SCAP
SOCIEDADE DE CIÊNCIAS AGRÁRIAS DE PORTUGAL

Assessing endophytic phase of *Gnomoniopsis smithogilvyi* and aerial spores in two chestnut groves of Trás-os-Montes and application of cuprous oxide on plant debris (leaves and burrs) to reduce disease inoculum

Avaliação da fase endofítica de *Gnomoniopsis smithogilvyi* e dos esporos aéreos em dois soutos de Trás-os-Montes e avaliação da aplicação de óxido cuproso em restos vegetais (folhas e ouriços) para reduzir o inóculo da doença

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https://doi.org/10.19084/rca.42951

Received/recebido: 2025.09.02
Accepted/aceite: 2026.02.12

ABSTRACT

Gnomoniopsis smithogilvyi is an emerging pathogen causing nut rot, bark cankers, and leaf necrosis, while also existing asymptomatically as an endophyte. This study evaluated the presence of *G. smithogilvyi* aerial spores in two chestnut orchards in Trás-os-Montes. And assessed, by isolation, the presence of *Gnomoniopsis* in leaves, burrs and branches and also in litter of the same type of fallen plant tissues maintained in outdoor net cages. Additionally, 3 net cages were treated with cuprous oxide for evaluation of its efficacy in reducing aerial inoculum. Air samples were collected weekly from May to October in orchards. Airborne spores were detected in both orchards and during all the sampling time with two distinct peaks, one in July at the blossoming time of chestnut trees and another in early autumn. The presence of *Gnomoniopsis* (endophytic cycle) was very high in leaves, burrs and branches, reaching 90%, 97% and 90 % respectively. *Gnomoniopsis survival* in plant fallen tissues is also very high and consistently higher on cages that receive cuprous oxide fungicide, which indicates limited efficacy to reduce aerial potential inoculum. Additional research is required to increase control of plant debris in the field, which could result in improved management of *Gnomoniopsis* in the chestnut orchards.

Keywords: plant pathogen, *Castanea*, infection, endophyte, cuprous oxide

RESUMO

Gnomoniopsis smithogilvyi causa podridão dos frutos, cancros na casca e necrose foliar, existindo ainda no castanheiro na forma assintomática como endófito. Este estudo avaliou a presença de esporos aéreos de *G. smithogilvyi* em dois soutos de Trás-os-Montes e a presença do fungo em folhas, ouriços e ramos, bem como nos tecidos vegetais caídos no outono e mantidos em gaiolas de rede ao ar livre. Adicionalmente, as folhas e ouriços em 3 gaiolas foram tratados com óxido cuproso (1 g/L) para avaliação da eficácia na redução do inóculo aéreo. Amostras de ar foram recolhidas semanalmente de maio a outubro nos soutos. Os esporos aéreos foram detetados em ambos os soutos e durante todo o período de amostragem, com dois picos distintos, um em julho, na época da floração dos castanheiros, e outro em outubro. A presença de *Gnomoniopsis* em folhas, ouriços e ramos, no campo, foi muito elevada, 90%, 97% e 90% respetivamente.

A sobrevivência de *Gnomoniopsis* nas gaiolas, também foi muito elevada e consistentemente mais elevada nas gaiolas que receberam óxido cuproso, o que indica a limitada eficácia na redução do potencial de inóculo. Estudos adicionais para melhorar a gestão das folhas e ouriços caídos no outono poderiam resultar numa melhor gestão do inóculo de *Gnomoniopsis* nos soutos.

Palavras-chave: fitopatogénio, *Castanea*, infeção, endófito, óxido cuproso

INTRODUCTION

The European sweet chestnut (*Castanea sativa* Mill.) is a culturally and economically significant species, particularly in mountainous regions of southern Europe (FAO, 2022). In Portugal, chestnut cultivation plays a key role in rural economies, especially in the Trás-os-Montes region, which accounts for 88% of the national chestnut-growing area and 82% of total production (INE, 2021). Chestnut fruit is a traditional and nutritious food source for Mediterranean populations (Vasconcelos *et al.,* 2010), and the multipurpose chestnut agroforestry system represents a high-value ecological and landscape heritage. However, despite recent increases in production, chestnut orchards face serious challenges due to a combination of diseases and pests that reduce both yield and quality. Among these are major diseases such as ink disease (*Phytophthora* spp.), chestnut blight (*Cryphonectria parasitica* (Murrill) Barr), and brown rot caused by *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest (= *Gnomoniopsis castaneae* Tamietti). In addition, pests like the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu), chestnut weevil (*Curculio elephas* Gyll.), and the chestnut moth (*Cydia splendana* (Hübner)) significantly impact yield and quality (CastedoDorado *et al*., 2023).

Among the most concerning emerging threats is *Gnomoniopsis smithogilvyi*, an emerging fungal pathogen of chestnut trees, responsible for fruit rot, bark cankers, and leaf necrosis (Lione *et al*., 2019; Dobry and Campbell, 2023). It also behaves as a latent endophyte, which complicates detection and control strategies. First reported in association with nut rot in Italy (Visentin *et al*., 2012), it has since spread to several European countries, including Portugal (Coelho and Gouveia, 2021). The pathogen has been reported to cause postharvest fruit rot, with incidences reaching up to 60% (Vannini *et al*., 2017), and it is frequently associated with plant organs such as flowers, leaves, burrs, and branches. In orchards infested with *D. kuriphilus*, galls may also act as fungal reservoirs, increasing disease pressure (Vannini *et al*., 2018). In addition to chestnut trees, this species has also been reported as a pathogen on other Fagaceae species (e.g., *Quercus ilex* L.) and on other agricultural and forest species occurring in Portugal, such as *Corylus avellana* L., *Fraxinus ornus* L., and *Pinus pinaster* Aiton (Dobry and Campbell, 2023). These hosts may play an important role in inoculum production and its dissemination to chestnut trees.

The epidemiology of *G. smithogilvyi* is complex. The fungus can overwinter in burrs, fallen leaves, and branches lying on the orchard floor, which act as key reservoirs for inoculum (Shuttleworth and Guest, 2017). Wind and rain play a role in the transmission of spores infecting developing flowers, wounded stems, and branches (EPPO, 2017; Shuttleworth and Guest, 2017). Management strategies for *G. smithogilvyi* are still limited and under development, as the pathogen has only recently gained attention due to its emerging status. To date, no fungicides are registered specifically for *G. smithogilvyi* control, and chemical control remains limited by regulations and the need to protect beneficial endophytes. Current approaches primarily focus on preventive cultural practices, such as removal of infected nuts, pruning of symptomatic branches, and destruction of fallen debris to reduce inoculum sources (Vannini *et al*., 2017; Dobry and Campbell, 2023). However, the removal of foliage is difficult to carry out, and it does not occur in most traditional orchards. In some regions, biological control strategies have been explored, including the potential role of endophytic or antagonistic fungi to limit *G. smithogilvyi* colonization, although results are still preliminary (Lione *et al*., 2019). Additionally, the reduction of insect vector populations, especially *D. kuriphilus*, may help limit *G. smithogilvyi* dissemination, since galls can serve as reservoirs for the fungus (Vannini *et al*., 2017). Both practices are difficult to implement in

many traditional or unmanaged orchards. In particular, the removal of leaf litter, where the fungus persists and sporulates, is labor-intensive and rarely practiced, leaving a significant inoculum source untreated. In this context, the application of chemical treatments to fallen leaves and burrs may offer an alternative management strategy.

Copper-based fungicides are widely used in organic and conventional agriculture due to their broad-spectrum antifungal properties. Nordox (Copper Nordox® 75 WG), a formulation of cuprous oxide, has shown effectiveness against several plant pathogens and may serve as a candidate for managing *G. smithogilvyi* in orchard litter. However, data on its specific efficacy against *G. smithogilvyi* under field or laboratory conditions remains scarce.

This study aimed to monitor the presence and dispersal of *G. smithogilvyi* spores in two chestnut orchards in Trás-os-Montes region (Portugal) using air sampling techniques and to evaluate the potential of copper-based fungicides, particularly cuprous oxide, to reduce the fungal inoculum on symptomatic leaves and burrs through both field exposure and *in vitro* assays.

MATERIALS AND METHODS

Study site and sample collection

For this study, two chestnut stands were selected in Sobreiró de Cima (Vinhais municipality, northeast of Portugal) [Site 1: 41°50'4.722" N, 7°03'48.186" W; Site 2: 41°50'11.699" N, 7°03'39.816" W] (Figure 1). Both sites cover 1 ha and have approximately 20-year-old trees belonging to the cv. Longal and spaced 10 × 10 m apart. The soil at both sites is managed with natural vegetation and maintained under rainfed conditions, which is typical for the region. At Site 2, weed control was carried out with a single cultivator pass in early July. At both sites, the trees were georeferenced and mapped using GIS analysis tools and ArcView® 10.1 software (Environmental Systems Research Institute, Inc.).

Sample collection for testing and fungal identification was carried out in two periods during 2024. In February, two plastic bags (each with a volume of 100 L) were filled with chestnut leaves and burrs randomly collected from the litter, composed of fallen leaves and burrs, on the chestnut orchard floor at each site, and transported to the laboratory. In September, six trees were selected at each site, and from each tree, five leaves, five burrs, and five branches were collected (a total of 15 samples per tree). These samples were transported in plastic bags to the laboratory for subsequent fungal identification.

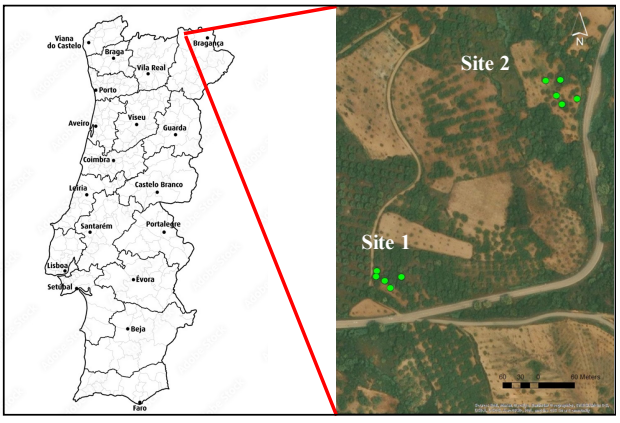

Figure 1 - Sample sites (Sobreiró de Cima, Vinhais- northeast of Portugal).

Detection of Gnomoniopsis spores in the field by air sampling

To collect *Gnomoniopsis* spores in the field (Sobreiró de Cima), samples were taken weekly from May to October 2024. From the litter under each tree, 200 m^3 of air was sampled at two different heights, approximately 50 cm above ground level and at canopy height, using a SAS air sampler (VWR) with two Petri dishes (90 mm in diameter) containing PDA. The sampler was disinfected with 70% ethanol before use at each tree.

In the laboratory, Petri dishes were incubated in the dark in a "Memmert IN55" incubator (Memmert Gmbh + Co.KG, Germany) at 25 ± 2 °C for two days, after which the number of bacterial and fungal colonies on each dish was counted. Colonies identified as *Gnomoniopsis* were transferred to new Petri dishes containing PDA for further identification. The pure cultures of *Gnomoniopsis* isolates were preserved in PDA, kept at 10ºC, and then deposited in the fungal collection of the Laboratory of Plant Protection at Instituto Politécnico

de Bragança (Portugal). The average number of colony-forming units (CFU) per plate for bacteria, non-*Gnomoniopsis* fungi, and *Gnomoniopsis* at canopy and ground level was calculated. CFU/plate data are presented as mean±standard deviation (mean±SD). Due to the great amount of *Gnomoniopsis* isolates obtained in this study and three *Gnomoniopsis* isolates obtained by SAS air sampling were selected for molecular confirmation.

Incidence of Gnomoniopsis on leaves, branches and burrs collected in the field

In October, six trees were selected at each study site. From each tree, five leaves, five branches, and five burrs were collected using pruning shears. The samples were placed in plastic bags and transported to the laboratory. For each leaf, branch, and burr, five tissue segments were used for the isolation of *Gnomoniopsis*. The isolation of the fungus is described in the next Section The incidence of *Gnomoniopsis* was calculated using the number of samples (leaves, branches, burrs) colonized by the fungus and the total number of samples, and expressed as a percentage.

Gnomoniopsis isolation and identification

From each sample, five sections were cut and dipped in 70% (v/v) ethanol for 2 minutes, then placed in 90 mm diameter Petri dishes containing PDA to allow mycelial growth. The plates were incubated in a "Memmert IN55" incubator at 25 ±2 ºC for seven days in the dark. Single colonies were then transferred to new PDA plates to obtain purified isolates for subsequent identification. The pure cultures of the fungus were preserved in PDA at 4 ºC and then deposited in the fungal collection of the Laboratory of Plant Protection at Instituto Politécnico de Bragança (Portugal).

Identification of purified *Gnomoniopsis* isolates was based on morphological characteristics and molecular methods. DNA extraction was performed using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, USA) following the manufacturer's instructions, and the ITS region was amplified using the universal primers ITS1 and ITS4 (White *et al*., 1990). The amplified ITS region was sequenced by Stabvida Laboratories (Caparica, Portugal), and the sequences were compared with published data in the GenBank databases using the BLAST (https://blast.ncbi.nlm.nih.gov/) search algorithm.

Phylogenetic analysis

The optimal tree is shown, as inferred using the Maximum Likelihood method and Tamura-Nei (1993) model (Tamura and Nei, 1993) of nucleotide substitutions. The initial tree for the heuristic search was selected by choosing the tree with the highest log-likelihood among Neighbor-Joining (NJ) trees (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths (above the branches) in the same units as those of the evolutionary

Table 1 - Reference isolates/strains and GenBank accession numbers for fungi included in phylogenetic analysis

Accession numbers	Isolate	Country	Reference
PX463349	Gs24.13	Portugal	under study
PX582109	Gs24.5	Portugal	under study
PX463347	GS24.4	Portugal	under study
JX094886	Gn TN3	Italy	Longa *et al*. (2012) unpub.
LT837820	LPPA F935	Spain	Gonzalez (2017) unpub.
OQ690676	Gc28	Turkey	Cakar (2023) unpub.
KY930638	TR45	Turkey	Akilli Simsek *et al*. (2018) unpub.
PX697973	MIFCC515	USA	Bastianelli & Miles (2025) unpub.
MW165483	Gs1	Portugal	Rodrigues & Possamai (2023) unpub.
LT593845	A2P4GS0	Italy	Mello (2017) unpub.
JQ910642	CBS1 30190 type strain	Australia	Crous *et al*. (2012)
KY695232	THDAS 2016 1120A	UK	Lewis *et al*. (2017)
JQ898296	CH3	Switzerland	Visentin *et al*. (2012)
GU320830	CBS 123202	USA	Walker *et al*. (2010)

distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA12 (Kumar *et al*., 2024) using 1000 bootstrap replicates. Referenced sequences and respective GenBank accession numbers are listed in Table 1.

Effect of cuprous oxide fungicide on reducing inoculum levels on leaves and burrs (litter on the chestnut orchard floor)

The leaves and burrs collected from the litter on the chestnut orchard floor at the sites in Sobreiró de Cima were divided into an equal volume and placed in eight net cages, which were left in the Agrária's orchard (Instituto Politécnico de Bragança) under natural environmental conditions. In February 2024, three of the cages were treated with cuprous oxide (Copper Nordox® 75 WG, Massó) at a concentration of 1 gL^{-1}, while the remaining five cages were left untreated.

Spores were detected on leaves and burrs from both copper-treated and untreated net cages between March and June, using weekly air sampling at 200 m^3 per cage. Sampling was performed with a portable SAS air sampler (VWR) equipped with two 90 mm Petri dishes containing Potato Dextrose Agar (PDA; 39 g/L, Difco). The sampler was sanitized with 70% ethanol before use at each cage. In the laboratory, Petri dishes were incubated in the dark in a "Memmert IN55" incubator (Memmert Gmbh + Co.KG, Germany) at 25 ± 2 °C for two days, after which the number of bacterial and fungal colonies on each dish was counted.

During this period, isolations were performed at two distinct times of the year, February and June, to confirm the presence of *Gnomoniopsis* in leaves and burrs left within the net cages. Samples were placed into plastic bags and transported to the laboratory. From each cage, three leaves or burrs were selected, and five isolations were conducted per leaf or burrs.

In vitro effect of the cuprous oxide on Gnomoniopsis isolates

This test aims to assess the *in vitro* effectiveness of cuprous oxide by determining the concentration that causes 50% growth inhibition (EC50) in *Gnomoniopsis* isolates. Three concentrations of cuprous oxide (100 mg/L, 200 mg/L, and 300 mg/L) were tested. Three isolates of *Gnomoniopsis* obtained in the fungal isolation performed in February were used in this test: Gs24.1, Gs24.6, and Gs24.11. The fungicides were added to PDA culture medium at the concentrations listed above.

The *in vitro* test included 4 replicates per concentration. Discs of mycelium from each *Gnomoniopsis* isolate, approximately 2 mm in diameter, were inoculated with a loop in the centre of each Petri plate (90 mm in diameter), and after inoculation, the plates were immediately placed in a "Memmert IN55" incubator at 25 ± 2 ºC. The reference standard for the fungus's growth was obtained on PDA without any added substances.

Data analysis

The mycelial growth (colony diameter) of the isolates was assessed by measuring colony diameter in two perpendicular directions after 6 days. Mycelial growth data are presented as mean±standard deviation (mean±SD). The percentage inhibition of mycelial growth was determined by the following equation: $I\ (\%) = (C - T)/C \times 100$, where I - the percentage of mycelial growth inhibition, C - mycelial growth of the control colony, and T - mycelial growth of the treatment colony.

Statistical analyses of *Gnomoniopsis* isolates for the different chemicals were carried out using one-way analysis of variance (ANOVA) followed by LSD and Tukey post hoc tests in Statistica 12 (StatSoft Inc., Tulsa, OK).

The effective concentration of active substance that cause a 50% reduction in mycelial growth relative to the control (EC_{50}) was estimated by a three-parameter log-logistic model (LL.3). This was performed using the functions drm and ED from the drc R package v. 3.0.1 (Ritz *et al*., 2015) based on the data from the tested fungicide concentrations for each isolate and respective mycelial growth.

The model selection procedure was based on the Akaike Information Criterion (AIC) and the "LL.3" with the lower asymptote fixed at zero using the

equation: f (x) = d – 0 / (1 + exp (b(log(x) –log(e)))), where b = Rate of decline (slope); d = Upper asymptote; 0 = Lower asymptote; e = EC_{50}, and x = Fungicide concentration or dose.

RESULTS

Detection of Gnomoniopsis spores in the field by air sampling

A total of 96 fungal colonies (54 at ground level and 41 at canopy level) and 14 bacterial colonies (10 at ground level and 4 at canopy level) were isolated from SAS plates across both sites. At all sampling times, the number of fungal colonies consistently exceeded that of bacterial colonies. Among the total fungal isolates, 50 colonies of *Gnomoniopsis* were identified, 13 from Site 1 and 37 from Site 2.

At Site 1, the highest number of CFU per plate for both non-*Gnomoniopsis* fungi (252.00 ± 40.82) (mean ± standard deviation) and bacteria (114.00 ± 70.75) was recorded at ground level on September 3 (Table 2). At Site 2, the highest number of CFU per plate for non-*Gnomoniopsis* fungi (202.20 ± 65.37) was observed on July 16, while the peak for bacteria (86.40 ± 61.78) occurred on July 23, also at ground level (Table 3).

Gnomoniopsis spores were collected in higher numbers at ground level at both sampling sites. Notably, a substantial number of *Gnomoniopsis* spores (28) were detected at ground level at Site 2, mainly during July. The highest spore counts were recorded on July 16 at both sites, with average values of 0.2 ± 0.52 CFU/plate at Site 1 and 0.35 ± 0.81 CFU/plate at Site 2 (Figure 2). As shown in Figure 2, two distinct peaks in *Gnomoniopsis* spore presence are evident: one in July, coinciding with the chestnut flowering period, and another in early autumn.

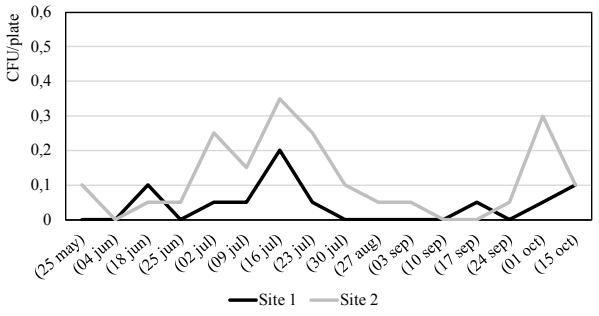

Figure 2 - Average number of colony-forming units (CFU) per plate of *Gnomoniopsis* isolates throughout the sampling period in Sobreiró de Cima.

Table 2 - Average number of colony-forming units (CFU) per plate for bacteria, non-*Gnomoniopsis* fungi, and *Gnomoniopsis* at canopy and ground level throughout the sampling period in the Site 1

Date	T (ºC)[1]	Canopy level			Ground level		
		Bacteria	Fungi[2]	Gnomoni.	Bacteria	Fungi[2]	Gnomoni.
21/05	15	1.40±0.84	208.30±43.75	0.00±0.00	3.80±0.00	181.70±67.75	0.00±0.00
04/06	25	5.70±3.40	116.00±76.45	0.00±0.00	30.50±26.26	158.60±61.95	0.00±0.000
18/06	16(*)	6.90±5.17	187.20±15.30	0.00±0.00	8.10±4.98	175.30±19.48	0.20±0.42
25/06	25	15.90±5.70	192.80±12.18	0.00±0.00	14.30±5.40	176.80±29.69	0.00±0.00
02/07	26	19.70±18.6	165.90±37.90	0.10±0.32	19.00±15.08	182.70±35.39	0.00±0.00
09/07	19	15.20±13.41	194.60±49.69	0.10±0.32	23.40±16.45	198.30±72.10	0.00±0.00
16/07	19	19.10±3.25	203.90±28.59	0.30±0.67	37.10±36.58	206.80±32.98	0.10±0.32
23/07	32	37.40±28.05	171.30±46.50	0.00±0.00	65.10±34.58	215.70±39.35	0.10±0.32
30/07	25	13.00±5.48	176.30±25.41	0.00±0.00	15.90±11.28	210.80±34.97	0.00±0.00
27/08	24	8.50±2.64	99.40±61.52	0.00±0.00	30.40±18.99	170.30±28.43	0.00±0.00
03/09	19	11.30±6.82	95.40±19.14	0.00±0.00	114.00±70.75	252.00±40.82	0.00±0.00
10/09	19	9.00±6.31	138.60±33.18	0.00±0.00	39.50±24.58	187.50±41.44	0.00±0.00
17/09	18	12.90±8.06	123.40±45.82	0.00±0.00	32.90±17.42	216.10±16.76	0.10±0.32
24/09	14	5.90±5.02	124.60±32.51	0.00±0.00	4.00±1.89	174.30±35.52	0.00±0.00
01/10	15(*)	1.50±0.71	96.60±34.28	0.00±0.00	3.10±1.37	181.60±32.30	0.10±0.32
15/10	14	17.60±22.16	137.80±30.63	0.10±0.32	27.10±26.39	141.60±27.78	0.10±0.32

[1] temperature at air sampling time; [2] non-*Gnomoniopsis* fungi, (*) rain; Gnomoni. - *Gnomoniopsis*

Table 3 - Average number of colony-forming units (CFU) per plate for bacteria, non-*Gnomoniopsis* fungi, and *Gnomoniopsis* at canopy and ground level throughout the sampling period in the Site 2

Date	T (ºC)[1]	Canopy level			Ground level		
		Bacteria	Fungi[2]	Gnomoni.	Bacteria	Fungi[2]	Gnomoni.
21/05	15	4.90±7.02	89.70±65.25	0.10±0.32	10.70±0.00	134.00±76.14	0.10±0.32
04/06	25	20.10±21.59	74.10±21.76	0.00±0.00	66.10±41.93	145.20±59.26	0.00±0.00
18/06	16(*)	7.60±3.50	159.70±24.49	0.00±0.00	15.50±7.38	154.40±24.51	0.10±0.32
25/06	25	10.60±2.84	174.80±11.60	0.00±0.00	15.10±11.63	168.70±21.55	0.10±0.32
02/07	26	19.10±17.64	116.00±14.48	0.00±0.00	20.50±14.49	133.30±33.04	0.50±0.97
09/07	19	11.00±8.29	114.80±42.61	0.00±0.00	70.80±38.42	145.50±34.05	0.30±0.48
16/07	19	21.60±11.74	121.60±23.23	0.20±0.42	80.50±48.61	202.20±65.37	0.50±1.08
23/07	32	28.20±33.06	40.00±30.62	0.10±0.32	86.40±61.78	151.50±80.11	0.40±0.52
30/07	25	9.10±5.61	115.10±10.49	0.00±0.00	39.30±14.46	159.70±45.13	0.20±0.42
27/08	24	5.30±2.45	52.10±7.64	0.00±0.00	38.80±23.30	173.00±72.54	0.10±0.32
03/09	19	27.90±20.34	87.30±20.84	0.10±0.32	81.00±35.48	149.00±62.74	0.00±0.00
10/09	19	11.60±7.40	104.50±39.10	0.00±0.00	74.20±68.22	183.10±64.71	0.00±0.00
17/09	18	10.90±5.80	73.20±24.04	0.00±0.00	36.30±29.29	110.00±35.05	0.00±0.00
24/09	14	4.30±1.49	125.50±18.14	0.10±0.32	5.60±4.12	154.40±38.32	0.00±0.00
01/10	15(*)	24.10±10.07	142.80±15.50	0.10±0.32	27.30±8.39	154.60±18.30	0.50±0.85
15/10	14	30.70±21.91	104.70±22.45	0.20±0.42	36.40±24.17	131.00±19.30	0.00±0.00

[1] temperature at air sampling time; [2] non-*Gnomoniopsis* fungi, (*) rain; Gnomoni. - *Gnomoniopsis*

Gnomoniopsis isolation and identification

In this study, 240 isolations were observed from chestnut burrs and leaves retrieved from litter and placed in net cages, 120 in February and 120 in June. From these, 175 fungal isolates were obtained. Among them, 76 isolates exhibited morphological characteristics consistent with *Gnomoniopsis*, while the remaining 99 belonged to other fungal genera. *Gnomoniopsis* isolates were recovered from all cages evaluated, in both February and June collections. The average number of *Gnomoniopsis* isolates per cage ranged from 0.33±0.58 (mean ± standard deviation) to 3.00±0.00 in February, and from 0.33±0.00 to 3.33±0.58 in June (Table 4).

From *Gnomoniopsis* isolates obtained from chestnut leaves or burrs in the net cages, 10 isolates were selected for molecular species identification. The remaining fungal isolates were not identified, as their characterization was beyond the scope of this study. Nine isolates were identified as *G. smithogilvyi*, with nucleotide identity in the ITS region ranging from 97.48 to 100% (Table 5). One isolate could not be identified, possibly due to degraded or insufficient DNA. From these *Gnomoniopsis* isolates, three isolates were deposited in GenBank, and the accession numbers are presented. Morphological analysis showed that the mycelium of *Gnomoniopsis* isolates was felty and dark grey, with concentric rings growing on PDA.

Table 4 - Average number of isolates obtained per net cage in February and June of 2024

	February 2024		June 2024	
Cages	*Gnomoniopsis* isolates	Other fungi	*Gnomoniopsis* isolates	Other fungi
1	2.00±2.65	3.00±2.65	0.33±0.00	2.00±1.73
2	2.00±1.00	3.00±1.00	1.00±1.00	4.00±1.00
3	1.00±1.00	2.33±0.58	1.33±0.58	0.33±0.58
4	2.33±1.53	2.67±1.53	3.33±0.58	2.00±1.00
5	0.33±0.58	3.67±1.53	1.00±1.00	0.67±0.58
6	2.00±2.00	2.33±1.53	2.00±2.65	1.33±0.58
7	3.00±0.00	2.00±0.00	0.33±0.58	0.33±0.58
8	2.66±0.58	2.00±1.00	0.67±1.15	1.00±1.00

Table 5 - Species identification and percentage of nucleotide identity of each identified isolate (left), and accession number of *G. smithogilvyi* isolates obtained in this study (right)

Isolates	Source	Cage	Species	% Identification*	Accession number (ITS)
Gs24.1	leaves	8	*Gnomoniopsis smithogilvyi*	99.59%	
GS24.3	leaves	2	*Gnomoniopsis smithogilvyi*	99.19%	
Gs24.4	leaves	2	*Gnomoniopsis smithogilvyi*	99.80%	PX463347
Gs24.5	leaves	2	*Gnomoniopsis smithogilvyi*	97.48%	PX582109
Gs24.6	leaves	5	*Gnomoniopsis smithogilvyi*	99.62%	
Gs24.7	leaves	6	*Gnomoniopsis smithogilvyi*	99.19%	
Gs24.11	burrs	1	*Gnomoniopsis smithogilvyi*	98.31%	
Gs24.12	burrs	1	*Gnomoniopsis smithogilvyi*	100.00%	
Gs24.13	leaves	6	*Gnomoniopsis smithogilvyi*	99.60%	PX463349
Gs24.16	burrs	1	No sequencing		

*compared to the CBS 130190 ITS reference collection, Accession number NR_166040 (Vu *et al.*, 2019).

In a phylogenetic analysis, 11 ITS sequences from Gnomoniopsis smithogilvyi isolates of different geographic origins were retrieved from GenBank. *Gnomoniopsis paraclavulata* was used as the outgroup to root the phylogenetic tree. The phylogenetic analysis revealed a very close relationship between the isolates obtained in this study and those retrieved from GenBank, including the type strain, confirming their genetic similarity (Figure 3).

Additionally, three *Gnomoniopsis* isolates (Gs24.21, Gs24.30, and Gs24.39) obtained from aerial spores via SAS air sampling were selected for molecular identification. Sequence comparison with the GenBank BLAST database confirmed that all three isolates belong to *G. smithogilvyi*, with nucleotide identity of the ITS region exceeding 99.40%.

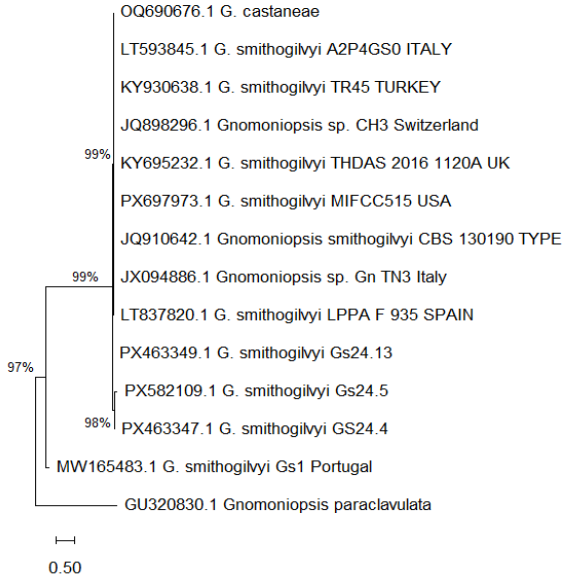

Figure 3 - Phylogenetic tree for *Gnomoniopsis smithogilvyi* isolates, ITS region for 14 sequences, in a total of 550 nucleotide positions.

Incidence of Gnomoniopsis on leaves, branches, and burrs in the field

From samples collected in September at Sobreiró de Cima (Vinhais), a total of 900 isolations were obtained, yielding 540 isolates of *Gnomoniopsis* across both study sites: 194 from leaves, 199 from burrs, and 147 from branches. All the tree tissues analysed showed the presence of *Gnomoniopsis*. Among the tissue types, burrs had the highest incidence of the fungus, with over 97% of samples testing positive. In contrast, branches showed the lowest incidence, with the fungus present in 80–90% of the samples (Figure 4).

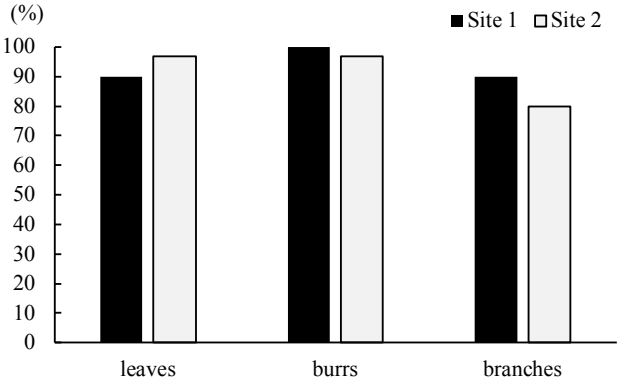

Figure 4 - Percentage of leaves, burrs, and branches with the presence of the *Gnomoniopsis* at each studied site in Sobreiró de Cima.

Effect of cuprous oxide fungicide on reducing inoculum levels on leaves and burrs

To assess the effect of cuprous oxide on *Gnomoniopsis* spore release, air sampling was conducted from February to June, with a total of 12 air samples collected from each cage. On three occasions, sampling was not possible due to rainfall. Fungal and bacterial growth was observed in all Petri dishes, with a total of 1854 bacterial colonies and 14135 fungal colonies recorded. However, *Gnomoniopsis* was not detected in any of the samples.

Additionally, the effect of a cuprous oxide fungicide on *Gnomoniopsis* inoculum levels was evaluated by isolating fungi from chestnut leaves and burrs left in net cages, sampled in February and June. A reduction in the number of *G. smithogilvyi*-positive isolations was observed in February and June (Figure 5). Interestingly, net cages that did not receive fungicide treatment consistently exhibited fewer positive isolations than those treated with copper, in both sampling periods.

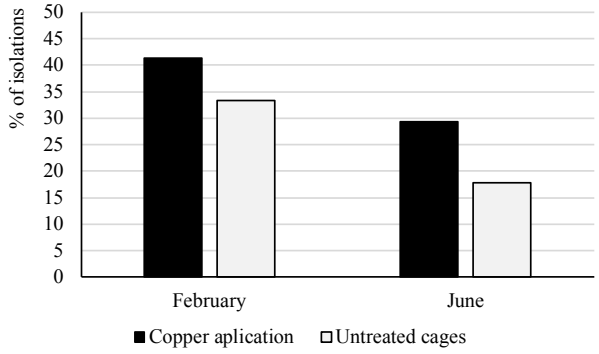

Figure 5 - Percentage of positive *G. smithogilvyi* isolations in treated and untreated cages.

In vitro effect of the cuprous oxide fungicide on Gnomoniopsis isolates

The mycelial growth data for the *Gnomoniopsis* isolates at different concentrations of cuprous oxide are shown in Table 6. Significant differences ($P < 0.05$) were observed between concentrations.

Growth inhibition increases with higher copper concentration. The effect of the lower concentration of cuprous oxide (100 μg/ml) varied between 8.25% (Gs24.1) to 11.18% (Gs24.6) of growth inhibition. At the highest cuprous oxide concentration (300 μg/ml), the growth inhibition ranged from 34.82% (Gs24.6) to 54.29% (Gs24.1).

Table 6 - Average mycelial growth (mean±standard deviation) of *Gnomoniopsis* isolates (Gs24.1, Gs24.6, and Gs24.11) on cuprous oxide concentrations

	Cuprous oxide			
Isolates	**0 mg/L**	**100 mg/L**	**200 mg/L**	**300 mg/L**
Gs24.1	7.86±0.09a	7.23±0.28b	5.03±0.35c	3.60±0.08d
Gs24.6	7.83±0.13a	6.95±0.47b	6.15±0.44c	5.10±0.36d
Gs24.11	7.88±0.05a	7.20±0.29b	6.68±0.33b	4.85±0.31c

The effective concentrations (EC_{50}) estimated for cuprous oxide fungicide are summarized in Table 7. EC_{50} varied between 269 mg/L and 516 mg/L values, which evidence relatively low toxicity and big differences between isolates. The lower and upper limits of the 95% confidence intervals for the EC_{50} estimates are also provided.

Table 7 - Estimated EC_{50} values of cuprous oxide fungicide for *Gnomoniopsis smithogilvyi* isolates

Active ingredient / *Gnomoniopsis* isolates	**Estimate (mg L^{-1})**	**Standard error**	**Lower**	**Upper**
Cuprous oxide				
Gs24.1	269.27	5.53	254.80	280.82
Gs24.6	516.42	52.16	399.43	631.22
Gs24.11	476.79	65.99	387.31	691.88

DISCUSSION AND CONCLUSION

In recent years, chestnut brown rot has become an increasing concern for farmers in Portugal. This disease has a significant economic impact, and researchers have sought to improve understanding of its biology, control, and management. In Portugal, however, studies on the detection of the fungus in leaf and burr litter, or on the application of chemical treatments to litter for disease control, are scarce or non-existent. Therefore, the present study aimed to detect *G. smithogilvyi* in asymptomatic chestnut tissues and litter in two orchards in the Trás-os-Montes region, assess the presence of *Gnomoniopsis* spores by air sampling, and evaluate

the efficacy of a copper-based compound in reducing the fungal inoculum.

The results of this study confirm the widespread occurrence of *G. smithogilvyi* in both orchards, with high incidence rates detected in asymptomatic burrs, leaves, and branches, confirming its endophyte behavior as reported by other authors (Crous *et al*., 2012). Burrs, in particular, exhibited the highest infection frequency, detected in over 97% of samples. Infected leaves and burrs eventually fall, forming the litter, which can serve as a source of air spores inoculum for future infections. Additionally, our study confirmed the presence of *G. smithogilvyi* isolates in all chestnut burrs and leaves collected from litter samples. The persistence of *G. smithogilvyi* in leaf and burr litter throughout winter and spring is consistent with earlier reports demonstrating its saprophytic survival in plant debris (Visentin *et al*., 2012; Lione *et al*., 2021), and highlights that orchard litter is a primary source of ascospore inoculum (Vannini *et al*., 2017). These findings emphasize the critical importance of managing infected litter, particularly burrs, to reduce inoculum pressure and mitigate the risk of floral infections in the following season.

The widespread colonization of burrs, leaves, and even branches suggests that *G. smithogilvyi* functions as both a necrotroph and a latent endophyte. Similar tissue colonization patterns have been described in other chestnut-growing regions (Maresi *et al*., 2013; Lione *et al*., 2019), where burrs and nuts were identified as the primary infection sites under humid conditions. Although the incidence in branches was slightly lower (80–90%), it still indicates the potential for systemic infection and suggests that woody tissues may act as secondary reservoirs. These findings are particularly relevant given the defoliation symptoms observed in the region. The presence of *Gnomoniopsis* in symptomatic leaves suggests its involvement in early-season infection cycles, potentially contributing to canopy decline and reduced fruit quality.

In this study, air samples collected from both sites during our trial detected *Gnomoniopsis* spores, probably originating from the infected leaves and burrs of litter. An important finding of this study was the detection of a distinct spore peak of *G. smithogilvyi* in July, coinciding with the chestnut flowering period. This observation is highly relevant, as floral infections are among the most dangerous infection routes, often leading directly to fruit rot and subsequent yield losses (Shuttleworth, 2012; Shuttleworth and Guest, 2017). The synchrony between ascospore release from overwintered burrs and leaves and the host's flowering stage creates a high-risk window of infection. If uncontrolled, infections initiated during blossom may explain the high incidence of nut rot and foliar necrosis later in the season (Lione *et al*., 2019; Bastianelli *et al*., 2022). Unlike earlier reports from New Zealand, where *Phomopsis* nut-rot airborne spores were not detected (Wadia *et al*., 2000), subsequent work in Italy using passive spore trapping confirmed the deposition of *G. smithogilvyi* propagules in orchards during flowering (Lione *et al*., 2021). Our findings similarly demonstrate that Portuguese conditions favor spore release at critical phenological stages. This highlights the need to target inoculum sources before flowering, either through orchard sanitation or the testing of effective chemical or biological suppressants. This highlights the need to target inoculum sources before flowering, either through orchard sanitation or the testing of effective chemical or biological suppressants (Shuttleworth and Guest, 2017; Bastianelli *et al*., 2022).

A key objective of this study was to assess the potential of cuprous oxide fungicide, cuprous oxide, in suppressing *G. smithogilvyi* aerial spores. Field results showed an unexpected outcome: fungicide-treated net cages yielded more positive *G. smithogilvyi* isolates than untreated net cages. While copper is commonly used to manage fungal diseases in chestnuts, its effectiveness against *G. smithogilvyi* appears limited, a conclusion echoed by research demonstrating superior efficacy of strobilurin–triazole combinations compared to copper-based treatments (SilvaCampos *et al*., 2022), and by reviews emphasizing alternative fungicides and biocontrol agents over copper for pre-harvest disease control (Lema *et al*., 2023). One possible explanation is that copper applications may disrupt the surrounding microbial community, which reduces antagonistic organisms and allows *G. smithogilvyi* to proliferate. Wightwick *et al*. (2013) observed similar dynamics in vineyards,

where repeated copper applications altered microbial balance, sometimes favoring target pathogens.

In vitro testing confirms that *G. smithogilvyi* isolates exhibit low sensitivity to the cuprous oxide fungicide, with EC_{50} values ranging from 269 to 516 mg/L. This finding is consistent with other research indicating moderate tolerance of *G. smithogilvyi* to copper and systemic fungicides (Silva-Campos *et al*., 2022). The fungus's facultative lifestyle and ability to persist in dead tissues may explain this resilience. The modest levels of inhibition observed *in vitro* further reinforce the limited field performance and suggest that copper compounds, at recommended doses, may not be effective for controlling *G. smithogilvyi* in either pre-harvest or post-litter settings.

While pre-harvest treatments have shown some success in reducing nut rot (Lione *et al*., 2019; Bastianelli *et al*., 2022), current management programs do not offer chemical solutions for treating infected litter on the orchard floor. Yet, this study and others demonstrate that litter serves as a key inoculum source, particularly during flowering. Given that litter removal is not widely practiced in Portuguese orchards, there is a clear need to develop new strategies, either through effective chemical agents or biological controls, to suppress inoculum in the field.

This study demonstrates that *G. smithogilvyi* is a persistent and widespread pathogen in chestnut orchards in Trás-os-Montes, capable of colonizing multiple tissue types and surviving across seasons. Burrs were confirmed as the most heavily infected tissues, underscoring their critical role in the disease cycle and their importance as a management target.

Copper-based fungicides, although widely used in orchard systems, proved largely ineffective against *G. smithogilvyi* in both field and laboratory settings. Their limited inhibitory effect and potentially counterproductive impact on microbial ecology question their utility for inoculum suppression in chestnut litter.

Airborne spore detection in the orchard atmosphere, coinciding with flowering, confirms the risk of floral infection from litter-borne inoculum. This highlights the importance of targeting pathogen sources before bloom to disrupt the disease cycle.

Given these findings, effective management of *G. smithogilvyi* will require an integrated approach that combines sanitation practices (such as the removal or composting of infected burrs and leaves), environmental monitoring to optimize the timing of control measures, and the development of alternative fungicides or biological control agents, while future research should focus on identifying more effective treatments, elucidating the pathogen's infection biology, and understanding the role of orchard microbial communities in disease suppression.

Funding: IPB Project – Experimental Program for Biological Control of Chestnut Blight, IPB no. 167, and PDR2020 – Prevenção da Floresta Contra Agentes Bióticos e Abióticos, CP/02/2022, Portugal.

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