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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.

Keywords: Rhizospheric fungi, *Aspergillus alliaceus, Trichoderma harzianum,* Chitinases, Biological control

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% are lost 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 don't 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, through the use of 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 the 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 some wheat

pathogens (*F. culmorum, F. graminearum,* and *Alternaria alternata*), (iii) improve chitinase production by the most effective isolates under SmF conditions by varying some 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 the excess soil was removed by slight shaking. The soil tightly adhering to the roots of the ten plants was then mixed together 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. This identification was guided by the work 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 (×400, OPTICA Axiom 2000, Italy), after staining with 1% (w/v) methylene blue solution.

The fungal isolates characterized by the highest chitinase activity (hyper producer strains) were identified by sequencing the beta-tubulin gene of 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.). Using the Basic Local Alignment Search Tool (BLAST), the sequenced data were matched against the Gene Bank database, 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 KH_2PO_4, 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 of 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 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 has 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, $FeSO_4{\cdot}7H_2O$ 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). The 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 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. The 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 resulting from the SmF process was estimated by the method outlined by Bradford (1976). Three milliliters of Bradford's reagent were mixed with 100 µL of enzyme extract, followed by homogenization. The 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 in 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 at 5 cm apart from each other and incubated for 7 days at 30°C (Khatri et al., 2017). Each treatment was repeated three times. The plates inoculated with only the test pathogens served as controls. The percentage inhibition of radial growth (PIRG) was calculated using the following formula:

$$\text{PIRG}\ (\%) = \frac{R_1 - R_2}{R_1} \times 100$$

Where R_1 represents the distance (measured in cm) from the inoculation point to the edge of the colony on the control dish (the radial diameter) and R_2 is the distance of fungal growth from the point of inoculation to the edge of the colony on the treated plates towards the antagonist (Saravanakumar and Wang, 2020). Certain 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 an 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 × 400 magnification in order

to estimate, for each confrontation, the morphological modifications of the phytopathogens (coiling, denaturation, penetration, and disintegration) caused by those of the selected antagonists.

Enhancement of chitinase production

To improve chitinase production by the hyper producer strains, the effects of some parameters of the SmF process, including the type of substrate, incubation time (24 h, 48 h, 72 h, 96 h, and 120 h), 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, were tested. 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).

The 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 then 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 the enhancement of 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 then incubated at 30°C for 168 h under static conditions. Fungal growth was determined by dry weight at 50°C after filtration through a preweighed Whatman filter paper No. 4. The percentage weight reduction (PWR) of the test fungus was calculated using the following formula:

$$\text{PWR}\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

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 the 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 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, the isolation of such organisms represents 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 among them. 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 not enough 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 was variable depending on 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, high diameters, while BSF4 (*Fusarium* sp.), MSF4', and MSF6 (*Penicillium* spp.) offered moderate chitinase activity. The remaining isolates presented low, very low, or total absence of activity, even after 168 h of incubation.

Notably, the release of chitinases on the medium by the best strains (23 isolates) improved with the incubation period, where 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 the different regions were able to secrete chitinases on the 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 diameter of the purple zone around fungal colonies, between 3 and 8 cm. 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. The selected strains presented good chitinase activity with two strains showing 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 after every 24 h of incubation until day 5. All strains were able to secrete chitinases in the liquid medium after 48 h of incubation. However, only five strains exhibited such activity after 24 h of incubation. The findings showed also that the maximum activities were reached after 48 h of incubation for MSF8 (0.145±0.006 IU/mL) and 96 h for MSF3 (0.173±0.011 IU/mL). Both strains MSF6 (0.123±0.013 IU/mL) and TSF5 (0.161±0.014 IU/mL) 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 remain 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.

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 have been 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 evaluate the antagonistic activity of the six most chitinase-producing isolates against three wheat pathogens, namely *F. graminearum*, *F. culmorum*, and *A. alternata*. Inhibition of the phytopathogen growth was evaluated through the determination of 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.

From the results presented in Table 3 and Fig. 3, it is clear 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 the three pathogens, with PIRG values ranging from 81.13±2.05% up to 87.71±1.45%, followed by MSF8 (*Aspergillus* sp.) (Inhibition rates between 68.27±1.17%–81.43±1.80%). 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 antagonist effect close 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.), the inhibition rates did not exceed 55% after 168 h of incubation, and they are significantly lower compared to 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 they made contact with its mycelium, then surrounded the pathogen colony and finally invaded it, probably showing a mycoparasitism mechanism for MSF3 strain, and a competition mechanism for MSF8. The

antagonism effect of the EndP2SF3 strain was also characterized by rapid growth toward the 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 are characterized by their mode of action, especially to occupy spaces before the arrival of phytopathogens, and to have mycoparasitic activity and good competitive ability (El-Debaiky, 2017; Boughalleb-M'Hamdi et al., 2018; Khan and Javaid, 2021). The high inhibitory potency of *Trichoderma* and *Aspergillus* isolates on 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 in agreement with those 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 *Trichoderma simmonsii* toward *Alternaria brassiceae* (PIRG=78%) (Kumari and Sharfuddin, 2022). In the same sense, Rahman et al. (2023) demonstrated the high efficiency of three *T. harzianum* isolates to suppress the growth of *F. oxysporum* f. sp. *lycopersicum* (values recorded between 88.80 and 92.50%). In addition, some *Aspergillus* species tested as biocontrol agents (*A. flavus*, *A. niger*, and *A. terreus*) induced a low to good reduction 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 based on microbial antagonism.

Antagonist-pathogen hyphal interactions

Microscopic observations of phytopathogen hyphae taken from the confrontation zone revealed several morphological modifications. The strain MSF3 (*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 were shrunken and lysed (Fig. 4a, b, and e). Our observations are in good agreement 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).

Whereas, MSF8 antagonist in contact with *F. culmorum* and *A. alternata* hyphae caused direct strong cell wall degradation (Fig. 4d), breakage of the host hyphae, and cell lysis (Fig. 4c). Moreover, a strong absence of phytopathogen spores with inhibition of their germination was observed in the proximity of antagonist hyphae. In contrast, hyphal interactions resulted in an increase in the number of antagonist conidia. The strong cell wall degradation and hyphal denaturation that occurred after MSF8 strain attacked *A. alternata* and *Fusarium* hyphae may be due to the production of lytic enzymes and secondary metabolites, which can be classified under 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 an activity of lytic enzymes, favoring mycoparasitism that prevents pathogen proliferation (Ferreira et al., 2020). The same 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 for different strains of *Trichoderma* vs. *A. alternata, Colletotrichum gloeosporioides,* and *Penicillium*

digitatum A21 (Ferreira et al., 2020). To the best of our knowledge, the present work illustrates 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 the hyper producers of chitinases and showed the highest antagonistic effect against wheat fungal pathogens; thus, they were selected for further studies.

Identification of fungal strains

Pure culture of MSF3 (*Trichoderma* sp.) initially appeared white and fluffy, later developed yellowish-green to dark green compact clusters, often in small areas or in concentric ring-like zones on the agar surface. The medium of mature colonies was characterized by an intense color of yellow to dark brown pigments. The mycelium was initially smooth, watery white in color, and sparse until floccose aerial mycelium was produced (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 sub-globose 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, becoming dark gray to black with age (Fig. 6a). Conidia are smooth-walled, subglobose to ovoid, cream to yellow or yellow ochre. The conidial colors tend to be in 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 the reference sequences present 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 of PQ564478 and PP235382, respectively. The 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).

Chitinase production by SmF process

For the enhancement of extracellular chitinase production by *T. harzianum* PQ564478 and *A. alliaceus* PP235382, some culture conditions controlling and influencing the SmF process were investigated. These factors included type of substrate, incubation time, 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 the 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). Interestingly, 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, which may be 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 the 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 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 certain maximum level after a time period and then decreases by further incubation (Paul et al., 2022). Most of the 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 a longer period of 96 h to peak its maximum. Thereafter, the 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 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 pH values in the acidic range (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). Probably, the presence of carbon sources caused the rapid production of acidic compounds in the medium, resulting in the 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 could have a mixed effect. It has been observed that the use of carbon sources (simple or complex) in combination with colloidal chitin can enhance the enzyme production or reduce it through catabolite repression. Nevertheless, some works 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 chitinases 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, the enzyme productivity was influenced by nitrogen supplements in the production medium. Among the various nitrogen sources used, the fermentation medium amended with casein peptone at 1% (w/v) significantly ($p \leq 0.01$) improved the production of chitinases by both fungal strains. It also allowed obtaining high biomass levels (0.13 g/100 mL for *T. harzianum* and 0.28 g/100 mL for *A. alliaceus*). This extra nitrogen additive contains various amino acids, minerals, and growth factors that can 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) in the culture medium is in good agreement 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 expression of the highest chitinase amount by *A. terreus* (Farag et al., 2014).

Based on the 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 of 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 enhancement of chitinase production and applied directly without prior concentration step. As evident from the experiments, it can be concluded that the crude enzymatic extracts of *T. harzianum* and *A. alliaceus* have the ability to degrade the cell wall of the tested pathogens and to inhibit the growth of their mycelia. This inhibition was more significant against *F. culmorum*. 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).

The effect of crude extracts on pathogen growth varied depending on the antagonist. The highest inhibition percentages were induced by *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 trivial reduction in fungal growth (less than 20%) was also reported by Gomaa (2012) using the partially purified chitinases produced by *Bacillus thuringiensis* and *B. licheniformis* against *Penicillum 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 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 on the basis of 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* as 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 period of incubation (48-96 h). The achieved activity levels 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 as potential biocontrol agents. Further experiments are needed 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.

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Not applicable.

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

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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		

Table 2. Primary screening (colored zone diameter in cm) of some fungal isolates on solid medium

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}

Note: 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.

Table 3. Mycelial growth inhibition (%) of three wheat pathogens by six fungal isolates after each incubation period

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}

Note: 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%).

Table 4. Inhibitory effect of crude enzymatic extracts of *T. harzianum* and *A. alliaceus* on the mycelial growth of wheat pathogens

	Percentage weight reduction (%)		
Antagonist	*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]

Note: 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.

Figure captions

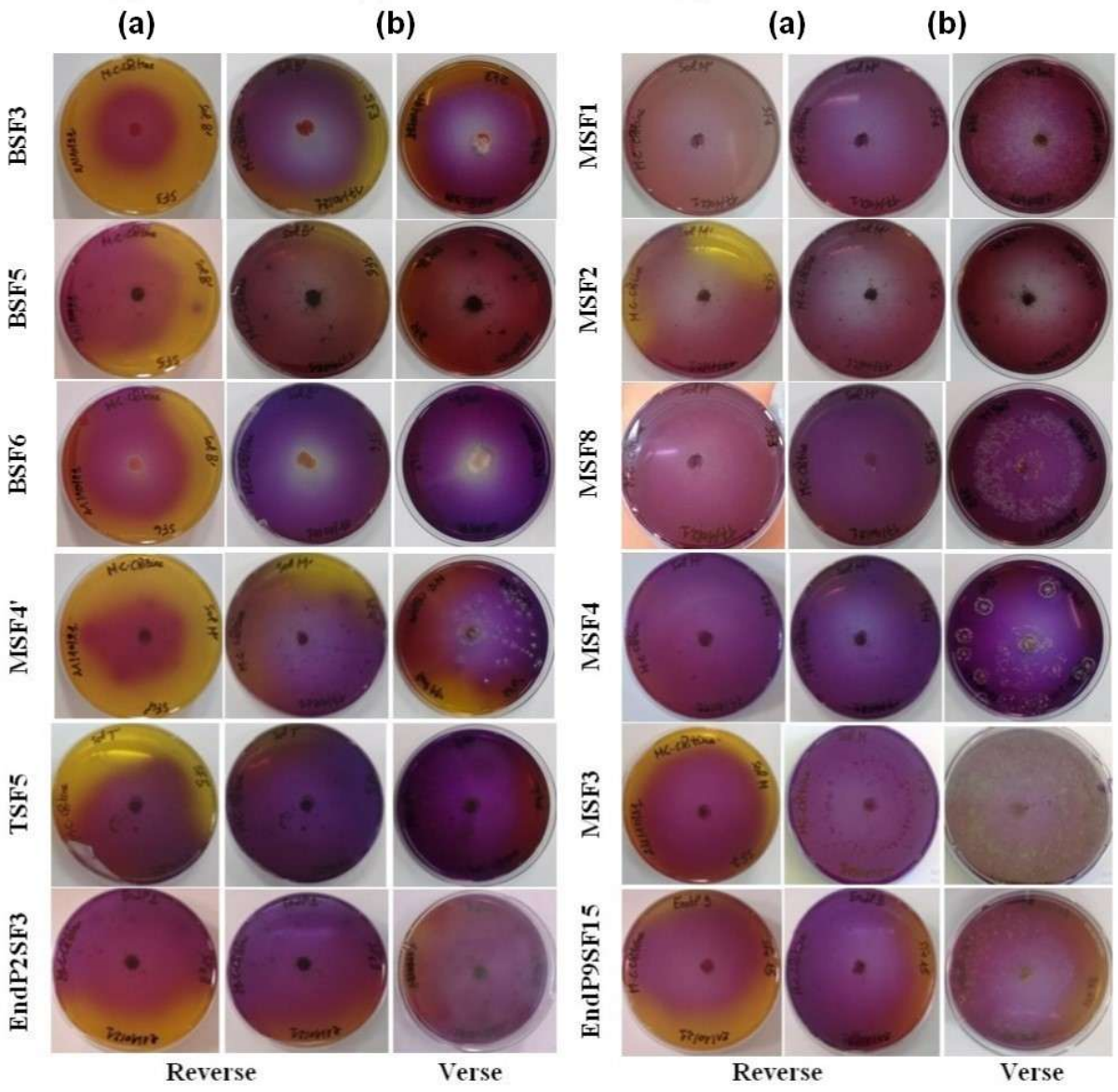

Fig. 1. Chitinase production by some fungal isolates after 72 h **(a)** and 168 h **(b)** of incubation on solid medium supplemented with colloidal chitin. The selection of chitinolytic fungal isolates was based on the largest diameter of the highly concentrated purple zone around the colonies after 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

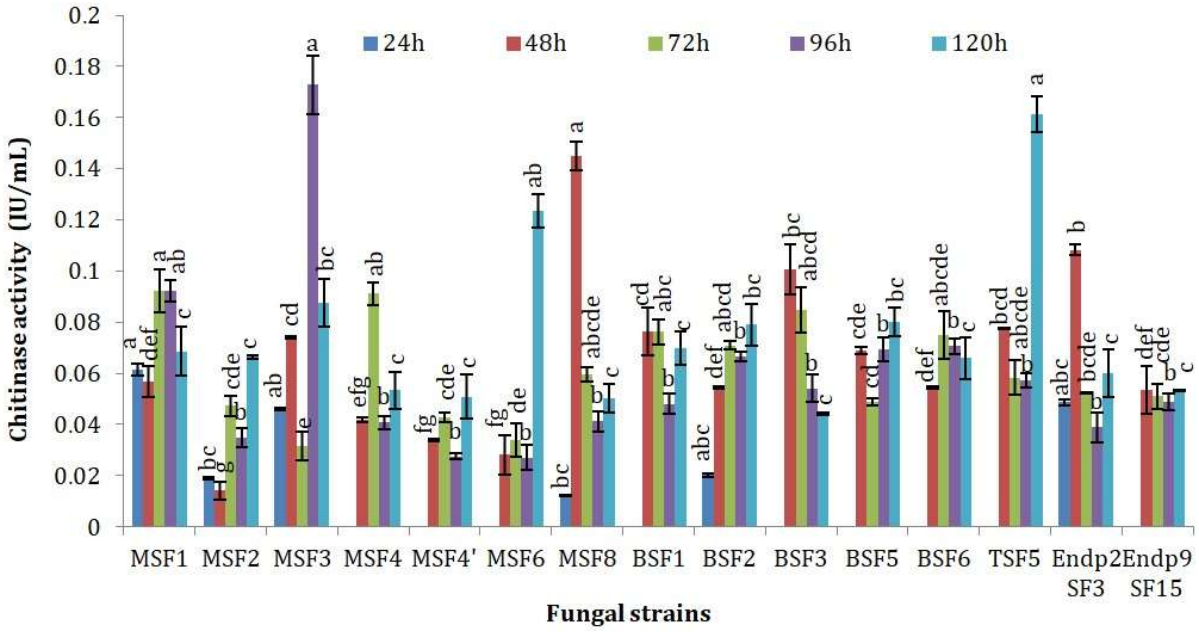

Fig. 2. Exochitinase activity of the selected isolates grown on liquid medium containing colloidal chitin as the substrate. Different letters for the same incubation time are statistically significant ($P \leq 0.05$, Tukey's test)

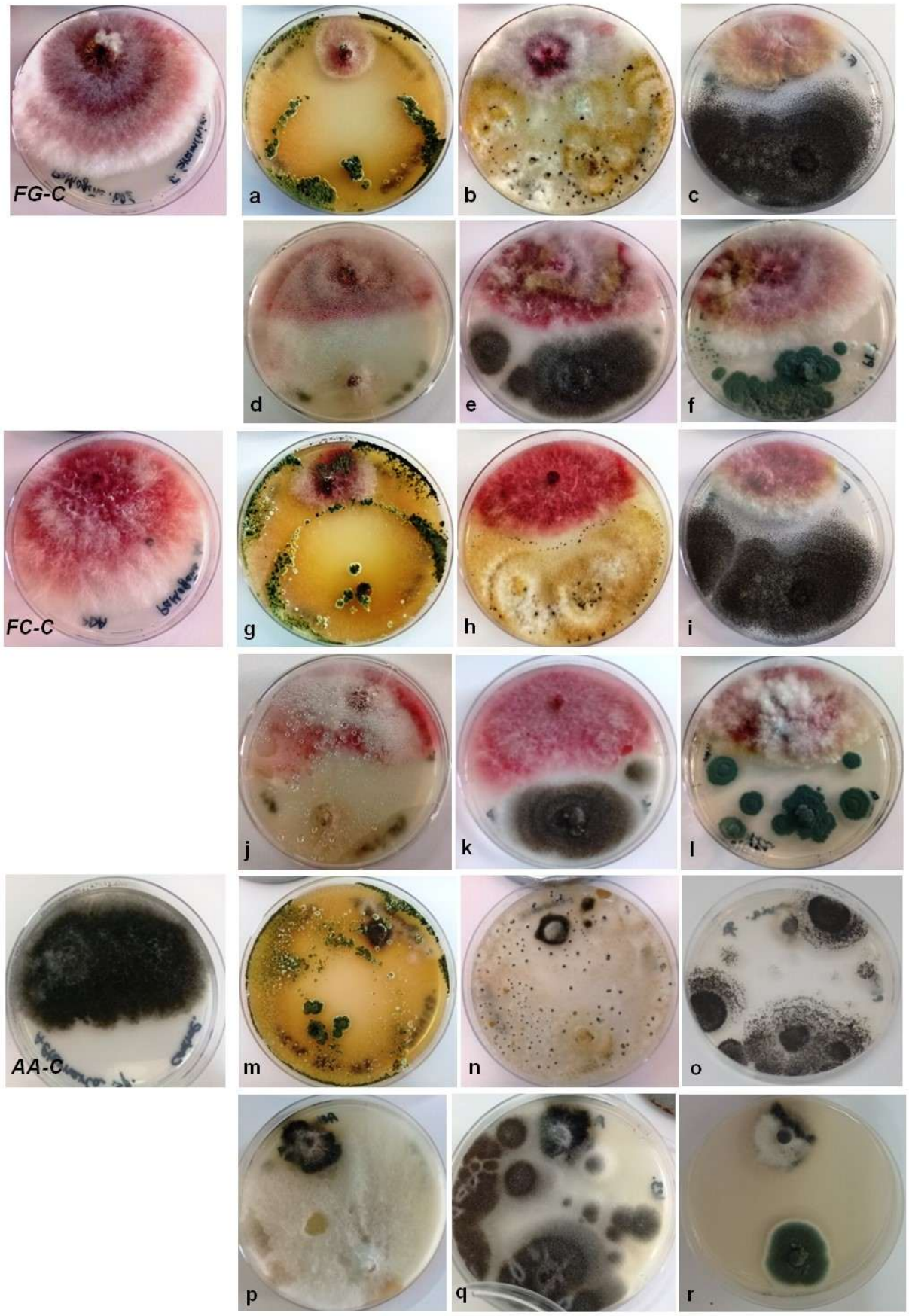

Fig. 3. *In vitro* antagonistic activity of six fungal strains against wheat pathogens on PDA

medium after 7 days of confrontation. 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* (**a–f**), *F. culmorum* (**g–l**), and *A. alternata* (**m–r**), respectively. ***Fg-c***, ***Fc-c***, and ***Aa-c*** represent the control growth of each phytopathogen

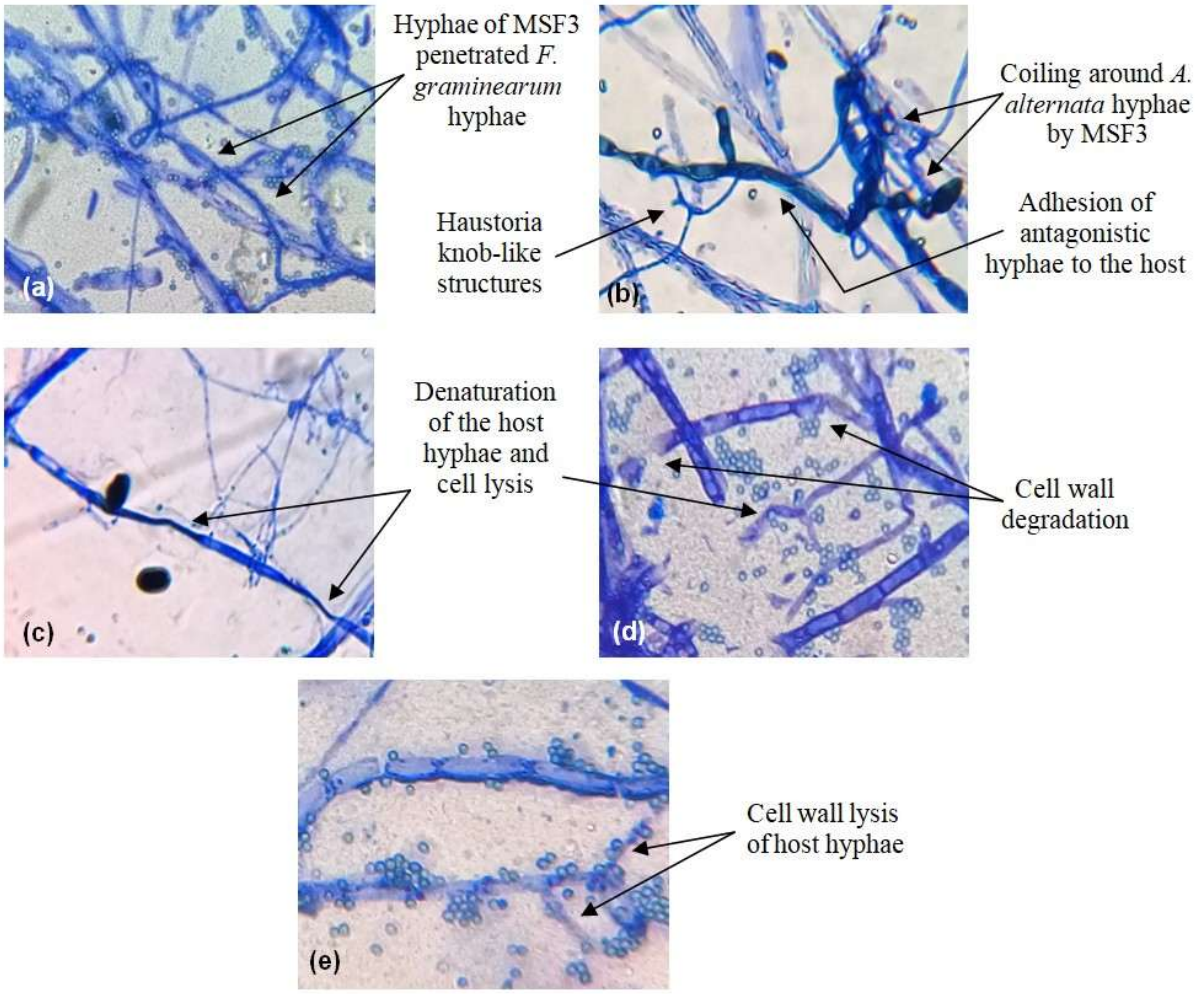

Fig. 4. Microscopic views of the contact zone between the mycelia of MSF3 vs. *F. graminearum* **(a)**, MSF3 vs. *A. alternata* **(b)**, MSF8 vs. *A. alternata* **(c)**, MSF8 vs. *F. culmorum* **(d)**, and MSF3 vs. *F. graminearum* **(e)**

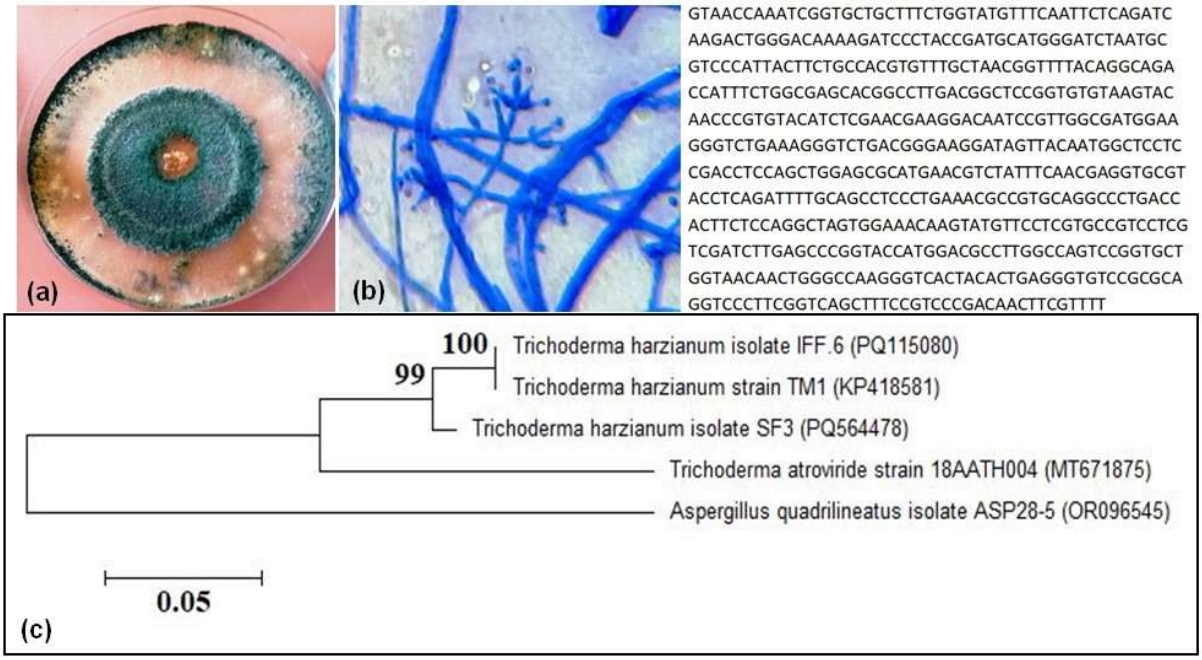

Fig. 5. Morphological features (**a**–**b**), sequence from the β-tubulin gene, and phylogenetic tree (**c**) of the MSF3 strain (SF3): *Trichoderma harzianum* PQ564478

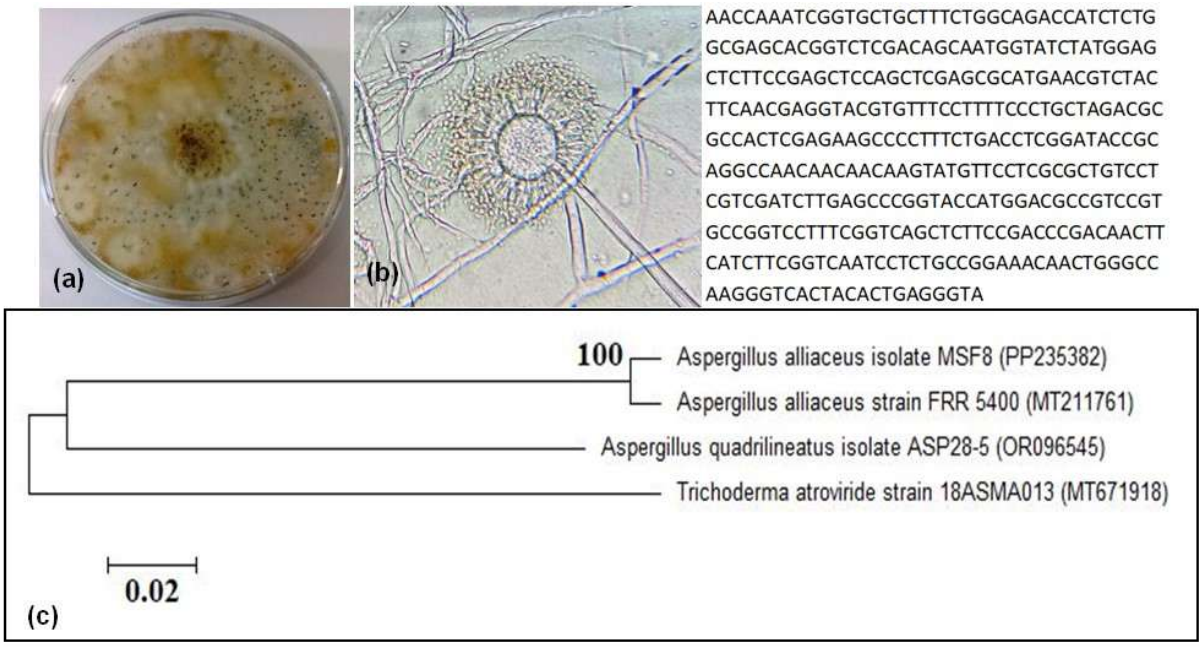

Fig. 6. Morphological characteristics (**a–b**), sequence from the β-tubulin gene, and phylogenetic tree (**c**) of the MSF8 strain: *Aspergillus alliaceus* PP235382

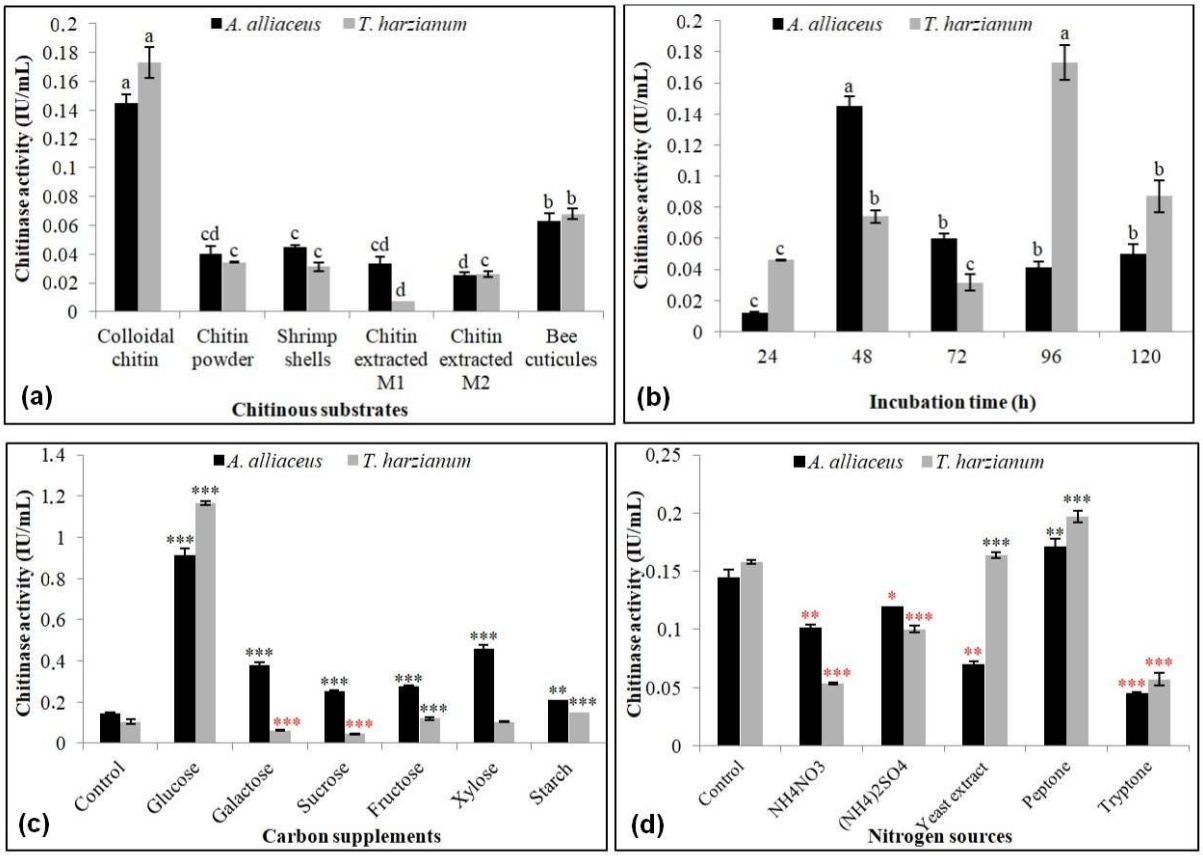

Fig. 7. Effect of inducer sources **(a)**, incubation time **(b)**, carbon supplements **(c)**, and nitrogen sources **(d)** on chitinase production by *A. alliaceus* PP235382 and *T. harzianum* PQ564478. Data with different letters are statistically significant ($P \leq 0.05$, Tukey's test), *: $P \leq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