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On the enzymes' actions of entomopathogenic fungi against certain indigenous and invasive insect pests

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Abstract

Background: Pathogenicity of entomopathogenic fungi (EPF) depends mainly on their ability to produce efficient enzymes, which degrade insect's integument and other cellular components. Lipases are the first enzymes secreted by these fungi, then proteases, phospholipases, and chitinases.

Results: Twenty-six fungal strains, isolated from the aphids, *Aphis* sp., Linnaeus (Homoptera, Aphididae), the tomato leaf minor, *Liriomyza trifolii*, Burgess (Diptera, Agromyzidae) (indigenous insects), the red palm weevil, *Rhynchophorus ferrugineus*, Olivier (Coleoptera, Curculionidae), and the peach fruit fly, *Bactrocera zonata*, Saunders (Diptera, Tephritidae) (invasive insects) were tested for their enzymatic activities. Results showed that the majority of these strains were able to produce lipolytic enzymes with the most active being *Aspergillus niger*, *Botryotrichum atrogriseum*, *Cochliobolus spicifer*, *Fusarium chlamydosporum*, and *F. proliferatum*. Phospholipase was successfully produced by 73.1% of the tested strains among which *Aspergillus flavus*, *A. niger*, *Mucor racemosus*, *Pochonia chlamydosporia* var. *catenulata*, and *Scopulariopsis brevicaulis* were the highest producers. Proteolytic enzymes were detected in cultures of all the fungal strains except *Aspergillus chevalieri* and *M. racemosus*. The best proteolytic strain was *S. brevicaulis* followed by *A. flavus*, *A. sydowii*, and *F. semitectum*. The chitinolytic ability of the fungal strains was generally weak and the relatively active species belonged to *A. flavus*, *A. niger*, *B. atrogriseum*, *F. chlamydosporum*, *F. solani*, *S. brevicaulis*, and *Nigrospora oryzae*. Quantitative determination of chitinase revealed that the enzyme concentration ranged from 3.478 to 6.44 IU/ml.

Conclusion: Most of the isolated fungi had enzymatic activities, but *A. niger*, *F. semitectum*, *F. solani*, *F. chlamydosporum*, *P. chlamydosporia var. catenulata*, and *S. brevicaulis*, were the most active ones.

Keywords: Entomopathogenic fungi, Pathogenicity, Enzymes, Production, Insect pests

Background

The common insect pest species can be classified into two groups (indigenous and invasive species). Aphids (*Aphis* spp., Linnaeus) (Homoptera: Aphididae) and the tomato leaf miner (TLM) (*Liriomyza trifolii*, Burgess) (Diptera, Agromyzidae) are examples of the indigenous insects, whereas the red palm weevil (RPW) (*Rhynchophorus ferrugineus*, Olivier) (Coleoptera: Curculionidae) and the

peach fruit fly (PFF) (*Bactrocera zonata*, Saunders) (Diptera: Tephritidae) are considered invasive insect pests in Egypt. Aphids cause economic losses by direct feedings, the transport of plant viruses and toxins.

Tomato is one of the major economic host plants of the tomato leaf miner (*L. trifolii*) which attacks its leaves, buds, stems, and fruits. The insect also attacks other members of the family Solanaceae such as potato and eggplant.

Rhynchophorus ferrugineus is an invasive species that is originated from Southeast Asia, invaded Middle East and several countries of the Mediterranean Basin during

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the last decades. The pest palm trees (El-Mergawy and Al-Ajlan 2011) can completely destroy the palm trees displaying a total loss of foliage and rotting of the trunk (Abelardo et al. 2010).

In Egypt 1995, the peach fruit fly (*B.zonata*) was recorded attacking a broad range of fruits but it was misidentified as *Bactrocera pallidus* (Aboul-Ela et al. 1998). Its identification was corrected as *B. zonata* and it was reported as a serious pest on many fruit crops attacking more than 50 host plants (El-Minshawy et al. 1999).

Most of the insects present a segmented cylindrical structure. The integument rigidity is caused by the three layers which form cuticle, epidermis, and basal membrane. The cuticle serves as a physical block against parasitoids and diseases. It is a structure formed by crystalline chitin nanofibers inside proteins, polyphenols, and lipids matrix. Its main component is chitin, a polysaccharide that is like cellulose. Epicuticle is the external layer of the insect's cuticle, waterproof, and acts as the first barrier against microbial attack. It is formed by heterogeneous mix of lipids, long chain alkenes, esters, and fatty acids. The importance of lipases in order to hydrolyze the ester bounds of lipoproteins, fats, and waxes at the interior of the insect integument is well known (Ali et al. 2009). They significantly confer the cuticle penetration and initial nutrients liberate.

Entomopathogenic fungi (EPF) are an important biological control agent of insects. More than 700 fungal species belonging to approximately 90 genera have been reported to infect living insects and kill them but few are involved in the biological control. The pathogenic ability of the fungus depends on the enzymatic equipment which comprises lipases, proteases, and chitinases (Sánchez-Pérez et al. 2014). Once the insect exoskeleton breaks down, the fungus produces great quantities of proteases, which degrade the proteinaceous material found in the epicuticle. The solubilized proteins are degraded releasing amino acids which serve as nutrients for the EPF (Wang et al. 2002). Phospholipases are enzymes responsible for degrading phospholipids of insect's cuticle. Fungal chitinases act synergistically with proteases in order to degrade the insect's cuticle. These enzymes are important virulence factors for the EPF. According to Kaur and Padmaja (2009), there is a certain correlation between the degree of pathogenicity and levels of enzymes that can be used to cause cuticular structure weakening. Establishing a relationship between the active secretion of hydrolytic enzymes and the virulence of EPF is very important in exploring and developing screening methods for identifying new isolates with increased virulence and also for the development of bioproducts based on them (Montesinos-Matías et al. 2011). The present work was conducted to determine the ability of 26 fungal strains isolated from indigenous

and invasive insects to produce the 4 types of hydrolytic enzymes including lipases, phospholipases, proteases, and chitinases.

Methods
Collection of insect samples
Eighty-five samples of targeted insect species were collected during the period from 2015 to 2017. Five different localities in Minia Governorate, Egypt, were chosen for insect sampling. These were Deir Mawas, Mallawi, Abu-Qurqas, Minia City, and Samalott. Samples were placed in polyethylene bags and transferred to the laboratory for identification as well as for mycological analysis. Identification of insect species was confirmed by specialists. Samples were included (70 aphids' samples) (4 immature stages of TLM), (6 mature and immature stages of RPW), and (5 immature stages of PFF).

Mycological analysis of insect samples
Insect samples were placed in sterile Petri dishes containing water soaked sterile filter paper to keep enough humidity for growth of the fungi associated with insects. The growing fungi were then cultured on Sabouraud's dextrose agar (SDA) at 28 °C for 7-10 days. Fungi were identified according to their phenotypic characters as described by Domsch et al. (2007), Bensch et al. (2012), and Ismail et al. (2015).

The identified fungal strains were preserved as slant cultures on SDA medium. For long-term preservation fungi were placed in 2 ml sterile vials containing 15% autoclaved glycerol water and kept under ultrafreezing at −70 °C.

Screening for production of fungal enzymes
Lipase production
Twenty-six fungal strains were tested using the agar medium described by Ulman and Blasins (1974). The medium contained (g/l) peptone, 10; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.2; Tween 80, 10 ml; and agar, 15). Tween 80 was autoclaved separately and added to the sterile and cooled basal medium prior to solidification. The medium was dispensed aseptically into 20 ml test tubes (10 ml each). The tubes were individually inoculated by 50 μl of fungal spore suspension and incubated at 28 °C for 15 days. The lipolytic ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate (in mm) was measured.

Phospholipase production
The fungal strains were grown on egg yolk agaras described by Samaranayake et al. (2005). The medium consisted of (g/l); 13 g powdered Sabouraud dextrose agar (SDA), 11.7g NaCl, 0.12g CaCl2, and egg yolk (10% in

Table 1 Incidence of fungal species isolated from the tested insects

Fungal species	Aphids (N=70)	Tomato leaf miner (N=4)	Red palm weevil (N=6)	Peach fruit fly (N=5)	Total (N=85)	% Incidence
Alternaria alternata	7	0	0	2	9	10.6
A. tenuissima	13	3	0	2	19	22.4
Aspergillus chevalieri	1	0	0	0	1	1.2
A. flavus	4	1	4	3	12	14.1
A. niger	26	2	1	3	32	37.7
A. rugulosus	1	0	0	0	1	1.2
A. sydowii	5	0	0	2	7	8.2
A. terreus	3	0	0	0	3	3.5
Botryotrichum atrogriseum	3	0	0	0	3	3.5
Cochliobolus spicifer	1	0	0	0	1	1.2
Epicoccum nigrum	2	0	0	0	2	2.4
Fusarium chlamydosporum	12	0	0	0	12	14.1
Fusarium proliferatum	12	0	2	0	14	16.5
F. semitectum	2	0	0	0	2	2.4
F. solani	11	0	1	0	12	14.1
F. subglutinans	0	0	1	0	1	1.2
F. verticillioides	1	0	0	2	3	3.5
Mucor racemosus	13	1	0	1	14	16.5
Nigrospora oryzae	0	0	0	1	1	1.2
Penicillium aurantiogriseum	1	0	0	0	1	1.2
P. oxalicum	0	0	0	1	1	1.2
Pochonia chlamydosporia var. *catenulata*	0	0	0	1	1	1.2
Scopulariopsis brevicaulis	14	0	1	0	14	16.5
Talaromyces pinophilum	0	0	1	0	1	1.2
Toxicocladosporium irritans	16	0	0	0	16	18.8
Verticillium sp.	7	0	1	0	8	9.4
Total no. of species	21	4	8	10	26	

sterile 0.85% saline water). The egg yolk was centrifuged at 500 g for 10 min at room temperature, and 80 ml of the supernatant was mixed with the liquefied sterilized medium. The medium was then distributed into 20 ml sterile test tubes (10 ml/tube). After solidification, the test fungi were inoculated (50μl of fungal spore suspension) and the cultures were incubated at 25 °C for 14 days. Extracellular phospholipase activity was detected by measuring the depth of precipitation zone below the fungal colony.

Protease production
Proteolytic activity of the fungal strains was detected using casein hydrolysis medium described by Paterson and Bridge (1994). The medium had the composition of (g/l): KH_2PO_4, 1.0; KCL, 0.5; $MgSO_47H_2O$, 0.2; $CaCI_2.2H_2O$, 0.1; glucose, 10, agar, 15, and distilled water to 1 L). After autoclaving at 121 °C for 20 min, 25 ml of 15% sterilized skimmed milk was added and the medium was distributed into 20 ml capacity test tubes (10 ml each). Each tube was inoculated by 50 μl of fungal spore suspension and incubated at 28 °C for 17 days. Proteolysis was observed as degradation of milk protein appeared as clear depth in the tube. The depth of clear zone below the fungal colony was measured (in mm) as indication of casein hydrolysis by fungi.

Chitinase production
The isolated fungal strains were screened for chitinase activity on chitin-agar medium (Jenin et al. 2016). The medium consisted of (g/l): $(NH4)_2 SO4$ (1 g), K_2HPO_4 (1 g), KCl (0.5 g), NaCl (5 g), $MgSO_4$ (0.5 g), $FeSO_4$ (0.01 g), agar agar (20 g), and colloidal chitin (5 g). A portion of isolated fungus inoculated on chitin agar tubes,

incubated at room temperature for 14 days. The chitinase activity was observed by the halo zone of clearances below the fungal colonies.

Quantitative estimation of chitinase activity

Five fungal strains, which showed a good chitinase activity, were selected for this part of study. These fungi were individually grown in 250 ml capacity flasks each contained 100 ml autoclaved liquid medium of the following composition (g/l): 0.5 g of NaCl, 0.5 g of CaCl2, 3 g of KH2PO4, 1 g of K2HPO4, 0.7 g of MgSO4.7H2O, 1.4 g of $(NH4)_2SO4$, and 5 g of colloidal chitin, pH was adjusted to 4.7. Culture filtrates were harvested after 14 days and the enzyme assay was performed. As described by Jha and Modi (2017) chitinase activity was determined by mixing 0.9 ml of 1% chitin, prepared in 50 mM Na-citrate buffer (PH 5.4) with 1 ml of filtered crude enzyme and the mixture was incubated at 50 ℃ for 5 min. The reaction mixture was stopped by addition of 2 ml of 3,5-dinitrosalicylic acid (DNSA) and the contents were boiled in a water bath for 10 min. The colored solution was centrifuged at 8000×*g* for 5 min at 4 ℃. The absorption of supernatant was measured at 540 nm spectrophotometrically. The reducing sugar was estimated from standard curve of glucose (T60 UV-Visible) spectrophotometer, PG Instruments). One unit of Chitinase, defined as the amount of enzyme that liberates 1 μmol of glucose equivalent per min under the previous assay conditions and chitinase concentration, was calculated as follows.

$$\text{Chitinase concentration} = \frac{\text{glucose concentration (g/l)}}{0.00018}\text{IU/l}$$

$$\text{Enzyme activity} = \text{Absorbance} \times \text{DF} \times (1/x)(1/y) \times (1/t) \times (1/\text{slope})$$

Where DF is the dilution factor for enzyme; x is the volume of enzyme used; y is the volume of hydrolysate used

Table 2 Enzymatic activities of isolated fungal species lipases, phospholipases, proteases, and chitinases expressed as depth of activity zone in mm

Fungal species	Lipases	Phospholipases	Proteases	Chitinases
Alternaria alternata	10	0	25	0
A. tenuissima	6	5	24	3
Aspergillus chevalieri	6	6	0	2
A. flavus	7	14	30	5
A. niger	20	22	20	5
A. rugulosus	15	4	24	2
A. sydowii	15	0	30	2
A. terreus	14	6	22	0
Botryotrichum atrogriseum	30	4	20	5
Cochliobolus spicifer	20	0	26	0
Epicoccum nigrum	7	11	22	2
Fusarium chlamydosporum	22	10	20	5
F. proliferatum	15	9	20	3
F. semitectum	6	10	29	5
F. solani	10	4	22	5
F. subglutinans	5	6	20	3
F. verticillioides	15	6	25	2
Mucor racemosus	0	22	20	0
Nigrospora oryzae	10	10	18	5
Penicillium aurantiogriseum	7	0	25	0
P. oxalicum	5	4	26	4
Pochonia chlamydosporia var. *catenulata*	10	20	20	5
Scopulariopsis brevicaulis	12	17	50	5
Talaromyces pinophilum	10	3	26	2
Toxicocladosporium irritans	10	0	25	3
Verticillium sp.	5	5	18	4

for assay of reducing sugars; t is the time of hydrolysis; slope is determined from a standard curve (R^2) (Moubasher et al. 2016).

Results

Incidence of fungal species on indigenous and invasive insect species

Twenty-six fungal species were isolated, identified, and used for evaluating their enzymatic activities. Results presented in Table 1 showed that the fungal highest incidence was for *Aspergillus niger* (37.7% of total insects) followed by *Alternaria tenuissima, Toxicocladosporium irritans, Fusarium proliferatum, Mucor racemosus Scopulariopsis brevicaulis, Fusarium chlamydosporum, Fusarium solani,* and *A. flavus* (14.1-22.4%). The remaining fungal species were less frequently isolated. The widest spectrum of fungal species was obtained from aphids (21 species) whereas the narrowest occurred on TLM (4 species). Invasive insects (RPW and PFF) produced 8 and 10 fungal species, respectively.

Enzymatic activities of fungal strains
Lipases
Data in Table 2 and Fig. 1 indicated that all the tested strains, except *Mucor racemosus* were able to produce lipolytic enzymes. The most active fungi were *B. atrogriseum, F.chlamydosporum, A.niger, C.spicifer,* and *F. proliferatum.*

Phospholipases
The most active fungal strains in a descending order were *A.niger > M. racemosus>P. chlamydosporia* var.

catenulata >S.brevicaulis >A. flavus as shown in Table 2 and Fig. 2. Fungal strains belonging to *Epicoccum, Fusarium,* and *Nigrospora* exhibited an intermediate phospholipase activity.

Proteases
With the exception of *A.chevalieri,* the tested fungal strains proved to be active producers of proteolytic enzymes. The best producers were *S. brevicaulis, A. flavus, A. sydowii, F. semitectum,* and *C.spicifer* (Table 2 and Fig. 3).

Chitinases
A total of 21 (80.8%) of the tested strains showed a positive activity (growth and clear zone) on the chitin-containing medium. Chitinolytic ability was generally low than other enzymatic activities (Table 2 and Fig. 4). The prominent fungal strains included *A. flavus, A. niger, B. atrogriseum, F. semitectum, F. solani, N. oryzae, P.chlamydosporia var. catenulata,* and *S. brevicaulis* (Table 3).

Discussion
In the present study, the common fungal species obtained from both indigenous and invasive insects were *A.niger, A. tenuissima, T. irritans, F. proliferatum, M.racemosus, S. brevicaulis, F. chlamydosporum, F. solani,* and *A. flavus* (14.1-37.7% of total insects). *Alternaria alternata* occurred a low incidence on aphids and PFF (10.6%). These results are more or less similar to previous reports from different countries. In Greece, Christias et al. (2001) isolated *Alternaria alternata* from dying and dead aphids. The fungus caused the most damage to the hemocytes but the adipose tissue and the gonads were also affected.
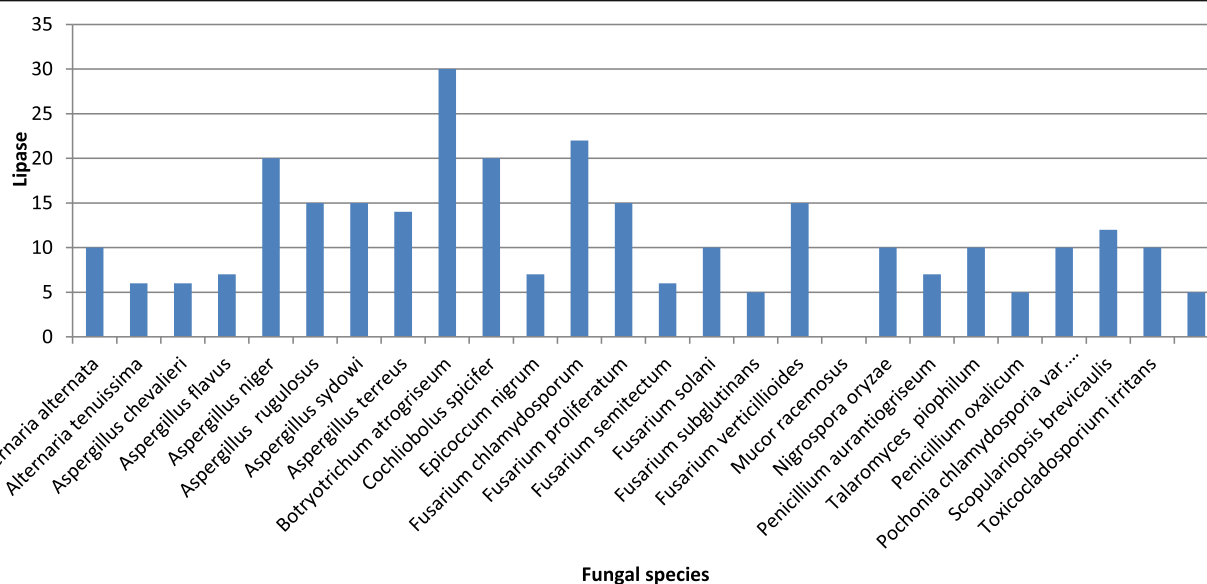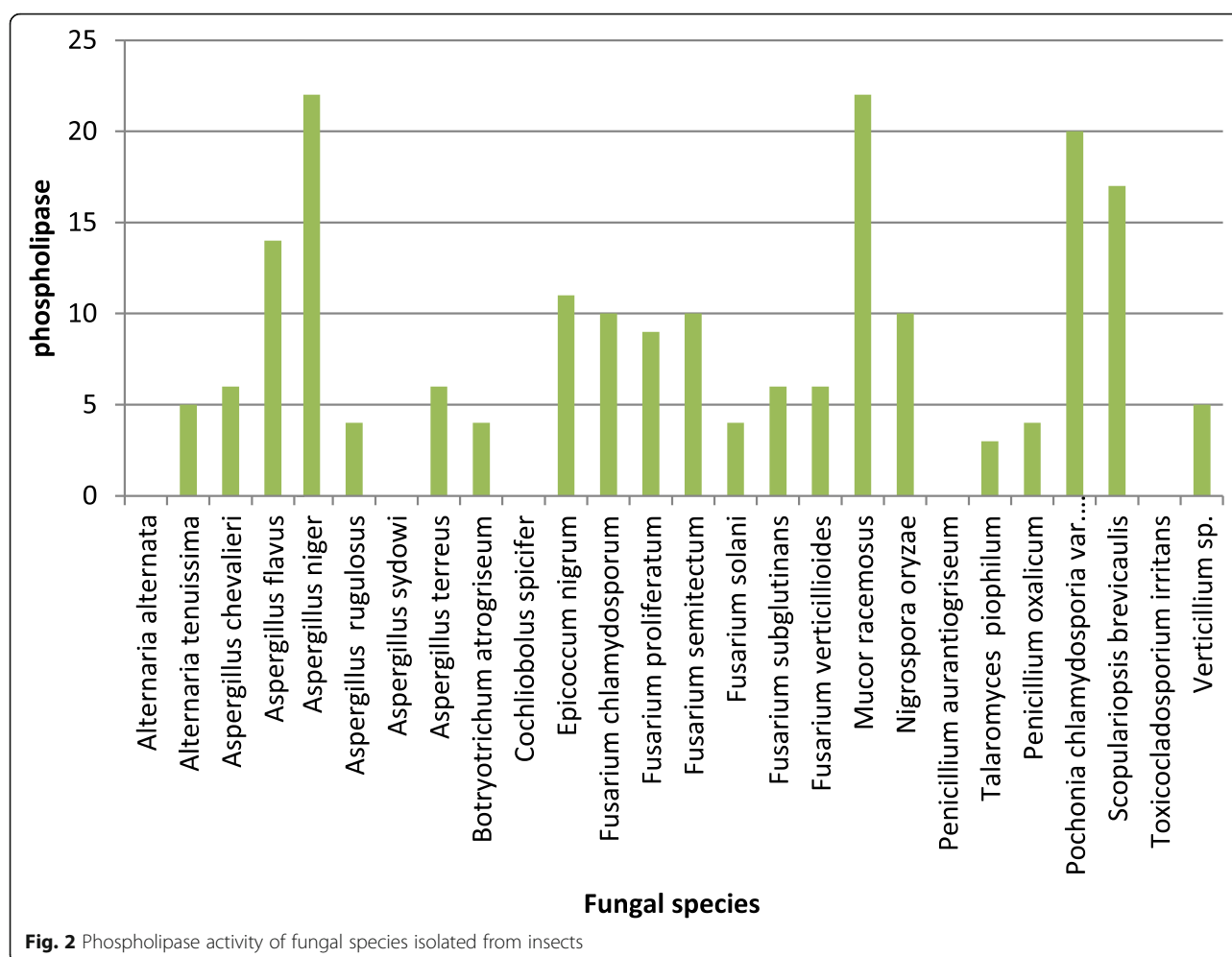

Fig. 1 Lipase activity of fungal species isolated from insects

Fig. 2 Phospholipase activity of fungal species isolated from insects

Six *Fusarium* species were isolated more frequently from aphids than from other insects. In India, Jayasimha et al. (2012) demonstrated that *F. semitectum* caused 79.90 and 64.40% mortality in nymphs and adults of okra aphid (*A. gossypii*), *respectively,* under laboratory and greenhouse conditions.

In Egypt, Abd El-Ghany et al. (2012) reported that *F. chlamydosporum* caused 58% mortality in the larval stage of *Galleria mellonella* L., showing the possibility of applying this fungal species as a biocontrol agent. More recently, Abdel Galil et al. (2019) found that *F. chlamydosporum, F. proliferatum, F. semitectum, F. solani, F. verticillioides, P. chlamydosporia* var. *catenulata, S. brevicaulis,* and *Verticillium* sp. exhibited high virulences against wheat and bean aphids causing 60-100% mortality of the tested aphids.

Pathogenic activity of EPF depends on the ability of degrading insect's cuticle components by active secretion of hydrolyzing enzymes including lipases, phospholipases, proteases, and chitinases (Abd El-Ghany et al. 2012). In the present work, most of the tested fungal strains were able to produce lipolytic enzyme with the most active

fungi being *B. atrogriseum, F. chlamydosporum, A. niger, C. spicifer,* and *F. proliferatum.* Lipases are responsible for the hydrolysis of ester bonds of lipoproteins, fats, and waxes found at the interior parts of the insect integument (Ali et al. 2009). Saleem (2008) tested the lipolytic activities of several fungal strains and found that *A. alternata, A. flaves, A. sydowi, A. terreus,* and *C. spicifer* were good producers of this enzyme.

Phospholipases are enzymes responsible for degrading phospholipids of insect's cuticle. Variation in phospholipases activity was observed among the 26 tested fungal strains. In the present study, the most active fungal strains were *A. niger, M. racemosus, P. chlamydosporia* var. *catenulata, S. brevicaulis,* and *A. flavus.* Fungal strains belonging to *Epicoccum, Fusarium,* and *Nigrospora* exhibited intermediate phospholipase activity. Ali et al. (2019) observed that clinical and environmental strains of *A. flavus, A. niger,* and *A. terreus* were active producers of esterase, phospholipase, and protease enzymes.

As shown from the obtained results, marked variations were observed in the proteolytic activities among the 26
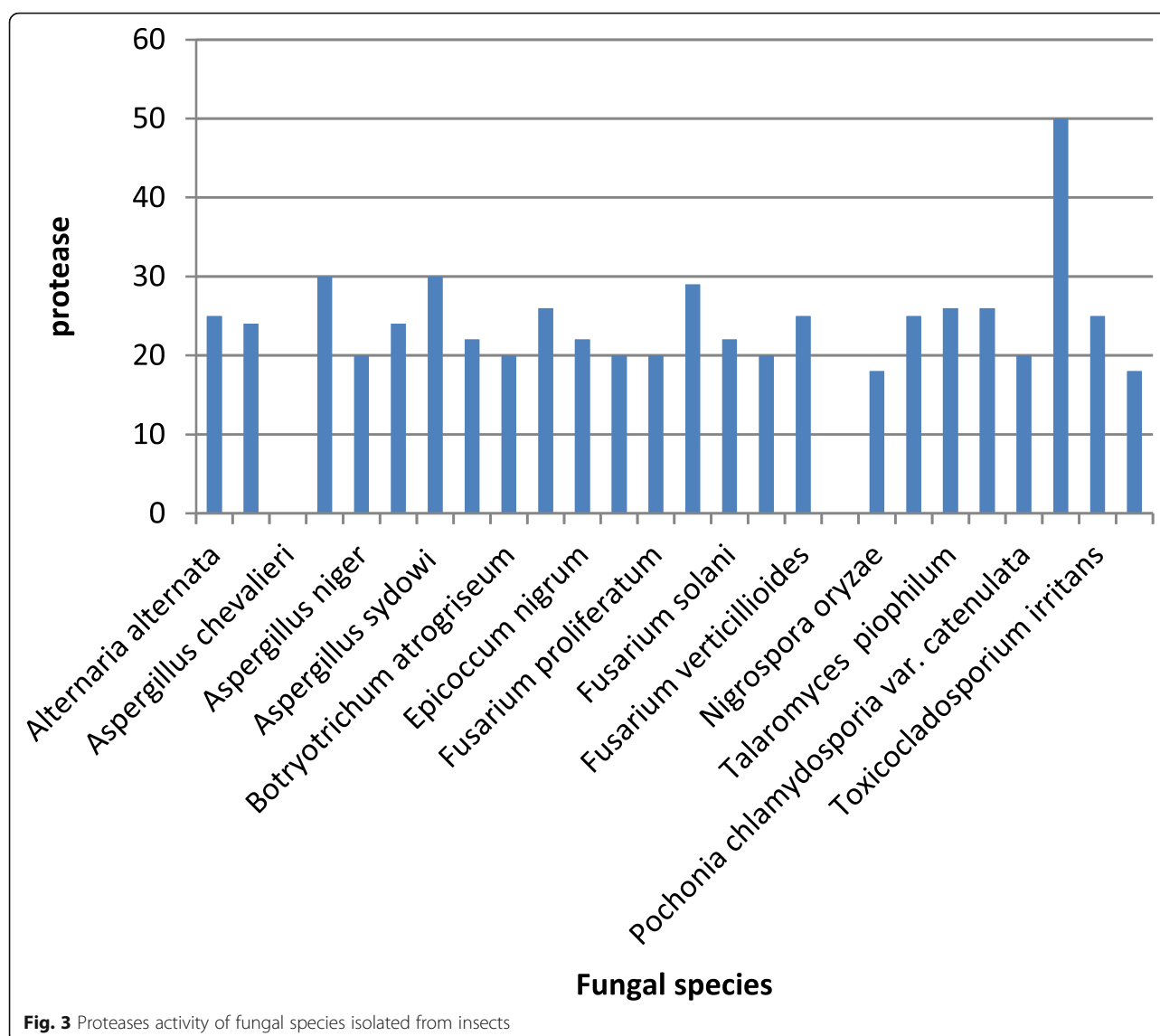
Fig. 3 Proteases activity of fungal species isolated from insects

tested fungal strains. The best producers were *S. brevicaulis, A. flavus, A. niger, A. sydowii, F. semitectum,* and *C. spicifer*. Shimizuy et al. (1993) emphasized that extracellular proteases were even found in insect hemolymphs. Working with *A. niger*, Milala et al. (2016) reported that the maximum proteolytic activity was achieved after 48 h of incubation at 40 °C, using casine as a substrate.

Chitinolytic abilities of the fungi tested in the present study were generally lower than other enzymatic activities. The prominent fungal strains included *A. flavus, A. niger, B. atrogriseum, F. semitectum, F. solani, N. oryzae, P. chlamydosporia var. catenulata,* and *S. brevicaulis*. These enzymes act synergistically with chitinases in the solubilization of the insect cuticle. Establishing a relationship between the production of hydrolytic enzymes and the virulence of EPF may be useful in developing of

screening methods for identifying new isolates with increased virulence and also for the development of bioproducts based on them (Montesinos-Matias et al. 2011). Chitinase was also produced by a halophilic strain of *A. flavus* isolated from Gulf of Suez, Egypt, with activity 620.54 U/l (Beltagy et al. 2018). Therefore, they suggested that the insecticidal effects of EPF could be directly linked with the activity of cuticle hydrolytic enzymes. Enzyme secretion by EPF may be involved in the degradation of cuticular polymers during pathogenesis, assisting in the penetration of the insect exoskeleton and providing nutrients for fungal growth. These enzymes can act synergistically, helping fungi to control insect pests and pathogens that attack productive crops, and offer potential economic benefit to agribusiness.
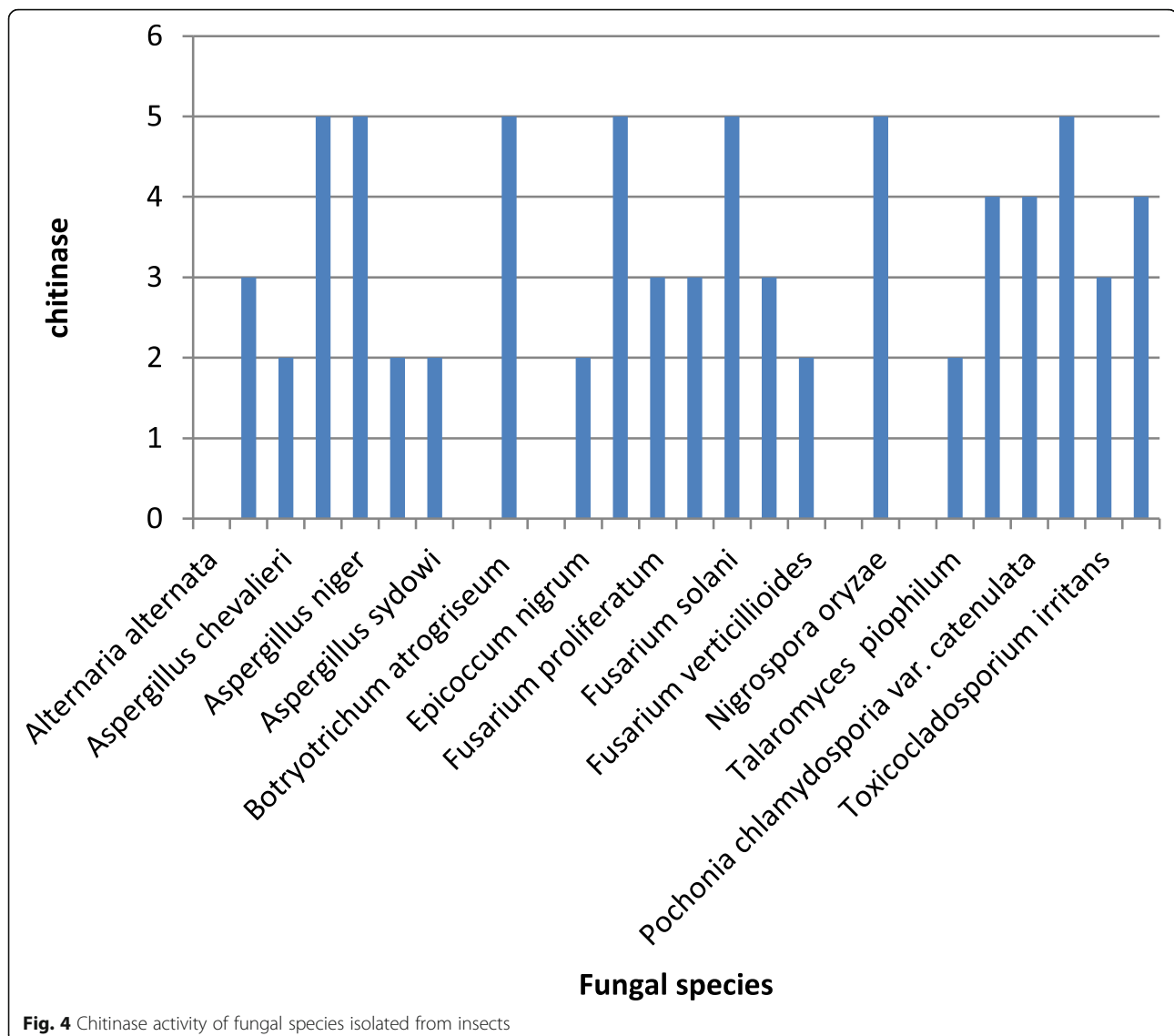
Fig. 4 Chitinase activity of fungal species isolated from insects

Conclusion

Aspergillus niger, B. atrogriseum, C. spicifer, F. chlamydosporum, and *F. proliferatum* were being the most active fungi for producing lipolytic enzymes. *Aspergillus flavus, A. niger, M. racemosus, P. chlamydosporia* var. *catenulata,* and *S. brevicaulis* were the highest producers of phospholipase. *Aspergillus chevalieri* and *M. racemosus* cannot produce protolytic enzymes. The best proteolytic strain was *S. brevicaulis* followed by *A. flavus, A. sydowii,* and *F. semitectum.* The chitinolytic ability of the fungal strains was generally weak and the relatively active species belonged to *A. flavus, A. niger, B. atrogriseum, F. chlamydosporum, F. solani, S. brevicaulis,* and *Nigrospora oryzae.*

Table 3 Concentration of chitinase produced by fungi isolated from insects

Fungal species	Glucose (g/l)	Chitinase conc. (IU/ml)	Chitinase activity (IU/ml/min)
Aspergillus niger	1.159	6.44	0.041
Fusarium semitectum	0.726	4.032	0.025
F. solani	0.980	5.443	0.034
Pochonia chlamydosporia var. *catenulata*	1.022	5.677	0.036
Scopulariopsis brevicaulis	0.626	3.478	0.022

Declarations

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