350 mL/ha | 25 June and 6 July | Blooming and burr formation |
| 2019 | Control | - | - | - | - |
| 2020 | Kalex® | endotherapy | 0.8 mL/tree | 23 June | Blooming |
| 2020 | Kalex Zn® | crown spray | 3 L/ha | 23 June and 9 July | Blooming |
| 2020 | Kalex Zn® | crown spray | 3 L/ha | 23 June; 9 July and 27 August | Blooming; burr development and kernel development |
| 2020 | Mystic® 430 SC | crown spray | 350 mL/ha | 23 June and 9 July | Blooming |
| 2020 | Mystic® 430 SC | crown spray | 350 mL/ha | 23 June; 9 July and 27 August | Blooming; burr development and kernel development |
| 2020 | Control | - | - | - | - |

#### 2.2.1. Inhibition of Mycelial Growth

Mycelial discs (6 mm) from actively growing colonies of the two *G. castaneae* isolates from the collection (GN01 and GNAm, GeneBank accession number MW494885 and OM818661, respectively) were placed in the center of a PDA plate. All the plates were incubated at 22 ± 2 °C. To calculate the percent inhibition, radial growth (mean of two perpendicular diameters) was expressed as the mean percentage of the growth in the control plates [16]. The mycelium plugs of the plates with 100% inhibition were removed at the end of the experiment and were plated on PDA without the fungicide and the plates were maintained for another 30 days at 22 ± 2 °C, to evaluate the fungicidal or fungistatic effect of the products.

#### 2.2.2. Inhibition of Conidia Germination

*G. castaneae* isolates (GN01 and GNAm) were grown on malt extract agar (MEA) and incubated for 15 days at 22 ± 2 °C. Conidial suspensions from each isolate were prepared by flooding the agar surface with approximately 15 mL of sterile distilled water (SDW) and scraping with a sterile spatula. The suspension was filtered through two layers of cheesecloth and adjusted with SDW to 100 conidia $mL^{-1}$, and 0.5 mL aliquot spread over a Petri plate containing medium with the product's addition. The plates with PDA only were used as controls. All the plates were incubated at 22 ± 2 °C to support the conidia germination. The number of colonies forming units (CFUs) from the conidial suspensions was assessed after four, eight, and 30 days. To calculate the percentage of inhibition, conidia germination was expressed as the mean percentage of germination in control plates [16].

### *2.3. Treatment Trials*

Trials were established in 2019 and 2020 in a young (15 years old; an average of 25 cm in trunk diameter and 3 m in height) sweet chestnut (*Castanea sativa*) plantation for fruit production located in Central Italy in the Province of Viterbo (42°17′35 N 12°08′37 E). Chestnut trees of the cultivar "Marrone Fiorentino" were grown in the orchard. A Randomized Complete Design (RCD) was adopted with a statistical unit of 3 adjacent trees along the row, and four replications per treatment. The units of replication were separated from each other by one tree along the row. Four and six treatments were considered in 2019 and 2020, respectively (Table 1). In 2019, two treatments were done with Kalex Zn® and Mystic® 430 SC (June and July); in 2020, a third repetition of the treatment with these products in August was also included. For each treatment, product doses (according to manufacturers' recommendation), application method, and data are summarized in Table 1. Endotherapy treatments were carried out using the Chemjet® Tree Injectors (Banyo Queensland, 4014 Australia), which are reloadable syringes for micro-injection and infusion systems developed for trees. Each syringe loads 20 mL of product. Each tree received two

injections at breast height and on the opposite side. Crown spray was carried out with a Trailer Mist-Blower Sprayer fitted with a "Top-Fan" with an instantaneous variable pitch fan unit, Model Trend by Caffini (Verona, Italy), with a tank of 2000 L (120–170 L $min^{-1}$).

### *2.4. Samples Processing*

At the end of the treatment campaigns, the clusters of burrs on branches were enclosed in a plastic net to avoid ripened fruits to fall on the ground. At harvest time, fruits were collected from the nets and transferred to the laboratory. In 2019, the fruits were stored at 20 ± 1 °C in moist chambers and the presence of *G. castaneae* conidiomata on the kernel surface was annotated after 14 days, through assessment of the characteristic cirri emerging from conidiomata [10]. Following that, 10% of fruits were randomly sorted from each sample tree, surface sterilized, split in half, and assessed for the presence of "brown rot" symptoms and *G. castaneae*, upon isolation in pure culture. In 2020, after harvest, it was decided to proceed immediately with visual scoring and isolation in a pure culture: all fruits were surface sterilized, then split in half; one half was assessed for the presence of "brown rot" symptoms and *G. castaneae* by isolation in the pure culture; the other half was stored at −20 °C for embryo DNA extraction. Isolation in pure was carried out as follows: after shell removal, each kernel was split in half with a sterilized razor blade. Half of the embryo of each healthy and symptomatic fruit was placed onto Petri dishes containing potato dextrose agar (PDA, Oxoid, Basingstoke, UK, 39 g/L) amended with streptomycin sulfate (0.06 g $L^{-1}$) (PDAs) and incubated at 22 ± 2 °C [6,17]. After 7 days of incubation, the plates were scored for the presence of *G. castaneae* colonies. The plates were re-scored after 30 days to confirm the absence of growth on fragments that scored negative on day 7. Identification was based on the colony and reproductive structure morphology, and confirmed by molecular barcoding according to the protocol reported by Morales-Rodríguez et al. [11].

### *2.5. DNA Extractions and qPCR Reactions*

In the 2020 trials, the detection and quantification of *G. castaneae* were done by newly developed molecular methods according to the protocols reported by Turco et al. [18]. DNA was extracted from six randomly sorted chestnuts from each of the twelve trees (4 blocks) of each thesis, using the NucleoSpin Plant II mini kit (Macherey Nagel, Düren, Germany), following the manufacturer's instructions. DNA concentration was measured with Qubit (Thermo Fisher, Waltham, MA, USA) using the High Sensitivity dsDNA Assay kit. DNAs were stored at −20 °C until further analysis. The qPCR reactions were performed as previously described in Turco et al. [18]. Briefly, 2 µL of DNA was used as a template in a reaction composed of 1× GoTaq Probe qPCR Master Mix (Promega, Madison, WI, USA), 0.5 µM of each primer, 0.3 µM probe, and ultrapure water to reach a final volume of 20 µL. Amplifications were performed in a RotorGeneQ (Qiagen, Hilden, Germany) with the first step at 95 °C for 4 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 45 s. Fluorescence was measured once per cycle at the end of the 60 °C segment and the Ct values were automatically calculated by the device. Two replicates per sample were tested and the Ct values obtained by qPCR amplification were used for the data analysis described below.

### *2.6. Assessment of Disease and Infection Indexes*

#### 2.6.1. Disease Indexes Based on Symptoms Expression

The assessment of brown rot symptoms was carried out according to an arbitrary visual scale in 5 classes: class 0, healthy fruits (0% brown rot), class 1 (10–30% brown rot), class 2 (30–50% brown rot), class 3 (>50% brown rot) and class 4 (100% brown rot) [6].

The incidence of brown rot (Ibr) was calculated as the ratio

$$\text{Ibr} = \frac{n}{N}$$

where $n$ is the number of symptomatic fruits and $N$ is the total number of fruits scored.

The severity of brown rot (Sbr) was calculated by the equation

$$\text{Sbr} = \frac{\sum(xi\ ni)}{n}$$

where $xi$ represents the disease class, in the number of diseased fruits in class $xi$, and $n$ the total number of diseased fruits [19].

#### 2.6.2. Infection Index Based on qPCR Results

Given that qPCR is a qualitative and quantitative detection method, both aspects were in the definition of an Infection index ($I_i$) suitable for statistical analysis. The limit of quantification (LOQ) of this assay was estimated at cycle threshold ($Ct$) 37, corresponding to about two pathogen cells in the analyzed sample [18]. Here, to avoid borderline misinterpretations of data, this limit was prudentially set to $Ct$ 35, thus assuming the null presence of the pathogen for any $Ct$ value beyond this threshold. Consequently, if $N_p$ is the number of samples positive for pathogen's infection ($Ct < 35$) and $N_t$ is the total number of chestnut samples analyzed for each treatment (i.e., 6 chestnuts per 12 plants, 2 reps, 144 in total), $\alpha_t$ represents the proportion of infected samples per treatment as follows:

$$\alpha_t = \frac{N_p}{N_t}$$

Additionally, detracting the actual $Ct$ measured in each sample ($Ct_i$) from the threshold 35, it is possible to define the coefficient $\Delta Ct_i$ that will return positive values only for infected samples ($Ct < 35$), increasing proportionally with the infection level.

$$\Delta Ct_i = 35 - Ct_i$$

Combining both the values, it is possible to calculate an Infection index $I_i$ as:

$$I_i = \Delta Ct \cdot \alpha_t$$

This index, pondering both the ratio of infected samples and their level of infection, summarizes in a single value the infection in each sample and thus, indirectly, the efficiency of each treatment under evaluation.

### *2.7. Residues in Fruits*

Three ripen fruits were sorted from each Kalex Zn®, Kalex® treated, and control trees. The residue of phosphonate was assessed using the official EURL-SRM (QuPPe) method by an external laboratory licensed for residues analysis in agriculture (AGRO-BIO-ECO Laboratori Riuniti s.r.l., Pomezia, Italy).

### *2.8. Statistical Analyses*

Statistics were carried out with GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA) (http://www.graphpad.com/ (accessed on 8 August 2022)). For the calculation of half-maximal effective concentration (EC50) for mycelial and conidia inhibitions, the non-linear regression module "agonist vs. response" of the GraphPad Prism version 8.01 was used where the "dose" was the "agonist", and the "inhibition" was the "response". The model comparison was carried out with the extra sum of squares F-test function of the "non-linear regression (curve fit)" module of GraphPad Prism version 8.01. Parametric, ANOVA, and Dunnett's multiple comparations were used to analyzed the result from isolation in the 2019 trial and incidence in the 2020 trial. Due to the lack of homoscedasticity of the data, the severity data were analyzed by Brown–Forsythe and Welch ANOVA test and Holm–Sidek's multiple comparations. Non-parametric, Kruskal–Wallis tests were used for the statistical significance of differences in the Infection index

followed by Dunn Multiple comparison tests. An ANOVA test for the equality of group variances and the D'Agostino and Pearson test for normality were carried out for the choice of ANOVA or Kruskal–Wallis tests.

## 3. Results

### *3.1. In Vitro Test*

The EC50 values of Kalex Zn®, Kalex®, and Mystic® 430 SC are shown in Table 2 for both mycelium and conidia of *G. castaneae* isolates GN01 and GNAm.

**Table 2.** Values of 50% effective concentration (EC50) for inhibition of radial growth and conidia germination of 2 isolates (GN01 and GNAm) of *Gnomoniopsis castaneae* by Mystic® 430 SC, Kalex Zn® and Kalex®.

| | EC50 (μL $L^{-1}$) | | | |
|---|---|---|---|---|
| | GN01 | | GNAm | |
| | Mycelium | Conidia | Mycelium | Conidia |
| Mystic® 430 SC | 0.06 | 0.017 | 0.06 | 0.01 |
| Kalex Zn® | $8 \times 10^2$ | $2 \times 10^3$ | $1 \times 10^3$ | $1.8 \times 10^3$ |
| Kalex® | $2.8 \times 10^3$ | $1 \times 10^4$ | $2.4 \times 10^3$ | $1 \times 10^4$ |

EC50 values of Mystic® 430 SC were by far the lowest, being 0.06 μL/L for mycelium of both isolates, and 0.017 and 0.01 for conidia of isolates GN01 and GNAm, respectively. The EC50 values for Kalex Zn® were 3.5 and 2.4 times lower than Kalex® for mycelium of GN01 and GNAm, respectively, and five times for the conidia of the two isolates. Mystic® 430 SC at 1 μL $L^{-1}$ completely inhibited the conidia germination and mycelial growth of both isolates. Kalex Zn® at 5 mL $L^{-1}$ completely inhibited the conidia germination and mycelial growth of both isolates. Finally, Kalex® at 5 mL $L^{-1}$ completely inhibited the mycelial growth of both isolates, while 100% inhibition of conidia germination was obtained only between 50 and 100 mL $L^{-1}$. The 100% inhibition of the mycelium resulted in a fungicidal effect of the products tested, with no fungistatic effect observed when mycelium plugs were plated on PDA without the fungicide.

### *3.2. Treatment Trials*

Production of conidiomata on fruits of the 2019 season was very low and irregularly distributed within the thesis; their assessment did not produce any reliable results. A total of 24 fruits per thesis (6 per block) were processed in isolation trials in the laboratory. In Figure 1, the percentage of isolation of *G. castaneae* for each thesis is shown as the average of the four blocks. Besides the low number of fruits processed for isolation, ANOVA was significant ($F = 6.104$; $p = 0.0025$). Dunnett's multiple comparison tests showed significant differences between each treatment and the control. After incubation in moist chambers (14 days), 94.6% of fruits were still asymptomatic, with no significant differences in Ibr and Sbr between theses.

In the season of 2020, a total of 864 were collected, 144 for each of the six theses (36 for each of the four blocks). In Figure 2, the Ibr and Sbr for each treatment are shown as the average of the values of the four blocks for each thesis. ANOVA was significant ($F = 5.782$; $p = 0.0002$). Dunnett's multiple comparison tests showed significant differences between each treatment and the control except for the treatment with Kalex® by endotherapy. No significant differences were found both for Kalex Zn® and Mystic® between the double treatments in June and July and the triple treatment in June, July, and August.

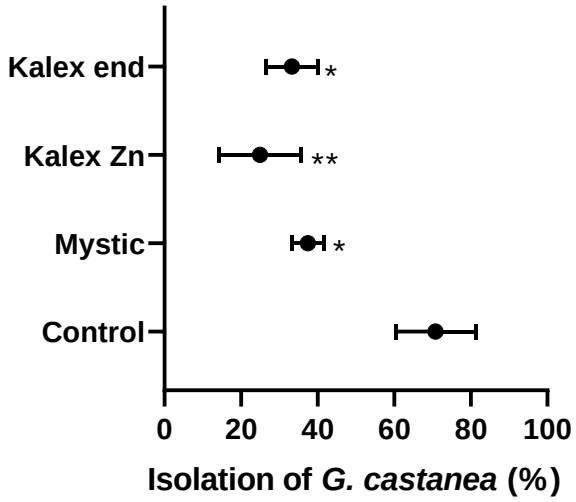


**Figure 1.** Percent of isolation of *Gnomoniopsis castaneae* from chestnut fruits of trees in the four treatments of 2019. Bars represent the SEM. Statistical differences with the untreated control at the Dunnett's Multiple comparison tests are evidenced by * = $p < 0.05$, and ** = $p < 0.01$.

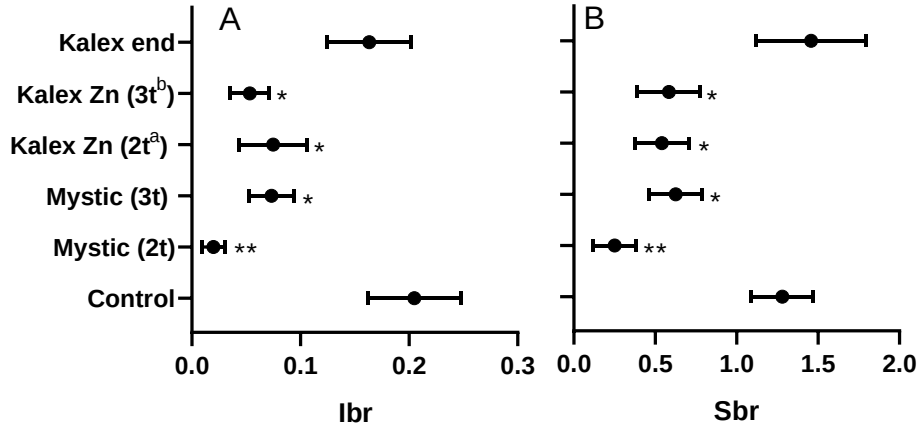


**Figure 2.** Incidence (**A**) and severity (**B**) of brown rot (Ibr and Sbr, respectively) of chestnut fruits collected from trees in the six treatment theses in 2020. Bars represent the SEM. Statistical differences with the untreated control at Dunnett's multiple comparisons (**A**) and Holm–Sidek's multiple comparison (**B**) tests are evidenced by * = $p < 0.05$, ** = $p < 0.01$. [a] two treatments (June and July), [b] three treatments (June, July and August).

In the case of tests carried out in 2020; the DNA of sampled fruits from each thesis (72) was analyzed by qPCR with a specific TaqMan probe [18], and the $I_i$ was calculated. In Figure 3, the $I_i$ is shown for each treatment. Again, significant differences were highlighted between each treatment and the control at the Kruskal–Wallis test.

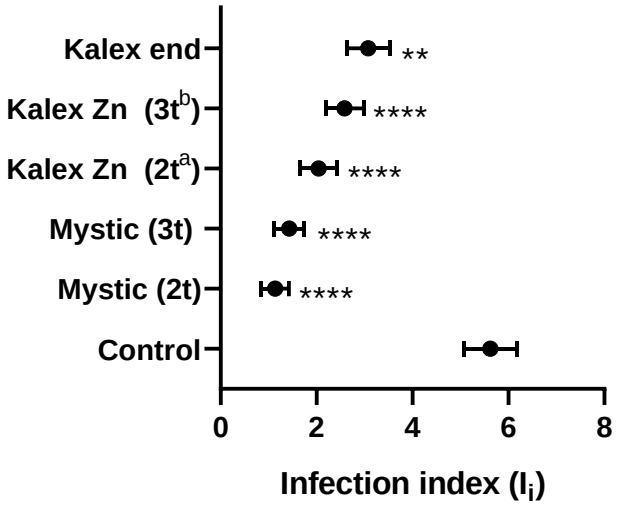


**Figure 3.** Infection index (Ii) based on qPCR-specific detection of Gnomoniopsis castaneae from chestnut fruits of trees in the six treatments in 2020. Bars represent the SEM. Statistical differences with the untreated control at the Dunn multiple comparison tests are evidenced by ** = $p < 0.01$, and **** = $p < 0.0001$. [a] two treatments (June and July), [b] three treatment (June, July and August).

### *3.3. Residues in Fruits*

The residues of phosphonate expressed as phosphonic acid and its salts are shown in Table 3.

**Table 3.** Residues of phosphonic acid and its salts in fruits treated with Kalex Zn® by crown spray, Kalex® by endotherapy, and untreated control. SEM, standard error.

| | **Phosphonic Acid and Its Salts (mg/Kg ± SEM)** |
|---|---|
| Kalex Zn® | 7.05 ± 0.2 |
| Kalex® | 19 ± 4.2 |
| Untreated control | 0.19 ± 0.03 |

## 4. Discussion

The present paper provides for the first time a comparative study on the efficacy of chemical treatments for the control of the "brown rot" of chestnut fruits by *G. castaneae* in orchards. Specifically, the efficacy of phosphonate salts to control a no-oomycete plant pathogen was demonstrated in comparison with the use of Tebuconazole (Mystic®).

Phosphonate salts have been widely used since the 1970s in agriculture, agroforestry, and forestry to control oomycetes and more recently fungal driven diseases [20]. Indeed, their application in the management of non-oomycete diseases, along with other functionalities, demonstrates their versatility in agriculture and more broadly. Guest and Bompeix [21] suggested phosphonate-based products as the optimal protectant for annual and perennial crops, being "systemically translocated in both the xylem and phloem, have protective and therapeutic activity, a complex mode of action involving several biochemical mechanisms, are persistent in the plant but ephemeral in the environment, leave no toxic residues and be cheap enough to provide economic returns to the grower". Furthermore, the lack in plants of an efficient enzymatic system that promotes the oxidation of phosphonate to phosphate [22,23] makes these products highly persistent in plants contributing to their success as protectants [20]. Their activity as crop protectants is related to three different 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's defense; and (iii) direct fungistatic effect towards pathogens [20,24]. Whether these mechanisms are all involved in protection against non-oomycetes plant pathogens still has yet to be clarified.

Kalex Zn® at the recommended doses (3 L/ha, crown spray) was efficient in controlling the disease with no statistical differences with Mystic® (recommended dose, 350 mL/ha, crown spray). The efficacy of Kalex Zn® was demonstrated in 2019 and 2020 experimental trials in orchards employing different methods of assessment, such as isolation of the pathogen in pure culture, assessment of symptoms, and detection of the pathogen by qPCR. In "in vitro" experiments, Kalex Zn® required a lower dose to inhibit radial growth and conidia germination of *G. castaneae* than Kalex®. In addition to phosphonate, zinc metal is also reported to have a sensible protective activity against plant pathogens and to represent a potential substitute for copper in commercial products [25]. Moreover, the fungistatic potential of nanoparticles of ZnO against fungal pathogens such as *Fusarium* sp., *Botrytis cinerea*, *Penicillium expansum*, *Aspergillus niger* and *Rhizopus stolonifer* has been reported [26–28].

The results of the use of Kalex® (K-phosphonate, 0.4 mL/trunk injection) were controversial. In the 2019 experimental trial, it was efficient in controlling the pathogen and its presence in fruits. However, in 2020, it did not significantly reduce the expression of symptoms, although it was associated with detection of the pathogen in fruits significantly lower than the untreated control. It must be pointed out that in 2019, trunk injections were applied immediately after the budburst, while in 2020, it was during blooming. One possible reason for failure in protection in 2020 could be related to the date of treatment corresponding to the blooming phase and the time needed to translocate the product to flowers to assure protection from infection. The time of translocation of trunk injected K-phosphonate in perennial crops was studied in avocado to protect against *Phytophthora cinnamomi.* In these studies, translocation in leaves was evident after 24 h, while a longer time was needed for detection in roots [29]. It must be clarified that the choice of trunk injection in the present study was determined by the fact this method (at the same doses) is widely used to rapidly translocate K-phosphonate to protect chestnut trees against the ink disease causal agents *P. cinnamomi* and *P. xcambivora* [11]. Whether a single treatment at the budburst phase might protect trees from both "ink disease" and "brown rot" must be further studied in experimental trials. Potassium phosphonate salts have been reported as effective in the infection caused by other plant pathogens such as *Hymenoscyphus fraxineus* [30], *Venturia inaequalis* [31], *Phytophthora infestans* [32] or *Plasmopara viticola* [33]

In the present study, the "in vitro" dose-dependent response of *G. castaneae* to Kalex Zn® and Kalex® (direct effect) was investigated, evidencing a fungicidal effect of both products, although at different doses. The doses showing a fungicidal effect "in vitro" are in the range of the concentration found in fruits. Thus, we can speculate that protection from brown rot is due to a direct fungicidal effect of the products instead of a resistance induction mechanism. However, a correlation between "in vitro" and "in planta" doses of phosphonate salts efficient for pathogen suppression is not always possible. Indeed, a suppression dose might depend on several factors including the media composition and pathogen development stage for "in vitro" tests, while the nutritional status of the plant can make the difference for "in planta" efficacy [20]. For instance, in the present study, the EC50 for inhibition of radial growth or conidia germination was different for both Kalex Zn® and Kalex®.

The results of the present study also support the epidemiological evidence given by Shuttleworth and Guest [7] about female flowers as the main site of infection. Indeed, the August treatment in addition to the spring one with both Mystic® and Kalex Zn® did not result in any significant reduction of disease expression or detection of *G. castaneae* in fruits compared to a single spring treatment at the blooming stage. This provides indirect evidence of the relevance of flowers as the site of infection [7] and the role of airborne inoculum as demonstrated by Lione et al. [34].

The use of phosphonate salts is differently regulated on a global scale. It is considered a systemic fungicide in Australia, the USA, and South Africa, where it is widely used in cultivated and natural ecosystems. In these areas, it is homologated on a wide range of plant hosts, including tree nuts and fruit trees, by trunk injection and/or crown spray. In

South America, phosphonate salts (either K or Zn salts) are still homologated as fertilizers [35], thus with almost no restrictions. In Europe, with the entry in force on July 2022 of the new regulation on fertilizers (REGULATION (EU) 2019/1009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 5 June 2019), any molecule based on phosphonates will be definitively banned from fertilizers and will be used only if homologated as a pesticide on target crops/plants. Now, in Europe, K-phosphonate is homologated as a systemic fungicide under the commercial name of Century® SL (BASF Italia Spa, Cesano Maderno, Italy) and is usable only on grapes by crown spray. Thus, the possibility of using phosphonate salts to protect against "brown rot" of chestnut fruits depends on country-based regulations and, in addition, (i) on their categorization as a fertilizer or fungicide; (ii) for those homologated as systemic fungicides, on the plant host range for which the product is registered; and (iii) on the modality of treatment for which they are authorized, basically crown spray and/or trunk injection.

A further aspect related to the use of phosphonate salts refers to the residues in the final product and the Maximum Residue Limit (MRL) according to local regulations. In the present study, according to the current bibliography [20], residues after crown spray treatments were lower than those after endotherapy. However, both values were far lower than, for instance, the MRLs recently issued by the EU regulation 2021/1807 that increase the limit for chestnut fruits to 1500 mg/kg (https://eur-lex.europa.eu/legal-content/IT/TXT/?uri=uriserv%3AOJ.L_.2021.365.01.0001.01.ITA&toc=OJ%3AL%3A2021%3A365%3ATOC) (accessed on 8 August 2022).

## 5. Conclusions

Most of the control strategies are applied post-harvest, and little is known about the use of fungicides to reduce nut infection in orchards early in the season. This is the first study showing the efficiency of products with the minimum environmental impact. Phosphonate salts, and at first Zn-phosphonate, are highly effective to protect chestnut fruits from the "brown rot" fungus *G. castaneae*. Its use in orchard management may complement the post-harvest processing of fruits by "heat treatments" in water that resulted efficient in completely inactivating *G. castaneae* in fruits [6]. Indeed, the adoption of the appropriate management from the orchard to the processing plant will assure the minimal impact of the disease on fruit stocks for the market. Although a still ongoing debate on phosphonate salts use and efficacy in agriculture [20,24,36], they can be considered an optimal fungicide in chestnut orchards because of the low environmental impact when used at the recommended doses, the high translocability and stability, and the multiple mechanisms of actions. Unfortunately, their availability on the market can be locally uncertain depending on the differences in categorization and authorization for their use. Further efforts should then be put into reinforcing the evidence of their efficacy also for no-oomycetes plant pathogens such as *G. castaneae*.

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