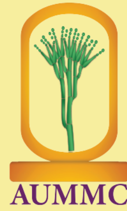

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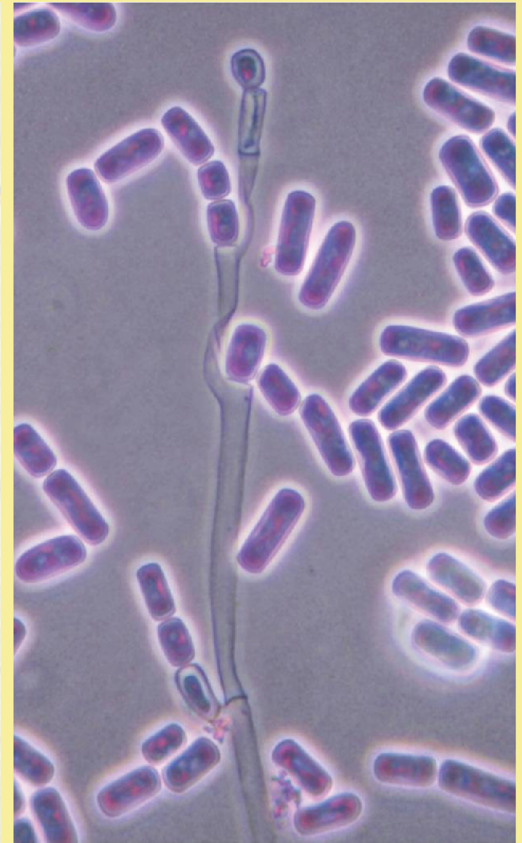

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Impact of fumigation with phosphine on viability of wheat grains stored for six months at two levels of moisture content, in addition to description of four new records of associated fungi and assessment of their potential for enzymatic production

Osama A. Al-Bedak[1*], Elmahdy A.E. Teama[2], E. A. Ali[2], M. T.Said[2], E. M. Shalaby[2] and Zainab A.M. Moharram[2]

[1] Assiut University Mycological Centre, Assiut University, Assiut, 71511, Egypt
[2]Department of Agronomy, Faculty of Agriculture, Assiut University, Assiut, 71511, Egypt

[*]Corresponding author: **Zainab A.M. Moharram**: z.a.moharram@gmail.com **Received 24/9/2020, Accepted 5/10/2020**

Abstract: In the current study, the effect of fumigation with three doses (3, 5 and 7 g/ton) of phosphine (PH_3) on wheat grains sustained at 10% and 14% moisture content (MC) for 6 months was evaluated. At the 10 % MC, percentage of germination and speed of germination index (SGI) of wheat grains as well as the average length of the shoot and primary root of seedlings showed marked increase in response to PH_3 treatment compared to control (non-fumigated grains). On the other hand, the percentage of abnormal seedlings decreased with the increase in PH3 doses. Nearly similar effects of PH_3 were observed on wheat grains and seedlings kept at 14% MC. Four fungal isolates expected to be new records on wheat grains were diagnosed on both morphological and molecular bases. Phylogenetic analyses revealed that two strains were classified as *Alternaria ventricosa* AUMC 14510 (MT974152) and *Cladosporium allicinum* AUMC 14511 (MT974153), which recorded here for the first time in Egypt. The other two strains showed lower similarity with related species in the GenBank and were described as potentially new species assigned as *Bipolaris* sp. AUMC 14512 (MT974154) and *Alternaria* sp. AUMC 14513 (MT974155). The amylolytic, cellulolytic and xylanolytic activities of these fungi were assayed in submerged fermentation. *A. ventricosa* and *Alternaria* sp. were the highest amylase producers registering specific activities of 65.5 IU/mg and 75.7 IU/mg respectively. *C. allicinum* was superior in the production of endoglucanase, exoglucanase and xylanase gaining 93.1 IU/ml, 64.46 IU/ml and 23.9 IU/ml for the three enzymes respectively.
Key words: Amylase, cellulase, deterioration, germination, phosphine, phylogeny, Seed-borne fungi, *Triticum aestivum*, xylanase.

Introduction

Wheat (*Triticum aestivum* L.) has been a staple food supply for mankind since time immemorial (Satish *et al.* 2010). It is considered the biggest agricultural product and nutritional component in the world and is one of the most essential cereals in terms of nutritional value (Abd El-Baky 2009). In Egypt, wheat is known to be the first important food crop (Ouda 2006). Due to its significance in the Egyptian diet, wheat is a strategic commodity in the region. It accounts for more than one-third of the daily calorie intake of Egyptian consumers and 45 percent of the overall dietary protein intake of Egyptians (Ali and Adams Jr 1996; Baka 2014). Production, processing and storage practices are the key factors influencing the efficiency of the crop. As the production of Egyptian wheat is expected to reach 8,495 million metric tons in 2020 (Abdelaal and Thilmany 2019) and the deterioration occurs during grain storage (Omar *et al.* 2011). Fumigation by phosphine, is therefore an important technique for effective control and management of infection of stored grain pests, especially in tropical countries such as Egypt. Phosphine is easily available, low expense with an easy treatment process and is approved as a free or clear grain residue in all markets. Phosphine disperses easily inside the environment and no fans are

required. It is safe to ship in the initial packaging and it is not believed to cause germination (Chadda 2016).

Wheat grains can also be infected by several fungal species in the field and during storage. These fungi are not only responsible for a notable decrease in yield but also a decline in marketable grain quality. Early identification of pathogens is a critical step in the evaluation and control of wheat research projects (Majumder *et al.* 2013). Common seed-borne fungi of wheat included species of the genera *Acremonium*, *Alternaria,* *Aspergillus*, *Bipolaris*, *Drechslera*, *Cladosporium,* *Curvularia,* *Fusarium,* *Mucor,* *Penicillium, Rhizopus, Stemphylium* and *Trichoderma* (Bhatti and Bhutta 2002; Rajput *et al.* 2005; Fakhrunnisa and Ghaffar 2006; Singh *et al.* 2011; Hajihasani *et al.* 2012; Majumder *et al.* 2013; Pathak and Zaidi 2013; Zrari 2013). These fungi are associated with a significant depletion of food, which leaves the crop uneconomical, and also develop mycotoxins that impair the overall nutritional value of grains (Galvano *et al.* 2001).

Aims of the present work

Current study was planned to assess the effect of phosphine fumigation on the germination and viability of wheat grains stored for six months at 10% and 14% moisture content, and to emphasize some enzymatic activities of certain newly reported fungal strains associated with the fumigated wheat grains.

Materials and methods

Collection of wheat grain samples and storage conditions

Wheat grains (*Triticum aestivum*) samples (Gameza 11) were collected from Department of Agronomy, Faculty of Agriculture, Assiut University, Egypt. The collected samples were kept in sterile plastic bags during transport to the laboratory and on the same day 1500 g aliquot was taken from each sample, thoroughly mixed, and maintained in a 2-Kg-capacity glass jars equipped with a sealed cover. The wheat samples studied were divided into two groups. Every category was packed at 10 % and 14 % moisture content (MC) for 6 months under greenhouse conditions (25.0±5.0 °C) during the period from April to September, 2019.

Source of phosphine and fumigation procedure

Commercial aluminum phosphide tablets (Quickphos) were used in this study. Phosphine (PH_3) was used at concentrations of 3, 5 and 7 g/ton for each MC treatment.

Evaluation of seed germination and viability

Random sample of 400 grains for each fumigation treatment at every MC value were allowed to germinate at the end of the storage time (6 months) according to the rules of International Seed Testing Association (ISTA) on top filter paper in sterilized Petri dishes (14 cm diameter) and each Petri dish contains 25 grains. The filter paper in each Petri dish was moistened with 6 ml sterile distilled water at zero time and 4 ml at the third day. The plates were incubated at greenhouse conditions at 20.0±2.0 °C for 10 days. The germinated grains were counted and the first count was specified as the number of germinated grains on the third and seventh day of the test, abnormal seedlings on the seventh day. Percentage of germination (G %) and speed of germination index (SGI %) as indications of grain viability were counted at the end of the third day of the test (Bartlett, 1937). Normal seedlings of each treatment were counted at the end of standard germination test and expressed as percentage according to the following equation:

$$G\,\% = \frac{\text{Number of normal seedlings}}{\text{Number of total seeds}} \times 100$$

Isolation of fungi associated with wheat grains

Direct plating technique (Golden *et al.* 1988) was used for isolation of fungi from wheat grains stored for 6 months with 3, 5 and 7 g/ton phosphine gas as aluminum phosphide. Ten grains were plated on Petri dishes each containing 20 ml of 1 % glucose-Czapek's agar with the following composition (g/l): Glucose, 10; Na_2NO_3, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005; Rose Bengal, 0.05; chloramphenicol, 0.25; agar, 15 and the final pH 7.3 (Ismail *et al.* 2017). The plates were incubated for 15 days at 25.0 °C. Pure cultures of the obtained fungal isolates were maintained on Czapek's agar slants at 4 °C as well as on cotton balls as described by Al-Bedak *et al.* (2019), in the culture collection of Assiut University Mycological Centre (AUMC) and the accession numbers were given.

Morphological identification of the new recorded fungi

Morphological features and growth rates of the fungal isolates were monitored on Czapek's agar (CZ, (Raper and Fennell 1965), malt extract agar (MEA, (Samson *et al.* 2010), and potato dextrose agar (PDA, (Smith and Onions 1994) at 25 °C. Microscopic features on PDA were examined in lacto-phenol cotton blue.

Molecular identification of the new recorded fungi

DNA extraction

DNA extraction was performed following the method of Moubasher *et al.* (2019), in which, a small piece of 7-day-old fungal mycelia grown on PDA plates at 25.0°C was transferred, individually to 2 ml-Eppendorf tube. A 800 µl CTAB buffer composed of 3 % CTAB, 1.4 M NaCl, 0.2 % Mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0 and 1 % PVP-40, were added to each tube. After incubation at 65.0 °C for 30 min, 800 µl of CI Mix with the composition of 24 ml chloroform and 1 ml isoamyl alcohol, were gently added and mixed with the tube contents. A clear supernatant was obtained by centrifugation at 10000 xg for 10 min. For DNA precipitation 2/3 volume of isopropanol (precooled at -20.0 °C) was added and mixed gently. The samples were incubated at 4.0 °C overnight, thereafter centrifugation at 13000 xg for 10 min. The supernatant was discarded and the pellet was pooled and washed with 200 µl washing buffer composed of 76.0 % ethanol and 10 mM ammonium acetate. The washing buffer was carefully decanted and the pellet was suspended in 200 µl TE buffer supplemented with 10 mg/ml RNase. After incubation at 37.0 °C for 30 min, 100 µl of 7.5 M ammonium acetate and 750 µl ethanol were added and mixed gently. Samples were centrifuged at 13000 xg for 10 min at room temperature. The supernatant was completely discarded and the pellet was suspended in 100 µl sterile distilled water.

PCR for rDNA and sequencing using ITS1 and ITS4 primers

The PCR reaction was carried out using the universal primers ITS1 and ITS4 (White *et al.* 1990) and SolGent EF-Taq. In the PCR tubes 1µl of DNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10x complete buffer and 40 µl of sterile ddH2O, 10 pmol of the primers ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') were added. Amplification was conducted using the following PCR conditions: denaturation at 95.0 °C for 15 min (one cycle), denaturation at 95.0 °C for 20 sec (30 cycles) then annealing at 50.0 °C for 40 sec and extension at 72.0 °C for 1 min (30 cycles), with a final extension step of 72.0 °C for 5 min. Prior to sequencing, the PCR products were purified using the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South

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Korea). The purified PCR products were confirmed on 1% agarose gel by electrophoreses, then eluted and sequenced in the forward and reverse directions using the same primers with the incorporation of ddNTP in the reaction mixture.

Alignments and phylogenetic analyses

Sequences of new recorded fungi in the present work and those retrieved from GenBank were aligned together using MAFFT (Katoh and Standley 2013). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Alignment gaps and parsimony uninformative characters were treated by BMGE (Criscuolo and Gribaldo 2010). Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 (Guindon *et al.* 2010). The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications (Felsenstein 1985). The best optimal model of nucleotide substitution for the ML analyses was determined using Smart Model Selection (SMS) version 1.8.1 (Lefort *et al.* 2017). The phylogenetic tree was visualized using Fig. Tree version 1.4.3 (Rambaut and Drummond 2012). The resulting tree was edited using Microsoft Power Point (2016) and saved as TIF file.

Amylase, cellulase and xylanase production in submerged fermentation (SmF)

The fungal strains were cultivated each in a 250 ml Erlenmeyer conical flask containing 50 ml Czapek's broth medium supplemented with 1 % soluble starch as sole carbon source for amylase production, or 1 % oat spelt xylan for cellulases (endoglucanase and exoglucanase) and xylanase production. The flasks were inoculated each with 1 ml spore suspension (1.5 x 10^8 spores/ml) from 7-day-old cultures. The flasks were then incubated for 7 days at 30.0 °C in shaking condition (150 rpm).

Extraction of enzymes

After the incubation period, the medium was filtered through Whatman No.1 filter paper after incubation, and the cell-free supernatant was obtained by centrifugation (10000 xg at 4 °C for 10 min) and used as enzyme source.

Enzymes assay and protein estimation

Amylase, endoglucanase, exoglucanase and xylanase production were determined by mixing 0.5 ml filtered crude enzyme with 0.5 ml of 1 % starch, CMC, avicel, and oat spelts xylan respectively (prepared in 50 mM Na-citrate buffer, pH 5.0). The reaction mixture was incubated at 50.0 °C for 20 min (Bailey *et al.* 1992) and the process was stopped by introducing2 ml of 3, 5-dinitrosalicylic acid (DNS) and boiling in a water bath for 10 min (Miller 1959). After cooling, the color absorbance was measured at 540 nm using UV-Visible spectrophotometer (T80+). The amount of reducing sugar liberated was quantified using standard curves of glucose (for amylase, cellulase), or xylose (for xylanase). One unit of the enzyme is defined as the amount of enzyme that liberates 1 µmol of the reducing sugar (glucose or xylose) equivalent per minute under the standard assay conditions (Ghose and Bisaria 1987).

Calculations of enzyme production and activity

Enzyme production was calculated as the following equations:

$$\textbf{Amylase \& cellulase production} = \frac{\text{glucose concentration (g/l)}}{0.00018}\ \textbf{IU/l}$$

$$\textbf{Xylanase production} = \frac{\text{xylose concentration (g/l)}}{0.00015}\ \textbf{IU/l}$$

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The enzyme activity was calculated according to the following formula (Moubasher *et al.* 2016).

$$\textbf{Enzyme activity} = \textbf{Absorbance x DF x}\left(\frac{1}{x}\right)\left(\frac{1}{y}\right)\left(\frac{1}{t}\right)\left(\frac{1}{slope}\right)$$

Where: DF = the dilution factor for enzyme; x = the volume of enzyme used; y = the volume of hydrolysate used for assay of reducing sugars; t = the time of hydrolysis; slope is determined from a standard curve. Soluble protein was estimated by Folin Lowry's method using bovine serum albumin as standard (Lowry *et al.* 1951). The specific activity was calculated by dividing the enzyme activity of the enzyme by the total protein.

Table 1: Effect of phosphine fumigation on viability and seedlings of wheat grains stored for 6 months at 10 % and 14 % MC

Moisture content	Phosphine concentration	G %	SGI	% of abnormal Seedlings	Primary root length (cm)	Shoot length (cm)
10%	Control	83.0	0.83	3.7	9.3	7.0
	3 g/ton	97.85	0.98	1.5	12.8	10.0
	5 g/ton	98.2	0.98	1.5	13.5	10.3
	7 g/ton	98.0	0.98	1.4	13.4	10.2
14%	Control	79.0	0.79	5.0	8.0	5.5
	3 g/ton	95.8	0.96	2.6	10.0	8.0
	5 g/ton	96.2	0.96	2.75	10.5	7.8
	7 g/ton	97.2	0.97	2.0	10.7	7.6

Results

Effect of different phosphine dosages on germination and viability of wheat grains

At 10 % MC and as the concentration of phosphine raises the level of seed germination, the speed of germination index and the length of the shoot and root increase, while the percentage of irregular seedlings decreased (Table 1).Results also showed that 14 % MC had deleterious impact on all seed parameters at the three concentrations of phosphine. Germination percentages and speed of seed germination index as well as the length of shoots and primary roots were declined. The percentage of abnormal seedlings was markedly higher at 14 % MC than at 10 % MC (Table 1).

Newly recorded fungi associated with wheat grains

The current findings revealed the isolation of four interesting fungal species belonging to the genera *Alternaria* (2 species), and *Bipolaris* and *Cladosporium* (one species each), from wheat grains stored for six months using phosphine (as aluminum phosphide). *Alternaria* sp. AUMC 14510 was isolated from wheat grains stored at 14 % MC and phosphine concentration of 5 g/ton, while *Cladosporium* sp. AUMC 14511, *Bipolaris* sp. AUMC 14512 and *Alternaria* sp. AUMC 14513 at 10 % MC and 7 g/ton phosphine concentration.

Descriptions of the newly recorded fungal strains

***Alternaria ventricosa* AUMC 14510**
GenBank MT974152
Phylogenetic analysis

Phylogenetic analysis of ITS dataset was performed to determine the taxonomic identity of *Alternaria* sp. AUMC 14510 relative to other members of *Alternaria*. The entire ITS dataset comprised 15 sequences, of which 14 sequences are related to *Alternaria* including sequence of *Alternaria* sp. AUMC 14510 obtained in this study and one for *Fusarium solani* TA-5 as the out

group. The maximum parsimony dataset consisted of 594 characters, of which 535 were constant characters (no gaps, no N), 57 variable characters which were parsimony-uninformative (10.7 % of constant characters), and 2 characters were counted as parsimony informative (0.4 % of constant characters). K80 was the perfect model for nucleotide substitution. The dataset for maximum parsimony yielded 3 most parsimonious trees with a tree length of 58 steps. The best scoring ML tree (Figure 1) with the final ML optimization likelihood value of –1210.4594 and tree size of 0.17125 was selected to represent and discuss the phylogenetic relationships among taxa. Estimated base frequencies were: f(A)= 0.25000, f(C)= 0.25000, f(G)= 0.25000, f(T)= 0.25000; Gamma shape parameter= 0.087; transition/transversion ratio= 1.961.The phylogenetic tree places the isolate AUMC 14510 on the same branch as *Alternaria ventricosa* CBS 121.546. The two species having 100% similarity of their ITS sequences (Figure 1). So, this isolate is identified here as *Alternaria ventricosa*, which is recorded for the first time in Egypt.

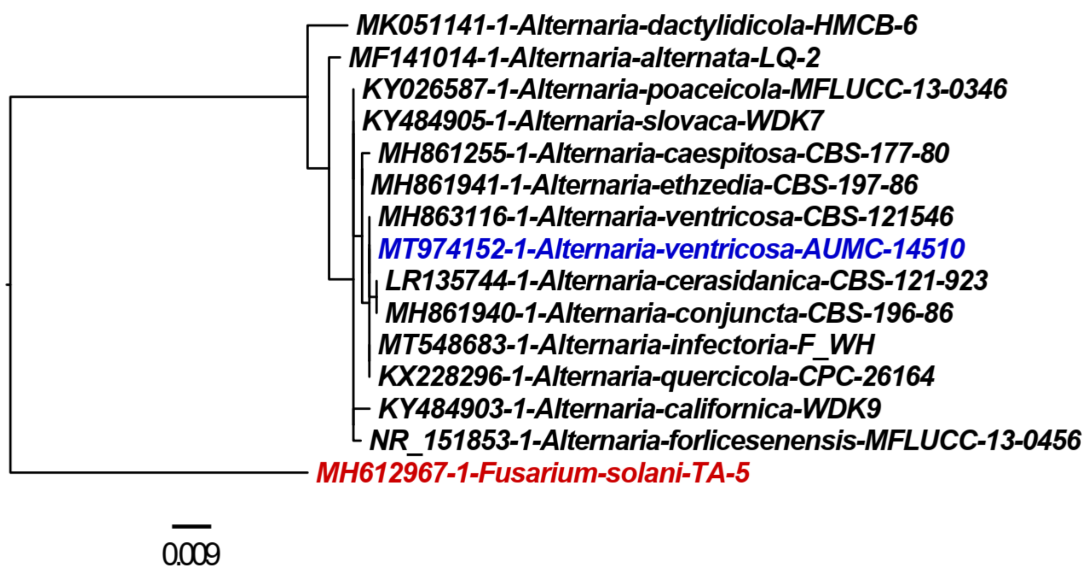

Figure 1: Phylogenetic tree generated from MP analysis based on ITS sequence data of ***Alternaria ventricosa* AUMC 14510** associated to other *Alternaria* related genes in the ITS gene sequences in GenBank. Sequences of *Alternaria ventricosa* AUMC 14510 in this study are in blue color. Bootstrap support values (100 replications) for ML/MP combination ≥50% are indicated at the respective nodes. The tree is rooted to *Fusarium solani* TA-5 (in red color).

Macroscopic and microscopic description

Colonies on Cz, MEA and PDA after 7 days at 25 °C attaining 60-65 mm, 55-60 mm and 60-65 mm in diameter respectively. On PDA colonies lanuginose, olive brown to bronze brown, centrally dark grey. Vegetative mycelium septate, pale brown to dark brown. Sporulation short, simple or branched chains of 2-8 conidia. Young conidia globose, ovoid, or ellipsoid, 7-13 × 5-9 µm. Mature conidia 28-55 × 14-17 µm produced on simple conidiophores commonly 50-80 µm long. The secondary conidiophore may be simple 1-geniculate and produces a conidial branch. The mature conidia have up to 7-9 transverse or oblique septa and 1-2 longitudinal or oblique septa in most of the body cells, with well-rounded or conical apex (Figure 2).

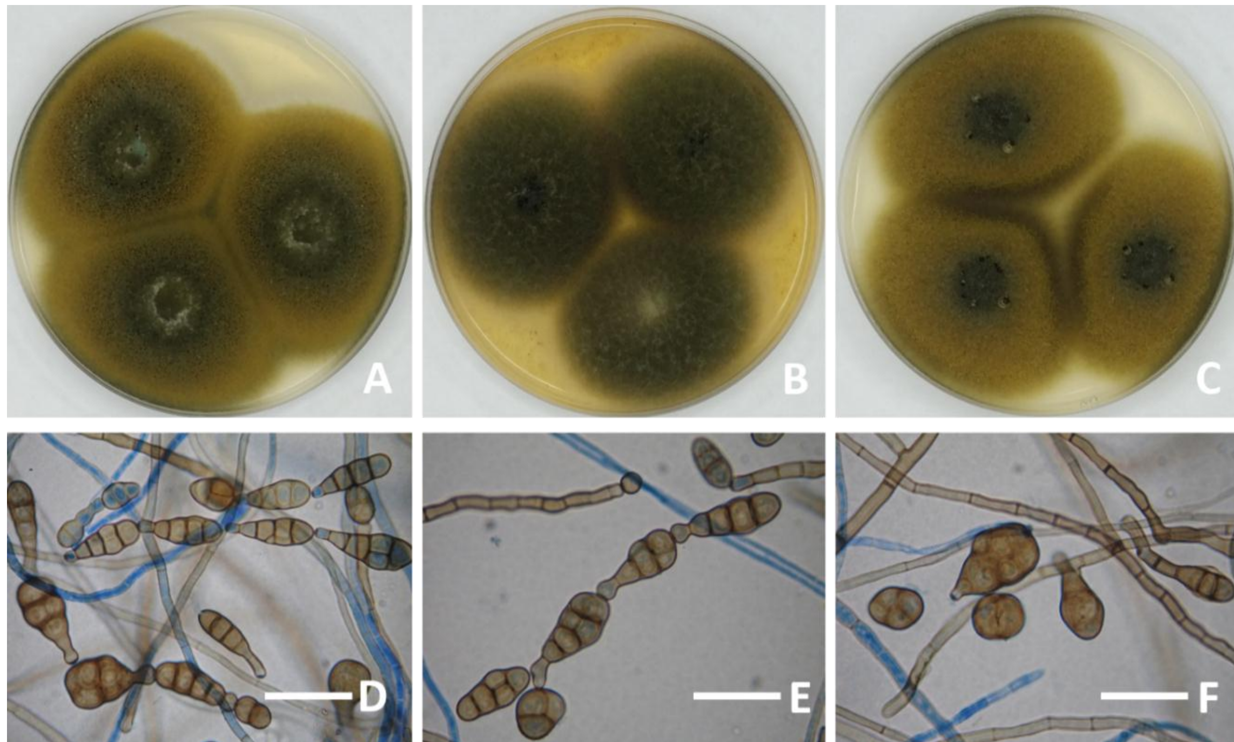

Figure 2: 7-day-old colonies of ***Alternaria ventricosa*** **AUMC 14510**: A-C, on Cz, MEA and PDA at 25 °C; D-E, simple conidiophores and chains of well-rounded or conical apex conidia; F, globose, ovoid, or ellipsoid young conidia (Scale bars = 50 µm).

Cladosporium allicinum **AUMC 14511**
GenBank MT974153
Phylogenetic analysis

Phylogenetic analysis of ITS dataset was employed to determine the taxonomic status of *Cladosporium* sp. AUMC 14511 relative to other members of *Cladosporium*. The entire ITS dataset comprised 11 sequences, of which 10 sequences are related to *Cladosporium* and one for *Aspergillus oryzae* AUMC 10329 as the out group. The maximum parsimony dataset consisted of 564 characters with 498 constant characters (no gaps, no N), 151 variable characters which were parsimony-uninformative (30.3 % of constant characters), and no characters were counted as parsimony informative. HKY85 was the perfect model for substitution of nucleotides.

The dataset for maximum parsimony yielded one tree with a tree length of 151 steps. The best scoring ML tree (Figure 3) with the final ML optimization likelihood value of –1299.5564 and tree size of 0.39068 was selected to represent and discuss the phylogenetic relationships among taxa. Estimated base frequencies were: f(A)= 0.21246, f(C)= 0.26726, f(G)= 0.27196, f(T)= 0.24833. Transition/transversion ratio= 1.332.The phylogenetic analysis revealed that *Cladosporium* isolate AUMC 14511 consistently clustered with *C. allicinum* CBS 188.54 endorsing high bootstrap value of 99 % ML (Figure 3). Therefore, this isolate identified here as *Cladosporium allicinum*, which is recorded for the first time in Egypt in the current study.

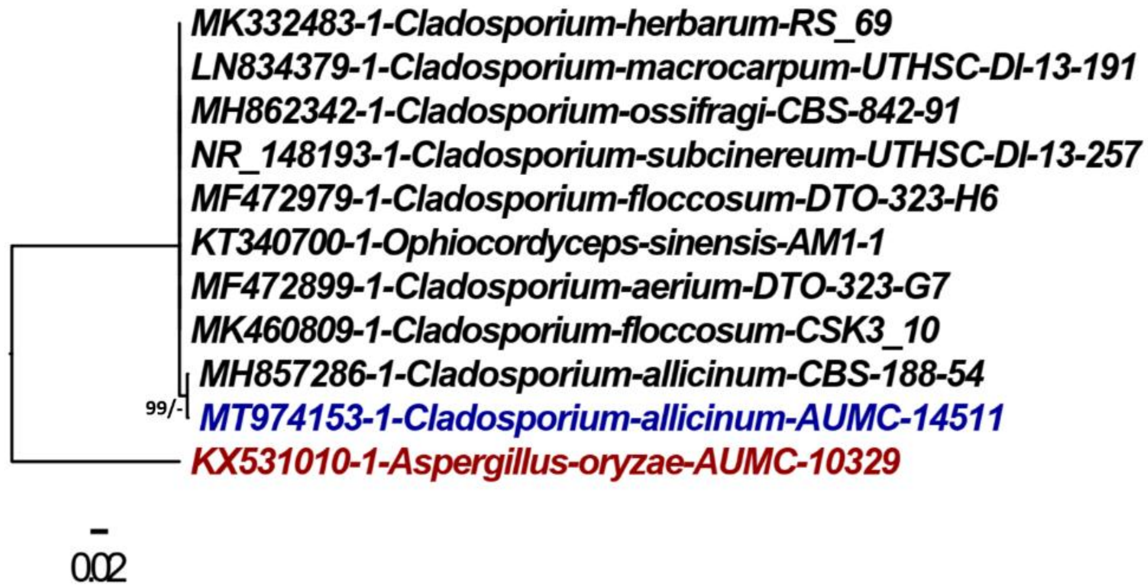

Figure 3: Phylogenetic tree generated from MP analysis based on ITS sequence data of ***Cladosporium allicinum* AUMC 14511** associated to other *Cladosporium* related genes in the ITS gene sequences in GenBank. Sequences of *Cladosporium allicinum* in this study are in blue color. Bootstrap support values (100 replications) for ML/MP combination ≥50% are indicated at the respective nodes. The tree is rooted to *Aspergillus oryzae* AUMC 10329 (in red color).

Macroscopic and microscopic description

Colonies on Cz, MEA and PDA after 7 days at 25 °C attaining diameters of 22-25 mm, 18-20 mm and 22-25 mm respectively. Olivaceous to dark olivaceous due to abundant aerial mycelium. Reverse black. Mycelium superficial, hyphae branched, 1.5–8 µm wide, broader hyphae usually slightly constricted at the septa and somewhat swollen, hyaline to subhyaline, almost smooth to somewhat verruculose or irregularly rough-walled (Figure 4). Conidiophores macronematous, arising as lateral or terminal branches from ascending hyphae, erect, straight to more or less flexuous, sometimes geniculate, nodulose, usually with small head-like swellings, unbranched, occasionally branched, commonly 100–300 µm, subhyaline to pale brown or pale olivaceous, smooth or somewhat verruculose. Conidiogenous cells integrated, usually terminal, with a terminal head-like swelling. Conidia catenate, formed in branched chains, straight to slightly curved, small terminal conidia subglobose, ovoid to obovoid or somewhat limoniforme, 4–9 × 2.5–3.5 µm, aseptate. Secondary ramoconidia ellipsoid to subcylindrical or cylindrical, 10–24(–31) × 3–5(–7) µm, 0–1(–3)-septate, subhyaline to pale brown or pale olivaceous, minutely verruculose to verrucose, smooth-walled or almost so, apex rounded or slightly attenuated towards apex and base, with protuberant, conspicuous hila (Figure 4).

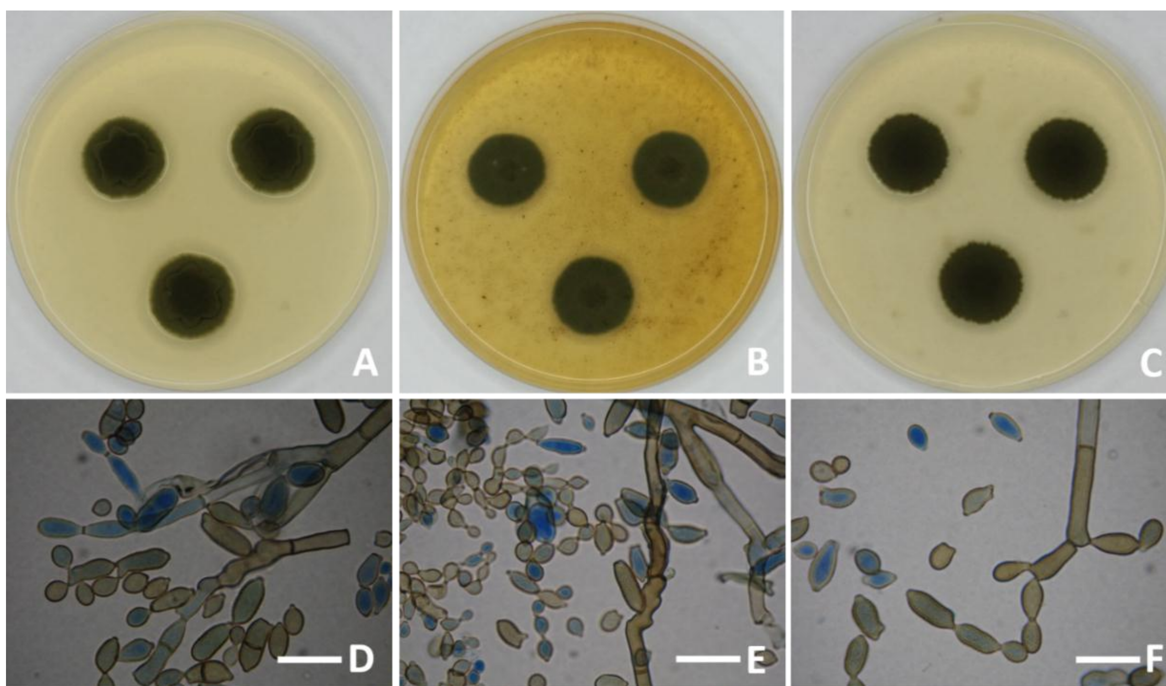

Figure 4: 7-day-old colonies of ***Cladosporium allicinum*** **AUMC 14511**: A-C, on Cz, MEA and PDA at 25 °C; D-F, geniculate, nodulose, with small head-like swellings conidiophores bearing chains of subglobose, ovoid, obovoid to limoniforme conidia (Scale bars = 10 µm).

Bipolaris **sp. AUMC 14512**

GenBank MT974154

Phylogenetic analysis

The generated sequence of *Bipolaris* sp. AUMC 14512 was compared with other fungal DNA sequences from NCBI's GenBank sequence database using a blast search. An alignment of ITS sequences was made using the sequence data of *Bipolaris* sp. AUMC 14512 and all the most similar *Bipolaris* sequences published in GenBank. Based on a megablast search in GenBank using the ITS sequence, it was revealed that the most similar records in GenBank were *Bipolaris sorokiniana* A14, M21 and MN01 [(GenBank accession number MK676000, MH592543 and MK103015 respectively; identities 574/574 (100%); no gaps; 100 % gene coverage].

Phylogenetic analysis of ITS dataset was employed to determine the taxonomic status of *Bipolaris* AUMC14512 relative to other members of *Bipolaris*. The entire ITS data set comprised 28 sequences, of which 25 for *Bipolaris* species including the isolate AUMC 14512 obtained in this study, 2 sequences for *Cochliobolus* and one for *Curvularia lunata* as an outgroup. The maximum parsimony dataset consisted of 587 characters with 386 constant characters (no gaps, no N), 45 variable characters which were parsimony-uninformative (11.7 % of constant characters), and 16characters were counted as parsimony informative (4.1 % of constant characters). TN93 was the perfect model for substitution of nucleotides. The dataset for maximum parsimony yielded one tree with a tree length of 63 steps. The best scoring ML tree with the final ML optimization likelihood value of -1737.5587and tree size of 0.36189was selected to represent and discuss the phylogenetic relationships among taxa (Figure 5).Estimated base frequencies were: f(A)=0.24961, f(C)=0.23881, f(G)=0.22801, f(T)=0.28358; Gamma

shape parameter=0.697; Transition/transversion ratio for purines=1.485 and transition/transversion ratio for pyrimidines=3.379.*Bipolaris* sp. AUMC 14512 in the phylogenetic tree was housed in a single division that suggested its uniqueness (Figure 5). For this strain, the phylogenetic study using ITS sequences was not sufficient for its recognition and could involve the sequencing of another gene to validate its taxonomic status.

The strain AUMC 14512 clustered in the phylogenetic tree within *B. salviniae*, *B. drechsleri* and *B. crotonis* clade (Figure 5). The phylogenetic tree places the isolate AUMC 14512 on the same branch as *Bipolaris crotonis* BRIP15875.

The two species having 99.5% similarity of their ITS sequences (Figure 1). The isolate AUMC 14512 can be distinguished from the three species within the clade by its smaller conidial size (up to 60 µm and 5-distoseptate) than those of *B. crotonis* (60-110 µm and up to 11 distoseptate), *B. salviniae* (100-170 µm and up to 14 distoseptate) and *B. drechsleri* (50-80 µm and up to 10 distoseptate). Also, the formation of synnemata-like stroma in the isolate AUMC 14512 can differentiate this species from the other three species in the clade. So, this isolate can be considered as potentially new species and need to another gene(s) for confirmation of its taxonomical status.

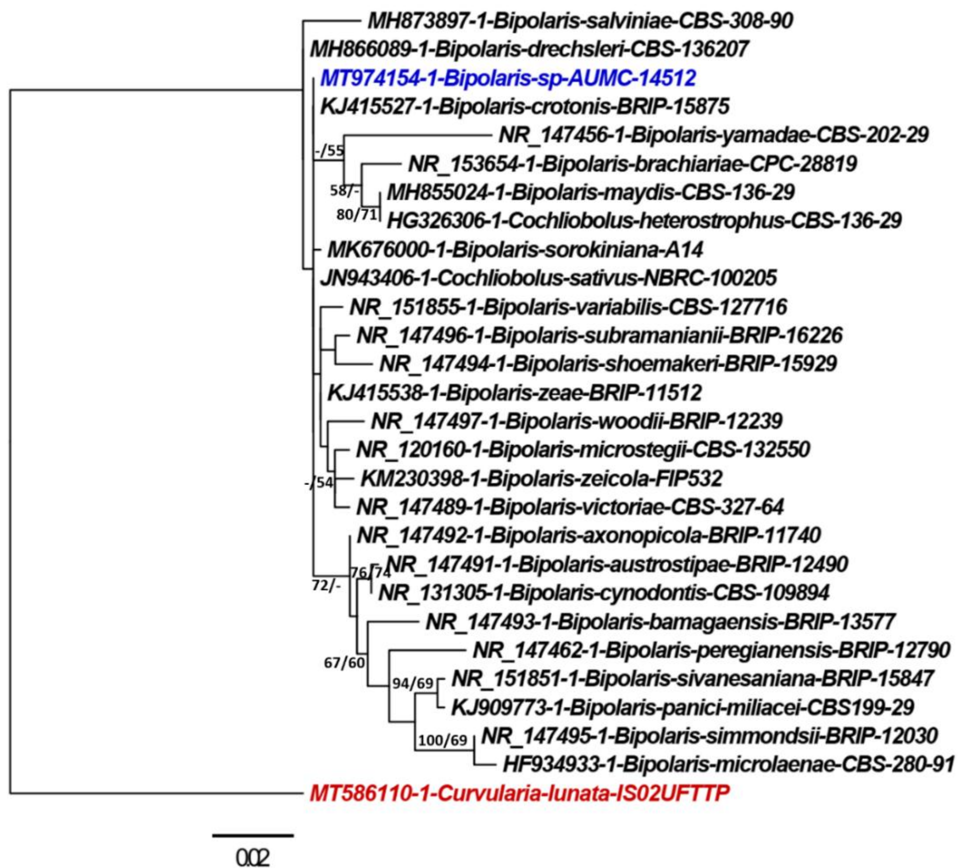

Figure 5: Phylogenetic tree generated from MP analysis based on ITS sequence data of ***Bipolaris* sp. AUMC 14512** associated to other *Bipolaris* related genes in the ITS gene sequences in GenBank. Sequences of *Bipolaris* sp. AUMC 14512 in this study are in blue color. Bootstrap support values (100 replications) for ML/MP combination ≥50% are indicated at the respective nodes. The tree is rooted to *Curvularia lunata* ISO2UFTTP (in red color).

Macroscopic and microscopic description

Colonies on Cz, MEA and PDA after 7 days at 25 °C attaining diameters of 50-55 mm, 65-70 mm and 75-85 mm respectively. Colonies on PDA whitish-grey when young, becoming grey at maturity, darker at the center. Margin irregular, effuse, velvety. Reverse black. Synnemata-like stromata formed in old cultures, up to 10 mm in length or more (Figure 6).Conidiophores macronematous, straight to flexuous, geniculate in upper part, simple, pale brown, septate, smooth-walled, 100–250 × 4–7 µm. Conidiogenous cells integrated, intercalary, with sympodial proliferation, monotretic or polytretic, dark brown, with circular scars. Conidia solitary, curved or straight, ellipsoidal, clavate, obclavate, obclavate-ellipsoidal, smooth-walled, pale to dark golden-brown, sometimes paler in end cells, 20–60 × 15–30 µm, up to 5 distoseptate with inprotuberant, dark brown to black hilum (Figure 6).

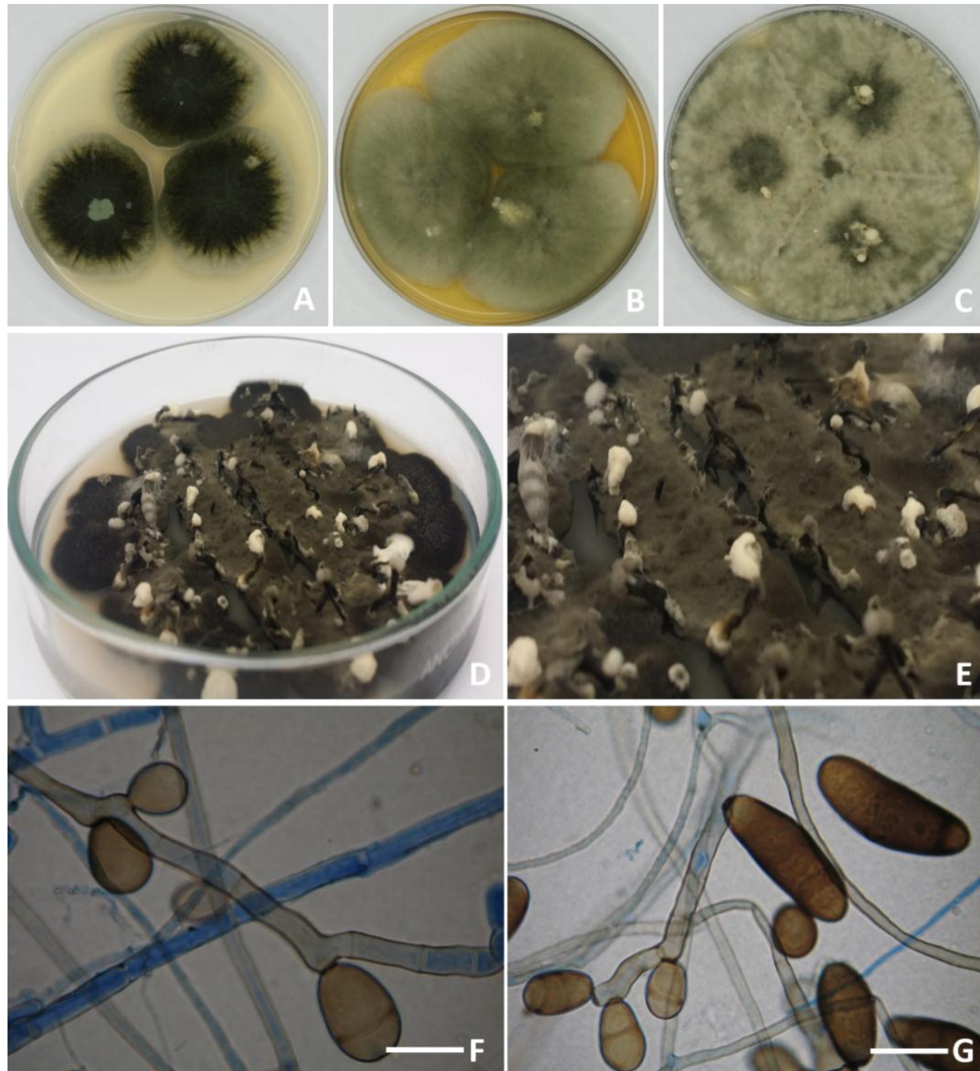

Figure 6: 7-day-old colonies ***Bipolaris* sp. AUMC 14512**: A-C, on Cz, MEA and PDA at 25 °C; D-E, synnemata-like stroma formed in old culture; F-G, geniculate conidiophores bearing clavate, obclavate to obclavate-ellipsoidal conidia (Scale bars = 20 µm).

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***Alternaria* sp. AUMC 14513**
GenBank MT974155
Phylogenetic analysis

The obtained sequence of *Alternaria* sp. AUMC 14513 was compared with other *Alternaria* sequences from NCBI's GenBank sequence database using a blast search. An alignment of ITS sequences was made using the sequence data of *Alternaria* sp. AUMC 14513 and all the most similar *Alternaria* sequences published in GenBank including the available type materials. The entire ITS data set comprised 19 sequences, of which 18 are *Alternaria* species including the isolate *Alternaria* sp. AUMC 14513 obtained in this study, and one sequence for *Fusarium solani* as an outgroup. The maximum parsimony dataset consisted of 568 characters with 514 constant characters (no gaps, no N), 3 variable characters which were parsimony-uninformative (0.6% of constant characters), and 1 characters were counted as parsimony informative (0.2% of constant characters). GTR was the perfect model for substitution of nucleotides. The dataset for maximum parsimony yielded one tree with a tree length of 3 steps. The best scoring ML tree with the final ML optimization likelihood value of -1078.0568 and tree size of 0.11403 was selected to represent and discuss the phylogenetic relationships among taxa (Figure 7).Estimated base frequencies were: f(A)=0.24748, f(C)=0.22793, f(G)=0.23687, f(T)=0.28773; GTR relative rate parameters: A-C=0.00119, A-G=1.33261, A-T=0.52475, C-G=1.27576, C-T=1.32181, G-T=1.00000.

In the phylogenetic tree, the strain AUMC 14513 was clustered within *A. alternata*, *A. burnsii*, *A. eichhorniae* clade. The phylogenetic tree places the isolate AUMC 14513 on the same branch as *Alternaria alternata* SQU 14008 and *A. burnsii* CBS 107.38 (type species). The three species having 100 % similarity of their ITS sequences (Figure 7).The presence of tuberculate conidia with chlamydospores-like to irregular shape can be easily distinguish this species from the other three species within the clade. Thus, it can be described as potentially new *Alternaria* sp. and another gene(s) should be sequenced for confirmation of its taxonomic status.

Macroscopic and microscopic description

Colonies after 7 days at 25 C on Cz, MEA and PDA attaining 70 mm, 70 mm and 80 mm in diameter. On PDA, colonies grey, centrally dark grey. Mycelium septate, branched, brown. Secondary conidiophores short, up to 50 µm. Chlamydospores abundant in aerial hyphae. Conidia in short chains of 1-3 conidia. Young conidia brown, ovoid to broad elliptical, 8-25 × 5-12 µm, without apical cell. Mature conidia chlamydospore-like to irregular in shape, becomes darker by maturation, 32-60 × 15-25 µm (Figure 8).

MN856340-1-Alternaria-eichhorniae-WZ-213
NR_136119-1-Alternaria-burnsii-CBS-107-38
KY684261-1-Alternaria-alternata-SQU-14008
MT974155-1-Alternaria-sp-AUMC-14513
MK905453-1-Alternaria-porri-VarAp-17
MK158222-1-Alternaria-brassicicola-MVR1
MH865506-1-Alternaria-burnsii-CBS-130264
NR_137143-1-Alternaria-destruens-ATCC-204363
-/58
MH861939-1-Alternaria-angustiovoidea-CBS-195-86
NR_111832-1-Alternaria-eichhorniae-ATCC-22255
NR_163686-1-Alternaria-alstroemeriae-CBS-118809
NR_135927-1-Alternaria-arborescens-CBS-102605
MH037553-1-Alternaria-destruens-S4S201
KJ728842-1-Alternaria-brassicae-SR/II/36
MH879771-1-Alternaria-arborescens-ALT-14
MH712187-1-Alternaria-longipes-AH20
MN004781-1-Alternaria-tenuissima-BAT4ITS
MN795638-1-Alternaria-alstroemeriae-P4-6-PE2-ab1
MH612967-1-Fusarium-solani-TA-5
0.006

Figure 7: Phylogenetic tree generated from MP analysis based on ITS sequence data of ***Alternaria* sp. AUMC 14513** associated to other *Alternaria* related genes in the ITS gene sequences in GenBank. Sequences of *Alternaria* sp. AUMC 14513 in this study are in blue color. Bootstrap support values (100 replications) for ML/MP combination ≥50% are indicated at the respective nodes. The tree is rooted to *Fusarium solani* TA-5 (in red color).

Figure 8:7-day-old colonies of ***Alternaria* sp. AUMC 14513**: A-C, on Cz, MEA and PDA at 25 °C; D-F, short secondary conidiophores bearing tuberculate, irregularly-shaped conidia (Scale bars = 20 µm).

Enzymatic activities of the newly recorded fungal strains

Amylolytic, cellulolytic and xylanolytic activities of *Alternaria*, *Bipolaris* and *Cladosporium* strains obtained in this study was explored in submerged fermentation. All the four tested strains could produce amylase, cellulase (endoglucanase and exoglucanase) and xylanase enzymes but with varied levels. *Alternaria ventricosa* AUMC 14510 and *Alternaria* sp. AUMC 14513 were the best producers gained 94.7 IU/ml and 82.9 IU/ml respectively, however the first strain (AUMC 14510) harbored a specific activity of 65.5 IU/mg next to the later (AUMC 14513) which gave 75.7 IU/mg specific activity. Their xylanase production was almost similar (5.5 IU/ml and 5.12 IU/ml) respectively. *Cladosporium allicinum* AUMC 14511 was the runner of *Alternaria* strains in amylase production (66.11 IU/ml) more than *Bipolaris* sp. AUMC 14512 (40.55 IU/ml), however the specific activity of the *Cladosporium* strain (48.35 IU/mg) was lower than that of *Bipolaris* strain (57.33 IU/mg). It was superior in endoglucanase, exoglucanase and xylanase production over the remaining strains. It gained 93.1 IU/ml endoglucanase, 64.46 IU/ml exoglucanase and 23.9 IU/ml xylanase production (Table 2; Figure 9).

Table 2: Production, relative activity and specific activity of amylase, endoglucanase, exoglucanase and xylanase produced by *Alternaria ventricosa* AUMC 14510, *C. allicinum* AUMC 14511, *Bipolaris* sp. AUMC 14512 and *Alternaria* sp. AUMC 14513 at 30 °C under submerged fermentation.

Enzymes produced		***A. ventricosa* AUMC 14510**	***C. allicinum* AUMC 14511**	***Bipolaris* sp. AUMC 14512**	***Alternaria* sp. AUMC 14513**
α-amylase	**Production** IU/ml	**94.7**	66.11	40.55	82.9
	Activity IU/ml/min	**7.14**	4.98	3.05	6.252
	Specific activity IU/mg protein	65.5	48.35	57.33	**75.7**
Endoglucanase	**Production** IU/ml	11.14	**93.1**	15.12	33.1
	Activity IU/ml/min	0.84	**7.0**	1.14	2.5
	Specific activity IU/mg protein	5.6	**53.85**	10.36	19.84
Exoglucanase	**Production** IU/ml	3.66	**64.46**	3.0	14.32
	Activity IU/ml/min	0.276	**4.86**	0.228	1.1
	Specific activity IU/mg protein	1.84	**37.4**	2.1	8.73
Xylanase	**Production** IU/ml	5.5	**23.9**	7.0	5.12
	Activity IU/ml/min	0.35	**1.512**	0.444	0.324
	Specific activity IU/mg protein	2.33	**11.63**	4.0	2.6

The highest value of production, activity or specific activity for each enzyme is shown in bold.

Discussion

In the current research, the effects of various doses of phosphine on wheat grains packed for 6 months at 10% and 14 % MC were reported. The present results showed that 14 % of MC had detrimental effect on all seed parameters at the three phosphine concentrations used in this study. This can be due to the development of fungal growth, as well as their enzymes at high MC levels, and these fungi or their enzymes may cause decay of wheat grains. As a result, grain germination and germination rate have been affected leading to the appearance of odd seedlings and abnormal shoot and

root lengths. In this context, it was reported that relative humidity played a major role in the release of phosphine. As moisture reduces the release of phosphine slows down (Sankarganesh *et al.* 2020). As a result, its effect on the prevention of grain degradation is growing. Few studies have been reported on the effect of phosphine on the growth of molds in stored wheat and corn grains (Hocking and Banks, 1991; Castro *et al.*, 2000).

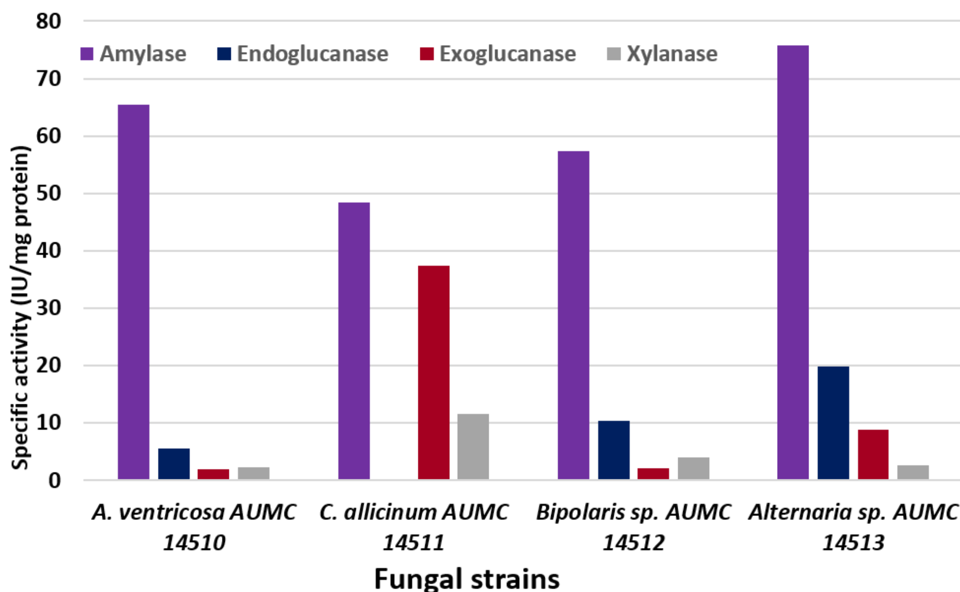

Figure 9: Specific activity of amylase, endoglucanase, exoglucanase and xylanase produced by *Alternaria ventricosa* AUMC 14510, *C. allicinum* AUMC 14511, *Bipolaris* sp. AUMC 14512 and *Alternaria* sp. AUMC 14513 in submerged fermentation at 30 °C.

These studies reported that phosphine only causes a slight decrease in the population of *Aspergillus flavus* in stored maize and *Eurotium chevalieri*, *A. flavus*, or *A. parasiticus* in stored wheat grains. As mentioned by Solanki *et al.* (2019) phosphine fumigation is known to have broad biological activity but its effect on the microbial community of stored wheat grains is largely unknown. In the present study, four promising fungi were recorded. Based on the sequence of their internal transcribed spacer (ITS) genes, two species of these fungi have been identified as *A. ventricosa* and *C. allicinum*, and the other two as possibly new species similar to *Bipolaris* and *Alternaria*. Wheat grains can be colonized by complex microbial species that have distinct functions in grain production and disease susceptibility (Baudisch *et al.* 2014).

Fungi associated with seeds and grains are associated with a significant depletion of food, which leaves the crop uneconomical, and also develop mycotoxins that impair the overall nutritional value of grains (Galvano *et al.* 2001). For example, particular species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* can cause spoilage in stored wheat grains and produce mycotoxins that significantly reduce crop value and are hazardous to animal and human health (Placinta *et al.* 1999). *Alternaria*, *Bipolaris* and *Cladosporium* are broadly distributed fungal genera including saprophytic, endophytic and pathogenic species. Species of *Alternaria* are regarded as

significant plant parasites, causing severe losses in a wide variety of crops. They are associated with a wide range of substrates, including crops, trees, agricultural products, soils as well as the air (Al-lami *et al.* 2020; Abdelrazek *et al.* 2020; Apangu *et al.* 2020; Van de Vijver *et al.* 2020; Masiello *et al.* 2020; Dewdney 2020; Aung *et al.* 2020; Abdel-Sater *et al.* 2020).

The genus *Bipolaris* includes a number of significant worldwide plant pathogens commonly associated with leaf spots, leaf blights, root rots, foot rots and other disease symptoms mainly in high value field crops including rice, maize, wheat and sorghum and on various other host plants (Ellis 1971; Manamgoda *et al.* 2014; Sivanesan 1987; Berbee *et al.* 1999; Rossman *et al.* 2013). *Bipolaris* species are known to occur on at least 60 other genera in Anacardiaceae, Araceae, Euphorbiaceae, Fabaceae, Malvaceae, Rutaceae and Zingiberaceae as either saprobes or pathogens (Ellis 1971; Sivanesan 1987; Manamgoda *et al.* 2011). The global distribution of common phytopathogenic species of *Bipolaris* may have resulted from the transfer of agricultural commodities including plants and seeds across geographical borders (Zhang *et al.* 2013; Manamgoda *et al.* 2014).

Because of *Cladosporium* species are well adapted to be spread easily in large numbers over long distances, they are cosmopolitan and widely present in all various types of plants, fungi and other debris, mostly isolated from air, soil, seeds, grains, food, paint, textiles and other organic matter (Ellis 1971; Ellis 1976; Yehia *et al.* 2020; Moubasher 1993; Ismail *et al.* 2017; Farr *et al.* 1989; Haas 2011; Flannigan *et al.* 2016; Bensch *et al.* 2012). Other species of this genus are plant pathogenic causing leaf spots and other lesions (Schubert and Braun 2004), or they occur as hyper parasites on other fungi (Heuchert *et al.* 2005). *Cladosporium* species are also known to be common endophytes (Riesen and Sieber 1985; Brown 1998; El-Morsy 2000) as well as phylloplane fungi (Islam and Hasin 2000; Inácio *et al.* 2002; Stohr and Dighton 2004; Levetin and Dorsey 2006).

The amylolytic, cellulolytic and xylanolytic activities of the fungal strains isolated in this research was quantified by submerged fermentation. Such enzymatic potential represent severe biochemical weapons for these fungi to deteriorate the grains of wheat particularly under favorable conditions of Moisture content. All strains had various degrees of enzymatic activity. Both *Alternaria ventricosa* and *Alternaria* sp. AUMC 14513 obtained in the current study were superior in the development of amylase enzyme registering 94.7 IU/ml and 82.9 IU/ml, respectively. These amounts are higher than those produced by *Alternaria tenuissima* At-Ch-5.6 (76.75 IU/ml) and At-UV-2.8 (63.12 IU/ml) (Shafique *et al.* 2009). *Bipolaris* sp. and *Cladosporium allicinum* obtained in the present study exhibited somewhat lower amylolytic activity than *Alternaria* species. *Cladosporium allicinum* AUMC 14511 was superior in endoglucanase, exoglucanase and xylanase development to 93.1 IU/ml, 64.46 IU/ml and 23.9 IU/ml for the three enzymes, respectively. To the best of our knowledge the amylolytic, cellulolytic and xylanolytic of *Alternaria ventricosa* and *Cladosporium allicinum* have not been previously detected.

Conclusion

The current study includes in-depth awareness of: First, degree of moisture content and phosphine concentration at which wheat grains should be retained, and second, fungi associated with wheat grains that may be strong sources of amylase, cellulase, and xylanase enzymes which have a wide range of valuable uses in many industries.

Compliance with ethical standards

The manuscript is original. No part of the manuscript has been published before nor is any part of it under consideration for publication at another journal.

Conflict of interest

The authors declare that they have no conflict of interest. All authors fully agree for submission of the manuscript.

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