Article

Could *Gnomoniopsis castaneae* Be Used as a Biological Control Agent against Insect Pests?

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Abstract: *Gnomoniopsis castaneae* is the cause of the chestnut brown rot but has been also regarded as an important mortality factor for the chestnut gall wasp *Dryocosmus kuriphilus*. The question to whether *G. castaneae* could serve as a natural biocontrol agent against insect pests is investigated in the present study. We used three serious insect pests as experimental model insects: *Plodia interpunctella* and *Trogoderma granarium*, which are important pests of stored products, and *Myzus persicae*, a cosmopolitan, serious pest of annual and perennial crop plants. Although chemical pesticides represent effective control means, they are also related to several environmental and health risks. In search for alternative pest management methods, scientific interest has been focused, inter alia, on the use of entomopathogenic fungi. While *Isaria fumosorosea* has long been recognized as an effective control agent against several pests, *G. castaneae* has been very little studied. The present study examined whether and to what extent *G. castaneae* and *I. fumosorosea* exhibit insecticidal activity against fourth-instar larvae of *P. interpunctella* and *T. granarium* and adults of *M. persicae*. Mortality was examined in interrelation with dosage and time exposure intervals. Both fungi exhibited pesticidal action. However, *G. castaneae* induced noteworthy mortality only at very high doses. In general, we concluded that *G. castaneae* failed to cause high insect pathogenicity at normal doses and may not be an efficient biocontrol agent compared with other entomopathogens. On the other hand, our study reiterates the pathogenic potential of *I. fumosorosea*. More studies are needed to further our insight into the potential of EF species as a component of IPM.

Keywords: biocontrol; *Gnomoniopsis castaneae*; *Isaria fumosorosea*; *P. interpunctella*; *T. granarium*; *M. persicae*; survival time

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1. Introduction

Entomopathogenic fungi (EPF) make up a specialized group of fungi which comprise numerous genotypes [1]. Within the framework of the biocontrol of insect pests in field crops and storage facilities, EPF fungi have attracted extensive interest in recent years [2]. EPFs inhabit a wide range of dwellings including urban environments, meadow land, dry, and agricultural places. They demonstrate high virulence against many insects and have minimal residual effects.

Insect pathogens have a biological cycle that is synchronized with that of the host but also adapted to the prevailing environmental conditions [3]. Infection can be restricted by low levels of relative humidity, shortage of nutrients in the cuticle, as well as the inability to recognize the host due to the absence of contributing factors [4,5]. The degree of the infection depends on the infectivity of the fungus, the defensive resilience of the insect, and several other biological and abiotic factors [3]. In the 1990s, *Isaria fumosorosea* Wize

(Hypocreales: Clavicipitaceae) was reported as the most common entomopathogenic fungus in Asia, Russia, the USA, South America, and India, infecting *Bemisia* spp. (Hemiptera: Sternorrhyncha) and *Trialeurodes vaporariorum* (Hemiptera: Sternorrhyncha) [6]. Depending on the strain, *I. fumosorosea* has a wide range of hosts and could be effective against several insect pests [7–10]. For example, it has been proved highly effective against *Plutella xylostella* L. (Lepidoptera: Plutellidae) [11–13]. In addition, it has been commercially available all over the world [14].

On the other hand, *Gnomoniopsis castaneae* G. Tamiatti (Sordariomycetes: Gnomoniaceae) has been recognized as the cause of the chestnut brown rot in Europe and Australasia [15–18]. The chestnut brown rot which has appeared in several chestnut areas around the world, could be the most threatening disease of chestnuts, causing losses to post-harvest yields as high as 60–70% [19]. Additionally, *G. castaneae* causes chestnut canker symptoms in several countries worldwide [20,21]. While its origin has not been definitively established yet, *G. castaneae* is a cryptic species which has been reported in Australasia and India on Japanese (*Castanea crenata* Sieb. Zucc. (Angiosperms: Fagales) and European (*C. sativa* Mill.) chestnut.

G. castaneae exists endophytically in most chestnut organs and tissues, in an asymptomatic manner unless favorable environmental conditions trigger its emergence [22,23]. For instance, the chestnut brown rot is aggravated by rainfall during flowering [24], most probably because flowers are infected by ascospores dispersed from dead burrs in the orchard [18]. In Italy and Switzerland, a noteworthy aggravation of chestnut brown rot took place after the invasion of the *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) chestnut gall wasp in the mid-2000s [25,26]. However, after studying the dead *D. kuriphilus* galls, Magro, et al. [27] reported that the galls and the associated leaves had in fact been killed by *G. castaneae* before the adults flew in. Upon this basis, the question arises as to whether *G. castaneae* could serve as a natural agent for the control of the chestnut gall wasp and other insect pests [28]. Apart from its profound phytopathogenic behavior, field observations and lab bioassays have demonstrated the possibility for *G. castaneae* to act also as an entomopathogenic fungus against chestnut pests, such as the chestnut gall wasp [27–31].

For this study, we recorded the survival of three major pests, the Indian Meal Moth *Plodia interpunctella* Hübner (Lepidoptera, Pyralidae), the Khapra beetle *Trogoderma granarium* Everts (Coleoptera, Dermestidae), and the Green Peach Aphid *Myzus persicae* Sulzer (Hemiptera, Aphididae). The present study was designed to investigate the potential of *G. castaneae* as a biocontrol agent in comparison to a common EPF species, *I. fumosorosea*.

2. Materials and Methods

2.1. Insects

In order to evaluate the potential insecticidal action of *G. castaneae* on many different targets, three very diverse insect species (one beetle, one moth, and one aphid) were selected for experimentation. *T. granarium* was kept on whole wheat (var. Mexa), and only adults of mixed sex (younger than two weeks old) were used for experimental purposes. Uninfested grains were infested with adults; after one week, the adults were taken away, and the infested grains were kept in ambient conditions to obtain individuals of standardized age.

M. persicae was obtained from the Plant Protection Institute of Patras, ELGO Dimitra and was maintained on the potted pepper plant variety Stavros Peloponnesus (*Capsicum annuum* L., Solanales: Solanaceae). We pre-germinated the pepper plants in 2 × 2 cm pots, sowing only one seed per pot at 1 cm in depth with Pindstrum plus peat substrate; we then transplanted them into 1 L pots with Pindstrum plus type peat substrate. Insects developed in a growth chamber (PHC Europe/Sanyo/Panasonic Biomedical MLR-352-PE), in stable conditions (27 ± 1 °C, 65 ± 5% RH, complete darkness). Throughout their developmental cycle, insects were in a room with pepper plants, at 25 ± 1 °C, 60–70% RH, and photoperiod 16/8 h (L/N) constantly.

Finally, the rearing of *P. interpunctella* took place in $25 \times 45 \times 30$ cm cages. The frame of the cages, their sides, their back, and their floor were made of medium-density fiberboard (MDF); two sides were covered with a plastic mesh, and the front of the cage, which served as an entrance to the interior, was covered with a small cross-section plastic mesh. To each cage corresponded 15 to 20 pairs of adult insects. Sugar water was offered to adults with 1 cm cotton slices, placed in a Petri dish with water, on the floor of the cage. In the cages with *P. interpunctella*, we placed transparent plastic cassettes containing 200 g of whole flour, 100 g of bran, 80 g of brewing yeast, 130 mL of pure honey, and 100 mL of glycerol as artificial spawning substrate. The cassettes remained in the cages for two days and were then transferred to a dark place until the insects completed their biological cycle. We initially covered the cassettes with tulle to prevent the new larvae from escaping. The larvae were age-distributed in transparent containers to ensure their smooth development and equal food intake. The remaining food was stored at 4°C until reused. Throughout their development, the insects remained in a room in stable conditions, at a temperature of $25 \pm 1^\circ\text{C}$, humidity of 60–70%, and photoperiod 16/8 h light/dark.

2.2. Fungal Isolates

G. castaneae was isolated for the first time in 2015, from symptomatic chestnuts collected from European sweet chestnut orchards in Greece [32]. A single-spore isolate of *G. castaneae* belonging in the fungal collection of the Forest Research Institute was used.

I. fumosorosea (strain name: Ir Achaia) was a local strain from the Achaia region in Southern Greece. We kept the dishes at a temperature of $25 \pm 2^\circ\text{C}$ in the dark to ensure that the fungi incubated and developed properly. We isolated the developed fungi once again to ensure a clear cultivation. The conidia were collected from cultures which had grown on SDA dextrose agar (Sabouraud Dextrose Agar, OXOID LTD). We kept the isolates at $25 \pm 1^\circ\text{C}$, in Petri dishes containing a nutrient SDA material and we renewed them monthly. After 15 days, we collected fresh conidia from the SDA cultures to transfer them to a 500 mL glass beaker containing 100 mL of sterile distilled water with 0.05% Tergitol NP9. We filtered the conidial suspension through 10–12 layers of sterile cloth to separate hyphal debris and then mixed it with a magnetic stirrer for 5 min. By means of a stereomicroscope, we examined bark samples for the teleomorphic stage of the fungus.

The viability of all test fungi was determined by spreading a 100 μL aliquot of a conidia suspension (1×10^6 conidia mL^{-1}), prepared with a sterile surfactant solution (0.1% v/v) of Tween 80, on SDA medium in Petri dishes (90×15 mm) and incubated in the dark at $25 \pm 1^\circ\text{C}$. Plates of *Isaria* and *Gnomoniopsis* were incubated for 16 and 18 h, respectively, prior to evaluation. Conidia were scored as viable if any germ tube was $2 \times$ longer than the diameter of the spore; a total of 100 conidia per sample were examined under $400\times$ magnification.

Conidial viability was calculated based on the formula below:

Viability (%) = $[G1/(G1 + G2)] \times 100$, where G1 refers to the number of germinated conidia, G2 is the number of non-germinated conidia, while the sum of G1 and G2 is equal to 100. Thus, the viable conidia percentage was determined by counting a total of 100 conidia per fungal sample. Fungal strains presenting $\geq 90\%$ viability was used in insect bioassays.

2.3. Bioassays

2.3.1. Virulence Bioassay for *T. granarium* and *P. interpunctella* Larvae

For *T. granarium* and *P. interpunctella*, we used 500 g of wheat which we placed in 0.45 L round glass jars. Two milliliters of conidial suspension were applied on the wheat at rates of 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , and 1×10^8 conidia per ml, using a Potter spray tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) at 1 kgf cm^{-2} . We adjusted the suspensions following Gurulingappa, et al. [33], using a Neubauer hemocytometer (TIEFE 0.100 mm $1/400$ 9 mm). After the application of the fungi, we returned the wheat lots to the jars and shook them manually for 30 s to ensure

that the fungi would be equally distributed to the contents. The controls consisted of separate series of lots which were sprayed with distilled water plus 0.05% Tergitol NP9. From each jar, we took three 20 g samples and placed them in cylindrical plastic vials which were 3 cm in diameter and 8 cm in height. The lid of the vials was open in the center, which was covered with a fine mesh; the interior of the “neck” of the vials was covered with Fluon (Northern Products, Woonsocket, RI, USA) to prevent the escape of individuals. Ten *T. granarium* and *P. interpunctella* fourth-instar larvae were allocated per each vial (we used separate vials for each species). We placed the vials in plastic boxes containing saturated solutions of sodium chloride to stabilize the relative humidity at 75%. The plastic boxes were finally placed in incubators set at 27.5 °C and 75% R.H. We checked mortality daily for a period of 7 days. Larvae treated with *I. fumosorosea* were used as positive control in this study.

2.3.2. Virulence Bioassay for *M. persicae*

In the case of *M. persicae*, we rinsed the leaves of the pepper plants with tap water for 15 min, we then washed them three times with distilled water, and we finally air-dried them in a sterile laminar-flow hood. Working in the sterile hood, the leaves of the pepper were placed on 1.5% agar in plastic 90 mm² petri dishes. The 1.5% agar was nutrient-free, but it supplied leaves with water so as to maintain 100% of relative humidity throughout the experiment. In the presence of aphids, the leaves sustained a satisfactory condition for at least 7 days. We sprayed the experimental leaves with the desired conidial suspension using a Potter spray tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) at 1 kgf cm^{−2}. We transferred the treated petri dishes into lid-covered plastic boxes. We applied 2 ml of conidial suspension at rates of 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , and 1×10^8 conidia per ml. Suspensions were adjusted as described. We determined the viability of conidia after 24 h, by performing a repeat germination test for each stock suspension following Goettel and Inglis [34]; this was done to secure the constancy of the viability assessments. In all cases, the viability of conidia was on average exceeding 95%. The controls consisted of separate series of lots which were sprayed with distilled water plus 0.05% Tergitol NP9. We placed 10 3-day-old aphid adults on each leaf, settled for 30 min after conidial treatment. We checked mortality daily for 7 days. *I. fumosorosea* was used as the positive control.

2.4. Quantification of Conidial Production on Insect Cadavers

After incubating the dead individuals in a humid chamber, five dead individuals covered with conidia (sporulated cadavers) were randomly removed from each sample of fungal treatment, surface-sterilized with sodium hypochlorite (1% v/v) for 30 s, rinsed twice in sterile distilled water for 30 s, and then the cadavers were placed in a humid chamber maintained at 26 °C with 14 h photophase for 14 days. Cadavers bearing conspicuous masses of aerial conidia were placed individually in 1.5 mL Eppendorf tubes with 1 mL of a sterile solution of 0.1% Tween-80 and vigorously vortexed for 1 min to dislodge the conidia from the insects' body. Subsequently, these same tubes were vortexed vigorously for 1 min and left in an ultrasonic bath for 2 min to enhance the suspension of conidia. Conidial density was calculated by hemocytometer counts at 40× magnification to determine the average of conidial production per insect. Three insect species and two repeated assays were used for each fungal strain, totaling 10 cadavers per treatment. Altogether, the conidial production was measured in 60 cadavers (2 treatments × 2 experimental replicates × 5 cadavers × 3 insect species).

2.5. Data Analysis

We arcsine-transformed the mortality values to stabilize variance. Using the general linear model of the SPSS (SPSS Inc., IL, New York, USA, ver. 25), we then analyzed the data with univariate ANOVA for a multi-factor analysis. Where significant F values occurred, we compared the means with the Bonferroni test. We calculated LC50 values by Probit

analysis with 95% confidence interval (CI) of the SPSS (SPSS Inc., IL, USA, ver. 25). The mean survival time of larvae and adults was determined with the Kaplan–Meier method.

3. Results

3.1. Virulence of the Tested Fungi on Larvae of *P. interpunctella*

Significant differences in larval mortality were detected among treatments ($F = 30.784$, $df = 1.1832$, $p < 0.001$), exposure time ($F = 7.022$, $df = 6.1832$, $p < 0.001$), and used EF concentrations ($F = 23.536$, $df = 6.1832$, $p < 0.001$). The main effects and interactions for all factors proved to be significant (Treatment * Exposure time: $F = 4.123$, $df = 6$, $p < 0.001$; Treatment*Concentration: $F = 2.570$, $df = 5$, $p = 0.028$; Exposure time * Concentration: $F = 8.464$, $df = 30$, $p < 0.001$; Treatment * Exposure time * Concentration: $F = 3.660$, $df = 30$, $p < 0.001$).

The mortality percentage depended on the dose of the used fungi. Final mortality percentages (7 days after exposure) of *P. interpunctella* larvae ranged from 10 to 86.5% with *I. fumosorosea* and from 6.7 to 50% with *G. castaneae*. Control treatment (using only ddH₂O) demonstrated minor mortality (3.3%) at the end of the experiment (Figure 1). The survival time of *P. interpunctella* larvae treated with *I. fumosorosea* and *G. castaneae* was significantly reduced in comparison to the survival time of the control treatment. More specifically, after exposure to *I. fumosorosea*, the survival time of the larvae ranged from 6.56 to 4.6 days, and after exposure to *G. castaneae*, from 6.73 to 5.96 days. For control larvae, the survival time was 6.9 days ($F: 3.667$; $df: 12$; $p = 0.030$).

Figure 1. Average ($\% \pm sd$) larval mortality of *P. interpunctella* treated with conidial suspensions of *I. fumosorosea* and *G. castaneae* at six concentrations ($1:10^3$, $2:10^4$, $3:10^5$, $4:10^6$, $5:10^7$, $6:10^8$).

3.2. Virulence of the Tested Fungi on Larvae of *T. granarium*

The final mortality (7 days after exposure) percentages of *T. granarium* larvae ranged from 10 to 73.3% with *I. fumosorosea* and from 3.3 to 36.7% with *G. castaneae* (Figure 2). Control treatment mortality amounted to 3.9% at the end of the experiment. The mortality percentage depended on the concentration of the used fungi. The survival time of *T. granarium* larvae treated with *I. fumosorosea* and *G. castaneae* was statistically significantly different in comparison to the survival time of control larvae. More specifically, after exposure to *I. fumosorosea*, the survival time of the larvae was 6.66–4.63 days, after exposure to *G. castaneae*, it was 6.82–6.23 days, while after the control treatment, it was 6.9 days ($F: 5.902$; $df: 12$; $p = 0.015$).

Figure 2. Average ($\% \pm \text{sd}$) larval mortality of *T. granarium* treated with conidial suspensions of *I. fumosorosea* and *G. castaneae* at six concentrations ($1:10^3$, $2:10^4$, $3:10^5$, $4:10^6$, $5:10^7$, $6:10^8$).

Significant differences in the mortality of *T. granarium* larvae were detected among Treatments ($F = 55.706$, $df = 1.1832$, $p < 0.001$), Exposure time ($F = 4.503$, $df = 6.1832$, $p < 0.001$), and used Concentrations ($F = 21.546$, $df = 6.1832$, $p < 0.001$). The main effects and interactions for all factors proved to be significant (Treatment * Exposure time: $F = 7.164$, $df = 6$, $p < 0.001$; Treatment * Concentration: $F = 3.748$, $df = 5$, $p = 0.003$; Exposure time * Concentration: $F = 5.195$, $df = 30$, $p < 0.001$; Treatment * Exposure time * Concentration: $F = 7.804$, $df = 30$, $p < 0.001$).

3.3. Virulence of the Tested Fungi in Adults of *M. persicae*

Significant differences in *M. persicae* adult's mortality were detected among Treatments ($F = 71.062$, $df = 1.1832$, $p < 0.001$), Exposure time ($F = 6.347$, $df = 6.1832$, $p < 0.001$), and used Concentrations ($F = 18.105$, $df = 6.1832$, $p < 0.001$). The main effects and interactions for all factors proved to be significant (Treatment * Exposure time: $F = 7.994$, $df = 6$, $p < 0.001$; Treatment * Concentration: $F = 4.430$, $df = 5$, $p < 0.001$; Exposure time * Concentration: $F = 4.668$, $df = 30$, $p < 0.001$; Treatment * Exposure time * Concentration: $F = 4.317$, $df = 30$, $p < 0.001$).

On day 7 after exposure, the final mortality percentages of *M. persicae* adults were 26.7 to 86.7% with *I. fumosorosea* and 6.7 to 36.7% with *G. castaneae* (Figure 3). For the control, larvae mortality was 6.7% at the end of the experiment. The mortality percentage was dose dependent. The survival time of *M. persicae* adults treated with *I. fumosorosea* and *G. castaneae* was statistically significant in comparison to the survival time of control adults. More specifically, the survival time of adults was 6.43–4.46 days after exposure to *I. fumosorosea* and 6.56–5.96 days after exposure to *G. castaneae*. For control adults, the survival time was 6.76 days ($F: 6.117$; $df: 12$; $p = 0.017$).

Figure 3. Average ($\% \pm \text{sd}$) adult mortality of *M. persicae* treated with conidial suspensions of *I. fumosorosea* and *G. castaneae* at six concentrations ($1:10^3$, $2:10^4$, $3:10^5$, $4:10^6$, $5:10^7$, $6:10^8$).

3.4. Lethal Concentration of the Fungi

Probit analysis results showed that the median lethal concentrations (LC50) of *I. fumosorosea* after a 7-day exposure were: 8.3×10^4 conidia/mL (for *P. interpunctella*), 3.1×10^5 conidia/mL (for *T. granarium*), and 6.5×10^4 conidia/mL (for *M. persicae*). The respective estimated values for *G. castaneae* were: 9.3×10^6 conidia/mL (for *P. interpunctella*), 1.5×10^9 conidia/mL (for *T. granarium*), and 1.8×10^8 conidia/mL (for *M. persicae*). Significant differences in LC50 values were recorded between the two different entomopathogenic fungi in all cases (Table 1).

Table 1. Pathogenicity of *I. fumosorosea* and *G. castaneae* against *P. interpunctella*, *T. granarium*, and *M. persicae*. The value of each χ^2 refers to the probability of the angular coefficient >0 . LC—values and slopes (in g/L) were estimated by the probit procedure.

Treatment	Insect	Slope	Intercept	R ²	LC50 (95% CI) Conidia/mL	Chi-Test (χ^2) Sig
<i>I. fumosorosea</i>	<i>P. interpunctella</i>	0.472	2.205	0.991	8.3×10^4 (14.7×10^3 – 4.6×10^5)	0.994
<i>G. castaneae</i>		0.448	1.431	0.951	9.3×10^6 (1.1×10^5 – 7.6×10^7)	0.653
<i>I. fumosorosea</i>	<i>T. granarium</i>	0.366	2.625	0.982	3.1×10^5 (3.5×10^4 – 2.7×10^6)	0.998
<i>G. castaneae</i>		0.254	2.412	0.934	1.5×10^9 (3.6×10^8 – 6.8×10^{10})	0.063
<i>I. fumosorosea</i>	<i>M. persicae</i>	0.331	3.073	0.983	6.5×10^4 (6.3×10^3 – 6.6×10^5)	0.997
<i>G. castaneae</i>		0.339	1.858	0.959	1.8×10^8 (9.9×10^6 – 3.4×10^{10})	0.845

3.5. Conidial Production by Sporulated Cadavers

Conidial production on the infected cadavers varied strongly among fungal strains ($F = 3902$, $df = 10$, $p < 0.0001$), with the *Isaria* strain producing higher numbers of conidia per cadaver for all tested species (6.5×10^8 conidia per *T. granarium*, 3.8×10^8 conidia per

P. interpunctella, 2.1×10^8 conidia per *M. persicae*). On the other hand, relevant *G. castaneae* post-mortem conidial production was 1.5×10^4 conidia per *T. granarium*, 2.3×10^4 conidia per *M. persicae* cadavers, and 1×10^5 conidia per *P. interpunctella* cadaver.

4. Discussion

Although EPFs are already available for commercial use [35–40], basic and applied research is still necessary to improve their virulence and enhance their role in Integrated Pest Management (IPM). Although the original habitat underlines the pathogenicity of EPF species [41], evaluation assays of potential isolates should not be targeted only at the original host, but also at various insect pests, as it was clearly indicated by our results.

We used two major stored product pests for experimentation, because there is a plethora of publications on the effect of EPF species against insect pests of stored products, often with contradictory results [2,37,40,42–50]. Demirci, et al. [51] stated that the entomopathogen *Isaria farinosa* achieved high pathogenic activity on the citrus mealybug, especially under humid conditions, and caused high mortality within six days of incubation. Also, Cherry, et al. [46] showed that different isolates of *M. anisopliae* and *B. bassiana* can achieve a satisfactory control of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) by immersion bioassay at 12 d, whereby *B. bassiana* proved more virulent than *M. anisopliae*. Khashaveh, et al. [47] claimed *B. bassiana* to be successful against stored wheat pests. In a similar vein, Wakefield, et al. [48] and Storm, et al. [49] reported that some *B. bassiana* isolates can achieve 100% mortality of the organophosphate resistant strain of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) after 10 d of treatment at 1×10^8 conidia/mL. In older studies, the mortality of stored grain pests reached 80–100% after 10–20 t the same doses [2,50]. Ekesi, et al. [52] showed that the efficacy of *M. anisopliae* and *B. bassiana*, was over 90% on treated larvae of the African tephritid fruit fly.

We used *I. fumosorosea* as a positive control given that it has demonstrated noteworthy virulence (70–100%) in numerous studies against many insect pests [53–55] and has often exhibited the highest pathogenicity in comparison with other EPF species [56]. The current study mainly focused on the entomopathogenic potential of *G. castaneae* in comparison with that of *I. fumosorosea*. Although *Gnomoniopsis castaneae* proved to be entomopathogenic, it caused low pathogenicity to *M. persicae* and *T. granarium*, but significant mortality levels, especially at the highest conidial concentration, to *P. interpunctella*. On the other hand, the *I. fumosorosea* native strain caused a mortality of 86.7% to *P. interpunctella*, 86.7% to *M. persicae*, and 73.3% to *T. granarium* at high conidial doses. On the other hand, Michalaki, et al. [57] found that *I. fumosorosea* was not effective against adults and larvae of *Tribolium confusum* (Coleoptera: Tenebrionidae) Jacquelin du Val and of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae).

The dependency of a fungal disease on conidial concentration normally indicates that a higher conidial density will often result in a faster control of the targeted insects. Consequently, where large insect pest populations exist, higher conidial concentrations are more effective to contain pest populations from incurring economic damages. However, low conidial concentrations can also be used in the initial developmental stages of pest populations to maintain them below economically damaging thresholds. Tuncer, et al. [58] and Kushiyevev, et al. [8] showed that the LT50 and LT90 values of *M. anisopliae* and *B. bassiana* isolates applied to females of the European gypsy moth and of the Black timber bark beetle decreased by increasing conidial concentrations. Shams, et al. [59] used probit analysis to estimate the LT50 of *B. bassiana* at 10.45 days against *C. maculatus* and *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). In our experiment, the survival time of larvae of *P. interpunctella* and *T. granarium* and of adults of *M. persicae* depended on the dose of the fungi. In the case of *G. castaneae*, the survival time of the tested insects was very high in relation to the survival time of *I. fumosorosea* in the same insects and at the same doses. This indicates that *G. castaneae* did not generate a high enough entomopathogenic impact on the tested insects.

The quality of nutrients in host cadavers may also be an important factor for in-spore production. This depends greatly on the enzymatic machinery of the fungus to utilize all components of the dead host. It has been postulated that a fungus producing large numbers of infective propagules could enhance fungal population density in host environments and the likelihood of conidial transmission or spread in the host population. However, we found a relationship between virulence and conidial production on cadavers, which means that highly lethal EPF strains such *I. fumosorosea* are necessarily prolific in producing offspring from cadavers, as they require shorter time and smaller inoculum doses to kill insects than low-virulence EPF strains like *G. castaneae*. In agreement with our results, the relationship between virulence and conidial production has been also verified for EPF strains infesting the Mediterranean corn borer *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) [60].

The insecticidal efficacy of EPF depends on many other factors such as the behavior of the insect, its age, its population density, nutrition, genetics, as well the degree of host physiology and morphology tolerance to biological control agents [61]. In this sense, variation in insect susceptibility to EPF could not be accounted for only in terms of the applied conidial concentration [62]. *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), which had been exposed to *B. bassiana*, was marked by reduced reproductive ability of its progeny [63]; *Aedes aegypti* L. (Diptera: Culicidae) larvae, which had survived infection from the fungal pathogen *Leptolegnia chapmanii* Seymour (Peronosporomycetes: Saprolegniales), had their reproduction disrupted, thus laying fewer eggs [64]. *Greater wax moth* larvae developed only low resistance to *B. bassiana* even after 25 generations of selection [65].

The entomopathogenic action of *G. castaneae* is not a surprise, as it has been considered the principal mortality factor for the oriental chestnut gall wasp and, especially, the main factor for the inability of adult wasps to emerge [66]. This insecticidal action of *G. castaneae* has been associated with various causes such as spore production, host population, and ecological factors [28,66].

5. Conclusions

In the current study, *G. castaneae* failed to cause high pathogenicity against larvae of *P. interpunctella* and *T. granarium* and adults of *M. persicae*. Drawing on these results, *G. castaneae* may not be the ideal EPF candidate to select for a biological control strategy. However, it should be taken under consideration that virulence is not the only desired characteristic of an effective biocontrol agent. Compatibility with other IPM methods (physical control, cultural measures, etc.), cost of mass production, difficulty in handling, ease of application, performance in challenging environments, shelf-life, formulation issues, and many other parameters should also be tested when evaluating a biocontrol agent. Apart from that, our study reiterates the pathogenic potential of *I. fumosorosea* against larvae of *P. interpunctella* and *T. granarium* and adults of *M. persicae* and indicates that it can be used as a biocontrol agent. Although this represents only a preliminary investigative stage into the use of *G. castaneae*, our study does not render promising results, but reinforces our knowledge of the insecticidal value of *I. fumosorosea*. More studies are needed to further our insight into the potential of *G. castaneae* species as a component of IPM.

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