Research Article

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Unravelling the effect of control agents on *Gnomoniopsis smithogilvyi* on a chestnut-based medium by proteomics

Micaela Álvarez,[a,b] Isadora Agostini,[c] Ana Sampaio,[d,e] Ángel Román,[f] Josué Delgado[a*] and Paula Rodrigues[c,g]

Abstract

BACKGROUND: *Gnomoniopsis smithogilvyi* is the major chestnut pathogen, responsible for economic losses and recently described as a 3-nitropropionic acid and diplodiatoxin mycotoxin producer. *Bacillus amyloliquefaciens* QST 713 (Serenade® ASO), *B. amyloliquefaciens* CIMO-BCA1, and the fungicide Horizon® (tebuconazole) have been shown to reduce the growth of *G. smithogilvyi*. However, they enhanced mycotoxin production. Proteomics can clarify the mould's physiology and the impact of antifungal agents on the mould's metabolism. Thus, the aim of this study was to assess the impact of Horizon®, Serenade®, and *B. amyloliquefaciens* CIMO-BCA1 in the proteome of *G. smithogilvyi* to unveil their modes of action and decipher why the mould responds by increasing the mycotoxin production. For this, the mycelium close to the inhibition zone provoked by antifungals was macroscopically and microscopically observed. Proteins were extracted and analysed using a Q-Exactive plus Orbitrap.

RESULTS: The results did not elucidate specific proteins involved in the mycotoxin biosynthesis, but these agents provoked different kinds of stress on the mould, mainly affecting the cell wall structures and antioxidant response, which points to the mycotoxins overproduction as a defence mechanism. The biocontrol agent CIMO-BCA1 acts similar to tebuconazole. The results revealed different responses on the mould's metabolism when co-cultured with the two *B. amyloliquefaciens*, showing different modes of action of each bacterium, which opens the possibility of combining both biocontrol strategies.

CONCLUSION: These results unveil different modes of action of the treatments that could help to reduce the use of toxic chemicals to combat plant pathogens worldwide.

Supporting information may be found in the online version of this article.

Keywords: brown rot; *Bacillus amyloliquefaciens*; tebuconazole; proteomics

1 INTRODUCTION

Gnomoniopsis smithogilvyi, the causal agent of chestnut brown rot, is currently considered the major chestnut pathogen, being responsible for numerous economic losses in areas where chestnut is traditionally cultivated, as in Trás-os-Montes, in the north-east of Portugal.[1] Recently, our research group demonstrated that, besides being a significant rot-causing agent of chestnut fruits, this pathogen was also able to produce two mycotoxins, the 3-nitropropionic acid (3-NPA) and the diplodiatoxin,[2] turning it also into a potential human and animal health issue. Both mycotoxins are neurotoxic for animals and humans.[3,4] The 3-NPA produces vomiting and stomach ache, dystonia, coma, and even death in

* *Correspondence to: J Delgado, Higiene y Seguridad Alimentaria, Instituto Universitario de Investigación de Carne y Productos Cárnicos, Facultad de Veterinaria, Universidad de Extremadura, 10003-Cáceres, España. E-mail: jdperon@unex.es (Delgado)*

a *Higiene y Seguridad Alimentaria, Instituto Universitario de Investigación de Carne y Productos Cárnicos, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain*

b *Sección Departamental de Nutrición y Ciencia de los Alimentos (Nutrición, Bromatología, Higiene y Seguridad Alimentaria), Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain*

c *Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Bragança, Portugal*

d *Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal*

e *Laboratório Associado Instituto para a Inovação, Capacitação e Sustentabilidade da Produção Agroalimentar, Universidad de Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, Vila Real, Portugal*

f *Departamento de Bioquímica, Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain*

g *Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Bragança, Portugal*

humans.[3] Diplodiatoxin induces irritability, imbalance, convulsions, and inhibition of brain acetylcholinesterase activity in animals.[4]

To minimize the rotting of chestnuts by *G. smithogilvyi*, new strategies are focused on the use of biocontrol agents to avoid the use of chemical fungicides such as tebuconazole, which is widely used in agriculture. Tebuconazole is a wide-spectrum azole fungicide and belongs to the class of sterol 14-α-demethylase inhibitors (DMI), whose main target is the fungal cell membrane. It acts by inhibiting the conversion of lanosterol to ergosterol as a result of the inhibition of the critical enzyme cytochrome P450 sterol 14-α-demethylase, leading to cell membrane disruption and the consequent arrest of fungal growth.[5,6] Like many of the DMIs intensively used in agriculture, worrying environmental contamination and fungal resistance to tebuconazole have been reported in both agricultural and clinical settings.[6–8] In this context, the bacterium *Bacillus amyloliquefaciens* has been successfully used as biofungicide in numerous crops.[9,10] The *B. amyloliquefaciens* strain QST 713, commercialised as Serenade ASO (BAYER®), is recommended against *Botrytis cinerea*, *Alternaria* spp., or *Fusarium spp.* in different agricultural and horticultural crops.[11–15] Another strain of *B. amyloliquefaciens*, CIMO-BCA1, has shown antifungal activity against *G. smithogilvyi* in chestnut-based agar[2] and *B. amyloliquefaciens* strain UASWS BA1 inhibited the growth of this pathogen in chestnut tree scions.[16] However, the effectiveness of an agent in reducing the fungal growth is not necessarily accompanied by a lower production of mycotoxins, as it has been described for *G. smithogilvyi* by Álvarez *et al.*,[2] and for other mycotoxin producers such as *Penicillium nordicum, Penicillium verrucosum* or *Aspergillus westerdijkiae*.[17–19] For *G. smithogilvyi*, tebuconazole (the active substance of Horizon®) and *B. amyloliquefaciens* stimulated the production of diplodiatoxin,[2] showing the inconvenience of using the antifungals at subinhibitory concentrations. Although capable of reducing fungal growth and rot losses, this can lead to an increased risk for consumers.

Proteomics has been described as a useful tool to study microbe–host or microbe–food interactions and to clarify the mould's physiology and the impact of the antifungal agents on the mould's metabolism.[20,21] In this sense, proteomics showed that the biocontrol agent *Debaryomyces hansenii* reduced the abundance of proteins involved in ochratoxin A biosynthesis and the cell wall integrity pathway (CWI) in the toxigenic moulds *Aspergillus westerdijkiae* and *Penicillium nordicum*.[21,22] Another study using citral as the antifungal agent, demonstrated that this compound altered the proteins involved in numerous metabolic pathways, such as nutrient intake, sterol biosynthesis, energy metabolism, and oxidative stress, in *Aspergillus ochraceus*.[23] This technique generates massive proteomic data that contributes to unveiling the modes of action of chemical and biocontrol agents against plant pathogens like *G. smithogilvyi*, potentially serving to establish a link between biosynthesis pathways and mycotoxin production. Thus, the main aim of this study was to test the impact of the chemical fungicide Horizon® and two biocontrol agents, Serenade® and *B. amyloliquefaciens* CIMO-BCA1, in the proteome of *G. smithlogilvyi*. The second objective was to unveil their principal modes of action and why the mould responds to the effect of these agents by increasing mycotoxin production.[2]

2 MATERIALS AND METHODS

2.1 *Gnomoniopsis smithogilvyi* inoculum

G. smithogilvyi MUM 21.93 deposited in the fungal collection of Micoteca da Universidade do Minho (Braga, Portugal) was used in the present study [GenBank accessions OK326920.1 (ITS) and OK323174 (TEF1-α gene)]. The mould was inoculated on potato dextrose agar (PDA; Liofilchem, Roseto degli Abruzzi, Italy) and incubated for 7 days at 25 °C in dark conditions. Mycelium agar plugs 6 mm in diameter were cut and used as inoculum.

2.2 Control agents

The chemical fungicide Horizon® (oil in water with 250 g/L tebuconazole (1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol); Bayer, Leverkusen, Germany; hereinafter designated as HOR) and Serenade® ASO (hereinafter designated as ASO; Bayer), composed of *B. amyloliquefaciens* QST 713 at 1.34% (1×10^9 cfu/g) were used as commercial agents.

The strain *B. amyloliquefaciens* CIMO-BCA1 deposited in the Microbial Culture Collection of the Centro de Investigação de Montanha Culture Collection (CIMOCC, IPB, Bragança, Portugal) under accession number CIMO 22PR001 was also used as a biocontrol agent.

2.3 Culture medium and experimental settings

Chestnut-based medium (CM) was used to simulate the chemical and nutritional conditions of chestnuts and was elaborated as described previously.[2]

The control agents were tested at subinhibitory concentrations to study their modes of action on *G. smithogilvyi* as described for the analysis of secondary metabolites in Álvarez *et al.*[2] An agar plug of *G. smithogilvyi* was put onto the medium at a distance of 6 cm of 10 µL of phosphate-buffered saline (PBS) to be used as negative control (C). Similarly, three batches were carried out in which PBS was replaced with the control agents, using concentrations previously tested[2]: one with 10 µL of HOR (0.4 µL/mL), another with 10 µL of ASO (10^7 cells/mL), and, finally, one with 10 µL of *B. amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL). For this, cell suspensions of both ASO and CIMO-BCA1 were prepared by cell counting using a Neubauer chamber. After 8 days at 25 °C, mycelium samples were collected from the edge of the inhibition zone, lyophilized, and frozen at −80 °C before protein extraction. The experiment was carried out in triplicate.

2.4 Morphological evaluation of fungal growth

The mycelium was observed macroscopically using a Leica MZ95 magnifying stereomicroscope and microscopically with a Leica DM500 microscope, both coupled to an ICC50W digital camera ICC50W and Leica Application Suite V4 software (Nußloch, Germany).

2.5 Comparative proteomic analysis

The proteomic analyses were carried out following the methodology described by Álvarez *et al.*[21] using three biological replicates. Briefly, the mycelium samples were lysed before sonication in a Branson sonifier TM 250 (Emerson, Barcelona, Spain) and then partially run in sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After this, the lysates were submitted to a reduction-alkylation process with dithiothreitol and iodoacetamide, and digested with trypsin (Promega, Madison, WI, USA). One microgram of peptides was analysed using a Q-Exactive Plus coupled to a Dionex Ultimate RSLCnano (Thermo Fisher Scientific, Waltham, MA, USA). The Top15 method for MS/MS scans was used to collect the data[24] and label-free comparative proteome abundance and data analysis was performed using MaxQuant software (v. 1.6.15.0[25]) and Perseus software (v. 1.6.14.0) to organise the data. For the peptide identification, a proteome

from *G. smithogilvyi* was retrieved from Home – Gnomoniopsis castanea Behrend v1.0 (doe.gov) https://mycocosm.jgi.doe.gov/Gnocas1/Gnocas1.home.html. This proteome was annotated by identifying the protein homologs to the Uniprot diaporthales sequences[26] using the reciprocal best-hit strategy with Blast. The maximum peptide/protein false discovery rates (FDRs) were set to 1%, selecting proteins identified with at least two peptides. Only proteins detected in at least two replicates from at least one treatment were retained in the final datasheet. Qualitative analysis was carried out by detecting proteins in at least two replicates of the same batch and none of the compared batch. The Student *t*-test was applied to quantitative analysis between treatments ($P < 0.05$).[27] The enrichment analyses were carried out using ClueGO software (v. 2.5.6[27]). The kappa score was set at 0.4 and the *P* value was obtained by the Bonferroni step-down method and established at $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Morphological evaluation of fungal growth

The results obtained in the macroscopic evaluation demonstrated a clear inhibition of *G. smithogilvyi* when confronted with the control agents (Fig. 1). Furthermore, the magnified image using the stereomicroscope revealed different responses on the mould's mycelium when faced with *B. amyloliquefaciens* strains (ASO and CIMO-BCA1) with respect to the mycelium growth under control conditions (Fig. 2(A)). The edges of the mycelium next to the inhibition zone were darkened in both cases (Fig. 2(B,D)), and showed an increase in the production of resistance structures. The microscopic observation of the mycelium denoted a shorter, swelled, and truncated mycelium, with increased pigmentation in both ASO and CIMO-BCA1 treatments, as displayed in Fig. 3. In the case of the mycelium co-cultured with ASO, an increase in conidiomata was observed (Fig. 2(C)) as a response to the stressful conditions. However, treatment with CIMO-BCA1 induced the formation of a denser mycelium (Figs. 2(E) and 3). Antifungal compounds at subinhibitory doses such as those applied in the present study could induce spore production and germination, as well as morphological mycelium changes as a defence mechanism in filamentous fungi. For example, limonene induced spore germination and tube elongation at low concentrations in the fruit pathogen *Penicillium digitatum* through the regulation of energy metabolism and ROS homeostasis, while at high concentrations it had a strong antifungal effect.[28] Since *G. smithogilvyi* and the biocontrol agents were not in direct contact, the different responses

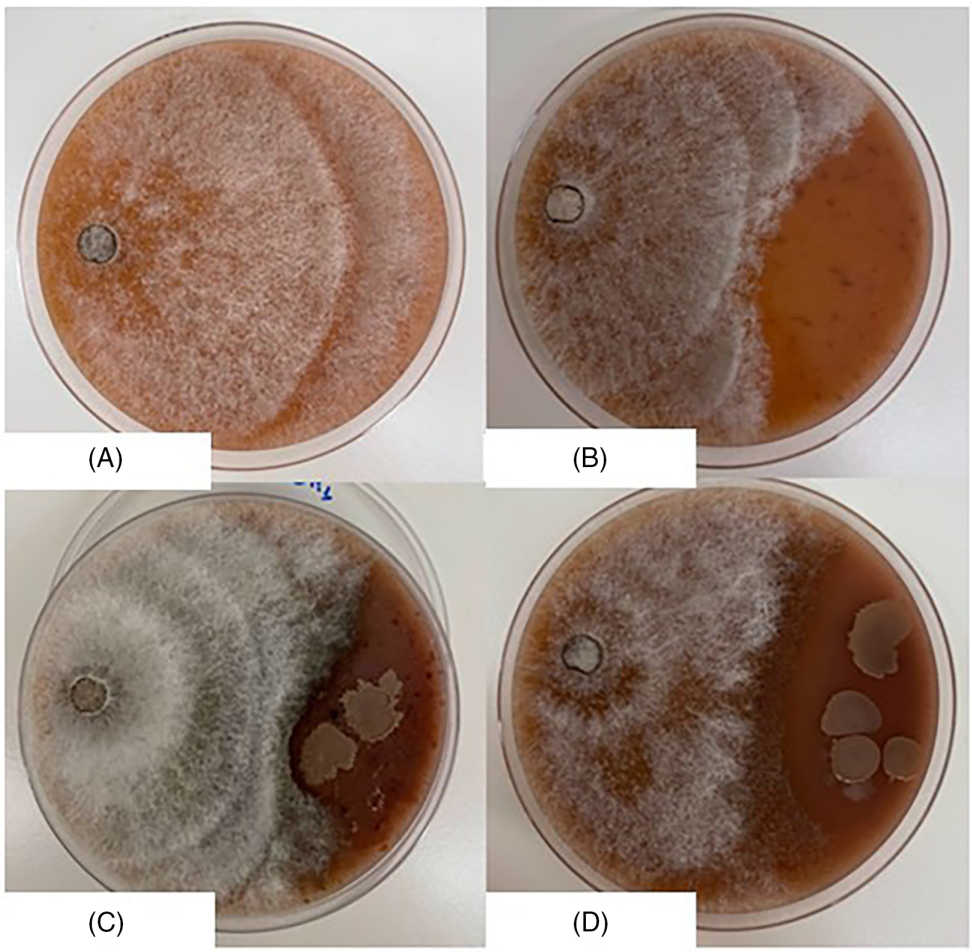

Figure 1. Macroscopic images of *Gnomoniopsis smithogilvyi* MUM 21.93 when confronted with control agents. (A) *G. smithogilvyi* MUM 21.93 and phosphate buffer saline. (B) *G. smithogilvyi* MUM 21.93 and Horizon® (tebuconazole, 0.10 g/L). (C) *Gnomoniopsis smithogilvyi* and CIMO-BCA1: *B. amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL). (D) *G. smithogilvyi* MUM 21.93 and ASO: *Bacillus amyloliquefaciens* QST 713 (10^7 cells/mL).

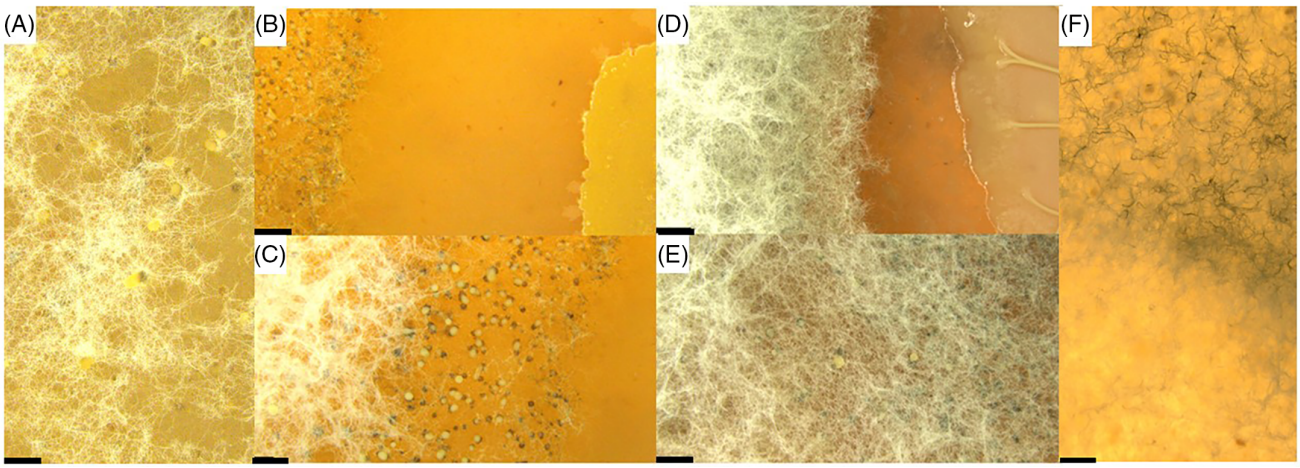

Figure 2. Macroscopic images of *Gnomoniopsis smithogilvyi* MUM 21.93 when confronted with biocontrol agents. (A) *G. smithogilvyi* MUM 21.93 (control). (B, C) *G. smithogilvyi* MUM 21.93 and ASO: *Bacillus amyloliquefaciens* QST 713 (10^7 cells/mL); (D, E) *G. smithogilvyi* MUM 21.93 and CIMO-BCA1: *G. smithogilvyi* MUM 21.93 and *B. amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL). (F) *G. smithogilvyi* MUM 21.93 and Horizon® (tebuconazole, 0.10 g/L) Bars indicate 500 μm.

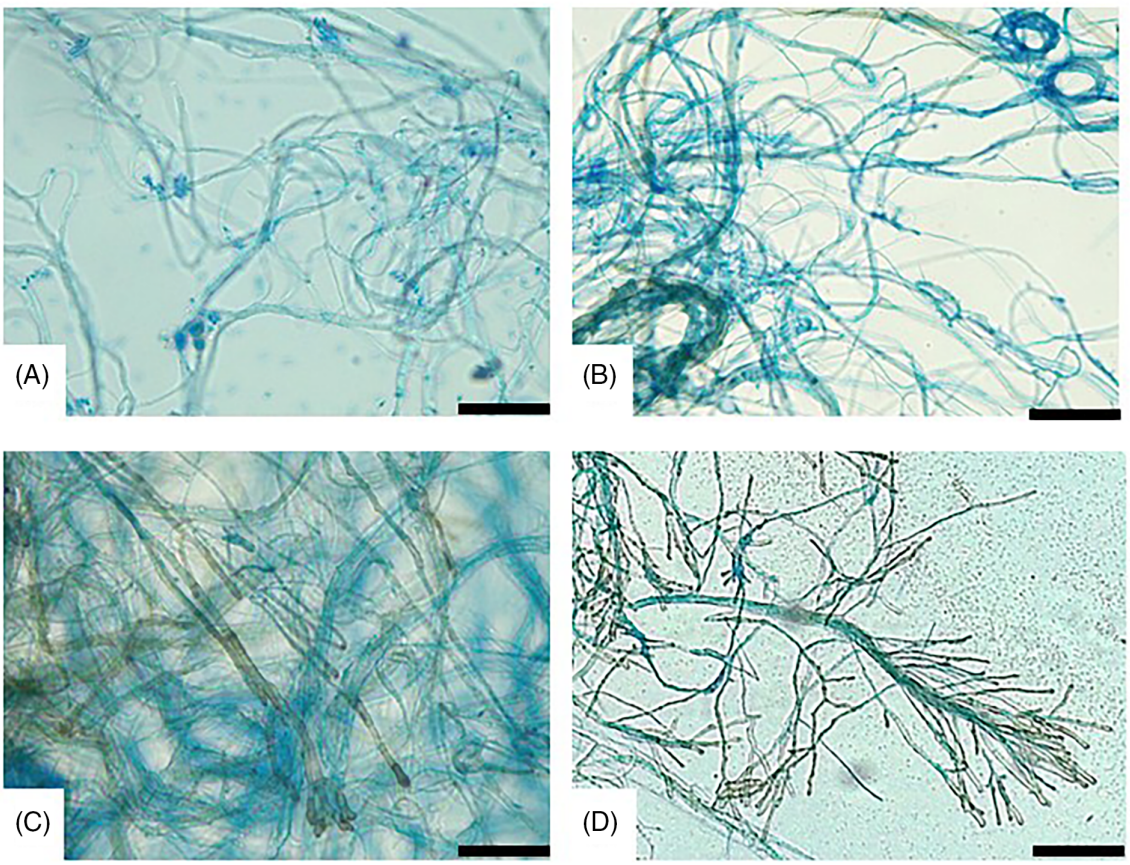

Figure 3. Microscopic images of *Gnomoniopsis smithogilvyi* MUM 21.93 when confronted with control agents. (A) *Gnomoniopsis smithogilvyi* MUM 21.93 and phosphate buffer saline. (B) *G. smithogilvyi* MUM 21.93 and Horizon® (tebuconazole, 0.10 g/L). (C) *G. smithogilvyi* MUM 21.93 and *B. amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL). (D) *G. smithogilvyi* MUM 21.93 and *Bacillus amyloliquefaciens* QST 713 (10^7 cells/mL). Bars indicate 50 μm.

could be due to the production of a dissimilar volatile profile or to the diffusion of different extracellular compounds in the medium, as previously described for other *B. amyloliquefaciens* strains.[29,30]

When facing HOR, *G. smithogilvyi* produced a particular morphological phenotype. Macroscopically, *G. smithogilvyi* produced a whitish and regular mycelium, similar to the control (Figs. 1 and 2(F)). On the other hand, at the microscopic level long, thin,

and highly coiled hyphae were observed (Fig. 3), showing to be different from all other treatments.

3.2 Proteomic analyses

The proteomic analyses were carried out by comparing the antifungal treatments (ASO, CIMO-BCA1, and HOR) with the negative control to elucidate the main proteins and metabolic pathways involved in their effects on the *G. smithogilvyi* proteome. A total of 1924 proteins were identified, with at least two peptides and FDR <1%. The number of proteins altered in abundance in *G. smithogilvyi* by the effect of the different control agents is given in Table 1 and Tables S1, S2, and S3.

3.2.1 C vs ASO

Regarding the proteins found when comparing C and ASO, the gene ontology enrichment analysis using ClueGO classified the affected metabolic routes or terms in more general groups. Most of the terms with proteins reduced in quantity were grouped in the 'L-methionine salvage from methylthioadenosine' pathway (15.22%; Fig. S1). Methionine biosynthesis is essential for plant infections in filamentous fungi.[31] Thus, this points to the fact that ASO alters the mechanisms involved in the mould's pathogenicity. The second group was the 'tetrahydrobiopterin biosynthetic process' (10.87%). The tetrahydrobiopterin (BH_4) is an essential cofactor that intervenes in numerous reactions and plays key roles in the cellular oxidative stress response in fungi and lipid biosynthesis regulation in the oleaginous fungus *Mortierella alpina*.[32]

The data showed that 33.33% of the terms increased in abundance comprised proteins related to the 'MICOS complex' (Fig. S2), which has a key function in the development and stability of mitochondrial cristae in eukaryotes, being involved in the aerobic respiration responsible for providing energy to the cell.[33] This complex of proteins is also essential for protein transport through the mitochondrial membrane. Additionally, 11.11% of terms belonging to 'Arp2/3 protein complex' group were increased in abundance (Fig. S2). The arp2/3 complex is involved in spore wall formation in fungi,[34] which explains the increase in conidiomata. This complex is also required for the integrity and mobility of actin patches and simultaneously controls the nucleation of actin polymerization and branching of the filaments.[35] Its increased quantity provoked by ASO may be responsible for the observed filament branching (Fig. 3).

Concerning some specific proteins that were altered in abundance (Table 2), the protein glutathione hydrolase was reduced on ASO treatment. This protein is involved in the antioxidant response of pathogenic fungi such as *Aspergillus nidulans*, indicating a major susceptibility of *G. smithogilvyi* to oxidative stress when ASO is present. An oxidative environment could trigger mycotoxin biosynthesis.[36,37] Furthermore, the chitin synthase was only found in the treatment with ASO, so this BCA stimulates the synthesis of chitin, a main constituent of the cell wall in moulds.[38] The actin-like protein 2 and actin-related protein 2/3 complex subunit were also increased in abundance in ASO. Due to the absence of available information in the literature about all the proteins identified in the present study, the activity of some of them was compared with their functions in different fungi, such as yeasts. For example, the aldo-keto reductase found in enhanced abundance has been identified as a stress response protein in yeasts,[39,40] which could indicate that ASO is causing stress on *G. smithogilvyi* that may activate mycotoxin production.[17,36,41]

3.2.2 C vs CIMO-BCA1

The enrichment analyses between the proteins found in C and CIMO-BCA1 showed a clearly different effect between CIMO-BCA1 and ASO on *G. smithogilvyi*, although both strains belong to the same species. There were 18.75% of terms that decreased in abundance when using CIMO-BCA1 and grouped in 'transcription factors binding' (Fig. S3), followed by 12.5% of the following

Table 1. Number of proteins altered in quantity in *Gnomoniopsis smithogilvyi* proteome when confronted with different antifungal agents

Treatment *vs* control	Proteins reduced in abundance	Proteins increased in abundance	Proteins only detected in the treatment	Proteins only detected in the control
ASO	54	21	24	30
CIMO-BCA1	74	99	65	16
HOR	99	79	6	113

ASO, *Bacillus amyloliquefaciens* QST 713 (10^7 cells/mL); CIMO-BCA1, *Bacillus amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL); HOR, Horizon® (active substance tebuconazole, 0.10 g/L).

Table 2. Changes in protein abundance of *Gnomoniopsis smithogilvyi* on a chestnut-based medium when confronted with ASO

Proteins	Function	Identification code	Log_2 fold change
Glutathione hydrolase	Antioxidant response	A0A2T3A832_9PEZI	−0.962
Chitin synthase	Cell wall components	A0A2T2ZY01_9PEZI	Only in ASO
Aldo-keto reductase	Stress response	A0A2T3AGS7_9PEZI	0.771
Actin-like protein 2	Cell wall components	A0A2P5HGQ4_9PEZI	0.308
Actin-related protein 2/3 complex subunit	Cell wall components	A0A2P5HZC1_9PEZI	0.457
Mannose-1-phospate guanyltransferase	Cell wall components	A0A2T3A7K1_9PEZI	0.727

ASO, *Bacillus amyloliquefaciens* QST 713 (10^7 cells/mL).

terms with proteins decreased in quantity: 'leucine metabolic process', 'glycine catabolic process', 'ether hydrolase activity' and 'response to extracellular stimulus'. In contrast, 40% of the terms found with proteins in higher quantity were grouped in the 'leucine metabolic process' (Fig. S4), showing that this pathway is deeply altered at different steps. Leucine biosynthesis is crucial for mould's virulence and iron and nitrogen acquisition in other fungal pathogens, such as *Aspergillus fumigatus*, suggesting this metabolic pathway is a target for antifungal treatments, including CIMO-BCA1.[42] Other groups whose terms included proteins that increased in abundance (20%) were 'COPII vesicle coat', 'Arp2/3 protein complex', and 'L-aspartate:2-oxoglutarate aminotransferase activity'.

Focusing on particular proteins of interest that had altered abundance, the cupin domain containing protein decreased in abundance (Table 3). This protein is responsible for maintaining the carbon/nitrogen balance and turnover in conidia in the pathogen *Fusarium oxysporum* f. *sp. cucumerinum*.[43] CIMO-BCA1 stimulated the production of Rho-GTPase activating protein 5, which mediates the signal cascade induced as a response to cell wall damage, leading to actin cytoskeleton biogenesis and ergosterol biosynthesis, the main sterol of the fungal cell membrane.[44] In addition, the acetyl CoA acetyltransferase (ERG10) found in higher relative quantity by CIMO-BCA1 treatment is involved in the first step of the ergosterol biosynthesis pathway, and its function is critical for cell viability.[44] Other proteins related to the CWI pathway, which is indispensable for hyphae growth, such as putative actin-related protein 3, actin-related protein 2/3 complex subunit 4, glycogen synthase, fimbrin, and chitin synthase, were increased in abundance in *G. smithogilvyi* when faced with CIMO-BCA1. Fimbrin is related to polarity during conidia germination, normal hyphal growth, and endocytosis in *A. nidulans*.[45] Summarising, the proteomic changes in *G. smithogilvyi* suggest that CIMO-BCA1 provokes mould cell wall damage and as a consequence of this stress, while *G. smithogilvyi* apparently responds by increasing its metabolic activity, leading to increased structures that form the mycelium. This explains why mycelium is denser in these samples (Fig. 2(E)) than in C. Another protein, the NmrA domain-containing protein, was only detected in C. The lower abundance of this protein, as occurred in CIMO-BCA1, increased the sensitivity to antifungals in *Aspergillus flavus* and diminished the invasive virulence of this mould.[46]

The differences between the targeted proteins by the two biocontrol agents described until this point—ASO and CIMO-BCA1—raise the possibility of finding new alternatives or combinations that our study proposes to better control this pathogen. The combination of these two biocontrol agents, with impact in the same pathway but in different proteins, could achieve a synergistic effect that should be explored in future studies.

3.2.3 C vs HOR

Gene ontology enrichment in C and HOR revealed 33.33% of the terms with decreased proteins in the 'omega peptidase activity' pathway (Fig. S5). Most of the terms that included proteins that increased in abundance were grouped in the 'regulation of mitotic nuclear division' (51.61%; Fig. S6). These results are in agreement with the expected effect of tebuconazole, the active substance in HOR, which is a sterol biosynthesis inhibitor that could be stimulating the mitosis[47] in *G. smithogilvyi* as a response mechanism of the mould to overcome that stress.

The proteins thioredoxin and thioredoxin-like fold domain-containing protein were found to decrease in abundance in HOR treatment (Table 4). The deletion of the gene that codified the thioredoxin decreased growth and the ability to form reproductive structures, such as conidiophores or cleistothecia.[48] Furthermore, the Hypa protein, which also decreased in quantity, is involved in hyphal morphogenesis through an apparent role in regulating endomembrane traffic, as described in *A. nidulans*.[49] Additionally, ergosterol synthesis is affected by the observed decrease in the protein ERG10.

The impact of tebuconazole provoked higher levels of proteins related to CWI. Among these, the Rho-type small GTPase increased. Other proteins related to ergosterol synthesis were increased in HOR treatment, such as the sterol 24-C-methyltransferase (ERG6), which transforms the zymosterol in fecosterol, one of the last steps in ergosterol synthesis. Although the roles of ergosterol are mostly associated with the regulation of the fungal cell membrane fluidity and structure, recent studies have recognized multiple cell targets of this compound, including its impact on cell wall structure and functionality.[50] In *A. fumigatus*, azole exposure up-regulated the *erg6* gene, and its mutations were related to azole-resistant isolates.[51] Because of the stress suffered in the cell wall, *G. smithogilvyi* produces more proteins involved in cell wall formation, such as fimbrin, actin-like protein 2, actin-related protein 2/3 complex subunit 4, and mannose-1-phosphate guanyltransferase, as a mechanism of survival.

Gs has been recently described as a mycotoxin producer when confronted with each of the control agents described (ASO, CIMO-BCA1, and HOR) at subinhibitory levels. As the proteomic analyses have shown, these agents provoked different kinds of

Table 3. Changes in protein abundance of *Gnomoniopsis smithogilvyi* on a chestnut-based medium when confronted with CIMO-BCA1

Proteins	Function	Identification code	Log_2 fold change
Cupin domain containing protein	Carbon/nitrogen balance	A0A2P5IFR2_9PEZI	−0.259
Rho-GTPase activating protein 5	Cell wall damage response	A0A194VDD3_9PEZI	Only in CIMO-BCA1
Acetyl CoA acetyltransferase	Ergosterol synthesis	A0A423VW16_9PEZI	0.209
Putative actin-related protein 3	Cell wall components	A0A0G2I5G9_9PEZI	0.274
Actin-related protein 2/3 complex subunit 4	Cell wall components	A0A2T3ALY6_9PEZI	0.347
Glycogen synthase	Cell wall components	A0A2T3ALA2_9PEZI	0.458
Fimbrin	Cell wall components	A0A2T3A2S2_9PEZI	0.667
Chitin synthase	Cell wall components	A0A2T2ZY01_9PEZI	Only in CIMO-BCA1
NmrA	Invasive virulence and antifungal resistance	A0A423WD71_9PEZI	Only in C

CIMO-BCA1, *Bacillus amyloliquefaciens* CIMO-BCA1 (10^7 cells/mL).

Table 4. Changes in protein abundance of *Gnomoniopsis smithogilvyi* on a chestnut-based medium when confronted with HOR

Proteins	Function	Identification code	Log_2 fold change
Thioredoxin	Redox system	Q1A3Q0_CRYPA	−2.671
Thioredoxin-like fold domain-containing protein	Redox system	A0A0G2ICU3_9PEZI	−0.872
Hypa	Hyphal morphogenesis	A0A2T3AJN4_9PEZI	−0.426
Acetyl CoA acetyltransferase	Ergosterol biosynthesis	A0A2T3A020_9PEZI	−1.569
Rho type small GTPase	Cell wall damage response	A0A2T2ZXV8_9PEZI	0.786
Sterol 24-C-methyltransferase	Ergosterol biosynthesis	A0A0G2HQM5_9PEZI	0.945
Fimbrin	Cell wall components	A0A2T3A2S2_9PEZI	0.581
Actin-like protein 2	Cell wall components	A0A2P5HGQ4_9PEZI	0.457
Actin-related protein 2/3 complex subunit 4	Cell wall components	A0A2T3ALY6_9PEZI	0.478
Mannose-1-phospate guanyltransferase	Cell wall components	A0A2T3A7K1_9PEZI	0.727

HOR, Horizon® (active substance tebuconazole, 0.10 g/L).

stress on the mould, mainly affecting the cell wall structures and the antioxidant response, which may trigger mycotoxin biosynthesis as a defence mechanism. This strategy to cope with stress has been reported for other mycotoxin producers, such as *Penicillium verrucosum*, *Penicillium nordicum*, *Penicillium expansum*, and *Alternaria* spp.[17,18,52,53]

Although these agents have been shown to increase the production of mycotoxins, the identified proteins are apparently not directly related to their synthesis. The main limitation of this study is that the proteins involved in their synthesis cannot be discussed due to the lack of knowledge about diplodiatoxin and the 3-nitropropionic acid biosynthesis pathway and the absence of a database with *G. smithogilvyi* proteome. In this sense, most studies based on the relationship between proteins and mycotoxin biosynthesis are limited due to the technical restrictions of the equipment to detect proteins that are produced in lower quantities, such as mycotoxin-related proteins, compared to proteins produced in higher quantities, such as energy-related or structural proteins.[54–56] This inconvenience combined with the absence of information about the proteome of *G. smithogilvyi* and the biosynthetic pathway of these two new mycotoxins hindered the approach to study the mycotoxin biosynthetic pathway(s).

In summary, the treatments ASO and CIMO-BCA1 have different antifungal modes of action against *G. smithogilvyi*, although these strains belong to the same bacterial species. This finding opens up the possibility of exploring the combination of these treatments, which deserves to be explored in future studies. On the other hand, HOR had an impact on *G. smithogilvyi* cell wall structures that is closer to the effect observed for CIMO-BCA1. This suggests that CIMO-BCA1 can replace the use of chemical compounds with an equivalent efficacy, although inhibitory concentrations should be used to totally inhibit the growth of *G. smithogilvyi* and explore its combined effect with ASO.

4 CONCLUSIONS

Proteomic analyses revealed that each agent in the present study uses different effective modes of action that impact the growth of *G. smithogilvyi*, although they all mainly alter different components of the fungal cell wall. Mycotoxin production by *G. smithogilvyi* could be a consequence of the stress suffered and displayed by the proteome changes, although further studies are needed to determine the induction of diplodiatoxin and 3-NPA because of the lack of data about their biosynthesis pathways. Additionally, it has been demonstrated that a biocontrol agent (CIMO-BCA1) can target different proteins related to fungal cell wall than ASO. This finding highlights the possible potential of the combined effect of these two low-impact environmental biocontrol agents, which will be studied in further experiments. Thus, these BCAs could contribute to *G. smithogilvyi* control and help to substitute the use of toxic chemicals such as tebuconazole to combat plant pathogens worldwide.

AUTHOR CONTRIBUTIONS

Conceptualisation: PR and AS. Methodology: PR, AS, IA, and JD. Formal analysis: PR, AR, MÁ, and JD. Investigation: PR, IA, and MÁ. Resources: PR. Writing—original draft preparation: PR and MÁ. Writing—review and editing: PR, MÁ, and JD. Supervision: PR and JD. Project administration: PR. Funding acquisition: PR and JD. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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