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Spatial Modulation of Multi-Fungal Antagonism in Integrated Insect and Pathogen Biocontrol

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Abstract

Biological control using fungi holds significant potential for managing pests and pathogens, especially when different agents are applied together. Several studies have investigated the interaction of fungi, focusing on competition and antibiosis in simple, single-pathogen systems. This neglects how spatial and temporal dynamics profoundly influence outcomes in complex host ecosystems. Using a 2D Petri dish system, this study investigated the impact of initial spatial positioning on interactions among key fungal biocontrol agents (*Trichoderma*, *Metarhizium*, and *Beauveria*) and the pathogen *Fusarium oxysporum*. Different fungal isolates were line-streaked on potato dextrose agar in various combinations and arrangements. Each fungal growth area in Petri dish was calculated by ImageJ after the incubation period and the interactions between them were assessed. In the pairwise interactions, *T. afroharzianum*, *T. guizhouense*, and *M. brunneum* isolates all demonstrated strong antagonism, consistently outcompeting *F. oxysporum*. *Beauveria bassiana*, however, was the least competitive, showing little antagonism against the pathogen. In three-way interactions, the initial arrangement of the fungi significantly influenced competitive outcomes, which may be isolate- or strain-specific. The antagonistic effects of *Trichoderma* were modulated by the presence and position of *Metarhizium* and *Beauveria*, highlighting that multi-way interactions cannot be predicted from pairwise interactions alone. Our results underscore the importance of initial spatial positioning, providing an essential basis for optimizing application methods and designing more effective, location-dependent microbial consortia.

Keywords: Fungal antagonism, co-application, spatial position, multi species interactions, biocontrol

Introduction

Effective biological control relies on a better understanding of how organismal interactions in the soil and root ecology significantly influence the efficacy of control agents. This efficacy hinges on complex ecological processes, particularly the competitive interactions within microbial communities. The fungal genera *Trichoderma*, *Beauveria*, and *Metarhizium* (Ascomycota: Hypocreales) are common soil inhabitants with diverse lifestyles, recognized for their potential as biological control agents. They target arthropod pests and other soilborne fungi like the plant pathogen *Fusarium oxysporum* (St. Leger and Wang 2020; Woo et al., 2023; Yao et al. 2023). Additionally, as endophytes, these fungi can also induce plant resistance to biotic and abiotic stresses such as water deficits, pest attack, salinity, and heat (Shapiro-Ilan et al. 2012; González-Mas et al. 2019; St. Leger and Wang 2020). *Beauveria* and *Metarhizium*, despite being primarily known as entomopathogens, have isolates that exhibit significant antagonistic potential against plant pathogens, effectively blocking pathogen progression in plant tissues (Barra-Bucarei et al. 2019; Hu and Bidochka, 2021; Sinno et al. 2021). These potential drives promising approaches, such as the co-application of different fungal species to enhance control, with ongoing studies focusing on identifying compatible fungal pairs (Krauss et al. 2004; Sasan and Bidochka 2013; Medina et al. 2020). Understanding the efficacy of such multi-agent systems, however, requires a deeper look into the spatiotemporal factors governing their establishment and interaction.

Fungal communities are known to be shaped by intense competition, particularly when species share overlapping niches, the outcomes of multi-species interactions often differ from simple pairwise comparisons. A crucial factor driving this complexity is spatial positioning, which is a well-established determinant of a fungus' competitive success (Boddy 2000; Hiscox et al. 2017, 2018). The dynamics of multi-species interactions are significantly influenced by the timing and location of establishment within an ecosystem. Early arrival or favorable

positioning can lead to competitive interactions, allowing one species to dominate and eliminate others (Pedersen and Fenton 2007; Rynkiewicz et al. 2015; Kinnula et al. 2017; Lello et al. 2018). This principle is clearly demonstrated in studies of entomopathogenic fungi. Insect cadavers typically host single species, even when exposed to diverse fungal inoculum, as competitive dominance leads to exclusive mycosis (Hughes and Boomsma 2004; Li et al. 2021; Shang et al., 2024; Costantin et al. 2025).

Despite the known importance of spatial dynamics in fungal ecology, the effects of spatial positioning on multi-way interactions, specifically among applied fungal biocontrol agents, have yet to be investigated. This gap is particularly relevant for biocontrol strategies, where the efficacy of beneficial fungi in controlling a pathogen may depend on their initial spatial arrangement in the soil environment. The simple pairing of two fungi in a laboratory setting does not adequately predict outcomes in more complex, real-world scenarios where multiple fungi are introduced and interact with a pathogen/competitor in a spatially constrained root environment. This study aims to investigate whether the efficacy of biocontrol agents (specifically *Trichoderma*, *Metarhizium*, and *Beauveria*) in a multi-species system is fundamentally dependent on their initial spatial positioning. This study examined the consequences of varied spatial inoculation patterns for biocontrol efficacy by investigating the following questions: i) How does the spatial arrangement of *Trichoderma*, *Metarhizium*, and *Beauveria* influence their pairwise and three-way interactions in vitro? and ii) Does the relative positioning of these three biocontrol agents affect the antagonistic activity of *Trichoderma* against *F. oxysporum*?

By addressing these questions, this research will demonstrate how initial spatial arrangement directly impacts competitive outcomes in multi-way interactions among biocontrol agents and a pathogen. These findings could help in developing more effective biopesticide formulations and optimizing application methods (like seed treatments or soil drenching) where the initial

placement of microbial inoculants is critical. This work provides a crucial step toward understanding how to move beyond simple pairwise compatibility and design more effective, multi-agent biocontrol strategies.

Material and methods

Fungi

The fungal isolates used were *Trichoderma afroharzianum* (Tr95), *T. guizhouense* (Tr118), *Beauveria bassiana* (Pa4), and *Metarhizium brunneum* (Met52). All fungi were maintained on potato dextrose agar (PDA) at 25°C in the dark for 10 days. The *Trichoderma* isolates were farm-sourced (Korkom 2022) while *B. bassiana* was isolated from infected *Pristiphora abietina* larvae (Biryol et al. 2021) in Türkiye. All were identified morphologically and molecularly identified in these respective studies.

Pairwise in vitro antagonistic interaction of biocontrol agents against Fusarium oxysporum

To initially assess the antagonistic potential of all four fungi, including the secondary fungicidal capabilities of the entomopathogens, pairwise interactions between *F. oxysporum* and each of the biocontrol agents (*T. afroharzianum*, *T. guizhouense*, *B. bassiana*, and *M. brunneum*). A 2D Petri dish assay was chosen as a simplified and controlled *in vitro* model to specifically isolate and examine the influence of initial spatial arrangement on fungal competitive interactions, minimizing the confounding variables present in more complex natural environments.

In this experiment, each pairing involved line-streaking the two fungal isolates at opposite ends of a Petri dish (9 cm diameter, area ≈ 63.6 cm^2) containing PDA medium (Marraschi et al. 2019; Otoya-Martinez et al. 2023) (Figure 1a). Fungi were inoculated using loops from 14-day-old sporulating pure cultures. Control groups included plates with *T. afroharzianum*, *T.*

guizhouense, *B. bassiana*, *F. oxysporum*, and *M. brunneum* inoculated alone. The inoculated Petri dishes were then incubated at 24°C in complete darkness for 14 days. Following incubation, the growth area of each fungus in the Petri dish was calculated using ImageJ software. The Petri dish diameter and a ruler were used as scale references for accurate measurement, and the interactions between the fungi were subsequently assessed. The antagonistic effects of *Trichoderma* on the other biocontrol agents (*Beauveria* and *Metarhizium*) were also assessed. Each treatment had five replicates, each consisting of a Petri dish containing PDA medium. This experiment was conducted twice.

The outcome of the interaction was assessed using a scale, where a score of **1**=antagonist (biocontrol agent) grew over almost the entire Petri dish (complete inhibition of pathogen); **2**= antagonist grew over approximately 3/4 of the plate, significantly restricting the pathogen's growth; **3**=antagonist and pathogen grew to roughly half of the plate each, indicating a more balanced competition; **4**= pathogen grew over approximately 3/4 of the plate, suggesting weak antagonism; and **5**=pathogen grew almost throughout the entire Petri dish, indicating no effective antagonism (Bell et al. 1982). Visual cues were also observed to assess the type of interaction, including: one fungus growing directly over the other, the presence of inhibition zones, and a dense, often raised and pigmented line of mycelia.

Three-way interactions among biocontrol agents and interaction with Fusarium oxysporum

In this experiment, the three-way interaction among the biocontrol agents (*T. afroharzianum*, *T. guizhouense*, *B. bassiana*, and *M. brunneum*) and their combined interaction with *F. oxysporum* were investigated. The different fungal isolates were inoculated onto the PDA surface by streaking them in lines with arrangements across different Petri dishes (9 cm diameter, area ≈ 63.6cm^2) (Figure 1b). The combinations *Trichoderma* + *Fusarium* + *Metarhizium* and *Trichoderma* + *Fusarium* + *Beauveria* were tested but the spatial positions

of these three fungi were interchanged across different replicate dishes. These inoculated Petri dishes were incubated under controlled environmental conditions for 10 days at 25 ± 1 °C. After incubation, the area (%) covered by each fungus in Petri dish was calculated from overhead photographs using ImageJ software programme and the interactions between fungi were assessed. This assessment included measuring the growth rate or colony size of each fungus in both monoculture and co-culture, alongside visual observation and software measurements of interactions such as overgrowth and physical contact. Each treatment had five replicates, each consisting of a Petri dish containing PDA medium. This experiment was conducted twice.

Statistical analysis

Data was analyzed in SPSS. Kruskal-Wallis' test followed by Dunn's test was employed to compare the antagonistic potential (Bell's score) of *T. afroharzianum, T. guizhouense, B. bassiana*, and *M. brunneum* against *F. oxysporum* in the pairwise experiments. In the three-way interactions, the area (%) covered by each fungus was compared using the General Linear Model with Tukey's test with spatial arrangement, fungi species and their interaction as factors.

Results

Pairwise in vitro antagonistic interaction of biocontrol agents against Fusarium oxysporum

In pairwise interactions, a significant difference was observed in the antagonistic effects of the fungal isolates against *F. oxysporum* ($\chi^2(9) = 83.11$, $P<0.001$) (Fig. 2, Table 1). Both *T. guizhouense* and *T. afroharzianum* exhibited strong and effective antagonism, almost completely outcompeting *F. oxysporum* (median score 1.0). They also showed a strong competitive advantage over *B. bassiana* (median scores 2.0). There were no significant

differences in the antagonistic effects between the two *Trichoderma* isolates against either *B. bassiana* or *F. oxysporum* (Fig. 2; Table 1).

Though not used in the direct control of *F. oxysporum*, *M. brunneum* displayed strong antagonism against this pathogen (Fig. 2) and showed a clear competitive advantage over *B. bassiana*. When co-inoculated with the *Trichoderma* isolates, *M. brunneum* displayed a nearly equal competitive interaction, forming a visible mycelial barrage at the zone of contact.

In contrast, *B. bassiana* showed the weakest antagonism against *F. oxysporum* (median score 4.0), failing to prevent the pathogen from covering most of the Petri dish. Furthermore, *B. bassiana* was consistently inhibited in its interactions with both *Trichoderma* spp. and *M. brunneum* (Table 1).

Three-way interactions among biocontrol agents and interaction with Fusarium oxysporum

The initial spatial positioning of fungal inoculations significantly influenced the competitive dynamics between the fungi in three-way interactions (Fig. 3 and 4). The final growth area of each fungus was not predictable from their pairwise interactions alone. The competitive interaction was significantly affected by spatial arrangement for *M. brunneum*, *T. guizhouense*, and *F. oxysporum* ($P<0.001$, Table 2), demonstrating that the growth of each species was highly dependent on its initial position relative to the others. When *T. guizhouense* was positioned in the middle, it dominated, covering 94% of the dish and suppressing both *F. oxsporum* and *M. brunneum*. Conversely, when *M. brunneum* was positioned between *T. guizhouense* and *F. oxysporum*, a nearly equal coverage was observed between *M. brunneum* and *T. guizhouense* ($P=0.932$). Similar spatial dependencies were observed in interactions involving *T. afroharzianum* and *M. brunneum*, where the final area covered by *M. brunneum* and *T. afroharzianum* was significantly impacted by their initial placement.

While the *Trichoderma* isolates consistently suppressed both *F. oxysporum* and *B. bassiana*, the presence and position of *B. bassiana* still had an effect. When *B. bassiana* was inoculated in the middle, the growth of *T. guizhouense* and *T afrohazianum* was visibly altered, as the *Trichoderma* isolates had to grow around the less competitive fungus to access the pathogen. This demonstrates that even a weak competitor can influence the growth and access to resources of a dominant species based on its spatial location. While spatial arrangement significantly impacted the growth of *B. bassiana*, it had no significant impact on the area covered by *F. oxysporum* and *T. afroharzianum*.

In all three-way interactions, *Trichoderma isolates* inoculated in the middle position displayed a competitive advantage, consistently dominating the other fungi. The results underscore that the competitive outcomes in multi-species systems are not fixed properties of the organisms but are a dynamic function of their spatial context (Fig.4).

Discussion

This study highlights the significant influence of spatial positioning on the competitive dynamics among the specific fungal isolates: *T. afroharzianum* (Tr95), *T. guizhouense* (Tr118), *B. bassiana* (Pa4), and *M. brunneum* (Met52), as well as their interaction with the plant pathogen *F. oxysporum*. Pairwise and three-way interactions were examined via co-inoculation in Petri dishes. It is important to note that the observed outcomes may be isolate- or strain-specific.

In pairwise interactions, *T. afroharzianum* and *T. guizhouense* consistently exhibited strong antagonism towards *F. oxysporum.* Similarly, *M. brunneum* presented antagonistic effects but was less competitive and dominating against *F. oxysporum*, compared with the *Trichoderma* species. Both *Trichoderma* isolates presented nearly equal effects with *M. brunneum*, when these two biocontrol agents were co-inoculated. A clear sharp line of demarcation, altered

textures, and a change in pigmentation was observed within the interaction zone between the *Trichoderma* and *Metarhizium* colonies. This phenomenon is attributed to the production of secondary metabolites by both fungi in response to their proximity. The assessed biocontrol fungal species have demonstrated antagonism *in vitro* and *in vivo* against plant pathogenic fungi including *Fusarium* spp. (Yang et al. 2011; Buensanteai and Athinuwat 2012; Correa-Cuadros et al. 2016; Marraschi et al. 2019; Tseng et al. 2020; Chen et al. 2021; Sinno et al. 2021). In pairwise assays, *B. bassiana* unexpectedly demonstrated weak antagonistic effects on *F. oxysporum*, largely mediated by the production of antifungal compounds. A distinct zone of inhibition was observed between the two fungi.

Furthermore, *Trichoderma* isolates and *M. brunneum* were competitively superior to *B. bassiana* in the Petri dish experiments. Supporting this, a recent study (Li et al. 2021) demonstrated that *M. robertsii* exhibited a strong competitive dominance within the insect host during dual infection, consistently outcompeting *B. bassiana* to the point of exclusive mycosis, regardless of initial inoculation ratios or infection order. However, *B. bassiana* grew faster and outcompeted *M. robertsii* in liquid culture. This one-sided mycosis in different environments and with different strains of the same species is intriguing as it highlights localized resource competition or antagonism within the host (Li et al. 2021). The selective pressures within an insect cadaver (host) differ significantly from those in a nutrient-rich medium. Similarly, the opportunistic fungal pathogen, *Aspergillus flavus* out-competed *M. anisopliae* var. *anisopliae* and sporulated better during their interaction within ants or termites (Hughes and Boomsma 2004; Chouvenc et al. 2012). Moreover, spatial considerations extend even within a single genus. Two closely related species, *B. brongniartii* and *B. pseudobassiana*, show partial niche separation in *Melolontha melolontha* (European cockchafer) infested sites in Switzerland (Pedrazzini et al. 2025). *B. brongniartii* is primarily soil-dwelling, infecting both adult and larval beetles, while *B. pseudobassiana* is mainly arboreal and pathogenic only to adults. This

ecological separation is likely driven by belowground competitive factors, including virulence and host specificity (Fernández-Bravo et al. 2016; Canfora et al. 2017; Pedrazzini et al. 2025). This specialization is notable because the species used in the study, *B. bassiana*, is a generalist, globally ubiquitous, and characterized by its wide host range across diverse habitats (Russo et al. 2024).

In the three-way interaction experiments, the presence of a second biocontrol agent (*Metarhizium* or *Beauveria*) influenced the antagonistic effect of *Trichoderma* spp. against *F. oxysporum*. *M. brunneum* and *Trichoderma spp.* maintained a strong antagonistic role against the pathogen in these three-way cultures. However, *M. brunneum* primarily dominated in treatments where it was inoculated between *F. oxysporum* and the *Trichoderma* isolates, or when *F. oxysporum* was in the middle (i.e., TMF and TFM treatments). Interestingly, both *T. afroharzianum* and *T. guizhouense* exhibit strong antagonistic effects against all fungi when they were inoculated in the middle of the three-way interaction, suggesting a powerful competitive advantage in that central position. In contrast, the three-way interactions involving *B. bassiana* further emphasized the competitive dominance of *Trichoderma spp*. Regardless of the initial arrangement, *T. guizhouense and T. afroharzianum* consistently suppressed both *F. oxysporum* and *B. bassiana*. A key observation was that *Trichoderma spp.* mycelia had to grow around *B. bassiana* and *M. brunneum* when these fungi was inoculated in the middle, indicating that direct contact or proximity might be a crucial factor in its antagonistic activity against *F. oxysporum*.

While in vitro results suggest that dense colonization by *M. brunneum* and *B. bassiana* could form a physical/chemical barrier, locally overriding the general competitive dominance of *Trichoderma* and affecting access to the pathogen, it is also important to consider alternative protective mechanisms. Research has shown that applying *Metarhizium* spp. and *B. bassiana* to a plant's roots or leaves can effectively protect it from both root- and leaf-infecting

pathogens. This protective effect is often indirect, as the fungi colonize the plant's internal tissues, triggering the host's own defenses and/or hindering pathogen progression (Barra-Bucarei et al. 2019; Hu and Bidochka, 2021; Sinno et al. 2021). *Metarhizium* species, for instance, are known to provide a "repellent barrier" against herbivores around plant roots, thereby deterring feeding (Villani et al. 1994; St. Leger 2008). *Metarhizium brunneum* and *Trichoderma* spp. can attract entomopathogenic nematodes towards plant roots, which can lead to increased pest control (Touray et al. 2025). However, a dense repellent barrier, while protecting the plant from herbivores, might still inadvertently impact the optimal root colonization or interaction dynamics of other beneficial fungi like *Trichoderma* if they compete for the same physical space or root exudates. Therefore, understanding the specific mechanisms (physical impedance vs. repellent activity) and the inter-fungal compatibility in such complex systems is crucial for designing effective multi-species biocontrol strategies.

Previous studies have extensively shown how the physical positioning of fungi on substrates like wood or soil influences resource access, territory defense, and inter-fungal interactions, with three-way and higher-order interactions in wood-decaying basidiomycete communities demonstrating that pairwise interactions often fail to predict outcomes in more complex scenarios (Sonnenbichler et al. 1994; Boddy 2000). Interactions at the level of individual hyphae ultimately determine the interactions observed at the mycelial level. Two primary types of direct hyphal interactions are interference, where one fungus directly inhibits the other's growth, and parasitism, where one fungus grows on and derives nutrients from another (Boddy 2000; Boddy and Heilmann-Clausen 2008; A′Bear et al. 2013). These studies have particularly explored three-way and higher-order interactions in wood decay basidiomycetes communities, revealing that pairwise interactions often do not accurately predict the outcomes of more complex scenarios. In the case of biocontrol fungi, factors like application timing, formulation, and the existing microbial community in the soil could all influence these spatial dynamics and

ultimately the success of biocontrol strategies. Field surveys show that despite exposure to diverse fungal inoculum, insect cadavers are typically colonized by single species due to competitive exclusion on insect cuticle prior to infection (Shang et al. 2024). Our study directly investigates how the inoculation position of biocontrol agents affects their antagonism against a pathogen, hence has a more direct applied focus on biological control in agriculture. Other biotic factors such as bacteria and invertebrates (like grazers) might influence or shape fungal interactions and community dynamics (Crowther and A'Bear 2012).

Natural environments comprise complex co-infection scenarios with multiple interacting species. Yet most biocontrol studies use simplified single-host or pairwise models, which often overlook this broader ecological context (Whipps, 2001; Wolinska and King 2009; Tollenaere et al. 2016; Schmid-Hempel 2021). This research addresses this gap by investigating three-way fungal interactions and demonstrating how the initial spatial arrangement of biocontrol agents critically influences their competitive outcome against the pathogen.

The current study, focusing on *in vitro* fungal interactions in Petri dishes, represents a simplified system. While invaluable for establishing foundational principles it is crucial to acknowledge that this 2D environment has limitations in fully replicating the inherent complexity of natural ecological niches such as soil or plant surfaces, where nutrient gradients, abiotic stressors (e.g., pH, moisture fluctuations), and a broader range of biotic interactions exist. Nevertheless, these findings underscore the critical role of spatial arrangement in determining the outcome of interactions among biocontrol agents and their efficacy against *F. oxysporum*, supporting the significance of proximity and initial contact points. The observed outcomes may be isolate- or strain-specific, necessitating future exploration of this variability. For long-term efficacy, EPF must maintain a positive reproductive balance, generating more infectious units from host cadavers than they lose. Persistence requires optimal environmental factors, a suitable host, and strong competitive ability to successfully complete the life cycle

and sustain the population (Pant et al. 2025). Future research should rigorously investigate the specific mechanisms driving these spatial competition dynamics, including the production of organic compounds and enzymes, and the potential for mycoparasitism. Examining the temporal aspects of these interactions, such as colonization rates and the timing of antagonistic compound production, would also provide invaluable insights. Ultimately, these findings have implications for a variety of real-world situations, such as developing more effective biopesticide formulations and optimizing application methods, especially in seed treatments or banded soil drenching, where the initial placement of microbial inoculants is a key variable.

References

A′ Bear AD, Murray W, Webb R, Boddy L, Jones TH (2013) Contrasting effects of elevated temperature and invertebrate grazing regulate multispecies interactions between decomposer fungi. PLoS ONE 8(10): e77610.

Barra-Bucarei L, France Iglesias A, Gerding González M, Silva Aguayo G, Carrasco-Fernández J, Castro JF, Ortiz Campos J (2019) Antifungal activity of *Beauveria bassiana* endophyte against *Botrytis cinerea* in two Solanaceae crops. Microorganisms 8(1): 65.

Bell DK, Wells HD, Markham CR (1982) *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. Phytopathol 72:379.

Biryol S, Araz N, Eski A, Aktürk R, Aksu Y, Çelik Göktürk B, Bilgin L, Demir I (2021) Biodiversity and pathogenicity of entomopathogenic fungi associated with the lesser spruce sawfly, *Pristiphora abietina*. Entomol Exp Appl 169:414–423.

Boddy L (2000) Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiol Ecol 31:185–194.

316 Boddy L, Heilmann-Clausen J (2008) Basidiomycete community development in temperate
317 angiosperm wood. In: Boddy L, Frankland JC, Van West P (eds) Ecology of
318 saprotrophic basidiomycetes. Elsevier Academic, London, pp 209–235

319 Buensanteai N, Athinuwat D (2012) The antagonistic activity of *Trichoderma virens* strain
320 TvSUT10 against cassava stem rot in Thailand. Afri J Biotechnol 11:14996–15001

321 Canfora L, Abu-Samra N, Tartanus M, Łabanowska BH, Benedetti A, Pinzari F, Malusà E
322 (2017) Co-inoculum of *Beauveria brongniartii* and *B. bassiana* shows *in vitro* different
323 metabolic behaviour in comparison to single inoculums. Sci Rep 7:13102.

324 Chen J, Zhou L, Din IU, Arafat Y, Li Q, Wang J, Wu T, Wu L, Wu H, Qin X, Pokhrel GR
325 (2021) Antagonistic activity of *Trichoderma* spp. against *Fusarium oxysporum* in
326 rhizosphere of *Radix pseudostellariae* triggers the expression of host defense genes and
327 improves its growth under long-term monoculture system. Front Microbiol 12:579920.

328 Chouvenc T, Efstathion CA, Elliott ML, Su N-Y (2012) Resource competition between two
329 fungal parasites in subterranean termites. Sci Nat 99:949–958.

330 Correa-Cuadros JP, Sáenz-Aponte A, Rodríguez-Bocanegra MX (2016) *In vitro* interaction of
331 *Metarhizium anisopliae* Ma9236 and *Beauveria bassiana* Bb9205 with *Heterorhabditis*
332 *bacteriophora* HNI0100 for the control of *Plutella xylostella*. SpringerPlus 5:2068.

333 Costantin EC, Roxinol JA, Braga PF, Elliot SL (2025) Insect-parasitic fungi as a model system
334 to investigate coinfections. J Invertebr Pathol 211:108358.

335 Crowther TW, A'Bear AD (2012) Impacts of grazing soil fauna on decomposer fungi are
336 species-specific and density-dependent. Fungal Ecol 5:277–281.

337 Datnoff LE, Nemec S, Pernezny K (1995) Biological control of *Fusarium* crown and root rot
338 of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biol
339 Control 5:427–431.

340 Fernández-Bravo M, Garrido-Jurado I, Valverde-García P, Enkerli J, Quesada-Moraga E
341 (2016) Responses to abiotic environmental stresses among phylloplane and soil isolates
342 of *Beauveria bassiana* from two holm oak ecosystems. J Invertebr Pathol 141:6–17.

343 González-Mas N, Cuenca-Medina M, Gutiérrez-Sánchez F, Quesada-Moraga E (2019)
344 Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions
345 between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. J Pest Sci
346 92:1271–1281.

347 Hiscox J, O'Leary J, Boddy L (2018) Fungus wars: basidiomycete battles in wood decay. Stud
348 Mycol 89:117–124.

349 Hiscox J, Savoury M, Toledo S, Kingscott-Edmunds J, Bettridge A, Waili NA, Boddy L (2017)
350 Threesomes destabilise certain relationships: multispecies interactions between wood
351 decay fungi in natural resources. FEMS Microbiol Ecol 93:fix014.

352 Hu S, Bidochka MJ (2021) Abscisic acid implicated in differential plant responses of
353 *Phaseolus vulgaris* during endophytic colonization by *Metarhizium* and pathogenic
354 colonization by *Fusarium*. Sci Rep 11(1): 11327.

355 Hughes WOH, Boomsma JJ (2004) Let your enemy do the work: within-host interactions
356 between two fungal parasites of leaf-cutting ants. Proc Biol Sci 271:S104–S106.

357 Kinnula H, Mappes J, Sundberg L-R (2017) Coinfection outcome in an opportunistic pathogen
358 depends on the inter-strain interactions. BMC Evol Biol 17:77.

359 Korkom Y (2022) Determination of the effects of *Trichoderma* spp., obtained from agricultural
360 areas in Aydin province, on charcoal Rot disease in strawberry and plant growth. Ph.D
361 Thesis, Aydin Adnan Menderes University.

362 Krauss U, Hidalgo E, Arroyo C, Piper SR (2004) Interaction between the entomopathogens
363 *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* and the
364 mycoparasites *Clonostachys* spp., *Trichoderma harzianum* and *Lecanicillium lecanii*.
365 Biocontrol Sci Technol 14:331–346.

366 Lello J, McClure SJ, Tyrrell K, Viney ME (2018) Predicting the effects of parasite co-infection
367 across species boundaries. Proc R Soc B: Biol Sci 285:20172610.

368 Li S, Yi W, Chen S, Wang C (2021) Empirical support for the pattern of competitive exclusion
369 between insect parasitic fungi. J Fungi 7:385.

370 Marraschi R, Ferreira ABM, da Silva Bueno RN, Leite JA, Lucon CM, Harakava R, Leite LG,
371 Padovani CR, Bueno CJ (2019) A protocol for selection of *Trichoderma* spp. to protect
372 grapevine pruning wounds against *Lasiodiplodia theobromae*. Braz J Microbiol
373 50:213–221.

374 Medina EQA, Oliveira AS, Medina HR, Rangel DEN (2020) Serendipity in the wrestle
375 between *Trichoderma* and *Metarhizium*. Fun Biol 124:418–426.

376 Otoya-Martinez N, Leite LG, Harakava R, Touray M, Hazir S, Chacon-Orozco J, Bueno CJ
377 (2023) Disease caused by *Neofusicoccum parvum* in pruning wounds of grapevine
378 shoots and its control by *Trichoderma* spp. and *Xenorhabdus szentirmaii*. Fungal Biol
379 127:865–871.

Pant B, Bilgo E, Mitra A, Safdar S, Diabaté A, Leger RS, Gumel AB (2025) Could malaria mosquitoes be controlled by periodic releases of transgenic mosquitocidal *Metarhizium pingshaense* fungus? a mathematical modeling approach. Appl Math Model 4:116540.

Pedersen AB, Fenton A (2007) Emphasizing the ecology in parasite community ecology. Trends Ecol Evol 22:133–139.

Pedrazzini C, Rehner SA, Stewart-Smith F, Boschi S, Widmer F, Enkerli J (2025) Partial ecological niche partitioning between *Beauveria brongniartii* and *Beauveria pseudobassiana* entomopathogens at *Melolontha melolontha* infested sites. J Invertebr Pathol 211: 108356.

Rigaud T, Perrot-Minnot M-J, Brown MJF (2010) Parasite and host assemblages: embracing reality will improve our knowledge of parasite transmission and virulence. Proc Biol Sci 277:3693–3702.

Russo ML, Vianna MF, Scorsetti AC, Ferreri N, de Abajo JM, Troncozo MI, Pelizza SA (2024) Entomopathogenic fungi as dual control agents against two phytopathogens and the lepidopteran pest *Rachiplusia nu* in soybean (*Glycine max* (L.) Merr). J Fungi 10:93.

Rynkiewicz EC, Pedersen AB, Fenton A (2015) An ecosystem approach to understanding and managing within-host parasite community dynamics. Trends Parasitol 31:212–221.

Sasan RK, Bidochka MJ (2013) Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. Canadian J Plant Pathol 35:288–293.

400 Schmid-Hempel P (2021) Evolutionary parasitology: the integrated study of infections,
401 immunology, ecology, and genetics, 2nd Edition. Oxford University Press, Oxford,
402 New York

403 Shapiro-Ilan DI, Bruck DJ, Lacey LA (2012) Principles of epizootiology and microbial control.
404 In: Vega FE, Kaya HK (eds) Insect pathology, 2nd Edition, Academic Press, San Diego,
405 pp 29–72

406 Shang J, Hong S, Wang C (2024) Fights on the surface prior to fungal invasion of insects. PLoS
407 Pathog 20(2):e1011994.

408 Sinno M, Ranesi M, Di Lelio I, Iacomino G, Becchimanzi A, Barra E, Molisso D, Pennacchio
409 F, Digilio MC, Vitale S, Turrà D (2021) Selection of endophytic *Beauveria bassiana*
410 as a dual biocontrol agent of tomato pathogens and pests. Pathogens 10:1242.

411 Sonnenbichler J, Dietrich J, Peipp H (1994) Secondary fungal metabolites and their biological
412 activities, V. investigations concerning the induction of the biosynthesis of toxic
413 secondary metabolites in basidiomycetes. Biol Chem Hoppe-Seyler 375:71–79.

414 St. Leger RJ (2008) Studies on adaptations of *Metarhizium anisopliae* to life in the soil. J
415 Invertebr Pathol 98:271–276.

416 St. Leger RJ, Wang JB (2020) *Metarhizium*: jack of all trades, master of many. Open Biol
417 10:200307.

418 Tollenaere C, Susi H, Laine A-L (2016) Evolutionary and epidemiological implications of
419 multiple infection in plants. Trends Plant Sci 21:80–90.

Touray M, Gutay Y, Ulug D, Cimen H, Gulsen SH, Korkom Y, Hazir S (2025) Aim for the roots! plants modulate the multitrophic interactions between entomopathogenic nematodes, fungi, and plants in the soil matrix. J Invertebr Pathol 212:108359.

Tseng Y-H, Rouina H, Groten K, Rajani P, Furch AC, Reichelt M, Baldwin IT, Nataraja KN, Uma Shaanker R, Oelmüller R (2020) An endophytic *Trichoderma* strain promotes growth of its hosts and defends against pathogen attack. Front Plant Sci 11:573670.

Villani MG, Krueger SR, Schroeder PC, Consolie F, Consolie NH, Preston-Wilsey LM, Roberts DW (1994) Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. Environ Entomol 23:502–513.

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52: 487-511.

Wolinska J, King KC (2009) Environment can alter selection in host-parasite interactions. Trends Parasitol 25:236–244.

Woo SL, Hermosa R, Lorito M, Monte E (2023) *Trichoderma*: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. Nat Rev Microbiol 21:312–326.

Yang X, Chen L, Yong X, Shen Q (2011) Formulations can affect rhizosphere colonization and biocontrol efficiency of *Trichoderma harzianum* SQR-T037 against *Fusarium* wilt of cucumbers. Biol Fertil Soils 47:239–248.

Yao X, Guo H, Zhang K, Zhao M, Ruan J, Chen J (2023) *Trichoderma* and its role in biological control of plant fungal and nematode disease. Front Microbiol 14:1160551.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The author declares that there is no conflict of interest

Research involving human participants and/or animals

This study does not contain any studies with human or animal subjects. No ethical approval is required.

colour figure

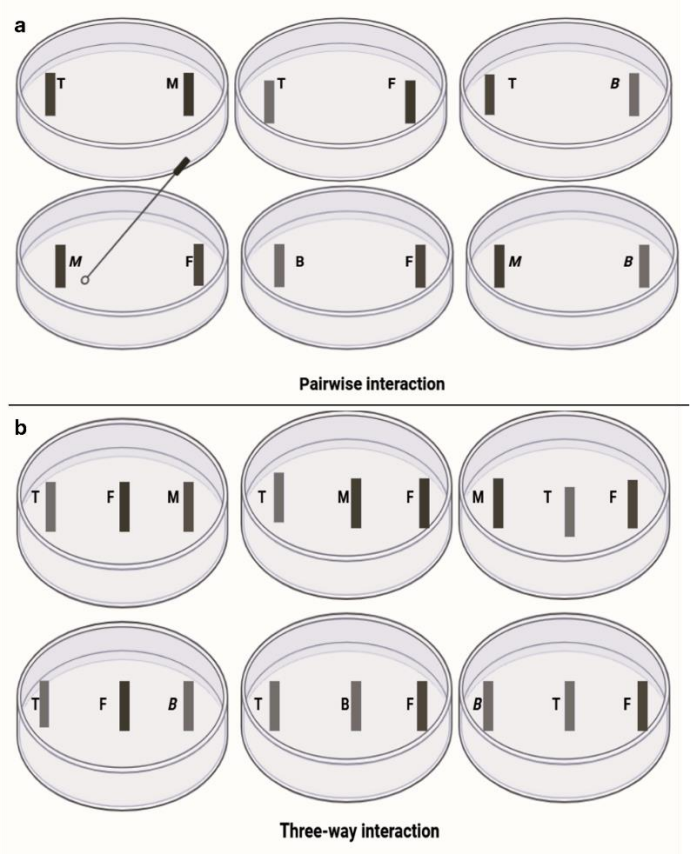

Figure 1. Inoculation arrangements of biocontrol agents and *Fusarium oxysporum during in vitro antagonistic interaction.* a) Pairwise interaction: Single biocontrol agents (T or M or B) versus F. b) Three-way interaction: Two biocontrol agents (e.g., T+M, T+B) versus F. *T=Trichoderma spp. M. Metarhizium brunneum, F= Fusarium oxysporum, B=Beauveria bassiana*

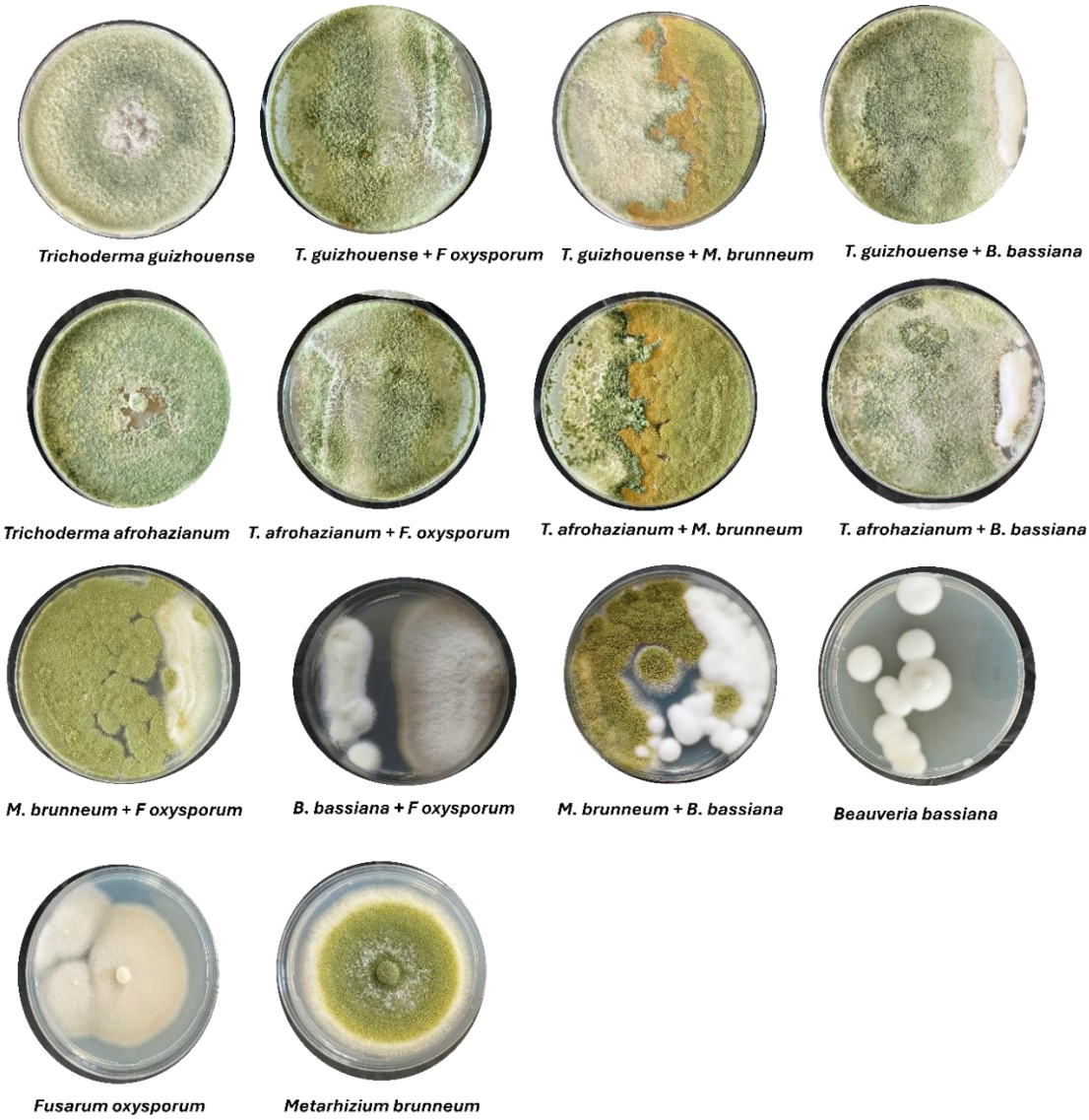

Figure 2 Pairwise *in vitro* antagonistic interaction of biocontrol agents against *Fusarium oxysporum* 14 days post inoculation. *Trichoderma afroharzianum*, *T. guizhouense*, *Beauveria bassiana*, *Metarhizium brunneum* and *F. oxysporum* were paired by inoculating the two fungal isolates in lines at opposite ends of a Petri dish (9 cm diameter) containing PDA medium and the outcome of the interaction was assessed 14 days post-inoculation.

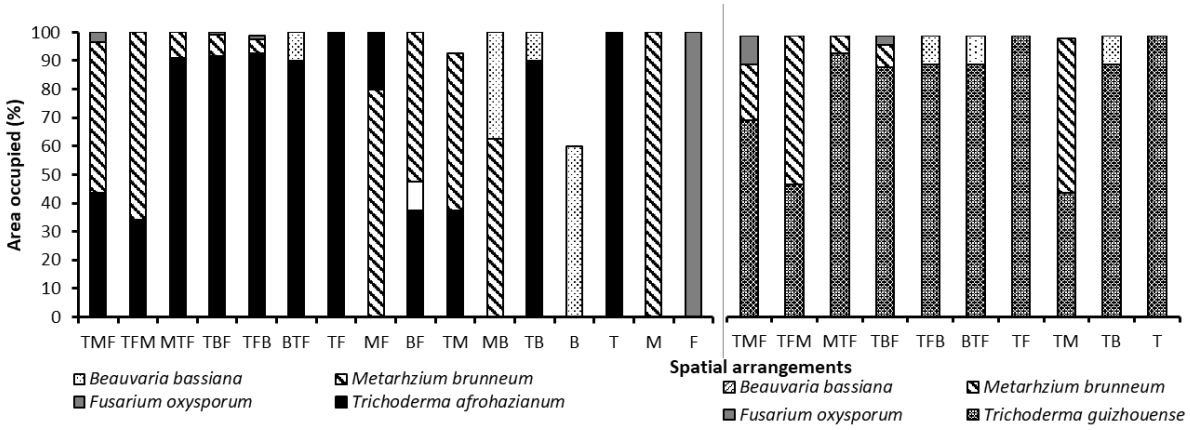

Figure 3 Representation of overall area covered by *Trichoderma afroharzianum*, *T. guizhouense*, *Beauveria bassiana*, *Metarhizium brunneum* and *Fusarium oxysporum* during pairwise and three-way *In vitro* interaction in Petri dishes. T=*Trichoderma* spp. M=*M. brunneum*, F= *F. oxysporum*, B=*B. bassiana.* Spatial arrangement abbreviations indicate *i*noculation order of inoculation

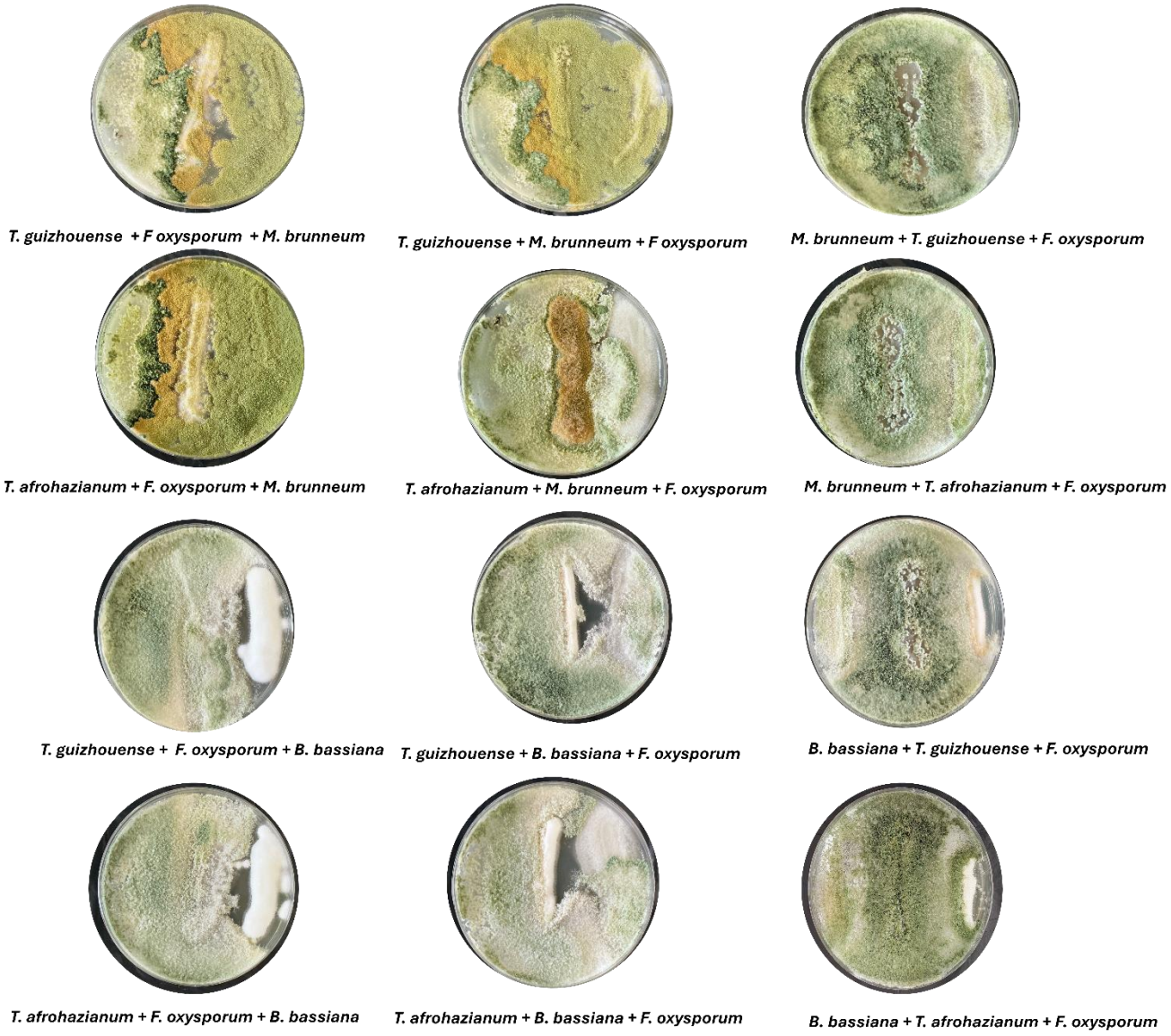

Figure 4 Three-way interaction among biocontrol agents and *Fusarium oxysporum.* Fungi were inoculated in threes in lines at opposite ends of a Petri dish (9 cm diameter) containing PDA medium and the outcome of the interaction was assessed 14 days post-inoculation.

table

Table 1 Evaluation of the pairwise *in vitro* antagonistic interactions between fungi.

Antagonism	***F. oxysporum***	***M. brunneum***	***B. bassiana***
Trichoderma afroharzianum	1.0 (1.0; 2.0) * a[#]	3.0 (3.0; 4.0) d	2.0 (1.0;2.0) a
T. guizhouense	1.0 (1.0; 2.0) a	3.0 (3.0; 4.0) d	2.0 (1.0; 2.0) a
Metarhizium brunneum	2.0 (1.0; 2.0) b	-	2.5 (2.0; 4.0) c
Beauveria bassiana	3.5 (2.0; 4.0) e	3.5 (3.0; 4.0) de	-

[*]Values represent the median antagonism score (minimum; maximum), with lower scores indicating stronger antagonism and higher scores indicating weaker antagonism.
[#]Different lowercase letters indicate statistical difference in the antagonism among all the treatments (Dunn's test, $p<0.05$).
Note: the interaction between the *Trichoderma* isolates was not assessed due to their similar colony morphology.

Table 2 Mean area covered by *Trichoderma afroharzianum*, *T. guizhouense*, *Beauveria bassiana*, *Metarhizium brunneum* and *Fusarium oxysporum* during three-way *in vitro* Interaction. Values given as % mean area (minimum; maximum) covered by individual fungi covered by that fungus across the spatial arrangement treatments. The two data sets represent different fungal communities*:* the top set includes the biocontrol agent *M. brunneum (M)* alongside *Trichoderma (T)* and the *F. oxysporum (F),* while the bottom set substitutes it with *B. bassiana (B).*

Spatial arrangement	***M. brunneum***	***F. oxysporum***	***T. guizhouense***	***M. brunneum***	***F. oxysporum***	***T. afrohazianum***
TMF	46.0 (20.0; 74.4)a[#]	7.5 (0.0; 24.0) a	46.5 (25.6; 70.0) a	53.0 (37.0; 70.0) a	3.4 (0.0; 10.0) a	43.6 (31.7; 51) a
TFM	48.0 (10.0; 70.0) a	0.0 (0.0; 0.0) b	52.0 (25.0; 95.0) a	66.0 (50.1; 80.0) b	0.0 (0.0; 0.0) a	34.0 (20.0; 33.9) b
MTF	6.6 (0.0; 21.2) b	0.0 (0.0; 0.0) b	93.4 (78.8; 100.0) b	9.0 (0.0; 0.0) c	0.0 (0.0; 0.0) a	91.0 (60.0; 100.0) c
	F=20.33; p<0.001	F=9.00; p=0.001	F=25.66; p<0.001	F=76.73;p<0.001	F=2.25;p<0.001	F=82.84;p<0.001
Spatial arrangement	***B. bassiana***	***F. oxysporum***	***T. guizhouense***	***B. bassiana***	***F. oxysporum***	***T. afrohazianum***
TBF	8.1 (4.4; 10.0) a	3.0 (0.0; 10.9) a	88.9 (84.1; 95.6) a	7.5 (5.0;10.0) a	1.0 (0.0; 5.0) a	91.5 (90.0; 95.0) a
TFB	10.0 (8.2; 12.0) a	0.0 (0.0; 0.0) b	90.0 (88.0; 92.8) a	10.0 (10.0; 10.0) b	0.0 (0.0; 0.0) a	90.0 (90.0; 90.0) a
BTF	10.0 (6.2; 12.0) b	0.0 (0.0; 0.0) b	90.0 (88.0; 93.8) a	10.0 (10.0; 10.0) b	0.0 (0.0; 0.0) a	90.0 (90.0; 90.0) a
	F=6.00; p=0.007	F=5.06; p=0.013	F=20.332; p=0.125	F=9.00; p=0.001	F=2.25; p=0.125	F=2.25; p=0.125

[#] Different lowercase letters within a column indicate a significant difference in the mean area covered by that fungus across the spatial arrangement treatments (General Linear Model with Tukey's test, $p<0.05$).