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Enzymatic Activities and Antagonistic Interactions of Aspergillus niger, Trichoderma harzianum and Mucor hiemalis Associated with Compressed Rabbit Dung

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Abstract:

Rabbit dung is a nutrient-rich coprophilous substrate that supports diverse fungal communities responsible for organic matter decomposition and nutrient cycling in soils. This study aimed to evaluate the production of selected extracellular enzymes (amylase, cellulase, phenol oxidase, urease and protease) by three soil fungi, Aspergillus niger, Trichoderma harzianum and Mucor hiemalis, during decomposition of gamma-sterilized, compressed rabbit dung, and to assess their mutual antagonistic interactions in dual and triple cultures. Enzyme activities were screened using plate assays on specific media, while antagonism was determined by growth inhibition and changes in dry weight loss of the substrate under different fungal combinations. The results indicated that T. harzianum exhibited the broadest enzymatic profile, producing strong amylase, cellulase, phenol oxidase and urease activities, whereas A. niger showed marked amylase and cellulase activities with limited phenol oxidase and urease, and M. hiemalis displayed restricted activity mainly for phenol oxidase and urease. Antagonism assays revealed that combinations involving T. harzianum and A. niger enhanced dry weight loss and energy depletion compared to single inoculations, while treatments including M. hiemalis tended to reduce overall performance. These findings highlight the functional diversity and competitive behavior of coprophilous fungi and suggest their potential for biotechnological applications in composting and waste valorization.

Key words: Fungal enzyme activity - Extracellular enzymes- Aspergillus niger- bgTrichoderma harzianum- Rabbit dung - Antagonistic interactions.

1. Introduction

In soils, decomposition of organic matter is mainly driven by microorganisms, especially fungi, that produce extracellular enzymes to degrade complex plant or animal residues into simpler compounds that can be metabolized by them (Burns et al., 2013; Allison et al., 2014; Li et al., 2024). Rabbit dung is an exceptional organic substrate with partially digested plant fibres, nitrogenous compounds, vitamins and minerals, which favours the colonization of coprophilous fungi (Webster, 1983; Valle et al., 1994; Verheggen et al., 2024). Rabbit dung experiences progressive bacterial and fungal decomposition, like that shown in Figure 1. The zygomycetes grow rapidly exploiting easily available soluble carbohydrate, followed by cellulolytic ascomycetes which break down structural polysaccharides, and later by basidiomycetes which break down lignin and other recalcitrant organic components (Webster, 1983; Kuthubutheen & Webster, 1986; Safar & Cooke, 1988). Therefore, rabbit dung is a dynamic micro habitat and the nutrient mineralization and carbon cycling within soil ecosystems is determined by a combination of fungal succession and extracellular enzymatic activity.

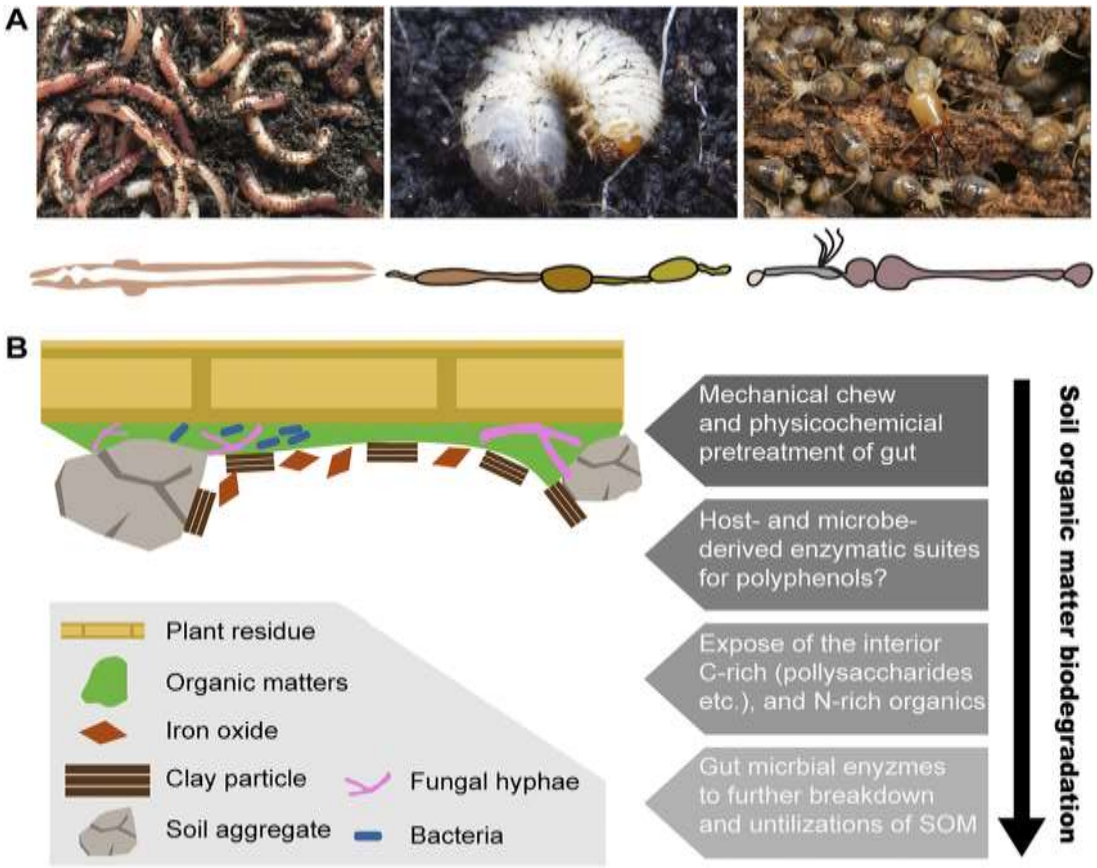

Figure 1. Biological Mechanisms of Soil Organic Matter Decomposition by Soil Fauna and Microorganisms

Extracellular enzymes such as amylase, cellulase, protease, phenol oxidase and urease play central roles in this decomposition process (Burns et al., 2013; Wang et al., 2022). Amylases hydrolyze starch into soluble sugars and are widely produced by Aspergillus species, including A. niger, which is considered a powerful industrial source of alpha-amylase (Siala et al., 2021; Brandi et al., 2024). Cellulases break down cellulose and hemicellulose into fermentable sugars; several soil fungi, including Mucor and Trichoderma species, have been reported as effective producers of cellulase and pectinase with promising

biotechnological applications (Alves et al., 2002; El-Shamy et al., 2003; Baskaran et al., 2017). Phenol oxidases (such as laccases) oxidize phenolic and lignin-derived compounds, while proteases target proteins and ureases hydrolyze urea, contributing to nitrogen mineralization and release of ammonium in the soil (Talbot et al., 2013; Li et al., 2024).

Fungi growing together on the same substrate often interact in antagonistic or competitive ways, either by secreting antifungal metabolites or by producing hydrolytic enzymes, and by faster colonization, that results in the suppression of other species (De Boer et al., 2005; Pena et al., 2025). The species Trichoderma are particularly well known for their antagonistic activity against other fungi and are broad-spectrum biocontrol agents (Vinale et al., 2008; Contreras Cornejo et al., 2016). The objectives of this study were to identify the enzymatic activity of these common soil fungi (A. niger, T. harzianum and M. hiemalis) on compressed rabbit dung on specific plates assays for amylase, cellulase, phenol oxidase, urease and protease, and to examine the antagonistic interaction between the fungi in dual and triple culture under standard gamma sterilization and incubation conditions, considering the loss of dry matter and visible interaction between the fungi. Although there is a vast amount of literature dealing with coprophilous fungal succession and enzyme production (Calaça et al., 2023), most studies have been concerned either with taxonomic characterisation or with enzyme profiling of single species under artificial conditions. Beyond that, there are less studies that compare enzymatic complementarity and antagonistic relationships of important decomposer fungi on a common, gamma-sterilized organic matrix like compressed rabbit dung. This gap is important because the ability of the fungi to decompose and the outcome of competition among species influence their impact on overall substrate mineralization, which are complex interspecific interactions that can either facilitate or inhibit mineralization (Peña et al., 2025). Thus, knowledge of the correlation between enzymatic repertoires and antagonistic behaviours under controlled conditions is fundamental for predicting changes in fungal community and for designing efficient microbial consortia for biotechnological applications. Furthermore, the rising awareness of sustainable waste valorization and the bio-based composting has rejuvenated the fungal enzymes as biocatalysts for converting proteinaceous and lignocellulosic wastes into valuable products (Manawasinghe et al., 2025; Lima et al., 2024). In this context the present study offers a comparative functional analysis of the three ubiquitous fungi of the soil, trying to correlate the enzymatic diversity with the interspecific competition and to find some of the best candidates for bioaugmentation strategies. The thesis is based on previous classical works on coprophilous fungi and dung decomposition and combines these with newer knowledge of fungal enzyme production and biocontrol behavior.

2. Literature reviews

2.1 Fungal Enzymes in Organic Matter Decomposition

Saprotrophic fungi produce the most important extracellular enzymes for the breakdown of complex organic material during manure decomposition. These enzymes help to break down cellulose and starch, proteins, compounds derived from lignin, and nitrogenous compounds, enhancing the mineralization of nutrients and compost maturity. Enzymes are important for their ability to reflect microbial metabolic functions and are widely recognized as reliable indicators of decomposition efficiency and soil health (Burns et al., 2013; Allison et al., 2014).

Recent research has shown that composting conditions have a significant effect on the performance of the enzymes of fungi. Cai et al. (2022) found that frequent watering was able to significantly improve

the enzymatic activity and fungal metabolic function in sheep manure composting in the Qinghai-Tibet Plateau. Similarly, Lu et al. (2022) demonstrated that optimizing the composting conditions of vegetable waste and pig manure improved the microbial succession and compost quality by enhancing the fungal activity. More recently, Zhang et al. (2026) also suggested an innovative approach to enhance compost quality by incorporating enzymes such as hydrolases and oxidoreductases obtained from spent mushroom substrate. Combined, these investigations highlight the crucial role of extracellular enzymes in the biodegradation of manure. However, most studies evaluated total microbial enzyme pools or composting performance rather than comparing the functional enzymatic capacities of individual fungal species.

2.2 Extracellular Enzyme Production by Decomposer Fungi

The extracellular enzyme repertoire varies greatly among filamentous fungi reflecting their growth habits and nutritional modes. Species of Trichoderma are one of the most efficient decomposers due to their capability to produce a wide range of hydrolytic enzymes such as cellulases, proteases, chitinases, and xylanases that enable them to break down the lignocellulosic materials quickly (Kredics et al., 2005; Harman et al., 2004). Recent reviews further highlighted the importance of Trichoderma spp. in the bioconversion of agricultural wastes and sustainable biomass utilization (Yi et al., 2022; Lima et al., 2024).

Similarly, A. niger is recognized as one of the most important industrial fungi due to its remarkable capacity to produce amylases, proteases, cellulases, and organic acids. It has been proven to be efficient in degrading agricultural residues and in the production of commercially valuable enzymes in many studies (Siala et al., 2021; Sohail et al., 2009; El-Shamy et al., 2003).

Species of Mucor, on the other hand, are characterized by a more limited set of enzymes. Alves et al. (2002) showed that there are significant differences between the Mucor species, as alvamylase and proteases were produced only in a few of the strains. Previous work in ecology also indicated that zygomycetous fungi were involved in the early stages of the decomposition of dung since they were known to utilize readily available nutrients more readily than highly recalcitrant polymers (Harper & Webster, 1964; Garrett, 1966). However, there are still few data available on the comparative enzymatic activity of M. hiemalis compared to T. harzianum and A. niger.

2.3 Dung-Inhabiting Fungal Communities and Functional Interactions

Animal dung is a living ecosystem with very particular fungal communities essential for the recycling of nutrients and decomposition of organic matter. Classical studies of fungal colonization of dung have shown that a well-defined succession occurs, fungal species replacing each other as dung availability and environmental conditions change (Valle et al., 1994; Wicklow et al., 1980; Webster, 1983). Molecular studies have, since, revealed that the community of fungi associated with dung is much more diverse than was previously thought (Herrera et al., 2011).

Recent reviews have focused on significant advances in fungal diversity documentation, but also pointed out the lack of research on fungal functional ecology. According to Calaça et al. (2023), the main focus of research published has been on taxonomy and biodiversity with less research on enzymatic activity, decomposition efficiency, and antagonistic interactions. Similarly, Sun et al. (2024) showed that the community composition of fungi differs significantly between various herbivore species, indicating that substrate properties play a major role in shaping fungal community assembly.

In addition to decomposition, fungi also influence the structure of the community and use of substrates. Enhancement and antagonism, including competition for nutrients, production of antifungal metabolites, and production of enzymes outside the cells, are known to be important ecological mechanisms that affect fungal succession (De Boer et al., 2005; Vinale et al., 2008). Although these advances have been made, very little work has been done to investigate production of extracellular enzymes and antagonistic interactions among the common decomposer fungi under standardized laboratory conditions.

This leaves large gaps in knowledge of the functional relationships between A. niger, T. harzianum and M. hiemalis during the decomposition of rabbit dung. These gaps are becoming more critical as fungi are becoming crucial biological tools for sustainable waste valorization, compost enhancement, and environmentally friendly agricultural systems (Manawasinghe et al., 2025; Hassan-Dalléac et al., 2026). Thus, the present study was conducted to compare the enzymatic profile and antagonistic interaction between the three ecologically important fungi under controlled laboratory condition with gamma sterilized compressed rabbit dung as presented in Table 1 and 2.

Table 1. Previous studies on fungal enzymes involved in manure decomposition

Study	Substrate	Main fungal group	Major findings	Limitation
Burns *et al*. (2013)	Soil	General fungi	Soil enzymes are key indicators of decomposition	Did not compare fungal species
Allison *et al*. (2014)	Soil organic matter	Soil microbes	Linked exoenzyme activity with organic matter degradation	No species-level comparison
Cai *et al*. (2022)	Sheep manure	Compost fungi	Water replenishment enhanced enzyme activity	Focused on compost performance
Lu *et al*. (2022)	Vegetable waste + pig manure	Compost fungi	Optimized fungal succession during composting	No enzymatic comparison among fungi
Zhang *et al*. (2026)	Mushroom substrate compost	Enzyme extracts	Improved compost quality using enzyme extracts	No investigation of living fungal interactions

Table 2. Comparative characteristics of the principal fungi investigated in previous studies

Fungus	Major enzymes reported	Ecological role	Representative references
Trichoderma harzianum	Cellulase, protease, chitinase, xylanase	Efficient decomposer and biocontrol agent	Harman et al. (2004); Kredics et al. (2005); Lima et al. (2024)
Aspergillus niger	Amylase, cellulase, protease, organic acids	Industrial enzyme producer and decomposer	Siala et al. (2021); Sohail et al. (2009); El-Shamy et al. (2003)
Mucor hiemalis	Amylase, protease (strain-dependent), limited hydrolytic enzymes	Early-stage colonizer	Alves et al.(2002); Harper & Webster (1964)

3. Materials and Methods

3.1 Research Design

A laboratory-based experimental design was used in this study and the role of three filamentous fungi, A. niger, T. harzianum and M. hiemalis in the decomposition of gamma-sterilized compressed rabbit dung was studied under controlled environmental conditions. The experimental method aimed at comparing the enzyme activity and antagonism of the selected fungal species, applying standard microbiological methodologies.

Two complementary experimental parts were used to implement the methodology. The first one was a study on the production of extracellular enzymes involved in organic matter degradation such as amylase, cellulase, phenol oxidase, protease, and urease. The enzymes chosen to represent the main biochemical systems responsible for the degradation of carbohydrates, cellulose, proteins, phenolic compounds and nitrogen containing substrates, respectively, are those selected (Burns et al., 2013; Allison et al., 2014). The production of enzymes was determined by standard plate assay methods that allow determination of the enzymes qualitatively and semi-quantitatively by determination of a halo or characteristic colour development, respectively (Conn et al., 1957; Garrett, 1966; Nicholson & Robinson, 1983; Seeliger, 1956).

Macroscopic and microscopic assays were used to examine the interactions between fungi during substrate colonization, as part of the second component. The antagonism between the different treatments (single, dual and triple inoculation) was assessed by microscopic analysis of interactions between hyphae and bioassays of culture filtrate effect on neighbouring species growth on gamma-sterilized compressed rabbit dung. These other mechanisms shed light on the direct and indirect aspects of fungal competition and coexistence (De Boer et al., 2005; Harman et al., 2004; Vinale et al., 2008).

Standardized conditions in the laboratory were used in all experiments to minimize experimental variation. All the conditions such as temperature, inoculation size, composition of the culture media and preparation of substrate were kept unchanged during the study. Three independent biological replicates were used in all experimental treatments and with uninoculated controls to guarantee the reproducibility and reliability of the results obtained. This is in line with recommended procedures for fungal physiological and ecological investigations (Burns et al., 2013; Allison et al., 2014). The entire experimental procedure used in this study is shown in Figure 2.

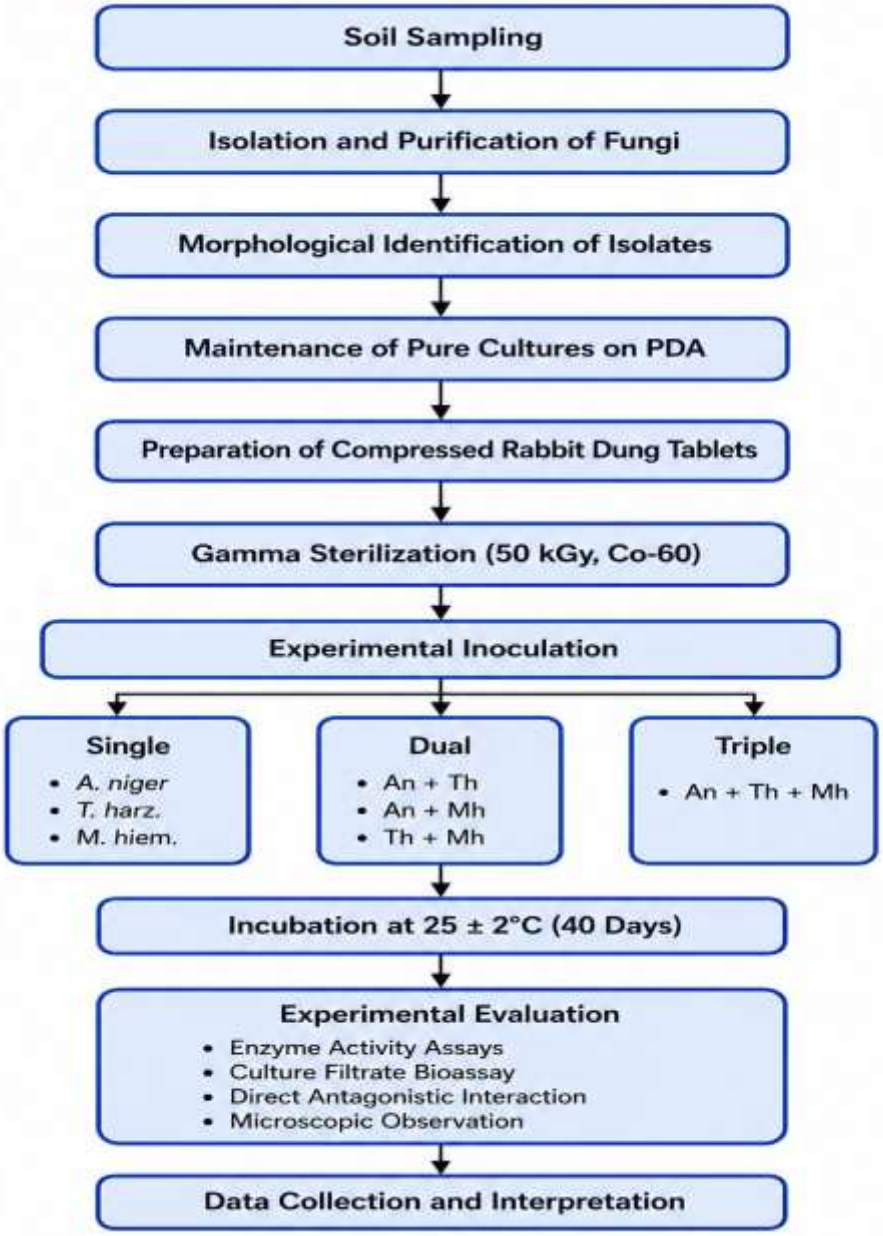

Figure 2. General workflow of the experimental methodology.

3.2 Fungal Isolates

In the present study, three filamentous fungal species have been investigated, such as A. niger, T. harzianum and M. hiemalis. The fungi selected because they are ecologically important decomposers and have different enzyme and competitive abilities in the biodegradation of organic substrates. The enzyme system of cellulolytic and proteolytic enzymes is very efficient in Trichoderma species and A. niger is one of the most prolific producers of extracellular hydrolytic enzymes, as shown previously. Mucor species, on the other hand, are usually involved in the initial composting phase of the substrate; they tend to have more limited enzymatic activity (Alves et al., 2002; Harman et al., 2004; Kredics et al., 2005; Lima et al., 2024).

In the cultivated areas of Misurata region in Libya, sterile hand shovels were used to take soils for using in agriculture. The samples were collected from the top 0–20 cm layer of soil, then were stored in sterile bags of polyethylene and immediately processed in Microbiology Laboratory, Faculty of Science, Misurata University within 24 h to maintain the viability of microorganism.

The results of previous studies showed that fungi tested belong to those commonly found in agricultural soils and rabbit droppings in the same area. Pure cultures were obtained by repeated subculturing on Potato Dextrose Agar (PDA) until a morphologically uniform culture was obtained. Conventional mycological criteria, such as the colony morphology, pigmentation, growth features, conidial morphology, sporulation structures and the characteristics of the hyphae, were used in the identification of the fungi, according to a standard taxonomic key and identification manual.

For viability of cultures for the duration of the experiment, the purified isolates were kept on PDA slants at 4°C and sub cultured on fresh slants prior to each experimental series (Table 3).

Table 3. Characteristics of the fungal isolates used in the present study

Fungal species	Original source	Ecological function	Major enzymatic characteristics reported in literature	Representative references
Aspergillus niger	Agricultural soil	Saprotrophic decomposer	High production of amylase, cellulase, protease and organic acids	Siala *et al*. (2021); Sohail et al. (2009)
Trichoderma harzianum	Agricultural soil	Decomposer and fungal antagonist	Strong cellulolytic, proteolytic and chitinolytic enzyme systems	Harman *et al*. (2004); Kredics *et al*. (2005); Lima *et al.* (2024)
Mucor hiemalis	Rabbit dung	Early-stage saprotrophic colonizer	Moderate production of hydrolytic enzymes with strain-dependent variation	Alves *et al*. (2002); Jamal *et al*. (2007)

3.3 Culture Media

Culture and assay media were made with analytical reagent grade chemicals and distilled water according to the normal microbiological methods. Media were autoclaved at 121ºC for 15 min, then cooled to about 50ºC and added amoxicillin/ampicillin (500 mg L^{-1}) if necessary to suppress bacterial contamination and then poured into sterile Petri dishes. The various media used were chosen depending upon the physiological needs of each experimental assay and the extracellular enzyme to be detected (Conn et al., 1957; Garrett, 1966; El-Gendy, 1966; Seeliger, 1956) (Table 4).

Table 4. Culture media used in the present study

Medium	Purpose	pH
Potato Dextrose Agar (PDA)	Isolation, purification and maintenance of fungal cultures	5.6
Sabouraud Dextrose Agar (SDA)	General fungal cultivation	5.6
Water Agar	Microscopic antagonism assay	—
Cellulose Agar	Cellulase activity assay	5.4
Starch Agar	Amylase activity assay	6.0
Phenol Oxidase Medium	Phenol oxidase assay	—
Milk Agar	Protease activity assay	—
Christensen's Urea Agar	Urease activity assay	6.8
Potato Dextrose Broth (PDB)	Preparation of fungal culture filtrates	5.6

3.4 Preparation of Gamma-Sterilized Rabbit Dung Tablets

Fresh rabbit dung was collected, air-dried, ground to a homogenous powder in a ball mill and compressed into tablets of the same size and shape as those used by Cooke (1984). The tablets were then subjected to gamma irradiation in 50 kGy of the Co-60 source for about 90 minutes to destroy the microorganisms which are present in the substrate to ensure the sterility of the product, without compromising the physical and chemical characteristics of the substrate. To conduct all experiments for the interaction of fungal species under controlled laboratory conditions, a standardized substrate was used: gamma-sterilized tablets.

3.5 Experimental Treatments

Sterilized rabbit dung tablets were placed in sterile 250 mL Erlenmeyer flasks each and inoculated with the fungal discs (1 cm diameter) which were actively growing. Seven experimental treatments were set up: three single inoculations (A. niger, T. harzianum and M. hiemalis), three two-way inoculations (A. niger + T. harzianum, A. niger + M. hiemalis and T. harzianum + M. hiemalis), and one triple inoculation (all three fungi). The fungal growth and interactions were observed at intervals of 8 days and the cultures were incubated for 40 days at 25 ± 2°C. Each treatment had three biological replicates.

3.6 Extracellular Enzyme Activity Assays

The enzymatic activity of A. niger, T. harzianum and M. hiemalis was tested with agar plate assays. The extracellular enzymes which were studied were amylase, cellulase, phenol oxidase, protease and urease. An 8 mm disc of mycelium obtained from a 3–4-day old PDA culture was centrally inoculated on each assay medium and grown at 25 ± 2°C for 72 h. For each fungal species three replicate plates were set up along with one uninoculated control. Standard methods used by Conn et al. (1957), Garrett (1966), Nicholson and Robinson (1983), El-Gendy (1966) and Seeliger (1956) were used to assess enzyme activity by measuring the diameter of the haloes produced by the hydrolytic enzymes and by recording the characteristic colour changes produced by phenol oxidase and urease.

3.7 Effect of Fungal Culture Filtrates

Indirect antagonistic interactions through diffusible metabolites were studied by preparing culture filtrates of individual fungi in Potato Dextrose Broth (PDB). The cultures were filtered through Whatman No. 1 filter paper and then sterilized by filtration through 0.2 µm membrane filters after four days of incubation. The sterile filtrates were added to the PDA medium and the target fungi were inoculated and cultured at 25 ± 2°C and growth was determined by measuring the colony diameter at regular intervals and comparing with the uninoculated controls to check the stimulation or inhibition of the fungi. For each treatment, three biological replicates were performed.

3.8 Microscopic Assessment of Direct Antagonistic Interactions

The direct antagonistic interaction between *A. niger* and *T. harzianum* and *M.hiemalis* was investigated by slide culture technique. A thin layer of water agar was spread on a sterile microscope slide and placed in moist chambers to keep the humidity high. Small pieces of mycelial growth (around 1 × 1 mm) of actively growing colonies were transferred onto the agar surface in different combinations in pairs at a distance of 1–2 cm. Slides were incubated at 25 ± 2°C for a period of time until the hyphal contact was observed and then were examined under the microscope at intervals. Overgrowth, hyphal interference, lysis or avoidance reactions were then examined in the interaction zone to characterise the nature and intensity of the fungal antagonism (Harman *et al*., 2004; Harper & Webster, 1964; De Boer *et al*., 2005).

3.9 Quality Assurance and Experimental Control

All experimental procedures were carried out in the laboratory under aseptic conditions to guarantee reliability and reproducibility of the experimental data. Culture media, glassware and instruments were sterilised prior to use and uninoculated controls were run with each experimental assay to ensure that there was no contamination. The standardisation of fungal inocula was done by cutting the fungus from the 3–4 day old PDA culture into 8 mm diameter mycelial discs and the incubation temperature (25 ± 2°C) and incubation periods were maintained throughout the study. All the qualitative and quantitative observations were made in triplicate for each experimental treatment, and the mean values were recorded. Quality control measures are standard microbiological, fungal physiology procedures to reduce experimental variation (Burns et al., 2013; Allison et al., 2014).

3.10 Data Recording and Statistical Analysis

The experimental observations like fungal growth characteristics, antagonistic response, diameter of haloes and qualitative enzyme activity were observed during the period of incubation. Three biological replicates were analysed for each treatment from which mean values were obtained. The results were then presented in tables and figures for comparative analysis of the enzymatic activities and antagonisms between the fungal species analysed. The analysis was descriptive and comparative of the responses observed in the treatments, applied under standardized experimental conditions, since the study was based mainly on microbiological assays (qualitative and semi-quantitative).

4. Results and Discussion

The enzyme activity studies showed that the three fungal species exhibited significant differences in their enzyme activity. T. harzianum had the widest enzymatic spectrum with high levels of amylase, cellulase, phenol oxidase and urease and did not produce detectable levels of protease. Overall enzymatic activity, T. harzianum was followed by A. niger which exhibited positive amylase and cellulase activity without producing phenol oxidase and urease. M. hiemalis showed only partial activity, and was urease positive but amylase, cellulase, phenol oxidase and protease negative under the conditions tested (Table 5).

Table 5. Enzymatic activity of the tested fungi (+ fungus produces the enzyme; – fungus does not produce the enzyme)

Enzyme	*A. niger*	*T. harzianum*	*M. hiemalis*
Amylase	+	+	–
Cellulase	+	+	–
Phenol oxidase	–	+	–
Urease	–	+	+
Protease	+	–	–

The culture filtrate experiments indicated that the media produced by A. niger and T. harzianum did not inhibit the growth of M. hiemalis when compared to the control, since the colony diameter of M. hiemalis was equal on the media obtained from the three fungi throughout the experiment. Similarly, filtrates of T. harzianum and M. hiemalis failed to produce any changes in the growth of A. niger, and the mean colony diameter of A. niger in all treatments was found to be the same as the control at all observation times.

The filtrates of A. niger and M. hiemalis, however, clearly affected the growth of T. harzianum. A. niger filtrate did not have any effect on the growth of T. harzianum with colony diameter of 70 mm after 48 h and then remaining constant on further incubation. The growth of T. harzianum was inhibited after 48 h when grown in the presence of M. hiemalis filtrate, with colonies growing to 65 mm after this point (Table 6).

Table 6. Effect of fungal culture filtrates on the growth of each fungus on the others (+ effect present; – no effect).

Fungi	*A. niger* filtrate	*T. harzianum* filtrate	*M. hiemalis* filtrate
A. niger	–	–	–
T. harzianum	+	–	+
M. hiemalis	–	–	–

Co culture with T. harzianum did not result in antagonism and both fungi grew normally with no inhibition visible. When T. harzianum was cultivated on the same culture dishes as A. niger, the growth of A. niger was checked and mycelium of T. harzianum surrounded the growth of A. niger. When microscopic examination of the contact zone between the two colonies revealed that hyphal growth of both fungi was inhibited before they were actually fused, this was an example of mutual growth inhibition at short distance.

Both fungi grew as usual and overgrew one another despite obvious growth swellings of the hyphae of M. hiemalis in the interaction zone (Figure 3).

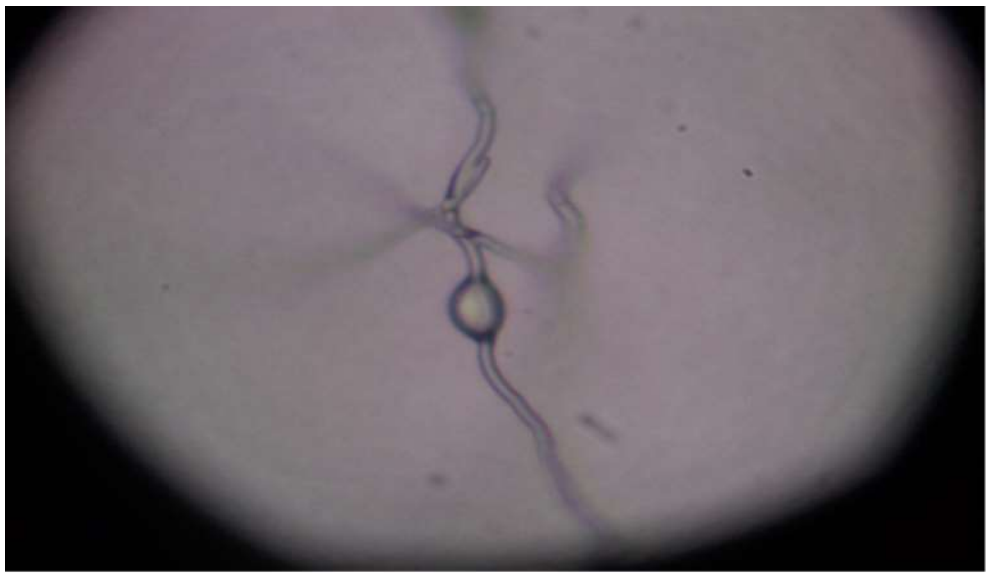

Figure. 3. Co-culture of *A. niger* and *M. hiemalis* on solid medium showing hyphal swellings in *M. hiemalis* at the interaction zone

5. Discussion

The enzymatic profile found in the present study is fairly similar to the previously reported profiles of Trichoderma, Aspergillus and Mucor species. The production of the widest range of hydrolytic and lignocellulolytic enzymes (amylase, cellulase, phenol oxidase and urease) is in accordance with many reports that Trichoderma spp. are good producers of the enzymes that facilitates soil and organic substrate decompositions and this make them good biocontrol agents (Harman et al., 2004; Kredics et al., 2005; Lima et al., 2024). The superior enzymatic performance of T. harzianum may be explained by the presence of well-developed secretory machinery and its ecological adaptation as an aggressive saprotroph which is able to quickly colonize the lignocellulosic substrates. Coordinated carbon and nitrogen acquisition strategies are evident in the simultaneous production of cellulase, amylase, phenol oxidase and urease, which improve the efficiency of substrate utilization (Burns et al., 2013; Talbot et al., 2013; Li et al., 2024).

One of the most remarkable properties of T. harzianum is its production of phenol oxidase (laccase like activity), which is a type of enzyme that breaks down lignin and other phenolic compounds, components that are found in plant cell walls and animal dung, that are recalcitrant (Nicholson & Robinson, 1983; Yi et al., 2022). This characteristic makes T. harzianum an important microorganism for the later stages of decomposing the more complex lignified materials once the simpler polymers have been easily hydrolyzed (Rao & Radhakrishna, 2008; Cai et al., 2022).

In the same way, the capacity of A. niger to produce amylase and protease for our tests was consistent with Sohail et al. (2009) and other research works indicating that A. niger strains are good producers of starch and protein degrading enzymes (Siala et al., 2021; Brandi et al., 2024). It is known that A. niger is highly applicable in industries for the production of enzymes like alpha amylase and proteases for use in food, feed and biofuel industries (Abo Nouh et al., 2022). It is important in the breakdown of organic matter at the initial stages because of its capacity to break down starch and proteins; in substrates with high nitrogen, e.g. rabbit dung.

On the other hand, the M. hiemalis, which have only urease activity, seems to be a weaker competitor than many ascomycetes, as earlier reported (Harper and Webster, 1964; Alves et al., 2002; Jamal et al., 2007) since zygomycetous fungi have simpler carbon and nitrogen sources. The reduction in the enzymatic capacity can be explained by the differences in gene regulation, and the absence of a full complement of lignocellulolytic genes, as noted in genomic studies of Mucor species (Jamal et al., 2007). It is ecologically important because it produces urease which liberates readily assimilated nitrogen source (ammonium) that can favor rapid growth during early colonization (Seeliger, 1956; Schimel & Bennett, 2004). This trait may enable M. hiemalis to exploit a niche different from that of other species and use simple nitrogenous compounds and soluble sugars instead of direct competition for polymeric carbon sources (Stark, 2025). Its specialization as a pioneer decomposer, not as a late successional lignocellulose degrader (LCD), is further supported by the lack of protease and phenol oxidase activity (Harper & Webster, 1964; Valle et al., 1994).

Enzyme activity varied among the three fungi indicating a complementarity of activities that may increase the efficiency of decomposition in natural ecosystems where the fungi are present together. The enzymes produced were the widest from T. harzianum, while the enzymes produced by A. niger were amylase and protease and the enzymes produced by M. hiemalis were urease. The enzymatic complementarity is in accordance with the classical model of coprophilous fungal succession proposed by Webster (1983), which suggests that different fungal groups are effective at breaking down different dung components as it decomposes over time (Harper & Webster, 1964; Calaça et al., 2023).

Having multiple enzymatic activities in each species implies that the fungi community as a whole might be better able to utilize substrates than individual species. This agrees with the latest studies that highlight the significance of functional diversity in microbial decomposition processes (Allison et al., 2014; Peña et al., 2025). The two enzymes T. harzianum (cellulase and phenol oxidase) and A. niger (amylase and protease) together with M. hiemalis (urease) have the potential to degrade the starch, cellulose, lignin, proteins and urea present in rabbit dung, which could potentially lead to the rapid release of nutrients and the breakdown of organic matter (Verheggen et al., 2024; Burns et al., 2013; Li et al., 2024).

The interaction assays were partially confirming and partially contradicting to previous results. There is not much antagonism between M. hiemalis and T. harzianum, nor is there strong antagonism between most of the filtrates and A. niger and M. hiemalis, which supports the idea that some coprophilous fungi are able to live together in a more or less symbiotic interaction when resources are plentiful (De Boer et al., 2005; Peña et al., 2025). This is consistent with the niche differentiation theory which suggests that species with different enzyme capacities may not compete directly for the same substrate fractions (Webster, 1983; Sun et al., 2024).

In contrast to the studies that highlight the role of Trichoderma as the predominant antagonist in many systems, where other fungi are inhibited by lytic enzymes and/or secondary metabolites (Wicklow & Yocom, 1980; Harman et al., 2004; Vinale et al., 2008), the clear inhibition of T. harzianum growth by the culture filtrates of A. niger and M. hiemalis is opposite. In our experiment, the ability of T. harzianum to produce enzymes was also better than that of A. niger and M. hiemalis, but the growth of T. harzianum was also affected by A. niger and M. hiemalis metabolites and/or nutrient depletion patterns, which may indicate that antagonistic hierarchies among coprophilous fungi can be context dependent and modulated by substrate composition and incubation conditions.

It is interesting that in the present study, culture filtrates of A. niger and M. hiemalis inhibited the growth of T. harzianum to significant levels. The difference could be due to the production of specific secondary metabolites or organic acids by A. niger which were toxic to T. harzianum under the experimental conditions (Siala et al., 2021; Brandi et al., 2024). A. niger is known to produce various organic acids such as citric acid, gluconic acid and antifungal compounds that can inhibit the growth of other microorganisms (Abo Nouh et al., 2022). Likewise, the ability of M. hiemalis to produce the metabolites of low molecular weight which inhibit the growth of T. harzianum as reported in other zygomycete–ascomycete interactions (Rawat, 2022). At the same time, the observed inhibition may be due to depletion of nutrients in the filtrates instead of direct toxicity, since these were collected after 4 days of fungal growth on a rich medium (PDB) that could have been depleted of key nutrients for T. harzianum (Stark, 2025).

The interaction zones were also examined under the microscope to gain further insights into the nature of antagonistic interactions. Direct hyphal fusion was not observed when T. harzianum was co cultured with A. niger; even though hyphal growth of both fungi was inhibited at short distance. This mechanism of fungal antagonism is known as "hyphal interference" or "contact inhibition" and is common in fungi, where a diffusible antifungal compound is produced or localized cell death occurs (De Boer et al., 2005; Peña et al., 2025). This observation agrees with the well-known ability of Trichoderma species to out-compete competitors through mycoparasitic activity by mechanisms that involve both enzymatic degradation and production of antibiotics (Harman et al., 2004; Han et al., 2025).

The distinct swellings found on the hyphae of M. hiemalis during co culture with A. niger indicate that morphologically M. hiemalis reacts to the presence of A. niger. These swellings are sometimes called "hyphal distortions" or "chlamydospore like structures" and are commonly observed in the context of stress reactions or defense mechanisms in fungi (Wicklow et al., 1980; Rawat, 2022). As a part of interspecific interactions, these structures can be used to survive adverse conditions or to isolate damaged hyphae that will not be able to spread antagonistic factors (De Boer et al., 2005; Stark, 2025).

Interestingly, the superiority of T. harzianum in the enzymatic activity did not equal unconditional antagonism in the antagonism experiments. T. harzianum inhibited the growth of A. niger in direct contact with it (Figure1) but the growth was still affected by soluble metabolites of A. niger as well as by those of M. hiemalis. The correlation between enzymatic ability and antagonistic effect is not linear, but rather there is a complex interplay between enzyme mediated competition for resources, production of antifungal compounds, and physiological interactions (De Boer et al., 2005; Peña et al., 2025).

T. harzianum can produce many extracellular enzymes which can help it in efficient use of substrates, thus increasing its competitive ability in solid substrates, but it can also produce inhibitory metabolites from other species (Stark, 2025). This complex interaction underscores the importance of integrated approaches involving both enzymatic and metabolomic studies and ecological analyses in order to develop a comprehensive understanding of the dynamics of fungal communities (Hassan Dalléac et al., 2026; Manawasinghe et al., 2025). Although the present investigation demonstrates only qualitative and semi quantitative investigations, it does offer a basis for more detailed investigations of the chemical and molecular basis of these interactions.

6. Biotechnological Implications

The synergistic and antagonistic interaction of A. niger, T. harzianum and M. hiemalis mentioned above is of great relevance when developing fungal consortia for waste valorization and composting. The wide range of enzymes produced by T. harzianum makes it an interesting bioaugmenting agent for the final stages of composting, where degradation of lignocellulose and recalcitrant nitrogenous compounds are required (Lima et al., 2024; Yi et al., 2022). In contrast, M. hiemalis may be beneficial during the first thermophilic step, increasing the urease activity and thus the speed of urea hydrolysis and release of ammonium (Manawasinghe et al., 2025). A. niger can be used as intermediate decomposer bacterium that produces amylase and protease activities that can degrade starch and proteins at an early stage during decomposition of the organic waste (Siala et al., 2021; Abo Nouh et al., 2022).

The inhibitory effect of A. niger and M. hiemalis filtrates on T. harzianum however indicates that it is not advisable to use all three species together in a single inoculum without optimization of inoculation time and proportions. A sequential inoculation method, using just the first two soluble substrates (M. hiemalis and A. niger) and then T. harzianum once these have been consumed, could be a solution to this antagonism and help to maximize the overall decomposition efficiency. This is in line with the recent strategy of designing synthetic microbial communities by taking functional complementarity into account, instead of just co culturing a subset of the community (Hassan Dalléac et al., 2026; Han et al., 2025). Moreover, the enzymatic activity of T. harzianum has shown to be of interest for its industrial applications, such as in the production of enzymes and bioremediation, and the acid-producing ability of A. niger can be used for the extraction of minerals and organic matter (Brandi et al., 2024; Lima et al., 2024).

7. Research Significance

This study is one of the rare ones that relate the production of extracellular enzymes to antagonistic interactions between three ecologically important fungi, A. niger, T. harzianum and M. hiemalis, during decomposition of the gamma sterilized compressed rabbit dung. The use of a standardized substrate and conditions allowed us to separate the effects of the enzymatic properties of fungi and interactions with other species from other factors like the indigenous microbial communities and substrate heterogeneity (Calaça et al., 2023; Sun et al., 2024).

The findings provide a mechanistic understanding of the process of fungal community assembly and functional ecology, which provides a scientific basis for predicting decomposition rates and nutrient mineralization in agricultural systems. In addition, the discovery of both synergistic and antagonistic relationships offers valuable insights for developing microbial inoculants for composting and other organic waste management practices to help achieve more sustainable and circular food and agriculture systems (Manawasinghe et al., 2025; Hassan Dalléac et al., 2026). The study also underscores the need for taking the context dependent interactions into account when assessing the biocontrol and bioconversion potential of ubiquitous soil fungi, which has been mostly ignored in previous research (Rawat, 2022; Stark, 2025). The findings will help expand the knowledge on fungal functional ecology and lay the foundation for developing sustainable enzyme-based approaches for organic waste management (Zhang et al., 2026; Cai et al., 2022).

8. Conclusion

The present study shows that the extracellular enzyme profile and the antagonistic activity between the three fungi, A. niger, T. harzianum and M. hiemalis, vary with the dung on which they are growing on gamma sterilized compressed rabbit dung. T. harzianum was the most enzymatically versatile species producing amylase, cellulase, phenol oxidase and urease indicating its potential as a significant decomposer and biocontrol agent. Enzymatic activity of A. niger was moderate (amylase, cellulase, protease) and M. hiemalis was only active in urease, as expected for an early colonizer in the presence of simple substrates. Antagonism assays showed that soluble metabolites from both A. niger and M. hiemalis inhibited the growth of T. harzianum in direct contact, suggesting that chemical signaling plays a critical role in fungal competitive interactions, which are complex. The results can be useful for the design of fungal inoculants for composting and waste valorization, as sequential inoculation appeared to be more effective than co inoculation. Future research is needed to determine the chemical composition of inhibitory metabolites, to test the effectiveness of the selected fungal consortia in non-sterile substrates and to assess the long-term effects of these fungi in soil nutrient cycling and soil health in the field. The work presented here adds to the knowledge of fungal functional ecology and lays the groundwork for the sustainable organic waste management through the use of enzymes.

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