

Article

Evaluation of Copper-Free Alternatives to Control Grey Mould in Organic Mediterranean Greenhouse Tomato Production

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Abstract: Grey mould caused by *Botrytis cinerea* is an endemic disease in greenhouse tomato crops in the Mediterranean Basin, where the scarcity of heating systems together with the winter weather conditions makes the use of fungicides necessary. The availability of fungicides for organic tomato production is limited, and traditionally, farmers have used copper-based formulations. In the present work, in vitro tests with twelve commercial formulations resulted in cinnamon extract and potassium hydrogen carbonate (PHC) showing high efficacy in the inhibition of *B. cinerea* growth. Both formulations were evaluated in on-farm greenhouse trials conducted for two seasons (2019/2020 and 2020/2021) in three greenhouses located in Almería, Spain. In terms of controlling *Botrytis*, PHC showed efficacy results comparable to or even better than those that have been obtained for copper oxychloride. Weather conditions outside and inside the greenhouse were conducive to the onset and development of the disease. Tomato variety selection and pruning practices (flush cuttings) were the main factors that reduced the use of copper-based formulations or any other fungicide to prevent grey mould infection. Smart and integrated management of the mentioned factors could lead to the substitution of copper to control *Botrytis* in the crop system studied here.

Keywords: *Botrytis cinerea*; cinnamon extract; copper oxychloride; fungicide; potassium hydrogen carbonate



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1. Introduction

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a ubiquitous fungus which has been reported in >1400 plant species spanning nearly 600 genera, including many economically important fruit, vegetable, ornamental, and field crops, although the host range is probably much larger nowadays [1,2]. Thus, *B. cinerea* is considered to be the second most important global plant pathogen by researchers and the scientific community [3], and is considered to be responsible for billions of dollars in annual losses worldwide [4]. It causes grey mould on almost all major open-field and greenhouse crops. On tomato (*Solanum lycopersicum* L.), the fungus infects the flowers, fruits, and leaves and can grow through the petiole into the stem, causing the plant to collapse [5–7]. High relative humidity, free moisture on plant surfaces, and moderate temperatures are considered the most important environmental factors that promote infection by the fungus [8–12]. In this regard, *B. cinerea* is considered an endemic pathogen for winter greenhouse tomato crops and is therefore one of the most common and economically important plant pathogens in the Mediterranean Basin [13]. The main winter tomato production area in Europe is found in Almería (southern Spain), where the greenhouse surface area reaches 32,554 ha,

including 3693 ha that were certified as organic in 2020 [14]. This is the main supplier of fresh tomatoes (organic and non-organic) during the coldest months of the year [15].

For the effective control of grey mould in tomato greenhouses, an integrated strategy based on a combination of different methods is required, and cultural practices such as reducing plant density, the avoidance of condensation when temperatures are around 16 °C, and appropriate pruning are recommended; however, these practices are insufficient if the climatic conditions favour the disease, making the use of fungicides necessary [1,10,16,17]. The availability of fungicides in organic agriculture is limited, and traditionally, organic farmers have used copper-based formulations [18]. Additional biological and non-biological protection products authorised for *B. cinerea* in organic greenhouse tomato crops are available; however, because the traditional copper formulations are cost-effective plant-protection products, the use of these alternatives is limited [19,20]. In this regard, more sustainable alternatives to copper are required since the repeated use of cupric formulations favours their accumulation in soils, with undesirable effects on agrosystems [21], including soil biota, and thus, the crops themselves [22–24]. These reasons make the reduction of copper-based plant-protection products essential in Europe [25,26]. Therefore, the search for affordable alternatives that are highly effective in controlling grey mould in Mediterranean greenhouse tomato production and in maintaining the profitability of the agrosystem while respecting the environment is a priority [19,27].

Within this framework, the broad aim of this study was to assess the antifungal effects of twelve copper-free formulations available on the market against *B. cinerea*. The formulations were selected after meetings with a board of 10 experts and included low- or no-copper mineral fertilisers, formulations based on basic substances or on plant extracts, and fungicides authorised for other crops in organic farming. Firstly, *in vitro* tests were carried out. Then, the two best options (the most promising in terms of efficacy in inhibiting the growth of *B. cinerea* and in terms of economic viability) were selected for on-farm greenhouse assessments. Additionally, to assess the selected alternatives to study the system solution, not only was the use of alternatives approached, but the use of two different varieties of tomatoes and two pruning procedures were also implemented in order to obtain information about the impacts of these factors on the integrated system.

2. Materials and Methods

2.1. *In Vitro* Screening of Alternatives to Copper as Fungicides against *Botrytis cinerea*

2.1.1. Pathogen Isolates

Botrytis cinerea isolates were obtained from the infested stems of tomato plants from 2 different greenhouses located in Almería province (Spain) in January 2019 (locations: 36°47'26.7" N 2°36'17.2" W; 36°51'52.8" N 2°16'58.6" W). From a total collection of 20 isolates, only two were chosen for this research. These isolates showed different behaviour: the first one (code: Botrytis 1) had faster mycelial growth and slower conidia generation (*vegetative* isolate), while the other isolate (code: Botrytis 2) showed the opposite features (*sporulative* isolate). However, both strains produced enough mycelium and conidia to allow precise evaluation of the effects of the products. Both strains were morphologically examined. Molecular identification was performed by sequencing amplicons of the internally transcribed spacer region (ITS) rDNA using universal primers ITS5 and ITS4 [28]. Both strains were identified as *Botrytis cinerea*. Isolates were maintained on potato dextrose agar (PDA) at 25 °C in the dark and were transferred to new PDA weekly. Subcultures were prepared 5 days before each assay.

2.1.2. Alternatives to Copper Tested

Commercially available formulations were the subject of study in this research. Products based on biological control agents were not included. The candidate formulations were selected after a survey of 10 expert local crop advisors, who were asked for their experience regarding any type of formulation showing efficacy to control airborne pathogens that affect

tomato. Twelve products were selected and differentiated into four groups determined according to their nature (Table 1):

- Mineral foliar fertilisers with low or no copper content (three products): copper gluconate, zinc, and silicon;
- Formulations based on basic substances as defined by the European Commission [26] (three products): *Equisetum arvense* (two formulations: Equisetum 1 and Equisetum 2) and chitosan;
- Formulations based on plant extracts (four products): cinnamon extract (two formulations: Cinnamon 1 and Cinnamon 2), *Mimosa*, and *Camelia*;
- Fungicides authorised for organic farming as defined by the European Commission [26] (two products): potassium hydrogen carbonate (PHC) and lime sulphur.

Table 1. Concentrations of the products (formulations) tested for the in vitro screening of alternatives to copper as fungicides against *Botrytis cinerea*.

Product (% Active Ingredient)	Commercial Name, Manufacturer	Minimum Dose	Maximum Dose
Copper gluconate 5%	Cobregluc, Bioera S.L.	2 mL/L	4 mL/L
Zinc 6%	Zibac, Nutricrop S.L.	2 mL/L	2.5 mL/L
Silicon (23%)	Hortosil, Nutricrop S.L.	2 mL/L	4 mL/L
Equisetum 1 (2%)	Equisetomax, Bioera S.L.	2 mL/L	5 mL/L
Equisetum 2 (2%)	Lykos, Nutricrop S.L.	3 mL/L	5 mL/L
Chitosan (3%)	Camaro, Nutricrop S.L.	1 mL/L	3 mL/L
Cinnamon 1 *	Verunze, Bioera S.L.	1 mL/L	1.5 mL/L
Cinnamon 2 *	Cinna, Hortalan Med S.L.	1.5 mL/L	3 mL/L
Mimosa *	Mimset, Hortalan Med S. L.	2 mL/L	3 mL/L
Camelia *	Simensis, Hortalan Med S.L.	3 mL/L	5 mL/L
Potassium hydrogen carbonate (85%) (PHC)	Armicarb®, Certis Europe B.V.	3 g/L	5 g/L
Lime sulphur (18.5%)	Polisulfuro de cal ORO, Químicas ORO S.A.	-	100 mL/L
Copper oxychloride (50%)	Codimur 50, Exclusivas Sarabia S.A.	-	4 g/L

- not tested; * active ingredient percentage not declared.

The efficacy of these products was tested at two concentrations (the minimum and maximum concentrations recommended by the supplier). In addition, copper oxychloride (50%) (henceforth called Cox) was tested as a reference formulation.

2.1.3. Effect of Alternatives to Copper on Mycelial Growth

In vitro mycelial growth inhibition tests were carried out to evaluate the inhibitory effect of the products on *B. cinerea*. Autoclaved PDA media were amended with appropriate volumes of product dilutions (Table 1). PDA plugs (6 mm in diameter) were axenically taken away from peripheral portions of 5-day-old *B. cinerea* colonies and were placed in the centre of amended PDA plates and incubated at 25 °C in darkness. The diameters of the colonies were measured after 5 days of incubation. Controls consisted of fungi transferred into non-amended Petri dishes containing only PDA. Reference controls consisted of fungi transferred into Petri dishes containing PDA amended with Cox 50% at a 4 g/L dose. Growth inhibition was calculated using the following formula, expressed as a percentage: % Inhibition = $((D - d)/D \times 100)$, where *d* is the diameter of the *B. cinerea* colony in the presence of the products tested, and *D* is the maximum diameter of the *B. cinerea* colony of the controls. Five repetitions (i.e., Petri dishes) were carried out for each tested product and concentration. Trials with each *Botrytis cinerea* isolate were performed twice over time. Data obtained from both trials were pooled for a more consistent calculation of averages and deviations.

In addition, when total fungal growth inhibition was observed in the in vitro mycelial growth inhibition tests, the viability of the fungi was tested to see if the formulation had a fungicidal or fungistatic effect. Thirty days after the beginning of the above-mentioned

in vitro tests, mycelium discs were transferred on PDA plates with no growth inhibitors and were incubated at 25 °C in darkness. Colony growth was checked 14 days later.

2.1.4. Analyses for the Presence of Fungicidal Pollutants in the Formulations

The products that showed similar or better efficacy than Cox in inhibiting the growth of the mycelia of *B. cinerea* were analysed for the presence of known fungicides in their formulations. These analyses were carried out to check whether or not the products considered in the study included declared fungicides that could influence the results. The tests were performed in the official Laboratory of Plant Health and Production, Almería of Junta de Andalucía. The products had previously been diluted to obtain the expected levels of fungicides that allow clear detection of the ingredients being searched for. Two official methods were used: (1) liquid chromatography–tandem mass spectrometry (LC-MS/MS), comprising 144 active ingredients (including 33 fungicides) and (2) analysis of dithiocarbamates via their degradation product, carbon disulphide (CS₂). In this way, a total of 43 fungicides were analysed (see Supplementary Materials, Table S1).

2.1.5. Cost of Alternatives to Copper Application

The cost per hectare of the application of each formulation at the tested concentrations was obtained by consulting three local retailers. Finally, they were compared according to their Cox application costs.

2.2. Greenhouse Evaluation of Two Selected Alternatives to Copper as Fungicides against *Botrytis cinerea*

2.2.1. Location and Experimental Greenhouses

The trials were conducted during two subsequent years (2019/2020 and 2020/2021 seasons) in three experimental greenhouses (GH) located at the Andalusian Institute for Research and Training in Agriculture and Fisheries (IFAPA) facilities in Almería (Spain; 36°48' N, 2°41' W; altitude 142 m). The climate of the study area is arid/Mediterranean with mild winters and dry, hot summers. The GHs are representative of the “raspa y amagado” Mediterranean greenhouse [29]. The maximum and minimum heights in the greenhouses are 3.9 and 2.3 m, respectively. The so-called GH1 and GH2 have been certified for organic production by the Andalusian Organic Farming Committee (C.A.A.E.) since 2006, while GH3 has been operating under a Global-GAP certification. GH1 and GH2 both have an area of 832 m² and are composed of four warehouses of 8 m wide by 26 m long, while GH3 is 1,800 m² and is composed of three warehouses that are 8 m wide by 75 m long. In all three GHs, the crop rows were aligned north–south (Figure 1).

The irrigation system was automated, with drippers positioned 0.5 m apart and a discharge capacity of 3 L h⁻¹. The greenhouses had a 200 µm thick polyethylene “triple layer” cover with theoretical transmissivity of 90% and thermal properties, as well as zenithal and lateral ventilation with an anti-insect mesh (20 × 10 threads/cm²). The internal ventilation in the three GHs was generated by four zenithal windows—two east-facing and two west-facing in GH1 and in GH2—while GH3 had two north-facing and two south-facing windows. The lateral ventilation of the three greenhouses consisted of roller windows on all four sides of the greenhouse. Likewise, the lateral and zenithal windows had deflectors in their lower part, improving air circulation in the area occupied by the crop [30].

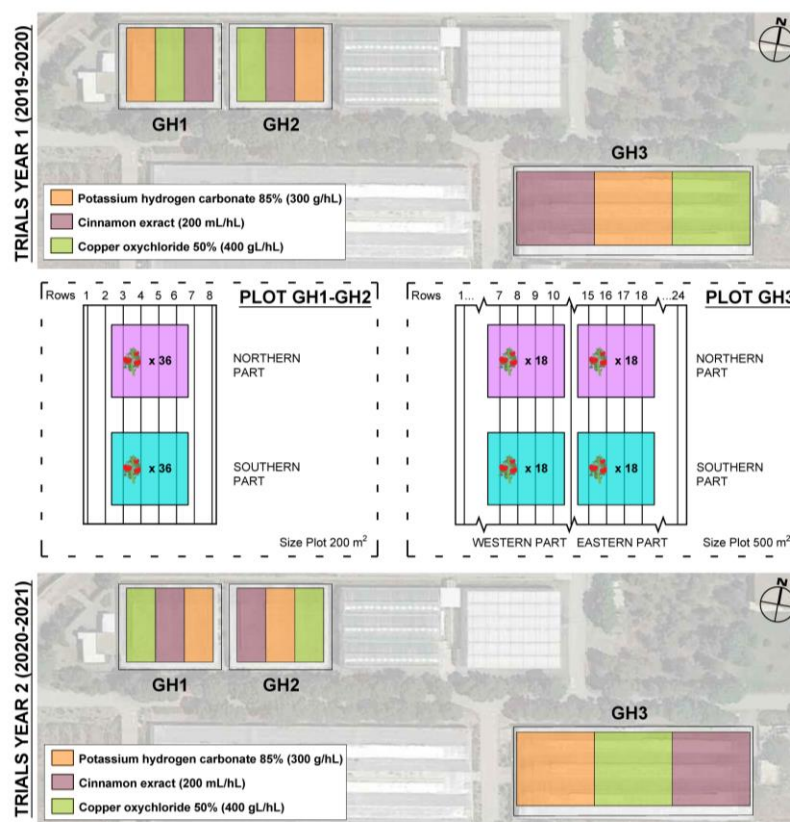


Figure 1. Experimental design and treatments in the seasons 2019/20 and 2020/21 (i.e., Season 1 and Season 2, respectively). The figure shows the distribution of the treatments in each experimental greenhouse (GH) as well as the divisions of the plots (including the number of plants evaluated in each division) that were considered as random replicates and that were thus considered elementary plots.

2.2.2. Plant Material and Crop Conditions

During the two subsequent seasons of trials, the same two tomato (*Solanum lycopersicum* L.) varieties were cultivated for fresh consumption. In GH1 and GH2, the winter cycles of tomato “Valenciano-type” plants grafted onto Armstrong[®] rootstock (Syngenta, Switzerland) were grown in the seasons of 2019/20 and 2020/21 (Season 1 and Season 2, respectively) with growth on two axes, with each being considered as an individual plant for sampling purposes, thus resulting in an overall density of 2 plants m⁻². In GH3, the tomato cv Caniles (Zeraim, Israel) was grown for the same seasons, with individual stem growth, at a density of 1.5 plants m⁻². The planting dates in GH1 and GH2 were 16 September 2019 and 25 September 2020 in Season 1 and 2, respectively, and crops finished growing on 25 March 2020 (191 DAP) and 13 April 2021 (200 DAP). In GH3, planting took place on 5 September 2019 and 20 September 2020 in Seasons 1 and 2, respectively, and crops finished growing on 13 May in both seasons (251 DAP and 235 DAP). Tomato plants were vertically trained with polypropylene strings, and the crops were managed according to the usual criteria of the area by considering irrigation endowment based on the moisture content of the soil in the reference treatment (−15 to −10 kPa) and via fertigation according to the crop’s phenological stage. In GH1 and GH2, crop management and pest control were guaranteed by adhering to Regulation (EU) 2018/848 on organic production. For correct and optimal pollination, bumblebee (*Bombus terrestris*) hives were introduced at a range of 1 hive/1,000 m² once the plants showed their first tomato branch, and were replaced each 4–6 weeks.

Climatic data outside the greenhouse were collected using a station included in the Andalusian network of climatic stations (<https://www.juntadeandalucia.es/agriculturaypesca/ifapa/riaweb/web/estacion/4/1>, accessed on 15 July 2022).

2.2.3. Treatments and Experimental Design

Due to the characteristics of the pathogen and the treatments studied, the experimental design required separation between treatments that was big enough to avoid interference from the pulverisation treatments. There were three treatments (the two products that were selected from the previous results of Section 2.1. In vitro screening of alternatives to copper as fungicide against *Botrytis cinerea* and a control treatment): (i) Cinnamon 2 extract applied at 200 mL/hL, (ii) potassium hydrogen carbonate 85% (PHC) applied at 300 g/hL, and (iii) copper oxychloride 50% applied at 400 g/hL as a control treatment.

The treatments were distributed differently in each of the greenhouses and during both seasons in order to avoid the influence of the location of the crops for each treatment on the expression of the disease (Figure 1). The size of the plots of the treatments in GH1 and GH2 was 200 m² and there were eight rows of tomato plants per plot. Edge rows numbers 1 and 2, as well as 7 and 8, were not considered in the evaluation, and only internal rows 3 to 6 were monitored. In total, 36 plants were randomly selected from the northern part of the plot and another 36 plants were selected from the southern part at the beginning of the trials. All of the monitored plants were located in the most internal part of the plots, thus favouring the proliferation of grey mould (Figure 1). The 36 plant groups were considered as random replicates and were thus elementary plots. The elementary plots from GH1 and GH2 were pooled, thus obtaining four replicates per treatment. In GH3, the protocol was similar, but due to the size of the plots (500 m²), the distribution was different (Figure 1). In this case, each plot included 24 rows of tomato plants, and only internal rows 7 to 10 and 15 to 18 were monitored. In total, 18 plants were randomly selected from the northern part, and another 18 plants were selected from the southern part at the beginning of the trials. Thus, in each of the four divisions of each plot in GH3, there were 18 plants. The divisions were considered as random replicates and were thus considered elementary plots.

For the application of the treatments, a unique protocol was followed, as the same treatment equipment was used for the three products. The protocol consisted of starting with 10 min of water application outside the greenhouse to wash the pipes and the sprayer device. Subsequently, the pipe was connected to the first application tank that contained the solution with the PHC product at the dose mentioned. The sprayer operator initiated the application from the west side of the plot by applying the product on all of the plants of row 1 before moving to the next row until rows 8 or 24 had been sprayed (depending on the greenhouse). Then, the pipes were connected to a water tank and the equipment was sprayed with water for ten minutes outside the greenhouse, and water was collected in a collection deposit. Subsequently, the second product applied was the cinnamon extract, which was applied at the mentioned dose using the same procedure of connecting the pipe to a new tank, and then, starting the application from row 1. At the end, the pipe and sprayer were washed with water as described before, and the last product (Cox) was applied. The time required for the whole treatment process meant that application had to take place over the course of two days: on the first day, GH1 and GH2 were treated simultaneously due to their proximity, and the next day, GH3 was treated. This procedure was designed to avoid contaminating the plants with the wrong product.

The applications were carried out using a manual lance with two nozzles (Novi Fan S.L., Almería, Spain) that works at high pressure (20 bar). This type of spray lance is widely used to apply plant-protection products in south-eastern Spain. The lance was coupled to a fixed sprinkler system consisting of a network of pipes distributed throughout the greenhouse, through which the pressurised product was circulated from a tank with a capacity of 1000 litres located in a facility outside the greenhouse.

The criterion followed for spraying the products was common in the region: treatments were applied after foggy or rainy days. In our case, we waited 3 to 5 days between humidity

events and spraying to favour grey mould proliferation. In Season 1 of the study (season 2019/2020), a total of five applications were carried out: during weeks 48 and 50 in the year 2019 and during weeks 2, 4, and 6 in 2020. Season 2 of the study (season 2020/21) was drier than the previous one, and due to the low incidence of *B. cinerea* in GH3, no treatment was applied. However, in GH1 and GH2, three applications were carried out at weeks 2, 7, and 10 in 2021 when signs of grey mould were observed.

2.2.4. Analysed Variables

The data evaluated in the present work were collected weekly and recorded from the onset of the grey mould symptoms, regardless the treatment or greenhouse in which symptoms appeared.

- Grey mould incidence

The incidence of *B. cinerea* was evaluated in the study by means of recording the number of symptoms due to the pathogen observed in each experimental plot, regardless of the location of the symptom in the plant and regardless of the number of symptoms per plant. Symptoms were recorded without considering any scale. This is an absolute value obtained from adding all of the symptoms observed. A disease curve of the accumulated incidences was built for each tomato variety and season. In addition, for each plant, the location of each symptom was recorded by considering four different parts: fruits, leaves, petioles, and the main stem (i.e., when grey mould totally surrounded it) (Figure 2). In this regard, the relative incidence of grey mould symptoms on the different locations of the plants at the end of the trials was calculated for each treatment and tomato type. In the case of the leaves, petioles, and fruits, once the symptoms had been recorded, they were cleaved, but the plants were not removed. To encourage drying and to prevent reinfection by the pathogen, clay was then applied to the wounds in the cut areas.



Figure 2. Recorded locations of grey mould symptoms: (A) tomato fruits; (B) leaves; (C) petiole (after pruning in the pictures); (D) main stem. Grey mould symptoms highlighted in yellow colour.

Every two weeks, four randomly chosen samples with grey-mould-affected tomato tissues were analysed for the isolation of *B. cinerea* to confirm the identity of the pathogen through microscopy.

Finally, when a plant died due to the girdling of the main stem produced by *B. cinerea*, it was considered a dead plant; thus, a mortality curve was calculated with this information until the end of the crop season.

- Crop production

Production was measured for all of the harvests. In GH1 and GH2, the first harvest was undertaken on 27 December 2019 (102 days after planting (DAP)) and 30 December 2020 (110 DAP) in Seasons 1 and 2, respectively, and the last harvest was undertaken on 25 March 2020 (191 DAP) and 13 April 2021 (200 DAP). In total, 14 harvests were undertaken in both seasons. In GH3, the first harvests were on 20 January 2020 (137 DAP) and 15 January 2021 (117 DAP), respectively, and the last harvests were on 13 May 2020 (251 DAP) and 14 May 2021 (236 DAP). In total, 9 and 11 harvests were undertaken in Seasons 1 and 2, respectively. The cumulative marketable tomato production was measured (kg m^{-2}) using an electronic balance with an accuracy of ± 0.01 kg.

2.3. Statistical Analyses

Mycelial growth inhibition tests were statistically analysed by means of a factorial analysis of variance (two-way ANOVA), including individual factors (factor 1, selected products at maximum and minimum doses; factor 2, *B. cinerea* isolates), as well as their interaction. Data on the variables assessed in the greenhouse trials were analysed via an analysis of variance (one-way ANOVA) to compare differences among treatments (three levels: cinnamon extract applied at 200 mL/hL, potassium hydrogen carbonate 85% applied at 300 g/hL, and copper oxychloride 50% applied at 400 g/hL) for each of the variables evaluated in the study (total incidences of *B. cinerea*, relative incidence of grey mould symptoms on different plant locations, plant mortality, and crop production) for both seasons and tomato varieties. Data from GH1 and GH2 (“Valenciano-type” tomato) were pooled for analytical purposes. Additionally, the effect of tomato variety and season on the incidence of grey mould linked to the location of the infection site was analysed by means of a factorial analysis of variance (two-way ANOVA. Factor 1, tomato variety, level 1: “Valenciano-type”; level 2: cv. “Caniles”; factor 2, Season, level 1: “2019/2020”; level 2: “2020/2021”). Previously, normality and homoscedasticity were tested using the Shapiro–Wilk and Levene tests, respectively. The Kruskal–Wallis one-way non-parametric test ($p = 0.05$) was performed when the normality or homoscedasticity of data were not evident ($p < 0.05$ Shapiro–Wilk or Levene tests, respectively). For these analyses, Fisher’s least significant difference (LSD) test was used to make comparisons of treatments (95% level of significance). Arcsine square root transformation was applied to percentages before the analyses. The statistical analyses were carried out using the statistical software package Statgraphics Centurion XVIII (Statgraphics Technologies, Inc., The Plains, VA, USA) for Windows (Microsoft Corporation, WA, USA).

3. Results

3.1. In Vitro Screening of Alternatives to Copper as Fungicides against *Botrytis cinerea*

3.1.1. Effect of Alternatives to Copper on Mycelial Growth and on Spore and Sclerotia Production

In general, mycelial growth inhibition greatly varied among formulations and increased with the dose of the products, although some of the formulations showed high and even total inhibition for both doses (Figure 3). All of the culturable replicates produced conidia and sporulating sclerotia in the same way as the controls. Only small differences depending on the isolate were detected. The general ANOVA showed a significant effect of the treatments ($p = 0.0000$), but no significant differences were found between the *B. cinerea* isolates ($p = 0.1620$); however, the interaction between both factors showed significance ($p = 0.0071$).

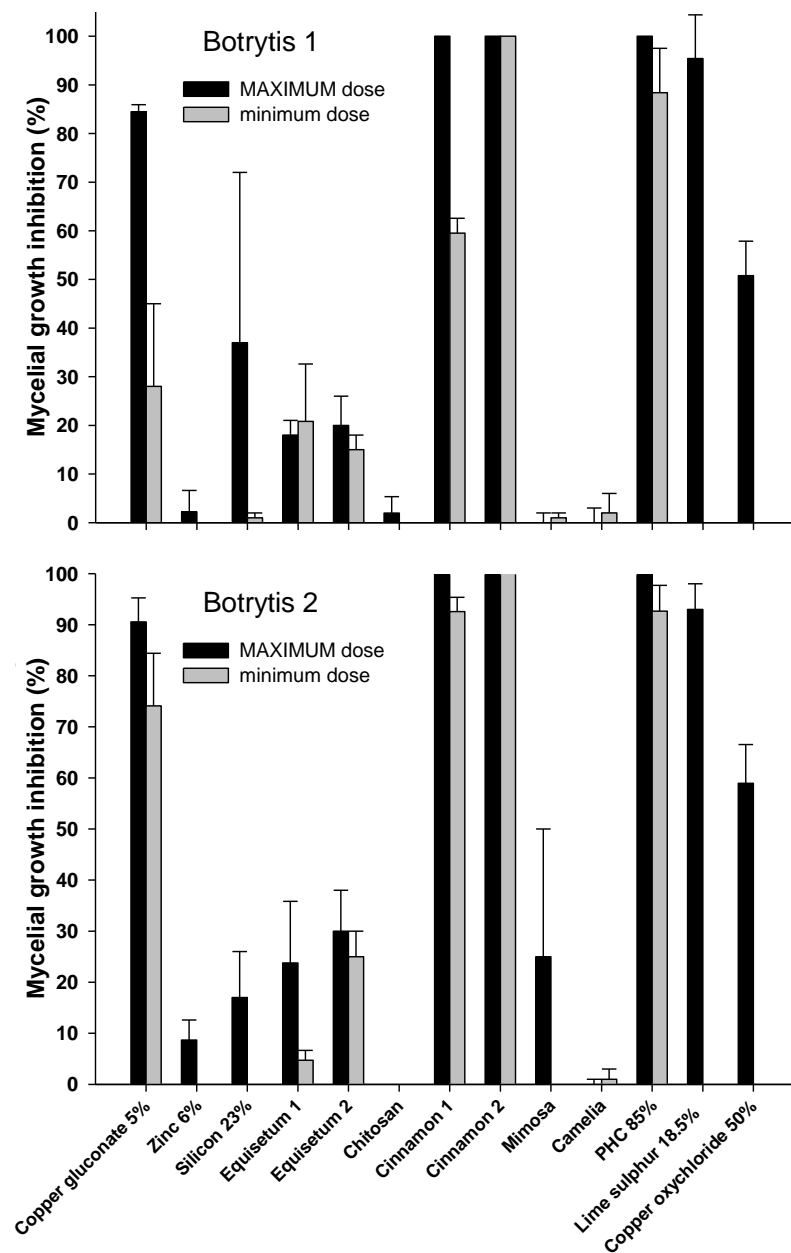


Figure 3. Mean inhibition (%) of mycelial growth and standard deviation of the mean for Botrytis 1 and Botrytis 2 isolates of *B. cinerea* grown on PDA amended with 12 selected products (three mineral fertilisers with low or no copper content: copper gluconate 5%, zinc 6%, and silicon 23%; three formulations based on basic substances: two formulations of *Equisetum* and chitosan; four formulations based on plant extracts: two formulations of cinnamon, mimosa, and camelia; two fungicides authorised for organic farming: potassium hydrogen carbonate 85% and lime sulphur 18.5%) at two doses (minimum and maximum doses recommended by the distributor).

The tests for mineral fertilisers showed differential efficacy for different products (Figure 3). Only copper gluconate (5%) was more inhibitory than Cox. This low-copper-content formulation inhibited over 80% of the growth of the two isolates when the maximum dose was tested. When the minimum dose of copper gluconate (5%) was tested, the efficacy on Botrytis 1 was lower and less effective than Cox. Regarding the other fertilisers, the tolerance of *B. cinerea* isolates to the “Zinc 6% product” was quite high. The case for the “Silicon 23% product” was different, as *B. cinerea* was only inhibited for the maximum dose.

On the other hand, none of the products of basic substances were more efficient than Cox (Figure 3). Both of the *Equisetum* products tested were slight inhibitors for *B. cinerea*, while chitosan had an almost negligible inhibitory effect on the Botrytis 1 isolate and no inhibitory effect on the Botrytis 2 isolate.

Regarding formulations based on plant extracts, both cinnamon products were more efficient compared to Cox. Thus, at maximum doses, these products completely inhibited the growth of two of the tested isolates (Figure 3). For the product called “Cinnamon 2”, even at minimum doses, the inhibition was 100%. On the other hand, *Mimosa* and *Camelia* extracts were less effective than Cox.

With regard to the fungicides authorised for organic farming, PHC showed the highest efficacy when the maximum dose was tested, i.e., 100% growth inhibition (Figure 3). Lime sulphur (18.5%) was quite inhibitory for *B. cinerea*.

3.1.2. Fungicidal vs. Fungistatic Efficacy of the Treatments

When the inhibitory effect of the products on *B. cinerea* reached 100% (Cinnamon 1 at the maximum dose; Cinnamon 2 at the minimum and maximum doses; and PHC at the minimum and maximum doses), PDA plugs from *B. cinerea* colonies which had been placed in the centre of the amended PDA plates were transferred to new unamended PDA plates to check for fungal culturability. All of the replicates of the isolate “Botrytis 1” were nonculturable for the maximum doses of Cinnamon 1, Cinnamon 2, and PHC. For the isolate “Botrytis 2”, 50% of the replicates were culturable after Cinnamon 1 application, as were 75% of the replicates after Cinnamon 2 application at maximum doses. On the other hand there was no culturability in PHC at the maximum dose. Minimum doses of “Cinnamon 2” inhibited 100% growth of *B. cinerea* isolates, but all the replicates were culturable.

3.1.3. Presence of Fungicidal Pollutants in the Products

The five products that showed similar or even better in vitro efficacy in inhibiting the mycelial growth of *B. cinerea* isolates than Cox were analysed for the presence of different active ingredients in their composition by means of two multi-target analyses.

None of the 159 active ingredients tested, including 43 fungicides, were detected in any of the products (copper gluconate, Cinnamon 1, Cinnamon 2, PHC, and lime sulphur).

3.1.4. Comparison of Product Application Costs

The real prices paid by the farmer for all the tested products are summarised in Table 2.

Table 2. Real costs per unit of treatment (ha) for the products evaluated. A volume application of 1000 L/ha has been assumed. Data are referenced for the year 2019.

Product	Unitary Price	Cost of Application (EUR/ha)		
		Minimum Dose	Maximum Dose	
Silicon 23%	12	EUR/5 L	5	10
Copper gluconate 5%	6	EUR/L	12	24
Copper oxychloride 50%	6	EUR/kg	12	24
Chitosan	11	EUR/L	11	32
Equisetum 2	7	EUR/L	20	33
Zinc 6%	16	EUR/L	31	39
Equisetum 1	9	EUR/L	19	47
Mimosa	17	EUR/L	38	56
Cinnamon 1	50	EUR/L	50	75
Cinnamon 2	24	EUR/L	39	78
Potassium hydrogen carbonate 85%	100	EUR/5 kg	60	100
Camelia	26	EUR/L	78	130
Lime sulphur 18.5%	70	EUR/25 L	-	300

Taking into consideration the *in vitro* efficacy of the products against the *B. cinerea* isolates and the relatively low cost of Cox, only copper gluconate (5%) is in the same price range per application and could be a feasible alternative in terms of economy. However, this product still contains copper. Looking at the non-copper alternatives, the cheapest alternatives are the minimum doses of cinnamon products, which obtained results similar to or better than Cox. In this case, the cost per application would be 208% and 163% for Cinnamon 1 and Cinnamon 2, respectively, compared to Cox. PHC (250% of the Cox price for the minimum dose) and lime sulphur (1250% of the Cox price) are both more expensive.

3.2. Greenhouse Evaluation of Two Selected Alternatives to Copper as Fungicides against *Botrytis cinerea*

Grey Mould Incidence

In GH1 and GH2, the cumulative incidence of grey mould only showed significant differences among the treatments in Season 2 at week 7 after disease onset, and the incidence was higher after treatment with cinnamon extract (i.e., Cinnamon 2) compared to the PHC and Cox treatments. (Figure 4). In the 2019/2020 season (Season 1), there were no significant differences among treatments, although a tendency toward higher incidence in the plots treated with cinnamon extract was observed (Figure 4).

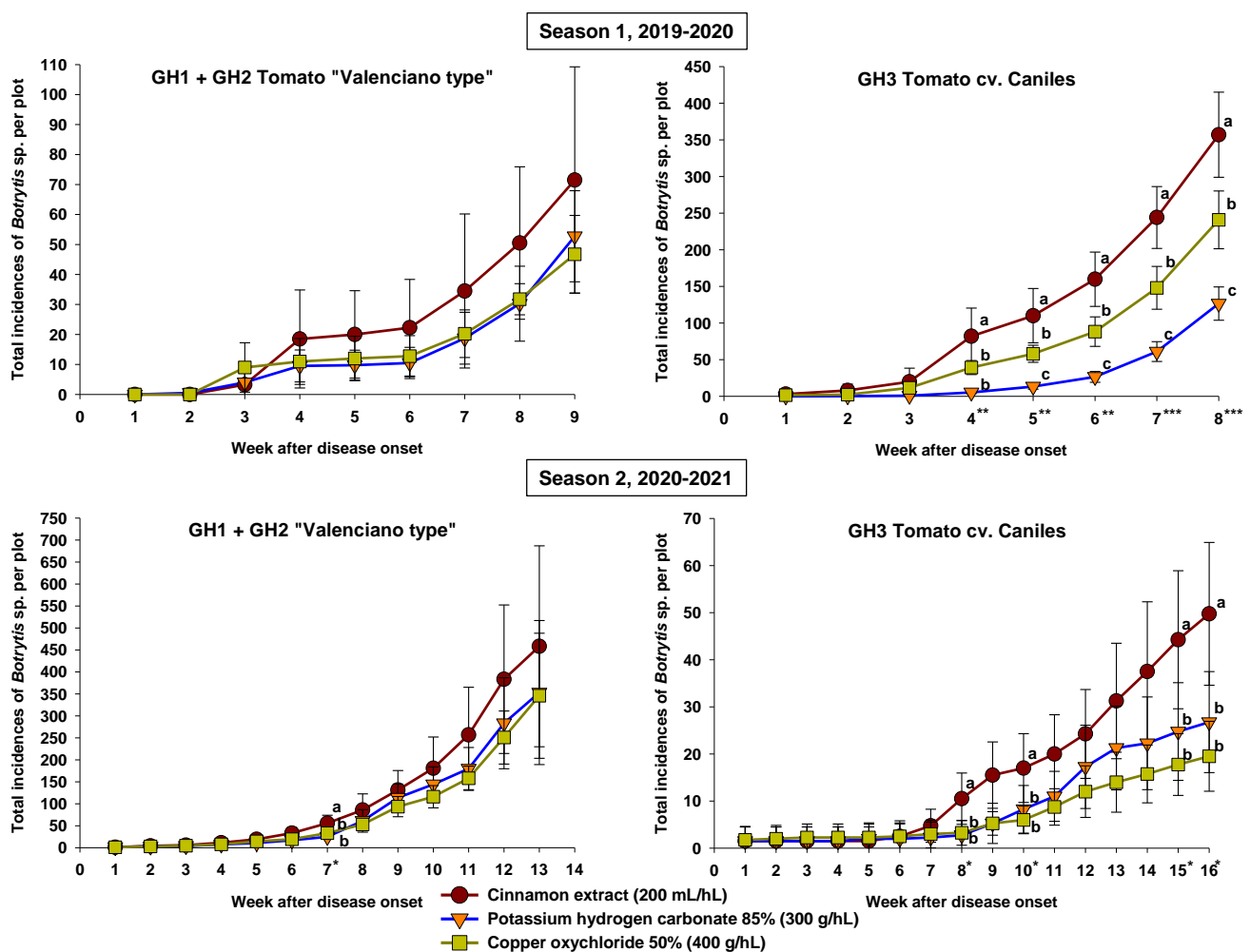


Figure 4. Accumulated incidence of grey mould on plants of tomato “Valenciano-type” and cv Caniles for Season 1 and Season 2 depending on treatment. Values are mean ± standard deviation; n = 4. Different letters in the same week indicate significant differences ($p \leq 0.05$, Fisher’s LSD test). “*”, “**”, and “***” indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively.

In GH3, the behaviour of the disease was inverse regarding the study season since in Season 1, the incidence was much higher than it was in Season 2. In this regard, in Season 1, the highest disease occurrence was recorded, regardless of the treatment, with the number of incidences of grey mould ranging from 126 to 357 on average. On the other hand, in Season 2, these incidences ranged from 20 to 50 depending on the treatment. In Season 1, there were significant differences among treatments, with the cinnamon extract showing the worst results again; however, in this trial, the best results were obtained for PHC, which had better results than the copper formulation (Cox). The differences were significant after the second week after disease onset and increased until the end of the crop season (Figure 4). In the Season 2, incidence was lower, but significant differences were also observed between cinnamon extract and the other two treatments (PHC and Cox) in weeks 8, 10, 15, and 16. Again, cinnamon extract showed the lowest efficacy in controlling grey mould infections (Figure 4).

- Distribution of symptoms in plants

The treatments did not significantly influence ($p > 0.05$) the relative incidence of grey mould symptoms in any of the four different locations on the plants (petioles, leaves, main stem, and fruits) in any of the years of study (Season 1 and Season 2), or in any tomato variety (“Valenciano-type” and cv Caniles) (Table 3). However, when the data of the different treatments were pooled, both the season of study and the tomato variety showed statistically significant differences ($p \leq 0.05$) for all of the locations on the plants (Table 4). The relative incidence of grey mould on fruits was higher for the “Valenciano-type” tomato. However, the tomato cv. Caniles showed higher percentages for the leaves, stems, and petioles. Regarding the seasons, the relative infection of the fruits, petioles, and stems was higher in Year 1 (2019/20), while in Year 2 (2020/2021), infection was higher in the leaves. In this regard, it should be noted that interactions were observed in the leaves, stems, and petioles, but not in the fruits (Table 4).

- Tomato plant mortality due to grey mould

Plant mortality due to grey mould was observed in Year 1 (2019/20), while in Year 2 (2020/21), no plants were killed by the fungus. The percentage of dead “Valenciano-type” tomato plants was very low, the lowest mortality was observed for Cox (1.4%), and around 5% mortality was observed for the other two treatments at the end of the experiment (Figure 5). The plants started to die at week 6 after the onset of the disease. There were no statistically significant differences at any recording date.

In GH3, the results were different. Mortality started 4 weeks after disease onset and continued for 4 weeks until the end of the experiment. It was significantly higher for the cinnamon extract treatment, and there were no differences between the other two treatments (Figure 5). The levels of mortality were very high for the treatment with cinnamon extract, with more than 25% of total plants being dead in the last month of the trial, while for the other treatments, the level only exceeded 5% in the last week.

- Crop production

In both seasons, none of the treatments studied as fungicides against *B. cinerea* significantly influenced the total marketable production at the end of the crop cycle, for either “Valenciano-type” tomato plants grown in GH1 and GH2 or for cv. Caniles tomato plants grown in GH3. In all cases, the total marketable production was within the range of common yields for these types of tomato. In this regard, the production of “Valenciano-type” tomato ranged from 12.66 to 14.65 and from 15.77 to 17.55 kg m⁻² in Years 1 and 2, respectively, while the production of cv. Caniles tomato plants ranged from 14.42 to 15.63 and from 14.69 to 15.18 kg m⁻² in the same seasons (Table 5).

Table 3. Relative incidence of grey mould symptoms on different locations of “Valenciano-type” and cv Caniles tomato plants for Season 1 and Season 2 depending on treatment. Percentages based on the final accumulated symptoms recorded per location in the whole plots.

Treatment	GH1 + GH2 Tomato “Valenciano-Type”				GH3 Tomato cv. Caniles			
	Petioles (%)	Leaves (%)	Stem (%)	Fruits (%)	Petioles (%)	Leaves (%)	Stem (%)	Fruits (%)
Season 1, 2019–2020								
Cinnamon extract (200 mL/hL)	4.9 ± 3.7	52.6 ± 10.6	4.3 ± 3.1	38.3 ± 11.0	35.4 ± 5.4	41.3 ± 6.4	17.5 ± 2.6	5.8 ± 4.8
Copper oxychloride 50% (400 g/hL)	7.7 ± 4.4	49.9 ± 17.5	3.5 ± 4.1	39.0 ± 10.7	25.0 ± 6.8	51.7 ± 7.9	15.3 ± 2.9	8.0 ± 1.3
Potassium hydrogen carbonate 85% (300 g/hL)	4.4 ± 2.2	47.8 ± 19.4	9.6 ± 4.2	38.2 ± 17.4	30.3 ± 9.7	45.3 ± 9.2	17.8 ± 8.6	6.7 ± 3.7
<i>p</i> -value	0.4684	0.9167	0.1165	0.9927	0.2088	0.2282	0.8382	0.5868
Season 2, 2020–2021								
Cinnamon extract (200 mL/hL)	5.8 ± 1.4	58.7 ± 4.8	0.7 ± 0.7	34.8 ± 6.1	11.0 ± 6.3	83.8 ± 8.1	0.0 ± 0.0	5.3 ± 3.7
Copper oxychloride 50% (400 g/hL)	6.1 ± 0.7	68.0 ± 5.0	0.9 ± 0.5	25.0 ± 4.8	14.9 ± 11.5	79.7 ± 14.5	0.0 ± 0.0	5.4 ± 3.9
Potassium hydrogen carbonate 85% (300 g/hL)	4.7 ± 0.3	59.8 ± 5.3	0.8 ± 0.4	34.7 ± 5.7	9.9 ± 4.6	85.7 ± 4.6	0.0 ± 0.0	4.5 ± 3.1
<i>p</i> -value	0.1517	0.0534	0.6524	0.0506	0.9639	0.9775	–	0.9111

Values are mean ± standard deviation; n = 4. No significant differences ($p > 0.05$) were detected in any case through Fisher’s least significant difference (LSD) test (95% level of significance).

Table 4. Effect of tomato variety and season on the incidence of grey mould linked to the location of the infection site.

Location	Tomato Variety			Season		Interaction (T. Variety × Season)	
	Valenciano-Type (%)	cv. Caniles (%)	<i>p</i> -Value	2019/2020	2020/2021	<i>p</i> -Value	<i>p</i> -Value
Petioles	5.6 ± 2.6	21.1 ± 12.1	0.0000	18.0 ± 14.0	8.7 ± 6.2	0.0001	0.0000
Leaves	56.1 ± 12.8	64.6 ± 20.8	0.0032	48.1 ± 12.0	72.6 ± 13.2	0.0000	0.0000
Stem	3.3 ± 4.0	8.4 ± 9.3	0.0048	11.3 ± 7.4	0.4 ± 0.5	0.0000	0.0000
Fruits	35.0 ± 10.3	5.9 ± 3.4	0.0000	22.7 ± 18.4	18.3 ± 14.5	0.0385	0.7100

Values are mean ± standard deviation; n = 4. Significant differences (95% level of significance) were detected when $p \leq 0.05$.

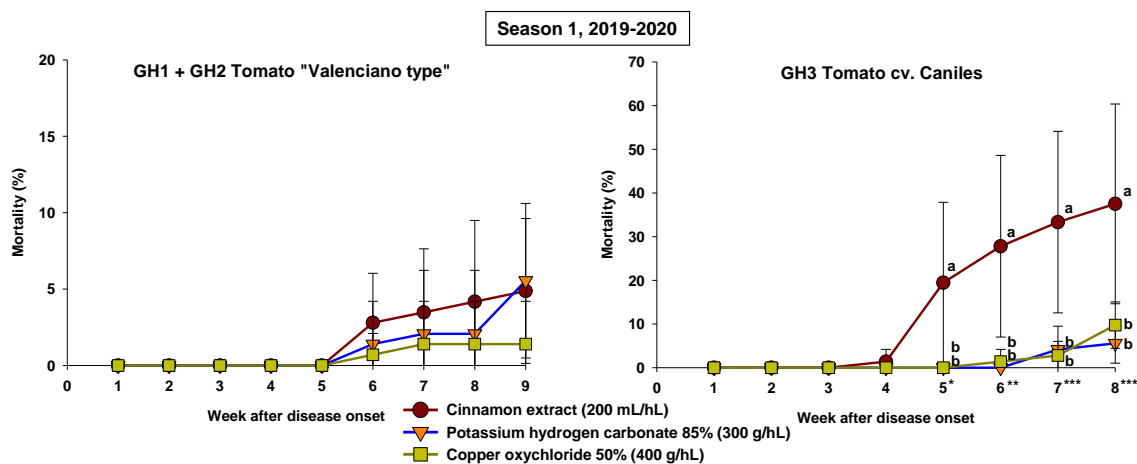


Figure 5. Accumulated mortality of “Valenciano-type” and cv Caniles tomato plants due to grey mould for Season 1 depending on treatment. Values are mean \pm standard deviation; $n = 4$. Different letters in the same week indicate significant differences ($p \leq 0.05$, Fisher’s LSD test). “*”, “**”, and “***” indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively. Note: in Year 2, there was no tomato plant mortality due to grey mould.

Table 5. Marketable production in the different trials for the different treatments.

Total Marketable Tomato Production (kg m^{-2})				
Trial	Cinnamon Extract (200 mL/hL)	Copper Oxychloride 50% (400 g/hL)	Potassium Hydrogen Carbonate 85% (300 g/hL)	p -Value
“Valenciano-type” Season 1 (2019/2020)	14.65 ± 2.07	13.81 ± 0.58	12.66 ± 0.39	0.2551
“Valenciano-type” Season 2 (2020/2021)	17.55 ± 1.18	15.77 ± 0.52	16.09 ± 1.82	0.2928
cv. Caniles Season 1 (2019/2020)	15.63 ± 1.84	14.42 ± 1.41	14.56 ± 1.11	0.4774
cv. Caniles Season 2 (2019/2020)	14.69 ± 1.36	15.08 ± 0.88	15.18 ± 0.34	0.5843

Values are mean \pm standard deviation; $n = 4$. No significant differences ($p > 0.05$) were detected in any case through Fisher’s least significant difference (LSD) test (95% level of significance).

4. Discussion

The reduction of copper-based plant-protection products with the final aim of phasing them out is a high priority in European policy [25,26] and in organic agriculture [19], mainly to avoid undesirable effects on agrosystems and ecosystems [21–23]. In this study, the antifungal effects of 12 commercially available products, including mineral fertilisers with low or no copper content, formulations based on basic substances, and formulations based on plant extracts and fungicides authorised for organic farming, were evaluated against the ubiquitous and polyphagous fungal pathogen *B. cinerea*, which affects tomato crops of the Mediterranean Basin and causes serious damage year after year [13]. Mediterranean greenhouses are generally not heated greenhouses, and cultural practices recommended for grey mould control are insufficient when the climatic conditions favour the disease [5,6]. In addition, *B. cinerea* is a pathogen that has shown a great ability to quickly adapt to chemical fungicides and to develop tolerant or resistant strains [31–34], and certain copper-based formulations may not be effective in suppressing *B. cinerea*-related plant diseases due to their ability to biotransform copper-based compounds [35].

In order to assess the efficacy of the 12 selected formulations through in vitro tests, two isolates of the pathogen were meticulously selected based on their growth and multiplication features. Thus, one of the isolates expressed fast vegetative growth, and the other was slower but showed fast initiation of sporulation. With this selection, we tried

to observe if the effect of the products was different depending on the “behaviour” of the isolate. In any case, the inhibitory products were equally effective. In this regard, among the tested copper-free products, only the cinnamon products, lime sulphur (18.5%), and potassium hydrogen carbonate resulted in more efficient inhibition of *B. cinerea* mycelial growth compared to copper oxychloride. Regarding the production of spores and sclerotia, unless the product showed fungicidal properties, none of the products prevented the production of these reproductive structures of the fungi. In all cases, *B. cinerea* isolates produced greyish mycelia and conidia, as well as black-coloured sclerotia, as is usually the case [36]. This fact suggested that the treated fungi would survive and reinitiate their germination and growth some time after the application of the products. However, some of the tested products killed the fungi when tested in vitro against *Botrytis cinerea* at the maximum dose concentration. In these cases, the efficacy of the treatment is supposed to be more persistent. The products that expressed themselves as fungicides were PHC and the cinnamon extracts. The first one is a product that has been registered and authorised as a fungicide for many horticultural crops to control powdery mildews. In this regard, our results are consistent with the results reported in several studies previously reporting the antifungal properties of potassium bicarbonate [37–39] and cinnamon [40–44] when tested in vitro against *B. cinerea*. In the case of cinnamon, not only has fungicidal activity been reported, but bactericidal, insecticidal, and nematocidal activities have been reported, as well [45].

PHC and cinnamon extract were the two most promising products in terms of efficacy in inhibiting the growth of *B. cinerea* when tested in vitro and in terms of economic viability; thus, commercial-sized greenhouse assessments were performed that included Cox as a control treatment. To our knowledge, these formulations have never been tested under real greenhouse conditions with no artificial pathogen inoculation. In our study, the conditions for the onset and spread of *B. cinerea* on tomato crops were different for the trials performed. Regarding the climatic conditions, the first season of the study (the 2019/20 season) was more humid, with an accumulated precipitation value of 194 mm against 179 mm in 2020/21 for the same period. Additionally, an earlier and higher number of rain events (four rainy days (over 10 mm/day)), were recorded in Year 1 (season 2019/20) compared to one rainy day in Year 2 (season 2020/21) for the same early period (100 days after planting) (Figure S1). These precipitation data are consistent for the most important environmental factors that promote and develop the disease caused by *B. cinerea* in non-heated greenhouses [7,9,11]. According to precipitation data, a higher incidence of *B. cinerea* and other fungal pathogens was expected during the first season. In the first year, higher incidence was observed for GH3 tomato crops (cv. Caniles). The crops in GH3 lasted for the same periods for Year 1 and Year 2, and the data were comparable. In the GH1 and GH2 crops (“Valenciano-type” tomato crops), the incidence of *B. cinerea* was higher in the second season, but this was probably because the crop lasted 3 weeks longer, increasing the chance to accumulate more pathogen incidence. In contrast, the duration of the wet periods was not long enough for effective infections by other pathogenic fungi such as *Fulvia fulva* or *Phytophthora infestans* on tomato plants.

Focusing on the parameters linked with the dissemination of grey mould in tomato plants during the commercial-sized greenhouse assessments, despite the absence of significant differences in some trials, there was a trend that was repeated in all of them: lower efficacy of the cinnamon extract to control the expansion of *B. cinerea* in the greenhouses. This result was more evident in GH3, mainly in the first season. In this trial, the best treatment for controlling the disease was PHC, which improved the results of Cox. The reason for cinnamon extract having lower efficacy despite being equally or more efficient than the two other treatments in the in vitro tests could be the higher volatilisation of the main fungicidal ingredients of cinnamon extracts (e.g., eugenol and cinnamaldehyde) [45,46], whereas copper and potassium salts remain on the plant surface once the treatment is dried. Additionally, similar observations were made by Sernaite et al. [43], who obtained excellent *B. cinerea* inhibition results in in vitro tests but obtained zero inhibition when applying

the same extract (12 mL/L dosage, i.e., 10 times more concentrated than the minimal fungicidal concentration found in their in vitro tests) to detached strawberry leaves that were previously infested with the fungus. Likewise, a recent article by Ebrahimi et al. [41] reported that cinnamon oil applied at three different concentrations inhibited the growth of *B. cinerea* through in vitro assays, as well as the development of grey mould on the tomato plants under greenhouse conditions, resulting in higher height, as well as higher fresh and dry weights of the plants when compared to untreated plants. However, in contrast to our study, Ebrahimi et al.'s [41] trials were performed with tomato seedlings grown in plastic pots (five repetitions, two plants each) covered with a plastic bag after being sprayed with cinnamon oil, and measurements were performed two weeks after the artificial inoculation of the pathogen. In our study, plants were grown directly in soil under on-farm greenhouse crop conditions for 6–7 months. Additionally, we applied cinnamon extract at a concentration of 2 mL/L, which is a low concentration to control *B. cinerea* in view of the results of the above-mentioned authors. In fact, in view of the results obtained in the first season of our study, we decided to apply cinnamon extract at a higher dose (3 mL/L), but after the first application, we returned to the lower concentration, as we found spotty phytotoxic symptoms on the leaves where the cinnamon extract was applied (Figure S2).

Concerning PHC, we used the lowest recommended dose all the time for economic reasons and to avoid phytotoxicity. At this dose, the results in the field trials were similar to or better than the control results obtained with Cox. The efficacy of PHC has been previously tested against *B. cinerea* in on-farm trials in organic grapevines fields, showing better results than a biocontrol product based on *Aureobasidium pullulans* and a blank control [47]. In our work, blank controls were not used, but Cox was used as the reference treatment, as it is one of the most common treatments used by “organic” growers to control the disease. On the other hand, Jabnoun-Khiareddine et al. [37] did not observe any reduction in the diameters of the lesions on tomato fruits caused by *B. cinerea* with the application of potassium bicarbonate in post-harvest trials. PHC is a naturally occurring inorganic compound that dissociates to potassium ions (K^+) and hydrogen carbonate ions (HCO_3^-) in water. Thus, PHC is not considered to be a persistent, bio-accumulative, or toxic compound, and risk assessments performed by EFSA concluded that it poses low risk to soil organisms, among others [48].

According to the results, the incidence of *B. cinerea* had no influence on marketable tomato production. This is something that we have observed in other experiments under similar conditions: low radiation, high crop densities, and low temperatures. When a plant dies, the physical space of the removed plant is gained by the adjacent plants in terms of radiation and aeration, and as a consequence, the productive parameters are increased [49]. In any case, in the trials included in this work, the production data were obtained for whole plots, representing a higher number of plants than the plants observed for symptomatology, i.e., the sample was larger and more representative of production than it was for *Botrytis* infection. Both of these facts influence the differences found between production and disease incidence.

The application of fungicides is only a part of the integrated strategy for the effective control of airborne diseases, and there are other factors that can help farmers to reduce their use during the crop season. In this regard, the plant material (tomato variety) used in our study showed a high impact on the distribution of the disease in plants, with a clear preference of the pathogen for infesting the fruits of “Valenciano-type” tomatoes. This could be due to the shape of these fruits, with indentations and cavities where the humidity is preserved for longer periods facilitating *B. cinerea* infection and growth (Figure S3), although other physiological features can also be part of these differences. In addition, a particular observation was made for the GH3 results from the first season concerning the type of pruning carried out. In this case, the petioles were cut to a length of 3–10 cm, while in the second season, they were flush cut. The results were quite evident and expected, as *B. cinerea* has necrotrophic behaviour, and wounds are a preferred place for infection [16].

Thus, in the second season, there were not any stem infections, whereas in the first season, stem infections were the reason for plant mortality, which reached 37.5% on average at the end of the crop season for plants from the plots to which cinnamon extract was applied.

In view of our results, to help avoid *B. cinerea* infections, it could be recommended to grow tomato cv. Caniles or commercial tomato cultivars with similar physiological and biological features using precise pruning with flush cutting. Additionally, the substitution of Cox with PHC seems to be a feasible alternative in the case of climatic conditions that are favourable to grey mould disease.

In this regard, the management of the climate in greenhouses together with optimal tomato variety selection and pruning practices, among other things, are the key factors in reducing the use of copper-based formulations or any other fungicide to prevent grey mould infections in winter tomato crops. The implementation of these practices by farmers would lead to less dependence on contentious inputs, making the transition to a more sustainable and profitable agricultural system possible. However, the absence of heating systems in most Mediterranean greenhouses makes the use of fungicides in winter greenhouse tomato crops for the control of grey mould necessary. In the present study, PHC appears to be an efficient copper-free alternative. However, because copper-based fungicides have a broad spectrum of disease control and are cost-effective plant-protection products, it is necessary to introduce several truly affordable alternatives to the European market to avoid undesirable effects on the agrosystem resulting from the use of copper-based formulations. In this sense, the existence of different crop systems, climates, greenhouses, and market conditioning makes it necessary to increase the number of case studies, with the aim of providing trusted knowledge that will be accepted by the end-users.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy13010137/s1>, Figure S1: Precipitation in the location of the experiments for the cropping periods of the 2019/20 (i.e., Year 1) and 2020/21 (i.e., Year 2) seasons. Figure S2: Symptoms of phytotoxicity after applying cinnamon extract at 3 mL/L. Figure S3: Tomato fruits of the varieties used. Left: “cv. Caniles” (GH3); right: “Valenciano-type” (GH1 + GH2). Table S1: List of fungicides analysed in the formulations.

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