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In vitro potential of *Aspergillus alliaceus* and *Trichoderma harzianum* as chitinase-producing biocontrol agents against *Fusarium* and *Alternaria*

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ABSTRACT

The biocontrol of phytopathogens, including fungal parasites, is considered a natural and eco-friendly alternative to chemical treatments. This study aimed to isolate, screen, and identify rhizospheric fungal strains from durum wheat cultivated in central Algeria, along with endophytic fungi from wheat weeds in the same region, for their ability to produce chitinase, an enzyme relevant for biocontrol. Of the 61 fungal isolates, 37.7% showed varying levels of enzyme activity during primary screening on solid medium. The fifteen most productive strains were selected for secondary screening through submerged fermentation (SmF) using colloidal chitin as the substrate. The chitinase activity assay revealed that *Aspergillus alliaceus* PP235382 and *Trichoderma harzianum* PQ564478 were the most potent after 48 h (0.145±0.006 IU/mL) and 96 h (0.173±0.011 IU/mL) of incubation, respectively. Furthermore, both strains exhibited the highest antagonistic activity against three wheat pathogens (*Fusarium graminearum*, *F. culmorum*, and *Alternaria alternata*) in dual culture, with inhibition rates between 68.27% and 87.71%, among six fungal strains selected for their high chitinase activity. Chitinase production by *T. harzianum* and *A. alliaceus* under SmF conditions was enhanced to achieve 1.167±0.011 IU/mL and 0.915±0.033 IU/mL, respectively, using a mineral medium containing 1% (w/v) colloidal chitin and 1% (w/v) glucose under the same incubation time as previously determined. The crude enzymatic extracts of the antagonists provided the best inhibitory potential against *F. culmorum*. The two newly isolated strains and their chitinolytic extracts could be used to control wheat fungal pathogens.

How to Cite

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INTRODUCTION

Plant pathogens are responsible for the large-scale destruction of various types of crops worldwide. Considerable agricultural losses occur annually due to diseases caused by plant pathogens, which affect productivity and reduce the commercial value of the product. Notably, 78% of losses occur in fruit crops, 54% in vegetable crops, and 32% in cereal crops (Silva et al., 2019). In this context, the FAO (Food and Agriculture Organization of the United Nations) estimates that 14% of global agricultural production losses are due to plant diseases, of which 42% are caused by fungal pathogens, the most prevalent, leading to drastic reductions in crop yields (Roca-Couso et al., 2021).

Fusarium is one of the most phytopathogenic fungi, inducing severe diseases that affect the roots, stems, and spikes of wheat plants at all growth stages. *Fusarium* crown rot (also known as foot or root rot) and *Fusarium* head blight are two serious fungal diseases of wheat worldwide. These diseases are primarily caused by two species, *F. culmorum* and *F. graminearum*, reflecting their high pathogenicity (Abdallah-Nekache et al., 2019; Hadjout et al., 2024). Furthermore, the *Alternaria* genus is the main causal agent of black point in wheat, resulting in significant economic losses. The fungus attacks the leaves, stem and fruit, reducing photosynthesis and inducing defoliation in severe cases (Mohammedi et al., 2022). The *Fusarium* and *Alternaria* genera can co-occur on wheat grains at harvest and persist through storage. This leads to the accumulation of a broad range of mycotoxins in the kernels, reducing their quality and posing health risks to humans and animals (Daichi et al., 2025).

Synthetic fungicides are still the principal method for managing fungal diseases in wheat. Unfortunately, these chemicals do not offer full protection against wheat phytopathogens like *Fusarium* and *Alternaria* (Mebdoua et al., 2025). Moreover, their excessive and irrational use can lead to the development of resistance in pathogens and pose significant risks to human health and ecosystem stability through environmental contamination (Hjort et al., 2014; Tyśkiewicz et al., 2022).

In recent years, interest in the use of biological control as an alternative to agrochemicals has increased (Mendoza et al., 2018). Biological control, using microorganisms and their metabolites, has been the subject of intense research worldwide and offers an environmentally friendly strategy to control phytopathogens (Kurniawan et al., 2018; Köhl et al., 2019). In this context, several rhizospheric and endophytic fungi, such as *Trichoderma*, *Penicillium*, *Aspergillus*, *Verticillium*, *Metarhizium*, *Beauveria*, *Lecanicillium*, *Aphanomyces*, *Neurospora*, and *Mucor*, have been studied for the development of new biopesticides (Gomaa, 2021). More recently, biological control has focused on microorganisms that produce mycolytic enzymes, specifically chitinases (EC.3.2.1.14), which are glycoside hydrolases that catalyze the hydrolysis of β (1,4)-glycosidic bonds between N-acetylglucosamine (GlcNAc) residues in chitin chains (Le and Yang, 2019; Sudha et al., 2020). These enzymes can degrade chitin, the main component of fungal cell walls, without harming the host plant (Kurniawan et al., 2018; Singh et al., 2021).

On the basis of the aforementioned data, we hypothesize that novel fungal strains exhibiting high chitinase activity can be isolated from underexplored environments, such as the rhizospheric soils of healthy cultivated durum wheat and its associated weeds. Previous studies have demonstrated that endophytes from weeds can be effective biocontrol agents (BCAs) against phytopathogens such as *Fusarium* and *Alternaria*. They have the ability to enhance the stress tolerance of plant hosts and protection against pathogens, as well as promote plant growth (Catambacan and Cumagun, 2021, Trung et al., 2021).

The present study aimed to: (i) screen fungal isolates from wheat rhizospheric soil and endophytes from durum wheat weeds in cereal-growing areas of central Algeria for chitinase production, (ii) assess the antagonistic activity of the selected strains against wheat pathogens (*F. culmorum*, *F. graminearum*, and *Alternaria alternata*), (iii) improve chitinase production by the most effective isolates under SmF conditions by varying fermentation parameters, and (iv) evaluate the biocontrol potential of the produced crude enzymatic extracts against the same phytopathogens under controlled conditions.

MATERIALS AND METHODS

Sample processing and isolation of antagonistic fungi

Rhizospheric soil samples of cultivated durum wheat (*Triticum turgidum* var. *durum*) were collected from various cereal regions in central Algeria, as detailed in Table 1. Ten plants, randomly selected at different locations in a single cultivated wheat field, were carefully uprooted (0–15 cm deep), and excess soil was removed by slight shaking. The soil tightly adhering to the roots of the ten plants was then mixed to form a composite sample. The collected samples were placed in sterile plastic bags and stored at 4°C until further processing (Singh and Lal, 2016).

In parallel, samples (leaves, stems and roots) of cultivated durum wheat weeds, namely *Glebionis coronaria* (L.) Spach, *Senecio leucanthemifolius* Poir, *Sinapis arvensis* L., *Papaver rhoeas* L., *Galium tricornutum* Dandy, *Fedia graciliflora* Fisch. & C.A. Mey, *Linaria triphylla* (L.) Mill., *Vaccaria hispanica* (Mill.) Rauschert, and *Bupleurum lancifolium* (Hornem) were collected by uprooting whole weeds, placed in plastic bags, and then stored at 4°C until used for the isolation of fungal endophytes (Hassanein et al., 2016).

Fungal strains were isolated from rhizospheric soil samples using serial dilution, followed by spread plating on Sabouraud medium supplemented with chloramphenicol (0.05 g/L) and amoxicillin (0.5 g/L). Incubation of Petri dishes was performed at 28±2°C for 5–7 days. The procedure described by Hassanein et al. (2016) was applied to isolate endophytic strains from the collected weed plant materials. The obtained colonies were purified by repeated transplantation on potato dextrose agar (PDA) medium under the same conditions. The purified strains were stored at 4°C until further examination.

Morphological and molecular identification

Preliminary identification of the purified fungal strains was based on their macroscopic and microscopic features, following the methods of Campbell et al. (2013) and Kidd et al. (2022). Macroscopic characteristics were examined directly on PDA medium after 5–7 days of incubation. Microscopic identification was conducted using the adhesive tape technique and observation under an optical microscope (400x, OPTICA Axiom 2000, Italy), after staining with 1% (w/v) methylene blue solution.

Table 1. Geographical and climatic properties of the different sampling regions

Province	Site	Location	Average altitude (m)	Climate	Bioclimatic stage
Bouira	Aomar	36°29′33′′N 3°46′16′′E	~438	Mediterranean	Sub-humid
	Ain El Hadjar	36°20′21′′N 3°48′23′′E	~632		Semi-arid
Tizi-Ouzou	Tizi Gheniff	36°35′14′′N 3°46′27′′E	~428		Sub-humid
Medea	Sedraia	36°14′34′′N 3°31′43′′E	~677		Semi-arid to Sub-humid
	Beni Slimane	36°13′37′′N 3°18′21′′E	~673		

Fungal isolates characterized by the highest chitinase activity (hyper-producer strains) were identified by sequencing the beta-tubulin gene from genomic DNA. The primer pairs, βt2a F (forward) (5'GGTAACCAAATCGGTGCTGCTTTC-3') and βt2b R (reverse) (5'ACCCTCAGTGTAGTGACCCTTGGC-3'), were used for partial amplification of the β-tubulin gene. A commercially available DNA extraction kit (Zymo Research Corporation, CA, USA) was used to extract the DNA. Polymerase chain reaction (PCR) was conducted with a 10 µL sample volume and reagents (buffer, deoxynucleotide triphosphates, $MgCl_2$, forward and reverse primers, *Taq* DNA polymerase). Sanger sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Thermo Fisher Scientific Inc.). The sequenced data were matched against the Gen Bank database using the Basic Local Alignment Search Tool (BLAST), which is available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). MEGA11 software was used to evaluate the beta-tubulin sequences of the isolates. The corresponding species of each isolate was subsequently identified.

Primary screening of chitinase production

The chitinase detection medium was a basal medium containing (g/L): 0.3 $MgSO_4.7H_2O$, 3.0 $(NH_4)_2SO_4$, 2.0 KH2PO4, 1.0 citric acid monohydrate, 15 agar, 200 µL Tween-80, 4.5 colloidal chitin, and 0.15 bromocresol purple, pH 4.70. Colloidal chitin was prepared from commercial chitin from shrimp shells (Sigma-Aldrich, Steinheim, Germany) according to the modified method of Hsu and Lockwood (1975). Fresh culture discs (6 mm) of the tested fungal isolates (5–7 days of incubation) were placed on the surface of the medium poured into Petri dishes using a thin metal clamp, and then incubated at 28±2°C. The presence of bromocresol purple in the medium causes its color to change from bright yellow to purple following chitinase-induced chitin degradation (Agrawal and Kotasthane, 2012).

Secondary screening of chitinase production by the SmF process

For this step, only strains that showed positive chitinase production as determined by the previous assay were included. The mineral culture medium used in this test had the following composition (g/L): colloidal chitin 10, $(NH_4)_2SO_4$ 4.2, NaH_2PO_4 6.9, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.3, Tween-80 0.2, $FeSO_4.7H_2O$ 0.005, $MnSO_4$ 0.0016, $ZnSO_4$ 0.0014, $CaCl_2.2H_2O$ 0.002, pH 6.0 (Shivalee et al., 2016). One hundred milliliters of this medium in 250 mL Erlenmeyer flasks were inoculated with 1 mL of fungal suspension containing 10^6 spores/mL. The inoculum size for each strain was estimated by counting spores under an optical microscope using a Malassez cell (Poly-Optik GmbH, Bad Blankenburg, Germany). The flasks were then incubated at 30°C with shaking (Stuart SSL2 reciprocating shaker, Staffordshire, UK) at 100 rpm/min for 5 days. Two milliliters of reaction medium were collected at different incubation times with an interval of 24 h (Mohiddin et al., 2021). The supernatant intended for the enzymatic assay was collected by centrifugation of the samples at 15000×*g* for 5 min (Sigma 1-14K, Osterode am Harz. Germany).

Chitinase activity assay

Chitinase activity was determined by measuring the release of reducing sugars from colloidal chitin according to the method of Monreal and Reese (1969). A mixture containing 200 µL of 0.5% (w/v) colloidal chitin in citrate-phosphate buffer (0.05 M, pH 6.6) and 200 µL of enzyme extract was incubated at 37°C in a water bath (WNB 14, MEMMERT GmbH+Co.KG, Schwabach, Germany) for 1 h. For the control, the sample was treated at 100°C for 15 min before adding the substrate to deactivate the enzymes. Reducing sugars (GlcNAc) were determined by adding 1 mL of dinitrosalicylic acid reagent (Sigma Aldrich, Steinheim, Germany) to the reaction mixture, which was then heated in boiling water for 5 min, cooled to room temperature, and then centrifuged at 4500×*g* for 5 min. Absorbance was measured with a UV–VIS spectrophotometer (Optima, SP-

3000 nano, Tokyo, Japan) at 540 nm. N-acetylglucosamine (1 mg/mL) was used as the standard. One unit of chitinase activity (ChiA) is defined as the amount of enzyme that releases 1 µmol of reducing sugar (GlcNAc) per min per mL of crude extract (Shivalee et al., 2016) and is expressed as IU/mL.

Protein content of enzymatic crude extracts

The concentration of proteins in the crude extracts from the SmF process was estimated by Bradford method (1976). Three milliliters of Bradford's reagent were mixed with 100 µL of enzyme extract, followed by homogenization. Absorbance was measured at 595 nm after 5–10 min. The calibration curve was plotted using bovine serum albumin (1 mg/mL) as a standard.

***In vitro* antagonistic activity**

The antagonistic capacity of the selected fungi exhibiting high chitinase activity according to the screening tests was evaluated *in vitro* using the dual culture technique involving direct confrontation in Petri dishes. This method was used to evaluate the antagonistic capacity of six fungal isolates against three wheat pathogens: *F. graminearum*, *F. culmorum*, and *A. alternata* on PDA medium. These pathogens were isolated from the aerial part of infected durum wheat and identified based on phenotypic (macroscopic and microscopic) and molecular analysis (only for *F. culmorum* PX843723 by sequencing the ITS region). The pathogen strains were maintained at 4°C on PDA slants and subcultured on the same growth medium in Petri dishes for 7 days at 30°C prior to subsequent *in vitro* antagonistic activity experiments.

To perform this test, 6 mm mycelial discs of fungal pathogenic strains and antagonist isolates cut from 7-day-old cultures, were placed on PDA medium 5 cm apart and incubated for 7 days at 30°C (Khatri et al., 2017). Each treatment was performed in triplicate. The plates inoculated only with the test pathogens served as controls. The percentage inhibition of radial growth (PIRG) was calculated using the following formula:

$$PIRG\ (\%) = [(R_1 - R_2)/R_1] \times 100$$

where R_1 is the distance (in cm) from the inoculation point to the edge of the colony on the control plate (radial diameter), and R_2 is the distance of fungal growth from the point inoculation to the edge of the colony on the treated plates towards the antagonist (Saravanakumar and Wang, 2020). The following inhibition criteria were adopted in our study to evaluate the antifungal activity of the tested isolates: no inhibition (PIRG = 0–20%), moderate inhibition (PIRG = 21–30%), strong inhibition (PIRG = 31–50%), and very strong inhibition (PIRG > 50%) (Brzezinska and Jankiewicz, 2012).

Light microscopic analysis of fungal interactions

To study the different hyphal interactions between the antagonistic strains tested and the phytopathogens, microscopic preparations were made after sampling in the interpenetration zone between the two confronted isolates. A mycelial fragment of the phytopathogen was adhered to adhesive tape, placed on a slide and then stained with a few drops of methylene blue. Observations were made using an optical microscope set at 400x magnification to estimate, for each confrontation, the morphological modifications of the phytopathogens (coiling, denaturation, penetration, and disintegration) caused by the selected antagonists.

Enhancement of chitinase production

To improve chitinase production by the hyper-producing strains, the effects of some parameters of the SmF process were tested, including the type of substrate, incubation time (24 h, 48 h, 72 h, 96 h, and 120 h), and the addition of carbon (glucose, fructose, xylose, galactose, sucrose, and starch) and nitrogen (ammonium sulfate, ammonium nitrate, yeast extract, casein peptone, and tryptone) sources at 1% (w/v) to the culture medium. The classical approach applied in this step of the study was the "one-factor-at-a-time" (OFAT) method. It relies on changing one parameter at a time while the other factors remain stable.

To study the effects of different chitinous materials, colloidal chitin was replaced by other substrates at the same concentration (1%, w/v). These included commercial chitin powder, shrimp shells, bee cuticles (cleaned, air-dried, and crushed), as well as lab-produced chitin from shrimp shells using two methods described by Yavari-Bafghi et al. (2019) (method 1) and de Queiroz Antonino et al. (2017) (method 2).

Submerged fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of culture medium (pH 6.0) (Shivalee et al., 2016) to which a source of C, N, or substrate was added at 1% (w/v), inoculated with a fungal suspension (10^6 spores/mL), and incubated at 30°C with constant shaking (80 rpm/min). For all these assays, liquid medium supplemented with 1% (w/v) colloidal chitin was used as a control. Crude enzymatic extracts were recovered by centrifugation at 4500×*g* for 15 min at 4°C (MPW-352R, MED. Instruments, Warszawa, Poland).

Effect of chitinases on the mycelial growth of wheat pathogens

The antifungal activity of the crude enzymatic extracts obtained after enhanced chitinase production was evaluated against the same three wheat pathogens (*F. graminearum*, *F. culmorum*, and *A. alternata*) according to the method of Abo-Zaid et al. (2021).

A fungal pathogen plug (6 mm diameter) from a freshly grown culture was inoculated into a mixture of 50 mL potato dextrose broth (PDB) and crude enzymatic extract (2%, v/v). Flasks containing only the pathogenic fungal plugs were used as controls. Cultures were incubated at 30°C for 168 h under static conditions. Fungal growth was determined by dry weight at 50°C after filtration through preweighed Whatman filter paper No. 4. The percentage weight reduction (PWR) of the test fungus was calculated using the following formula:

$$PWR\ (\%) = [(W_1 - W_2)/W_1] \times 100$$

where W_1 is the weight (g) of the pathogenic fungus tested in a control flask, and W_2 is the weight (g) of pathogenic mycelia in the presence of the enzyme extract.

Statistical analysis

Results are expressed as the mean ± SD, and the measurements were repeated at least three times. Statistical analysis of the collected data was performed using JMP® Pro 13.2.1 software (SAS Institute Inc., USA). The difference between enzymatic activities and antagonism percentages was considered statistically significant when the calculated p-value was ≤0.05 according to the One-way ANOVA analysis followed by the Tukey–Kramer HSD test. In addition, the effects of carbon and nitrogen sources and substrate type were statistically evaluated using the Student's t-test.

RESULTS AND DISCUSSION

Isolation of fungal antagonists

Effective biological control generally uses naturally occurring antagonists that can effectively reduce the activities of plant pathogens (Silva et al., 2019). Microorganisms as BCAs are most commonly sorted by screening rhizospheric strains or endophytes for their ability to inhibit the growth of target pathogens (O'Brien, 2017). Therefore, isolating such organisms is the first step in any biological control program.

Isolation from rhizospheric soils of healthy cultivated durum wheat taken from three cereal-producing regions of central Algeria (Bouira (B), Tizi-Ouzou (T), and Medea (M)) yielded a collection of 40 fungal isolates. Morphological identification revealed that the isolates belonged to the following genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Alternaria*, *Trichoderma*, and *Absidia*, with differing levels of abundance. Similarly, 21 fungal endophytes were isolated from weed samples of cultivated durum wheat (*Fusarium* spp., *Alternaria* spp., *Aspergillus* spp.). The phenotypic characteristics of the other strains were insufficient to identify their genera.

Primary screening of chitinase production on solid medium

The detection of chitinases produced by the fungal isolates on solid medium revealed that 23 strains were able to secrete these enzymes among the 61 isolates screened. The effectiveness of the isolated antagonists varied with their ability to synthesize chitinases and degrade chitin during a short incubation period. As shown in Table 2 and Fig. 1, the best-producing isolates with high and very high chitinase activity observed after 72 h of incubation were MSF3 (*Trichoderma* sp.), MSF4 (*Penicillium* sp.), MSF8 (*Aspergillus* sp.), EndP2SF3 (*Aspergillus* sp. section *Nigri*), TSF5 (*Aspergillus* sp.), and EndP9SF15 (*Aspergillus* sp. section *Flavi*), which showed a concentrated purple color with large areas ranging from 5.9±0.70 to 7.9±0.0 cm. The strains BSF6, BSF3 (*Fusarium* spp.), and BSF5 (*Aspergillus* sp. section *Nigri*) were characterized by less intense purple zones and slightly smaller diameters in comparison to the first isolates. Other strains, such as BSF1 (*Rhizopus* sp.), BSF2 (*Absidia* sp.), MSF1 (*Rhizopus* sp.), and MSF2 (*Aspergillus* sp. section *Nigri*) developed pale zones with large diameters, while BSF4 (*Fusarium* sp.), MSF4', and MSF6 (*Penicillium* spp.) showed moderate chitinase activity. The remaining isolates presented low, very low, or no activity, even after 168 h of incubation.

Notably, the release of chitinases onto the medium by the best strains (23 isolates) increased with the incubation period. After 7 days, some isolates developed a dark purple color with an increase in zone diameter (i.e., BSF6, TFS5, MSF4, MSF8). The results of this test revealed that 40% of the rhizospheric fungi and 33.33% of the endophytes isolated from different regions were able to secrete chitinases on solid medium under the test conditions.

Our results are consistent with those obtained by Muhanna (2019), who screened *Trichoderma viride*, *F. oxysporum*, *F. solani*, *A. alternata*, *Alternaria solani*, and other strains, resulting in a purple zone diameter of 3 to 8 cm around fungal colonies. Another work, in which 16 isolates of *Trichoderma* were screened on solid bromocresol medium containing colloidal chitin, confirmed chitinase production at different concentrations and diameters ranging from 1.7 to 9 cm (Sayed et al., 2019).

Secondary screening of chitinase production on liquid medium

According to the results of the first screening test, fifteen fungal strains were selected for secondary screening on a liquid medium comprising colloidal chitin as the sole source of carbon and energy.

Table 2. Primary screening (colored zone diameter in cm) of some fungal isolates on solid medium [The diameter of the fungal disc (0.6 cm) was eliminated. The diameter of the reverse side of the Petri dish was 8.5 cm. Values not connected by the same letter in the same column for the same incubation period are significantly different ($p \leq 0.05$) as determined by the test of Tukey]

Strains	72 h of incubation	168 h of incubation	Strains	72 h of incubation	168 h of incubation
Rhizospheric isolates					
BSF1	7.9±0.00^{a}	7.9±0.00^{a}	MSF3	6.4±0.73^{ab}	7.9±0.00^{a}
BSF2	7.9±0.00^{a}	7.9±0.00^{a}	MSF4	7.9±0.00^{a}	7.9±0.00^{a}
BSF3	4.5±0.55^{cde}	7.5±0.30^{a}	MSF4'	4.2±0.58^{de}	4.9±0.66^{bc}
BSF4	3.0±0.52^{ef}	5.4±0.25^{b}	MSF5	2.5±0.10^{f}	4.3±0.30^{c}
BSF5	5.9±0.70^{bc}	7.9±0.00^{a}	MSF6	4.6±0.60^{cd}	5.2±0.75^{b}
BSF6	5.7±0.63^{bcd}	7.9±0.00^{a}	MSF7	2.0±0.00^{f}	5.4±0.80^{b}
MSF1	7.9±0.00^{a}	7.9±0.00^{a}	MSF8	7.9±0.00^{a}	7.9±0.00^{a}
MSF2	6.4±0.82^{ab}	7.9±0.00^{a}	TSF5	5.9±0.80^{bc}	7.9±0.00^{a}
Endophytic isolates of weeds					
EndP2SF3	6.9±0.68^{ab}	7.1±0.70^{a}	EndP10SF17	2.4±0.50^{f}	5.4±0.45^{b}
EndP6SF11	1.4±0.10^{f}	7.9±0.00^{a}	EndP9SF15	5.9±0.40^{bc}	7.4±0.40^{a}

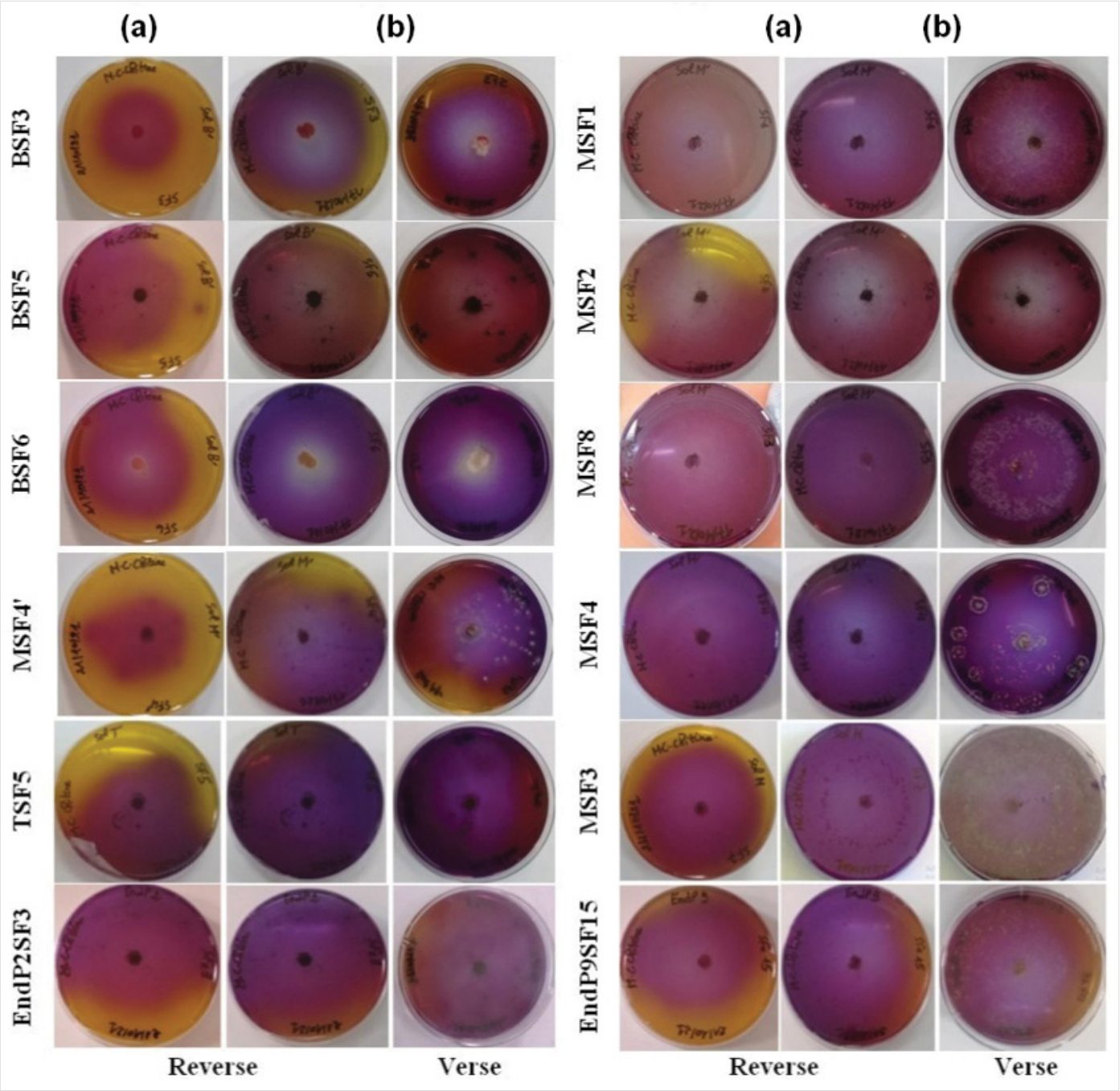

Figure 1. Chitinase production by some fungal isolates after 72 h **(a)** and 168 h **(b)** of incubation on solid medium supplemented with colloidal chitin. The selection of chitinolytic fungal isolates was based on the largest diameter of the highly concentrated purple zone around the colonies after 72 h of incubation **(a)**, which reflects high chitinase production. After 168 h of incubation **(b)**, an improvement in the diameter and concentration of the purple color was observed, indicating that the strains continue to consume colloidal chitin, but with different enzymatic production abilities

The selected strains showed good chitinase activity with two strains exibiting moderate activity after 72 h of incubation, and were as follows: MSF3 (*Trichoderma* sp.), BSF2 (*Absidia* sp.), EndP9SF15 (*Aspergillus* sp. section *Flavi*), MSF8, TSF5 (*Aspergillus* spp.), BSF5, MSF2, EndP2SF3 (*Aspergillus* spp. section *Nigri*), BSF1, MSF1 (*Rhizopus* spp.), BSF3, BSF6 (*Fusarium* spp.), MSF4, MSF4' and MSF6 (*Penicillium* spp.).

As illustrated in Fig. 2, the chitinase activity of each strain was determined every 24 h of incubation until day 5. All strains were able to secrete chitinases on liquid medium after 48 h of incubation. However, only five strains exhibited such activity after 24 h of incubation. The findings also showed that maximum activities were reached after 48 h of incubation for MSF8 (0.145±0.006 IU/mL) and after 96 h for MSF3 (0.173±0.011 IU/mL). Both MSF6 (0.123±0.013 IU/mL) and TSF5 (0.161±0.014 IU/mL) strains exhibited good levels of chitinase activity after 120 h of incubation. Moreover, after 48 h, BSF3 and EndP2SF3 crude extracts showed moderate chitinase activity of 0.101±0.019 and 0.108±0.004 IU/mL, respectively. However, these activities remained lower than the previous ones.

Therefore, based on the primary and secondary screening results, the six strains - MSF3 (*Trichoderma* sp.), BSF3 (*Fusarium* sp.), EndP2SF3 (*Aspergillus* sp. section *Nigri*), MSF4 (*Penicillium* sp.), MSF8, and TSF5 (*Aspergillus* spp.) - were selected to test their antagonistic effect against three wheat phytopathogens.

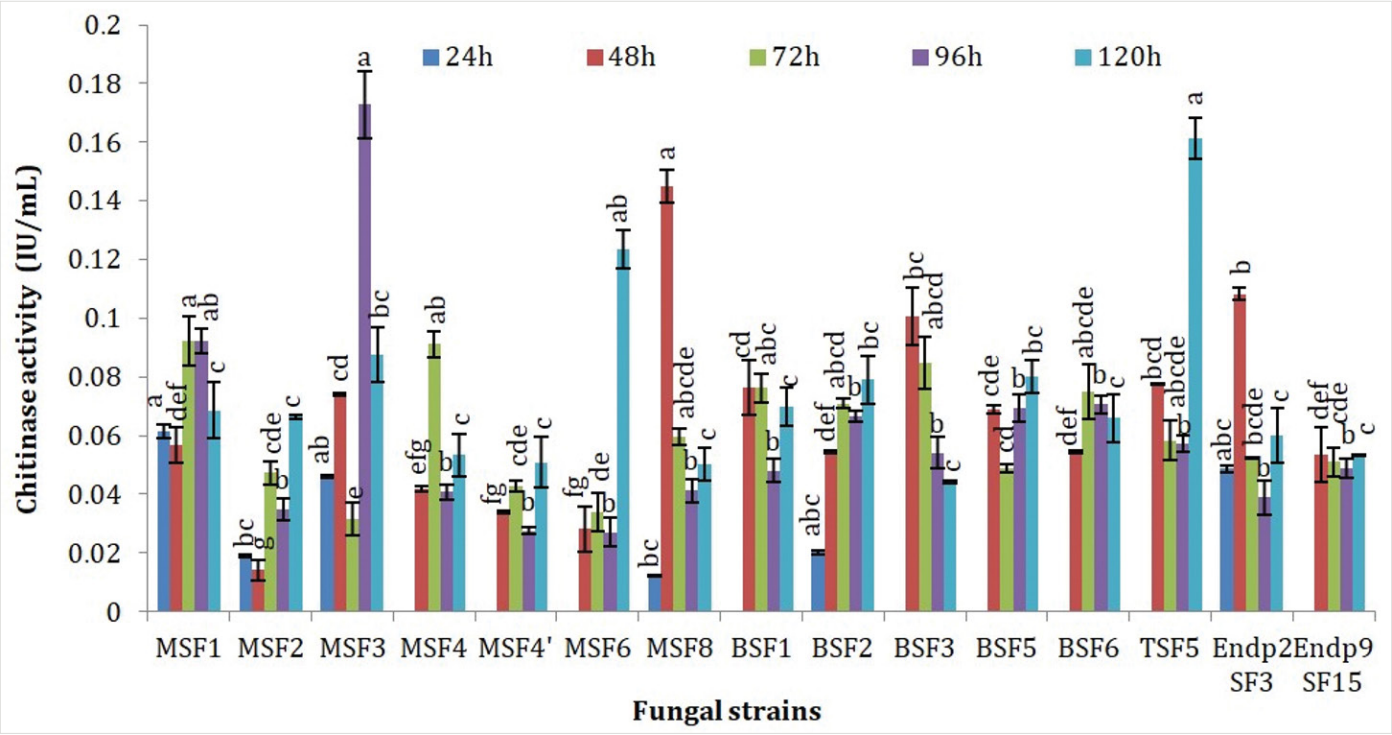

Figure 2. Exochitinase activity of the selected isolates grown on liquid medium containing colloidal chitin as the substrate. Different letters for the same incubation time are statistically significant ($p \leq 0.05$, Tukey's test)

The results obtained for MSF3 and MSF8, as the most potent strains, are in accordance with the findings of Wasli et al. (2009) for *Trichoderma virens* (ChiA= 0.147 U/mL) and Ornela and Guimarães (2024) for *Aspergillus niveus* LH0306 (ChiA = 0.140 IU/mL). These activities were achieved after 96 h of incubation under SmF conditions using colloidal chitin and shrimp shells as the fermentation substrates, respectively. In contrast, low chitinase activities were reported for other fungal and bacterial strains (Brzezinska and Jankiewicz, 2012; Stoykov et al., 2014; Herdyastuti et al., 2021).

Antagonistic activity in dual culture

The use of any BCA is based on a good knowledge of the processes involved in its antagonistic activity. The mycelial confrontation assay in dual culture is often used to assess the ability of antagonistic germs (El-Debaiky, 2017; Boughalleb-M'Hamdi et al., 2018).

During this work, particular interest was given to evaluating the antagonistic activity of the six highest chitinase-producing isolates against three wheat pathogens, namely *F. graminearum*, *F. culmorum*, and *A. alternata*. Inhibition of phytopathogen growth was evaluated by determining the PIRG applied by each antagonist, calculated after 72, 120, and 168 h of incubation, and the isolates with the greatest antagonistic activity were sorted.

The results presented in Table 3 and Fig. 3 show that all antagonistic isolates significantly inhibited the mycelial growth of the three phytopathogens, but to different degrees. This effect occurred from the third day and reached a maximum (total inhibition) after 168 hours of confrontation, with MSF3 (*Trichoderma* sp.) and MSF8 (*Aspergillus* sp.) being the most effective. The first strain strongly inhibited the growth of all three pathogens, with PIRG values ranging from 81.13±2.05% to 87.71±1.45%, followed by MSF8 (*Aspergillus* sp.) (inhibition rates between 68.27±1.17%–81.43±1.80%).

Table 3. Mycelial growth inhibition (%) of three wheat pathogens by six fungal isolates after each incubation period [Values not connected by the same letter in the same column are significantly different ($p \leq 0.05$) as determined by the Tukey test. No inhibition (PIRG = 0–20%), moderate inhibition (PIRG = 21–30%), strong inhibition (PIRG = 31–50%), and very strong inhibition (PIRG > 50%)].

Pathogens / Antagonist	*F. graminearum*			*F. culmorum*			*A. alternata*		
	72 h	120 h	168 h	72 h	120 h	168 h	72 h	120 h	168 h
MSF3	37.50±1.22^{a}	66.66±1.47^{a}	81.13±2.05^{a}	47.36±1.60^{a}	69.23±1.93^{a}	82.75±2.10^{a}	59.26±1.74^{a}	80.43±1.38^{a}	87.71±1.45^{a}
MSF8	15.00±0.62^{b}	51.66±1.33^{b}	73.58±1.75^{b}	28.24±1.15^{b}	49.74±0.78^{b}	68.27±1.17^{b}	47.65±1.85^{b}	72.26±1.32^{b}	81.43±1.80^{b}
EndP2SF3	13.12±0.21^{c}	50.00±1.50^{b}	70.31±1.21^{b}	21.57±1.04^{cd}	46.15±0.84^{c}	69.08±1.19^{b}	41.66±1.66^{c}	67.00±1.55^{c}	79.22±1.25^{b}
BSF3	00.00±0.00^{d}	16.66±0.33^{c}	54.96±1.28^{c}	10.52±0.10^{e}	13.84±0.24^{e}	52.52±1.10^{c}	16.66±0.86^{e}	42.00±1.42^{d}	65.55±1.62^{c}
MSF4	00.00±0.00^{d}	16.66±0.42^{c}	31.57±0.70^{d}	23.68±0.73^{c}	38.46±0.85^{d}	52.75±1.15^{c}	10.00±0.35^{f}	22.00±0.74^{f}	55.55±1.74^{d}
TSF5	00.41±0.02^{d}	12.77±0.96^{d}	43.40±1.33^{e}	20.35±0.60^{d}	14.05±0.70^{e}	50.28±1.49^{c}	33.33±0.93^{d}	30.00±0.67^{e}	64.44±1.42^{c}

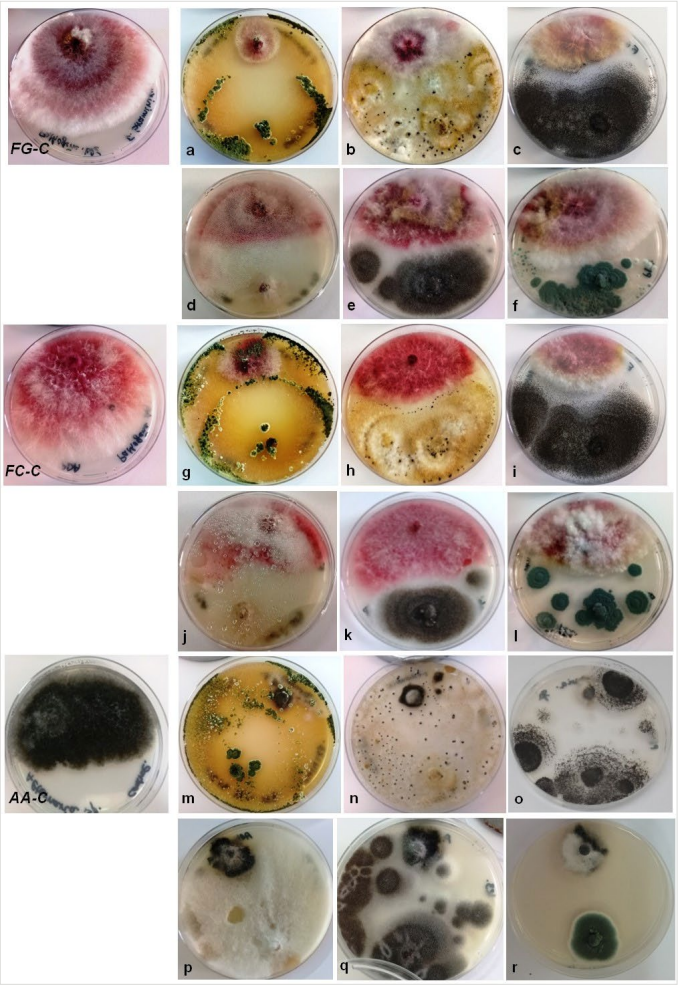

Figure 3. *In vitro* antagonistic activity of six fungal strains against wheat pathogens on PDA medium after 7 days of confrontation; **(a–f)** MSF3 (*Trichoderma* sp.), MSF8 (*Aspergillus* sp.), EndP2SF3 (*Aspergillus* sp. section *Nigri*), BSF3 (*Fusarium* sp.), TSF5 (*Aspergillus* sp.), and MSF4 (*Penicillium* sp.) strains against *F. graminearum*; **(g–l)** *F. culmorum*; and **(m–r)** *A. alternata*, respectively; (Fg-c, Fc-c, and Aa-c represent the growth control of each phytopathogen)

Furthermore, both antagonists exhibited significant antifungal activity against *A. alternata* after only 5 days of incubation. The endophytic strain EndP2SF3 (*Aspergillus* sp. section *Nigri*) had an antagonistic effect similar to that of the MS8 strain against the three pathogens tested. Finally, in the presence of BSF3 (*Fusarium* sp.), TSF5 (*Aspergillus* sp.), and MSF4 (*Penicillium* sp.), inhibition rates did not exceed 55% after 168 h of incubation, and were significantly lower than those of the previous strains ($p \leq 0.0001$). As an exception, BSF3 and TSF5 strains exerted a good inhibitory effect against *A. alternata* with values of 65.55±1.62% and 64.44±1.42%, respectively. Accordingly, *A. alternata* was found to be the most sensitive among the tested phytopathogens. As shown in Fig. 3, most of the isolates tested exhibited antagonistic action through contact.

The inhibitory efficacy of MSF8 and MSF3 can be attributed to their rapid growth towards pathogens. They occupied the growth space of the pathogen until making contact with its mycelium, then surrounded the pathogen colony and finally invaded it, probably showing a mycoparasitism mechanism for the MSF3 strain and a competition mechanism for MSF8. The antagonistic effect of the EndP2SF3 strain was also characterized by rapid growth toward pathogens. In addition, this strain was able to reduce the color of *Fusarium* colonies from burgundy to yellow, which was particularly evident on the reverse side of the colonies. Hence, MSF8 and MSF3 could serve as excellent antagonists based on the growth inhibition results.

Several researchers reported that *Trichoderma* and *Aspergillus* species were characterized by their mode of action, especially their ability to occupy spaces before the arrival of phytopathogens, their mycoparasitic activity and strong competitive ability (El-Debaiky, 2017; Boughalleb-M'Hamdi et al., 2018; Khan and Javaid, 2021). The high inhibitory potency of *Trichoderma* and *Aspergillus* isolates against phytopathogenic fungi has been demonstrated in many studies.

The biocontrol efficacy of MSF3 (*Trichoderma* sp.) and MSF8 (*Aspergillus* sp.) against *Fusarium* and *Alternaria* species is consistent with those that determined for five *Trichoderma* species (*T. atroviride*, *T. harzianum*, *T. virens*, *T. asperellum*, and *T. koningiopsis*) against *F. graminearum* (inhibition rates ranged between 70% and 80%) (Saravanakumar and Wang, 2020), and for *Trichoderma simmonsii* against *Alternaria brassiceae* (PIRG=78%) (Kumari and Sharfuddin, 2022). Similarly, Rahman et al. (2023) demonstrated the high efficacy of three *T. harzianum* isolates in suppressing the growth of *F. oxysporum* f. sp. *lycopersicum* (values between 88.80% and 92.50%). Additionally, some *Aspergillus* species tested as biocontrol agents (*A. flavus, A. niger*, and *A. terreus*) induced low to good reductions in the mycelial growth of *F. oxysporum* f. sp. *melonis*, *F. solani* f. sp. *cucurbitae*, and *F. solani* f. sp. *melonis* colonies (Boughalleb-M'Hamdi et al., 2018). Likewise, Khan and Javaid (2021) reported that *A. flavipes* displayed moderate antifungal activity against *Macrophomina phaseolina*, with percent inhibition values of 53%. The differences observed between isolates illustrate the variability of these antagonists and clearly demonstrate the need for rigorous screening before developing a control strategy based on microbial antagonism.

Antagonist-pathogen hyphal interactions

Microscopic observations of phytopathogen hyphae from the confrontation zone revealed several morphological modifications. The MSF3 strain (*Trichoderma* sp.) exhibited a mycoparasitism mechanism against *F. graminearum* and *A. alternata* by coiling around the pathogen hyphae, adhering to their hyphae, and penetrating through the formation of haustoria knob-like structures leading to cell lysis. After invading the host hyphae to derive nutrients, they became shrunken and lysed (Fig. 4a, b, and e). These observations are consistent with those obtained in the study of hyphal interactions of *T. harzianum* against *A. alternata*, A. solani, Botrytis cinerea, Sclerotium cepivorum, and Sclerotinia sclerotiorum (El-Debaiky, 2017), of T. viride against *F. oxysporum* f. sp. niveum (Boughalleb-M'Hamdi et al., 2018), and T. afroharzianum against Diplodia seriata (Kovács et al., 2021).

The MSF8 antagonist in contact with *F. culmorum* and *A. alternata* hyphae caused direct and strong cell wall degradation (Fig. 4d), breakage of host hyphae, and cell lysis (Fig. 4c). Moreover, a strong absence of phytopathogen spores and inhibition of their germination were observed near the antagonist hyphae. In contrast, hyphal interactions resulted in an increase in the number of antagonist conidia. The strong

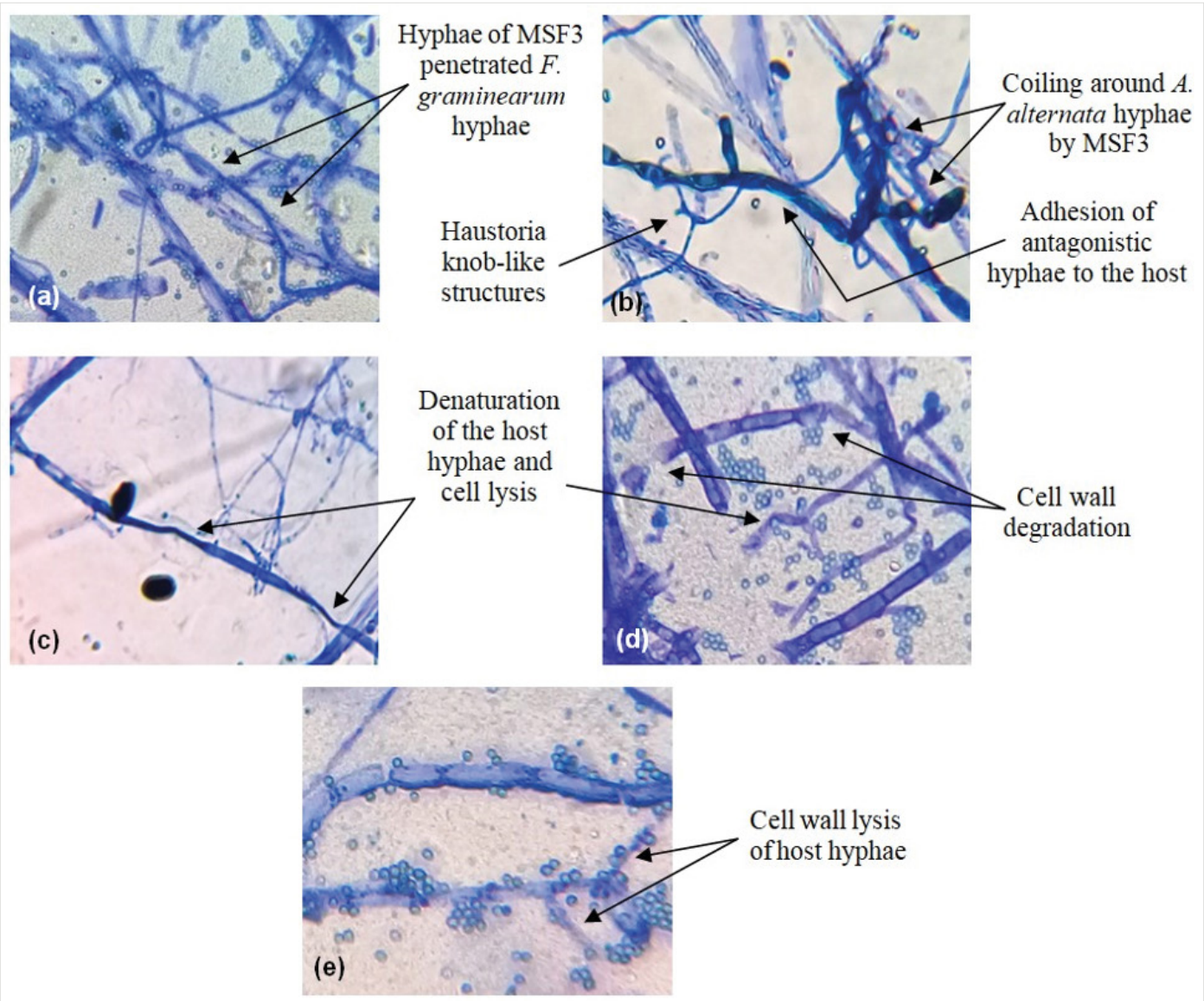

Figure 4. Microscopic views of the contact zone between the mycelia of (a) MSF3 vs. *F. graminearum*; (b) MSF3 vs. *A. alternata*; (c) MSF8 vs. *A. alternata*; (d) MSF8 vs. *F. culmorum*; and (e) MSF3 vs. *F. graminearum*

cell wall degradation and hyphal denaturation observed after the MSF8 strain attacked *A. alternata* and *Fusarium* hyphae may be due to the production of lytic enzymes and secondary metabolites, which can be classified as an antibiosis mechanism according to El-Debaiky (2017).

Moreover, the phenomena observed for both strains, such as hyper-sporulation, conidia binding to phytopathogen filaments, and their lysis, are closely linked to lytic enzyme activity, favoring mycoparasitism that prevents pathogen proliferation (Ferreira et al., 2020). Similar findings have been reported for *A. piperis* vs. *A. alternata* (El-Debaiky, 2017), *A. flavus* vs. *F. oxysporum* f. sp. *melonis* (Boughalleb-M'Hamdi et al., 2018), and different strains of *Trichoderma* vs. *A. alternata*, *Colletotrichum gloeosporioides*, and *Penicillium digitatum* A21 (Ferreira et al., 2020). To the best of our knowledge, this work demonstrates for the first time the inhibitory effect and hyphal interactions of *A. alliaceus* against *Fusarium* and *Alternaria* wheat pathogens.

Accordingly, the two strains - MSF3 (*Trichoderma* sp.) and MSF8 (*Aspergillus* sp.) - were hyperproducers of chitinases and showed the highest antagonistic effect against wheat fungal pathogens; thus, they were selected for further studies.

Identification of fungal strains

A pure culture of MSF3 (*Trichoderma* sp.) initially appeared white and fluffy, later developing yellowish-green to dark green compact clusters, often in small areas or concentric ring-like zones on the agar surface. The medium of mature colonies was characterized by an intense yellow to dark brown pigmentation. The mycelium was initially smooth, watery white, and sparse until floccose aerial mycelium formed (Fig. 5a). The conidiophores are pyramidal and highly branched, usually in groups of three or four. Conidiophore branches are usually paired. Phialides are bulbous and typically short and broad in the middle. Conidia are globose to subglobose with a pale green color (Fig. 5b). These morphological features are confirmed by Siddiquee (2017) who described the macroscopic and microscopic features of various *Trichoderma* species.

The culture of MSF8 (*Aspergillus* sp.) was characterized by its white, yellow to yellowish-brown color, with white floccose mycelium, dense or sparse. The macroscopic examination of MSF8 plates revealed the presence of rigid sclerotia, initially white, turning dark gray to black with age (Fig. 6a). Conidia are smooth-walled, subglobose to ovoid, cream to yellow or yellow ochre.

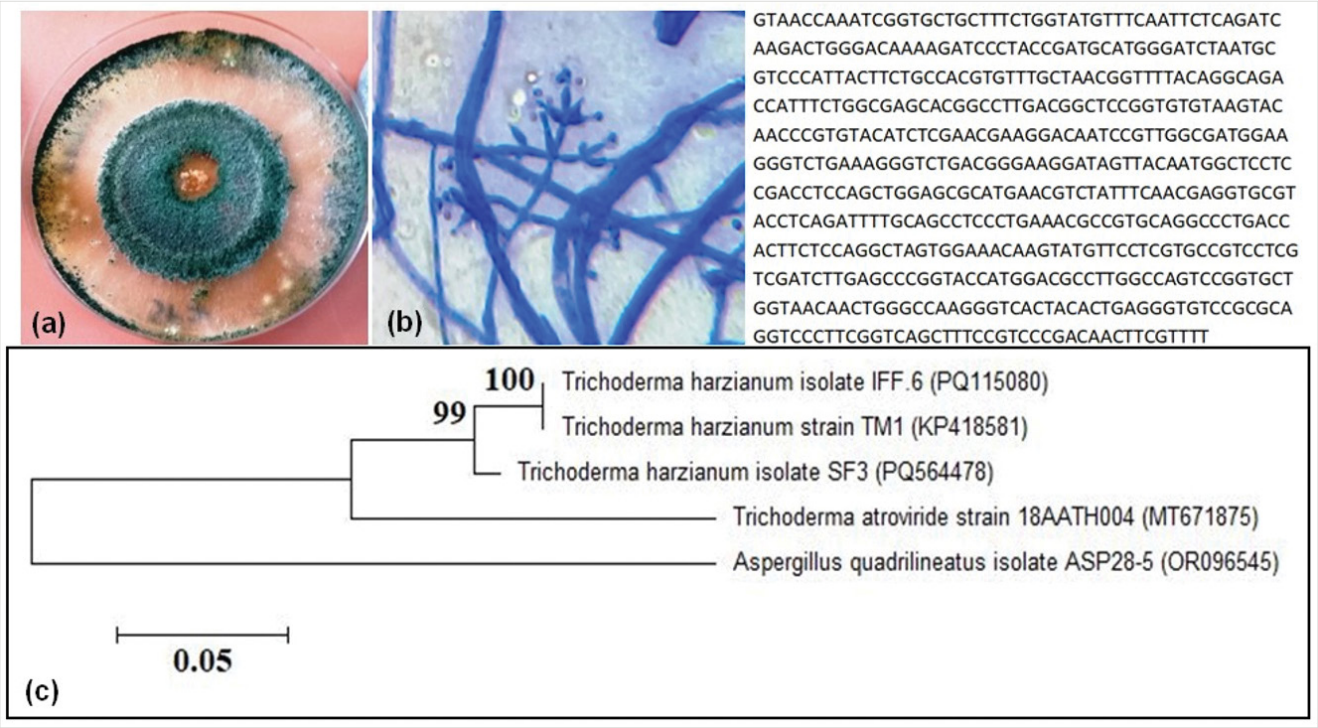

Figure 5. Morphological features **(a–b)**, sequence from the β-tubulin gene, and phylogenetic tree **(c)** of the MSF3 strain (SF3): *Trichoderma harzianum* PQ564478

The conidial colors tend to be deeper gold shades. Conidial heads are biseriate on large vesicles, often uniseriate on small vesicles, with metulae or phialides covering at least the upper half of the vesicle (Fig. 6b). This identification was supported by the study of Klich (2002).

Analysis of the β-tubulin gene sequences was used for species identification of the selected strains MSF3 and MSF8. The PCR products obtained after purification and sequencing are shown in Fig. 5 and 6. Partial amplification of the β-tubulin gene using βt2a and βt2b primers yielded products of 357 bp and 541 bp for MSF8 and MSF3, respectively. The generated sequences were analyzed and aligned to reference sequences in the NCBI database using the BLAST tool. Based on the analyses performed accordingly, the strains MSF3 (SF3) and MSF8 were identified as *Trichoderma harzianum* and *Aspergillus alliaceus*, with GenBank accession numbers PQ564478 and PP235382, respectively. Phylogenetic trees constructed with MEGA11 Software revealed that *T. harzianum* PQ564478 had 99% similarity with *T. harzianum* PQ115080 and *T. harzianum* KP418581 (Fig. 5c). In the case of *A. alliaceus* PP235382, 100% similarity was found with the strain *A. alliaceus* MT211761 (Fig. 6c).

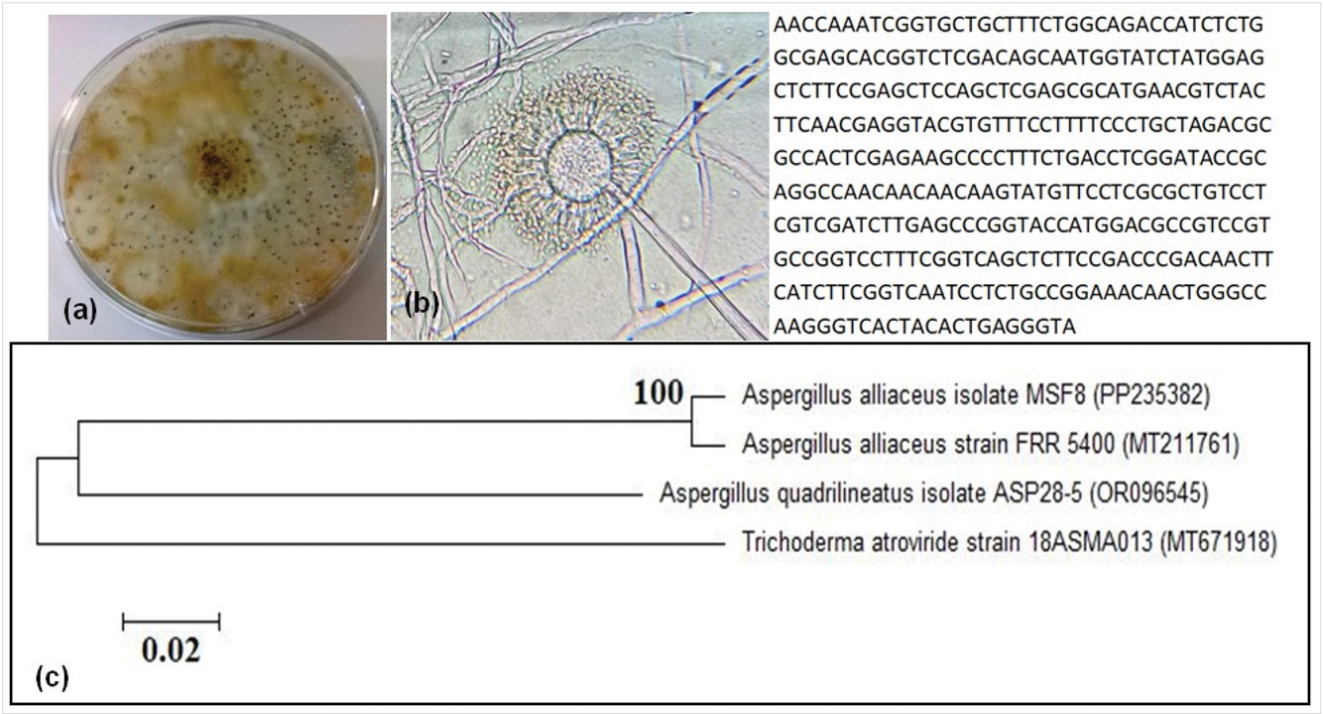

Figure 6. Morphological characteristics **(a–b)**, sequence from the β-tubulin gene, and phylogenetic tree **(c)** of the MSF8 strain: *Aspergillus alliaceus* PP235382

Chitinase production by the SmF process

For the enhancement of extracellular chitinase production by *T. harzianum* PQ564478 and *A. alliaceus* PP235382, several culture conditions controlling and influencing the SmF process were investigated. These factors included the type of substrate, incubation time, and addition of various carbon and nitrogen sources.

Effect of substrate on chitinase production

The major factor affecting chitinase production and its activity is the type and form of chitin (Gomaa, 2021). Among all the chitinous substances tested, colloidal chitin at 1% (w/v) was the most suitable for chitinase production by both strains (Fig. 7a). Bee cuticles also provided good activities and seemed to be more favorable for chitinase production compared to shrimp shells and chitin powder. Chitin extracted at laboratory scale by two methods (M1 and M2) yielded the lowest enzyme production, possibly due to its more rigid structure than the other substrates and its low solubility, which limited the release of oligomers in the medium. Compared to colloidal chitin, all substrates tested significantly decreased chitinase production by both strains ($p \leq 0.0001$).

Different chitinous substances, such as shrimp shell waste, shellfish waste, crab shells, or commercial chitin, were used in the chitinase production process as the principal carbon, nitrogen, and energy sources (Karthik et al., 2017; Singh et al., 2021; Paul et al., 2022). However, the presence of a small amount of GIcNAc in colloidal chitin appears to stimulate enzyme production more effectively, making it the best inducer of chitinase expression in comparison to other substrates (Stoykov et al., 2014; Karthik et al., 2017). Our results are in agreement with these conclusions. Several studies have shown that this substance is more suitable for fungal chitinase production (Loc et al., 2020; Xie et al., 2021; Wang et al., 2023).

Effect of incubation time on chitinase production

The incubation period has a significant influence on chitinase production, as it increases to a maximum level after some time and then decreases with further incubation (Paul et al., 2022). Most bacterial and fungal sources generally require 48–96 h for maximum chitinase production (Karthik et al., 2017; Singh et al., 2021).

As shown in Fig. 7b, chitinase activity was present in the culture filtrate after 24 h of incubation for both strains. The maximum chitinase activity for *A. alliaceus* was recorded after only 48 h of incubation, while *T. harzianum* required 96 h to reach its peak. Thereafter, enzyme productivity decreased significantly ($p \leq 0.01$), probably caused by nutrient depletion in the fermentation medium and accumulation of inhibitory products, leading to inactivation of the enzymatic secretory

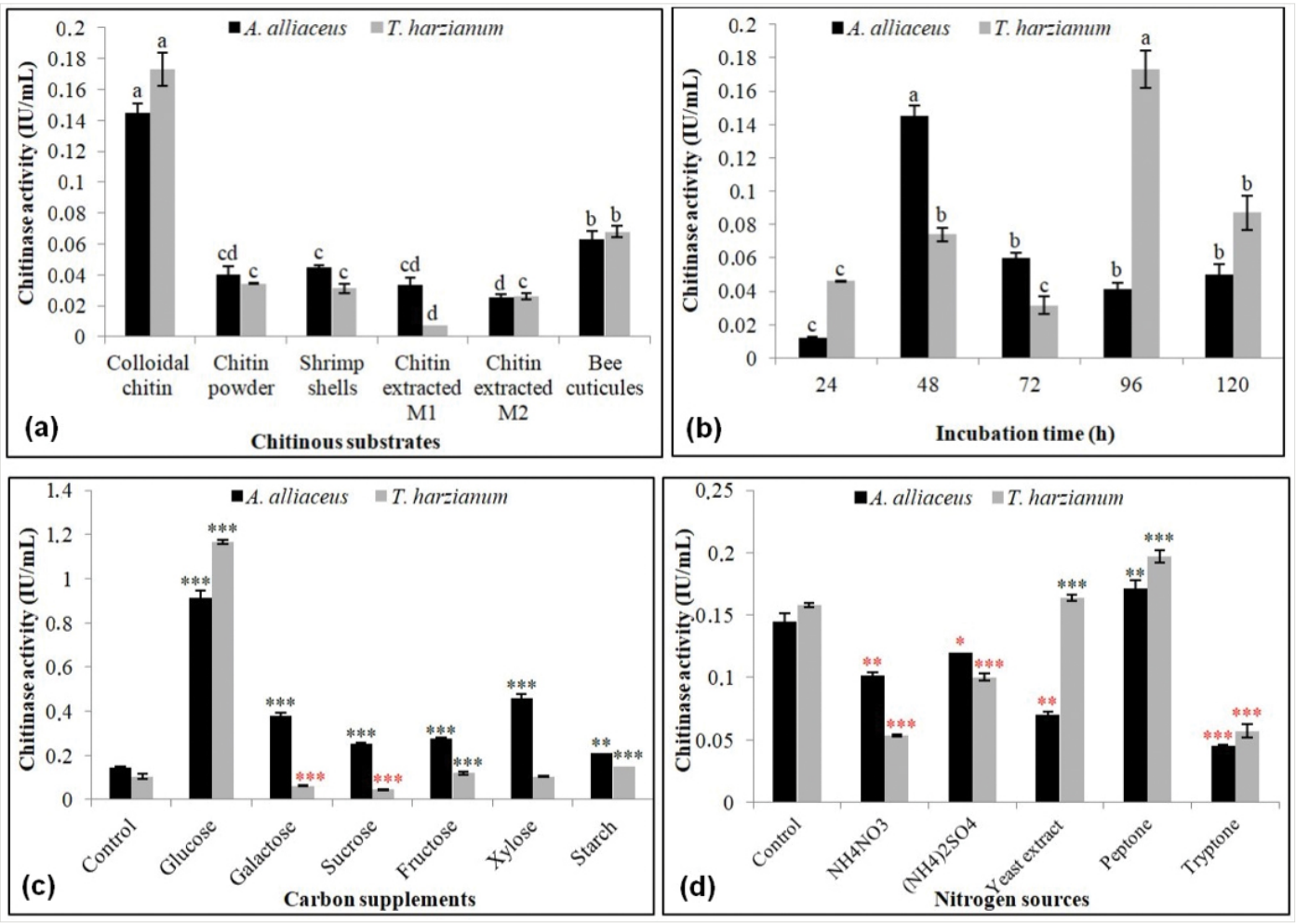

Figure 7. (a) Effect of inducer sources; **(b)** incubation time; **(c)** carbon supplements; and **(d)** nitrogen sources on chitinase production by *A. alliaceus* PP235382 and *T. harzianum* PQ564478 [Data with different letters are statistically significant ($p \leq 0.05$, Tukey's test), *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (Student's t-test), *negative effect, *positive effect]

mechanism or degradation of the enzyme itself (Karthik et al., 2017; Singh et al., 2021; Paul et al., 2022). Several *Trichoderma* and *Aspergillus* strains also produced maximum chitinase after 96 h of fermentation on liquid medium (Khatri et al., 2017; Loc et al., 2020; Jati et al., 2022; Wang et al., 2023; Ornela and Guimarães, 2024). In other cases, longer periods have been found to achieve optimal production, such as 120 h for *A. terreus* (Farag et al., 2014), 144 h for *A. niger* LOCK 62 (Brzezinska and Jankiewicz, 2012), and 192 h for *A. niveus* (Alves et al., 2018).

Effect of carbon supplements on chitinase production

The nature and concentration of the carbon source play significant roles in the production of chitinases (Karthik et al., 2017). The results clearly displayed that *A. alliaceus* was able to utilize all carbon sources and produce chitinases in considerable concentrations compared to the control (Fig. 7c). The enzymatic levels increased significantly ($p \leq 0.01$), ranging from 1.45 to 6.31-fold higher than those observed in the absence of carbon source supplementation. Meanwhile, the addition of galactose ($p=0.0034$) and sucrose ($p=0.0024$) showed a negative effect on enzyme production by *T. harzianum*. Glucose was the most suitable carbon source for chitinase production by both strains; it led to a 6.31-fold and 6.74-fold increase in chitinase yield for *A. alliaceus* and *T. harzianum*, respectively. In addition, crude enzymatic extracts recovered from media enriched with simple and complex sugars were characterized by acidic pH values (between 3.68–4.82 for *T. harzianum* and 4.34–5.62 for *A. alliaceus*), while the controls were stable at neutral values (6.26–6.30). The presence of carbon sources probably caused the rapid production of acidic compounds in the medium, resulting in a dramatic decrease in pH.

Our results are similar to those of Sandhya et al. (2004) and Farag et al. (2014). The addition of carbon sources other than chitin to the production medium can have a mixed effect. It has been observed that the use of carbon sources (simple or complex) in combination with colloidal chitin can enhance enzyme production or reduce it through catabolite repression. Nevertheless, some works have reported that this supplementation may not affect chitinase production (Karthik et al., 2017; Mohiddin et al., 2021). In the case of *Penicillium oxalicum* k10, corn starch and glucose had a significant positive effect on chitinase production (Xie et al., 2021), while the addition of galactose and arabinose resulted in increased chitinase production by *T. harzianum* BT3 (Mohiddin et al., 2021).

Effect of nitrogen sources on chitinase production

Nitrogen sources also play an important role in chitinase synthesis. Various organic and inorganic nitrogen sources, such as yeast extract, corn steep liquor, peptone, malt extract, ammonium and nitrate salts, offer potential advantages in this regard (Karthik et al., 2017; Singh et al., 2021).

As illustrated in Fig. 7d, enzyme productivity was influenced by nitrogen supplements in the production medium. Among the nitrogen sources used, the fermentation medium supplmented with casein peptone at 1% (w/v) significantly ($p \leq 0.01$) improved the production of chitinases by both fungal strains. It also resulted in high biomass levels (0.13 g/100 mL for *T. harzianum* and 0.28 g/100 mL for *A. alliaceus*). This additional nitrogen source contains various amino acids, minerals, and growth factors that support fungal growth and promote chitinase production. Conversely, ammonium nitrate and ammonium sulfate as inorganic nitrogen sources had a negative effect ($p \leq 0.05$) on enzyme expression. The presence of tryptone and yeast extract also resulted in a decrease in enzyme production ($p \leq 0.01$). However, yeast extract slightly improved the expression of chitinases by *T. harzianum* ($p=0.0004$) compared to casein peptone ($p=0.0000$).

The highest chitinase activity of our strains recorded with casein peptone at 1% (w/v) on the culture medium, is consistent with that reported for chitinase production by *T. harzianum* TUBF 966 (Sandhya et al., 2004). Casein enhanced chitinase production by *T. harzianum* BT3 (Mohiddin et al., 2021), and tryptone was most favorable for *P. oxalicum* k10 (Xie et al., 2021). Ammonium sulfate induced the expression of the highest chitinase amount by *A. terreus* (Farag et al., 2014).

Based on previous findings, chitinase activity was significantly enhanced, and the highest production was obtained in the presence of colloidal chitin (1%, w/v) and glucose (1%, w/v) as supplements in the fermentation mineral medium, after 48 h of incubation for *A. alliaceus* (0.915±0.033 IU/mL) and 96 h for *T. harzianum* (1.167±0.011 IU/mL). The specific activity was also increased by factors ranging from 3.06 to 3.60, reaching values of 3.64 IU/mg for *T. harzianum* and 4.05 IU/mg for *A. alliaceus*.

Antifungal activity of the crude enzymatic extracts

Crude extracts of *A. alliaceus* and *T. harzianum* were tested for antifungal activity against three wheat pathogens. These extracts were obtained after the enhancement of chitinase production and applied directly without a prior concentration step. The experiments show that the crude enzymatic extracts of *T. harzianum* and *A. alliaceus* can degrade the cell walls of the tested pathogens and inhibit the growth of their mycelia. This inhibition was more significant against *F. culmorum*. The enzymatic extract of *T. harzianum* provided the highest inhibition percentages (9.72–12.68%), while that of *A. alliaceus* was less effective against *F. graminearum* (6.00±0.085%) and *A. alternata* (4.50±0.10%) (Table 4).

Table 4. Inhibitory effect of crude enzymatic extracts of *T. harzianum* and *A. alliaceus* on the mycelial growth of wheat pathogens [Values not connected by the same letter in the same column are significantly different ($p \leq 0.05$) as determined by the test of Tukey]

Antagonist	Percentage weight reduction (%)		
	F. graminearum	*F. culmorum*	*A. alternata*
T. harzianum PQ564478 (MSF3)	9.72±0.080[a]	12.68±0.091[a]	10.80±0.10[a]
A. alliaceus PP235382 (MSF8)	6.00±0.085[b]	12.26±0.095[b]	4.50±0.10[b]

The effect of crude extracts on pathogen growth varied depending on the antagonist. The highest inhibition percentages were induced by the *T. harzianum* extract. Species of this genus are known for the antifungal activity of their filtrates and their effectiveness in biological control of phytopathogens (Loc et al., 2020; Mohiddin et al., 2021; Olowe et al., 2022). A slight reduction in fungal growth (less than 20%) was also reported

by Gomaa (2012) using partially purified chitinases produced by *Bacillus thuringiensis* and *B. licheniformis* against *Penicillium chrysogenum*, *Pythium* sp., *Rhizoctonia solani*, and *Rhizoctonia* sp. The preliminary results obtained with the crude extracts of our species are encouraging and need to be improved by optimizing the fermentation conditions to increase the activity of the target enzymes.

CONCLUSION

In this study, chitinase-producing fungi were isolated from wheat rhizosphere soil and wheat weed samples collected in central Algeria. Six isolates were selected from a collection of 61 rhizospheric and endophytic strains based on their good capacity to produce chitinases efficiently in both solid and liquid media within a short incubation period. These strains were tested *in vitro* against three wheat pathogenic fungi, *F. graminearum*, *F. culmorum*, and *A. alternata*, using the confrontation method. The two most active antagonists, identified as *A. alliaceus* PP235382 and *T. harzianum* PQ564478, demonstrated high antifungal efficacy, with inhibition percentages reaching 87.71% (*T. harzianum*) and 81.43% (*A. alliaceus*) against *A. alternata*, the most sensitive pathogen in dual culture. Microscopic observations revealed alterations in the mycelia morphology of the three phytopathogens and a significant reduction in spore germination. The production of chitinases by both strains under SmF conditions was greatly improved by adding glucose at 1% (w/v) to the fermentation medium containing colloidal chitin (1%, w/v) as substrate within a short incubation period (48-96 h). The activity levels achieved were 1.167±0.011 IU/mL for *T. harzianum* and 0.915±0.033 IU/mL for *A. alliaceus*. The resulting crude enzymatic extracts displayed apparent *in vitro* biocontrol activity against the targeted phytopathogenic fungi. Overall, *T. harzianum* PQ564478 and *A. alliaceus* PP235382, as well as their enzymatic extracts, can be considered potential biocontrol agents. Further experiments are required to optimize the production of chitinases by both strains, and to test their protective efficacy *in vivo*.

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Samira Bensmail: Methodology, Investigation, Data curation, Visualization, Software, Writing–original draft, Writing–review & editing. **F. Halouane-Sahir** and **S. Lahiani:** Conceptualization, Supervision, Project administration. **Souhila Bensmail:** Resources, Formal analysis, Writing–original draft, Writing–review & editing. **A. Bennacer:** Investigation, Resources, Visualization, Writing–review & editing. **S. Mebdoua, A. Reghmit,** and **Z. Oukali:** Resources, Visualization. All authors read and approved the final manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS AND PERMIT APPROVALS

Not applicable.

DATA AVAILABILITY STATEMENT

The data for this study are available from the corresponding author.

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No funding was received to assist with the preparation of this manuscript.

AI USE STATEMENT

The DeepL tool was used only for language editing purposes (grammar correction and language refinement). All scientific content, analysis, and conclusions remain the sole responsibility of the authors.

SAŽETAK

In vitro potencijal *Aspergillus alliaceus* i *Trichoderma harzianum* kao biokontrolnih agensa koji proizvode hitinazu u suzbijanju patogena pšenice rodova *Fusarium* i *Alternaria*

Biološka kontrola fitopatogena, uključujući gljivične parazite, smatra se prirodnom i ekološki prihvatljivom alternativom kemijskim tretmanima. Cilj ovog istraživanja bio je izolirati, provesti odabir i identificirati gljivične sojeve iz rizosfere durum pšenice uzgajane u središnjem Alžiru, kao i endofitne gljive iz korova pšenice u istoj regiji, s obzirom na njihovu sposobnost proizvodnje hitinaze, enzima važnog za biološku kontrolu. Od ukupno 61 izoliranog gljivičnog soja, 37,7% pokazalo je različite razine enzimske aktivnosti tijekom primarnog odabira na čvrstom hranjivom mediju. Petnaest najproduktivnijih sojeva odabrano je za sekundarni odabir putem potopljene fermentacije (SmF) uz korištenje koloidnog hitina kao supstrata. Analiza aktivnosti hitinaze pokazala je da su *Aspergillus alliaceus* PP235382 i *Trichoderma harzianum* PQ564478 bili najaktivniji nakon 48 h (0,145 ± 0,006 IU/mL), odnosno 96 h (0,173 ± 0,011 IU/mL) inkubacije. Nadalje, oba soja pokazala su najvišu antagonističku aktivnost protiv triju patogena pšenice (*Fusarium graminearum*, *F. culmorum* i *Alternaria alternata*) u dvostrukoj kulturi, s postotkom inhibicije između 68,27% i 87,71%, među šest odabranih sojeva s visokom aktivnošću hitinaze. Proizvodnja hitinaze kod *T. harzianum* i *A. alliaceus* u uvjetima SmF dodatno je povećana, dosežući 1,167 ± 0,011 IU/mL i 0,915 ± 0,033 IU/mL, korištenjem mineralnog medija koji sadrži 1% (m/v) koloidnog hitina i 1% (m/v) glukoze, uz isto vrijeme inkubacije kao prethodno utvrđeno. Sirovi enzimski ekstrakti antagonista pokazali su najveći inhibitorni učinak na *F. culmorum*. Ova dva novo izolirana soja i njihovi hitinolitički ekstrakti mogu se koristiti za kontrolu gljivičnih patogena pšenice.

Ključne riječi: rizosferne gljive, *Aspergillus alliaceus*, *Trichoderma harzianum*, hitinaze, biološka kontrola

REFERENCES

Abdallah-Nekache N., Laraba I., Ducos C., Barreau C., Bouznad Z., Boureghda H. (2019). Occurrence of *Fusarium* head blight and *Fusarium* crown rot in Algerian wheat: identification of associated species and assessment of aggressiveness. Eur J Plant Pathol 154 (3): 499–512. https://doi.org/10.1007/s10658-019-01673-7

Abo-Zaid G., Abdelkhalek A., Matar S., Darwish M., Abdel-Gayed M. (2021). Application of bio-friendly formulations of chitinase-producing *Streptomyces cellulosae* Actino 48 for controlling peanut soil-borne diseases caused by *Sclerotium rolfsii*. J Fungi 7 (3): 167. https://doi.org/10.3390/jof7030167

Agrawal T., Kotasthane A.S. (2012). Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India. SpringerPlus 1 (1): 1–10. https://doi.org/10.1186/2193-1801-1-73

Alves T.B., de Oliveira Ornela P.H., de Oliveira A.H.C., Jorge J.A., Guimarães L.H.S. (2018). Production and characterization of a thermostable antifungal chitinase secreted by the filamentous fungus *Aspergillus niveus* under submerged fermentation. 3Biotech 8: 1–10. https://doi.org/10.1007/s13205-018-1397-6

Boughalleb-M'Hamdi N., Ben Salem I., M'Hamdi M. (2018). Evaluation of the efficiency of *Trichoderma*, *Penicillium*, and *Aspergillus* species as biological control agents against four soil-borne fungi of melon and watermelon. Egypt J Biol Pest Control 28: 1–12. https://doi.org/10.1186/s41938-017-0010-3

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1-2): 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brzezinska M.S., Jankiewicz U. (2012). Production of antifungal chitinase by *Aspergillus niger* LOCK 62 and its potential role in the biological control. Curr Microbiol 65: 666–672. https://doi.org/10.1007/s00284-012-0208-2

Campbell C.K., Johnson E.M., Warnock D.W. (2013). Identification of pathogenic fungi, 2nd Edition. John Wiley & Sons, Ltd., Publication. https://doi.org/10.1002/9781118520055

Catambacan D.G., Cumagun C.J.R. (2021). Weed-associated fungal endophytes as biocontrol agents of *Fusarium oxysporum* f. sp. *cubense* TR4 in Cavendish banana. J Fungi 7 (3): 224. https://doi.org/10.3390/jof7030224

Daichi M.B., Masiello M., Haidukowski M., De Girolamo A., Moretti A., Bencheikh A., Rouag N., Somma S. (2025). Assessing *Alternaria* species and related mycotoxin contamination in wheat in Algeria: A food safety risk. Toxins 17 (6): 309. https://doi.org/10.3390/toxins17060309

de Queiroz Antonino R.S.C.M., Lia Fook B.R.P., de Oliveira Lima V.A., de Farias Rached R.Í., Lima E.P.N., da Silva Lima R.J., Peniche Covas C.A., Lia Fook M.V. (2017). Preparation and characterization of chitosan obtained from shells of shrimp (*Litopenaeus vannamei* Boone). Mar Drugs 15 (5): 141. https://doi.org/10.3390/md15050141

El-Debaiky S.A. (2017) Antagonistic studies and hyphal interactions of the new antagonist *Aspergillus piperis* against some phytopathogenic fungi *in vitro* in comparison with *Trichoderma harzianum*. Microb Pathog 113: 135–143. https://doi.org/10.1016/j. micpath.2017.10.041

Farag A.M., Al-Nusarie S., Taghreed S. (2014). Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terrus*. Afr J Biotechnol 13 (14): 1567–1578. https://doi.org/10.5897/AJB20 14.13628

Ferreira F.V., Herrmann-Andrade A.M., Calabrese C.D., Bello F., Vázquez D., Musumeci M.A. (2020) Effectiveness of *Trichoderma* strains isolated from the rhizosphere of citrus tree to control *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Penicillium digitatum* A21 resistant to pyrimethanil in post-harvest oranges (*Citrus sinensis* L. (Osbeck)). J Appl Microbiol 129 (3): 712–727. https://doi.org/10.1111/jam.14657

Gomaa E.Z. (2012). Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: Their potential in antifungal biocontrol. J Microbiol 50 (1): 103–111. https://doi.org/10.1007/s12275-012-1343-y

Gomaa E.Z. (2021). Microbial chitinases: properties, enhancement and potential applications. Protoplasma 258 (4): 695–710. https://doi.org/10.1007/s00709-021-01612-6

Hadjout S., Zouidi M., Bougrine H., Belkendil A., Zeghmar A., Ouaret W., Soufan W., Belhouadjeb F.A. (2024). Aggressiveness assessment of two *Fusarium* spp. on durum wheat grain coleoptiles under controlled conditions. Phyton 93: (11). https://doi.org/10.32604/phyton.2024.056982

Hassanein N.M., El-Gendy M.M., Abdelhameed N.M. (2016). Endophytic fungi of some medicinal plants in Egypt. Egypt Acad J Biol Sci G Microbiol 8 (1): 65–78. https://doi.org/10.21608/eajbsg.2016.16479

Herdyastuti N., Fauziah R.W., Prabowo Y.Y., Apriliana I.A. (2021). Diversity of chitinolytic bacteria from shrimp farms and their antifungal activity. J Nat Sci Biol Med 12 (3): 317–324. https://doi.org/10.4103/jnsbm.JNSBM_12_3_6

Hjort K., Presti I., Elväng A., Marinelli F., Sjöling S. (2014). Bacterial chitinase with phytopathogen control capacity from suppressive soil revealed by functional metagenomics. Appl Microbiol Biotechnol 98: 2819–2828. https://doi.org/10.1007/s00253-013-5287-x

Hsu S.C., Lockwood J. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 29 (3): 422–426. https://doi.org/10.1128/am.29.3.422-426.1975

Jati W.W., Abadi A.L., Aini L.Q., Djauhari S. (2022). Screening of *Trichoderma* spp. isolates based on antagonism and chitinolytic index against *Xylaria* sp. J Trop Plant Pests Dis 22 (1): 55–67. https://doi.org/10.23960/j.hptt.12255-67

Karthik N., Binod P., Pandey A. (2017). Chitinases. In: Current developments in biotechnology and bioengineering: production, isolation and purification of industrial products (Pandey A., Negi S., Soccol C.R., eds), Elsevier B.V., pp. 335–368. https://doi.org/10.1016/B978-0-444-63662-1.00015-4

Khan I.H., Javaid A. (2021). *In vitro* screening of *Aspergillus* spp. for their biocontrol potential against *Macrophomina phaseolina*. J Plant Pathol 103 (4): 1195–1205. https://doi.org/10.1007/s42161-021-00865-7

Khatri D.K., Tiwari D.N., Bariya H.S. (2017). Chitinolytic efficacy and secretion of cell wall degrading enzymes from *Trichoderma* spp. in response to phyto-pathological fungi. J App Biol Biotech 5 (6): 1–8. https://doi.org/10.7324/JABB.2017.50601

Kidd S., Halliday C., Ellis D. (2022). Descriptions of medical fungi. 4th Edition. CABI Publishing. p. 376. https://doi.org/10.1079/9781800622340.0000

Klich M.A. (2002). Identification of common *Aspergillus* species, 1st Edition. Centraalbureau voor schimmelcultures, Utrecht, The Netherlands. p. 116.

Köhl J., Kolnaar R., Ravensberg W.J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Front Plant Sci 10: 845. https://doi.org/10.3389/fpls.2019.00845

Kovács C., Csótó A., Pál K., Nagy A., Fekete E., Karaffa L., Kubicek C.P., Sándor E. (2021). The biocontrol potential of endophytic *Trichoderma* fungi isolated from Hungarian grapevines. Part I. Isolation, identification and *in vitro* studies. Pathogens 10 (12): 1612. https://doi.org/10.3390/pathogens10121612

Kumari S., Sharfuddin C. (2022). Antagonistic potential of *Trichoderma simmonsii* isolated from Bihar against pathogenic fungus *Alternaria brassiceae*. J Mycopathol Res 60 (2): 245–249. https://imskolkata.org/pdf/june22_2/sweta.pd

Kurniawan E., Panphon S., Leelakriangsak M. (2018). Potential of marine chitinolytic *Bacillus* isolates as biocontrol agents of phytopathogenic fungi. In: IOP Conference Series: Earth and Environmental Science (IOP Publishing), Universitas Airlangga, Indonesia, 217 (1): 012044. https://doi.org/10.1088/1755-1315/217/1/012044

Le B., Yang S.H. (2019). Microbial chitinases: properties, current state and biotechnological applications. World J Microbiol Biotechnol 35 (9): 144. https://doi.org/10.1007/s11274-019-2721-y

Loc N.H., Huy N.D., Quang H.T., Lan T.T., Thu Ha T.T. (2020). Characterisation and antifungal activity of extracellular chitinase from a biocontrol fungus, *Trichoderma asperellum* PQ34. Mycology 11 (1): 38–48. https://doi.org/10.1080/21501203.2019.1703839

Mebdoua A., Sadrati N., Mohammedi S., Saidi A. (2025). Sustainable control of *Fusarium verticillioides* in wheat using plant extracts and microorganisms. Cogn sustainabil 4 (2). https://doi.org/10.55343/CogSust.138

Mendoza D.Y.P., Mancilla A.G., Suárez J.J.A., Guzmán M.D.P.R., Barradas O.G., Delira R.A. (2018). Characterization of the antifungal activity of three rhizobacterial strains against *Rhizoctonia solani*. Span J Agric Res 16 (4): 16. https://doi.org/10.5424/sjar/2018164-13334

Mohammedi A., Trachi S., Ayad D., Messgo-Moumene S., Boureghda H., Bouznad Z. (2022). Antagonistic potential of *Trichoderma* spp. evaluated under *in vitro* and *in vivo* conditions against *Alternaria* spp. responsible for early blight of tomato in Algeria. Bulg J Agric Sci 28 (4): 626–635. https://agrojournal.org/28/04-08.pdf

Mohiddin F.A., Padder S.A., Bhat A.H., Ahanger M.A., Shikari A.B., Wani S.H., Bhat F.A., Nabi S.U., Hamid A., Bhat N.A. (2021). Phylogeny and optimization of *Trichoderma harzianum* for chitinase production: Evaluation of their antifungal behaviour against the prominent soil borne phytopathogens of Temperate India. Microorganisms 9 (9): 19–62. https://doi.org/10.3390/microorganisms9091962

Monreal J., Reese E.T. (1969) The chitinase of *Sarratia marcescens*. Can J Microbiol 15: 689–696. https://doi.org/10.1139/m69-122

Muhanna N.A. (2019). Semi-Solid Agar Medium for Detection of Fungal Enzymes. Egypt J Pathol 47 (2): 99–119. https://doi.org/10.21608/EJP.2019.134008

O'Brien P.A. (2017). Biological control of plant diseases. Australas Plant Pathol 46: 293–304. https://doi.org/10.1007/s13313-017-0481-4

Olowe O.M., Nicola L., Asemoloye M.D., Akanmu A.O., Sobowale A.A., Babalola O.O. (2022). Characterization and antagonistic potentials of selected rhizosphere *Trichoderma* species against some *Fusarium* species. Front Microbiol 13: 985874. https://doi.org/10.3389/fmicb.2022.985874

Ornela P.H., Guimarães L.H.S. (2024). Purification, characterization and antifungal activity of the *Aspergillus niveus* chitinase produced using shrimp shells. Appl Biosci 3 (2): 220–232. https://doi.org/10.3390/applbiosci3020015

Paul M.K., Umesh B.T., Mathew J. (2022). Recent advances and technologies in chitinase production under solid-state fermentation. Biosci Biotechnol Res Asia 19 (4): 815–825. https://doi.org/10.13005/bbra/3033

Rahman M., Borah S.M., Borah P.K., Bora P., Sarmah B.K., Lal M.K., Tiwari R.K., Kumar R. (2023). Deciphering the antimicrobial activity of multifaceted rhizospheric biocontrol agents of solanaceous crops viz., *Trichoderma harzianum* MC2, and *Trichoderma harzianum* NBG. Front Plant Sci 14: 1141506. https://doi.org/10.3389/fpls.2023.1141506

Roca-Couso R., Flores-Félix J.D., Rivas R. (2021). Mechanisms of action of microbial biocontrol agents against *Botrytis cinerea*. J Fungi 7 (12): 1045. https://doi.org/10.3390/jof7121045

Sandhya C., Adapa L.K., Nampoothiri K.M., Binod P., Szakacs G., Pandey A. (2004). Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation. J Basic Microbiol 44 (1): 49–58. https://doi.org/10.1002/jobm.200310284

Saravanakumar K., Wang M.H. (2020). Isolation and molecular identification of *Trichoderma* species from wetland soil and their antagonistic activity against phytopathogens. Physiol Mol Plant Pathol 109: 101458. https://doi.org/10.1016/j.pmpp.2020.101458

Sayed M., Abdel-Rahman T., Ragab A., Abdellatif A. (2019). Biocontrol of root-knot nematode *Meloidogyne incognita* by chitinolytic *Trichoderma* spp. Egypt J Agronematol 18 (1): 30–47. https://doi.org/10.21608/EJAJ.2019.52842

Shivalee A., Divatar M., Sandhya G., Ahmed S., Lingappa K. (2016). Isolation and screening of soil microbes for extracellular chitinase activity. J Adv Sci Res 7 (02): 10–14. https://sciensage.info/index.php/JASR/article/view/270

Siddiquee S. (2017). Morphology-based characterization of *Trichoderma* species. In: Practical handbook of the biology and molecular diversity of *Trichoderma* species from tropical regions. Fungal Biology, Springer Cham., pp. 41–73. https://doi.org/10.1007/978-3-319-64946-7_4

Silva R.N., Monteiro V.N., Steindorff A.S., Gomes E.V., Noronha E.F., Ulhoa C.J. (2019). *Trichoderma*/pathogen/plant interaction in pre-harvest food security. Fungal Biol 123 (8): 565–583. https://doi.org/10.1016/j.funbio.2019.06.010

Singh R.V., Sambyal K., Negi A., Sonwani S., Mahajan R. (2021). Chitinases production: A robust enzyme and its industrial applications. Biocatal Biotransformation 39 (3): 161–189. https://doi.org/10.1080/10242422.2021.1883004

Singh Y., Lal N. (2016). Isolation and characterization of PGPR from wheat (*Triticum aestivum*) rhizosphere and their plant growth promoting traits *in vitro*. Indian J Biol 3(2): 139–144. https://doi.org/10.21088/ijb.2394.1391.3216.8

Stoykov Y.M., Pavlov A.I., Krastanov A.I. (2014). Chitinase biotechnology: production, purification, and application. Eng Life ScI 15 (1): 30–38. https://doi.org/10.1002/elsc.201400173

Sudha S., Priyanka Sharon P., Revathi Yadav K., Sherly Priyanka R.B. (2020). Optimization of chitinase production from lake sediment inhabitant *Bacillus thuringiensis* strain LS1 and *Bacillus cereus* strain LS2. Asian J Pharm 14 (2): 175–182. https://doi.org/10.22377/ajp.v14i2.3611

Trung D.Q., Anh L.T., Thuy N.T., Van D.M., Hang T.T. (2021). Endophytic bacteria isolated from a weed plant as a potential biocontrol agent against stem end rot pathogen of pitaya in Vietnam. Egypt J Biol Pest Control 31 (1): 106. https://doi.org/10.1186/s41938-021-00451-0

Tyśkiewicz R., Nowak A., Ozimek E., Jaroszuk-Ściseł J. (2022). *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. Int J Mol Sci 23 (4): 2329. https://doi.org/10.3390/ijms23042329

Wang J., Zhu M., Wang P., Chen W. (2023). Biochemical properties of a cold-active chitinase from marine *Trichoderma gamsii* R1 and its application to preparation of chitin oligosaccharides. Mar Drugs 21 (6): 332. https://doi.org/10.3390/md21060332

Wasli A.S., Salleh M.M., Abd-Aziz S., Hassan O., Mahadi N.M. (2009). Medium optimization for chitinase production from *Trichoderma virens* using central composite design. Biotechnol Bioprocess Eng 14: 781–787. https://doi.org/10.1007/s12257-008-0127-z

Xie X.H., Fu X., Yan X.Y., Peng W.F., Kang L.X. (2021). A broad-specificity chitinase from *Penicillium oxalicum* k10 exhibits antifungal activity and biodegradation properties of chitin. Mar Drugs 19 (7): 356. https://doi.org/10.3390/md19070356

Yavari-Bafghi M., Babavalian H., Amoozegar M.A. (2019). Isolation, screening and identification of haloarchaea with chitinolytic activity from hypersaline lakes of Iran. Arch Biol Sci 71 (1): 71–81. https://doi.org/10.2298/ABS180525049Y