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Original Research Article

Cellulase activity of some phytopathogenic fungi isolated from diseased leaves of broad bean

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A B S T R A C T

Forty five fungal species and two varieties belonging to 23 genera were collected from 50 samples of broad bean diseased leaves collected from Qena governorate in Egypt on glucose-Czapek's, dichloran-chloramphenicol-malt extract and dichloran-chloramphenicol-peptone agar at 28°C. The most common genera were *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mycosphaerella* and *Penicillium*. The most prevalent species were *Alternaria alternata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Cladosporium cladosporioides*, *Fusarium merismoides*, *Mycosphaerella tassiana* and *Penicillium chrysogenum*. Among 8 dematiaceous hyphomycetes phytopathogenic fungi screened for their abilities to produce both exo- and endo-β-1,4-glucanase enzymes (C_1 and C_x), 5 species were high C_1 activity corresponding 2 species for C_x enzyme. However 2 and 3 species were moderate activity for C_1 and C_x enzymes respectively. The remaining species were low activity in both C_1 and C_x enzymes. The highest cellulase activity was recorded by *Alternaria citri* and *Cochliobolus spicifer* for C_1 and by *Alternaria alternata* and *A. citri* for C_x enzyme. Maximum production of C_1 enzyme by *A. citri* and *C. spicifer* were obtained after 6 days of incubation at 30°C with initial pH 6 in culture medium containing lactose and calcium nitrate as carbon and nitrogen sources respectively. For C_x enzyme the highest enzyme production by *A. alternata* and *A. citri* were recorded after 8 days of incubation at 30°C with initial pH 6 in culture medium containing carboxymethyl cellulose and sodium or calcium nitrate as carbon and nitrogen sources respectively.

Keywords

Leaf-surface fungi; broad bean; Cellulase activity.

Introduction

Broad bean (*Vicia faba* L.) is one of the most important leguminous crops grown in winter season in different types of Egyptian soils. Also it is considered as the basic source of protein for human and animal consumption (Ibrahim et al. 2007 and Abou El-Yazied and Mady 2012). It is an excellent source of protein (20-25%),

complex carbohydrates, calcium, phosphorus, lysine, methionine-cystine, dietary fiber, choline, lecithin and minerals. Therefore, broad bean has significant importance for human and animal foods (Rabey et al. 1992 and Bulletin 2004). This strategic crop is suffering from many destructive diseases. It is attacked by more than 100 pathogens in the Mediterranean region (Gaunt 1983). Several species cause a range of plant diseases, such as leaf spot, vascular wilt, root and stem rot, seedling blight, cereal ear rot and fruit rot (Logrieco et al. 2003, Senthil et al. 2010 and Saleem et al. 2012a). Diseases are the most important limiting factors which cause great annual losses and sometimes complete crop failures (Hanounik and Bisri 1991). Broad bean is adversely affected by numerous fungal diseases leading to a steady reduction in the cultivated area in many countries (Torres et al. 2006). Leaf spot is foliar disease caused by *Alternaria*. The disease spread by conidiospores, is common and serious in regions with heavy rainfall, high humidity and temperatures (Peralta et al. 2005). In Egypt, leaf spot of broad bean is caused by *Alternaria alternata* (Mahmoud 1985 and Saleem et al. 2012a), whereas in Japan a new leaf spot disease of broad bean is caused by *Alternaria tenuissima* (Rahman *et al*. 2002). El-Said et al. (2006) observed that *Alternaria*, *Aspergillus* and *Cladosporium* were the most common fungal genera on leaf surface of broad bean cultivated in Upper Egypt. From the above genera the most common species were *Alternaria alternata, A. petroselini, A. citri*, *Aspergillus niger, A. flavus* and *Cladosporium cladosporioides*. Kayini and Pandey (2010) isolated 38 epiphytic and endophytic phyllosphere fungi from living leaves of *Alnus nepalensis*, *Castanopsis hystrix* and *Schima walichii* in a subtropical forest of north east India. *Alternaria alternata, Cladosporium cladosporioides*, *Fusarium oxysporum* and *Pestalotiopsis* sp. were the dominant colonizers of three forest tree leaves. In many parts of the world numerous investigations have been carried out on leaf surface fungi of several plants (Lee and Hyde 2002, Osono 2002, 2006, Akimitsu et al. 2003, El-Naggar and Abdel-Hafez 2003, Sadaka and Ponge 2003, Gunasekera 2004, Hemida 2004, Murace and Cellitin 2005, Rekosz-Burlaga and Garbolinska 2006, Osono and Taked 2007, Evueh and Ogbebor 2008, Osono and Hirose 2009 and Mukhtar et al. 2010a,b).

Hydrolytic enzymes play an important role in the pathogenicity of plants by facilitating fungal penetration through the host cell wall (Wanjiru et al. 2002). The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Han et al. 2003 and Keegstra 2010). Cellulose consists mainly of long polymers of β 1-4, linked glucose units (Somerville 2006). Cellulase enzymes complex are consists of three major enzymes components which are endo-β-(1-4)-D-glucanase, exo-β-(1-4)-D-glucanase and β-glucosidase that works synergically in complex cellulose degradation (Saha 2004 and Kim et al. 2008). Generally, fungi produce three major types of cellulolytic enzymes: endoglucanase, exoglucanase and cellobiase (Klyosov 1990). These enzymes are extracellular and inductive in nature (Enari 1983). The ability to produce cellulases are widespread among fungi and became the subject of extensive investigation (Moharram et al. 2004, Baig 2005, El-Said et al. 2005, Narasimha et al. 2006, Fang et al. 2008, Shanmugam et al.

2008, Abu Bakar et al. 2010, Gautam et al. 2010, Sherief et al. 2010, Amir Ijaz et al. 2011, Juwaied et al. 2011, Sarkar et al. 2011, Qurat-ul-Ain et al. 2012 and Saleem et al. 2012b). Ahmed et al. (2009) observed that the optimum conditions for maximum production of cellulase by *Trichoderma harzianum* were 120 hours of incubation at 28°C and 150 rpm with CMC as carbon source and culture initially adjusted to pH 5.5. Ja'afaru and Fagade (2010) studied cellulase activity in *Aspergillus niger* YL128. *A. niger* produced large amounts of cellulase enzyme when grown in culture initially adjusted to pH 5, with sugarcane bagasse as carbon source and ammonium dihydrogen phosphate or ammonium sulphate as nitrogen source at 9 days after incubation at 30°C. This article aimed to study the potential of some facultative phytopathogenic fungi to produce cellulase enzymes which consider one of the most important degrading enzymes in plant pathogenicity as well as different environmental and nutritional factors affecting secretion of cellulases.

Materials and Methods

Collection of broad bean leaf samples

Fifty infected leaves samples of broad bean (*Vicia faba* L.) were collected from different fields in Qena governorate, in Upper Egypt (Fig. 1). Each sample was put in a sterile polyethylene bag and transferred to mycological laboratory for fungal analysis.

Mycological analysis of broad bean leaves

Determination of phyllosphere fungi

Known weight of infected leaves of broad bean was placed in sterile conical flasks containing 100 ml sterile distilled water. Flasks were shaken by hand in a rotating motion for 10 minutes. Ten ml of the suspension were transferred into another flask (250 ml) containing 90 ml sterile distilled water, then the flask was shaken for five minutes. One ml of the final dilution was transferred to each sterile Petri-dish followed by addition of about 15 ml liquefied agar medium. Three media were used, twelve plates were used for each sample (4 plates for each type of medium). The plates were incubated at 28°C for 7 days and the developing fungi were identified and counted. The numbers of colonies were calculated per g fresh weight of leaves.

Determination of leaf surface (phylloplane) fungi

Infected leaves of broad bean were cut into small pieces across lesions (about 1 cm^2 each), subjected to a series of washing with sterile distilled water. They were thoroughly dried between sterilized filter papers. Four segments were placed on the surface of the agar medium in each plate. Three medium were used. Four replicates were prepared for each sample as well as for each type of medium. Cultures were incubated at 28°C for 7 days, and the developing fungi were identified and counted. The numbers of colonies were calculated per 16 leaf segments.

Composition of media used for isolation of fungi

Glucose-Czapek's agar medium

Glucose-Czapek's agar medium (g/L; sodium nitrate, 3.0; potassium dihydrogen phosphate, 1.0; magnesium sulphate, 0.5; potassium chloride, 0.5; ferrous sulphate,

0.01; glucose, 10.0 and agar 15.0) was used for isolation of glucophilic fungi. Rose bengal (0.1 mg/ml) and chloramphenicol (0.5 mg/ml) were used as bacteriostatic agents (Smith and Dawson 1944 and Al-Doory 1980).

Dichloran-chloramphenicol-malt extract agar (DCMA) medium

The medium consists of (g/L; malt extract, 10.0; chloramphenicol, 0.1; dichloran, 0.2 (0.2% solution in 1 ml ethanol); agar 15.0).

Dichloran-chloramphenicol-peptone agar (DCPA) medium

The medium consists of (g/L; peptone, 15.0; potassium dihydrogen phosphate, 1.0; magnesium sulphate, 0.5; chloramphenicol, 0.29; dichloran, 0.2 (0.2% solution in 1 ml ethanol); agar, 15.0).

Cellulase activity of some phytopathogenic fungi

Screening of fungi for cellulase activity

Eight facultative phytopathogenic fungi (related to 5 genera) of Dematiaceous hyphomycetes isolated from diseased leaves of broad bean were screened for their abilities to produce exo- and endo-β-1,4-glucanase enzymes on solid medium. Fungi were cultured on Eggins and Pugh (1962) medium with the following composition (g/L): $(NH4)_2SO_4$, 0.5; L-asparagine, 0.5; KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.2; Yeast extract, 0.5; cellulose, 10; agar, 15. pH was adjusted to 5.4 using acetate buffer (0.2 N acetic acid, 30 ml and 0.2 N sodium acetate, 170 ml). Cultures were incubated at 28°C for 7 days. Using a sterile cork borer, 10 mm diameter discs were cut to inoculate 50 ml sterile liquid medium (in 250 ml Erlenmeyer conical flasks) of Eggins and Pugh medium (1962) for exo-β-1,4-glucanase (C_1) production and Prasad and Verma (1979) medium for endo-β-1,4- glucanase (C_x). The latter medium contained the following ingredients (g/L): NH_4NO_3, 2.1; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; Carboxymethyl cellulose (CMC), 10.0. After 7 days of incubation at 28°C, the cultures were filtered and the filtrates were used to detect the activity of cellulase enzymes.

Assay for exo-β-1,4-glucanase

Using a sterile cork borer, 3 cavities (10 mm diameter) were made in plates containing solid Eggins and Pugh medium. 0.1 ml of culture filtrate was dropped in each of these cavities followed by incubation at 28°C for 24 hours, then the plates were flooded with chloro-iodide of zinc solution and the clear zones around cavities were measured.

Assay for endo-β-1,4-glucanase

Cavities (10 mm diameter) were made in plates containing solid medium of Dingle et al. (1953) of the following composition (g/L): carboxymethyl cellulose (CMC), 10; agar, 15; pH was adjusted to 5.4. 0.1 ml filtrate obtained from fungal cultures grown on Prasad and Verma medium was dropped in each cavity. After 24 hours of incubation at 28°C, plates were flooded with chloroiodide of zinc solution and the clear zones around cavities were measured.

Factors affecting cellulase production
Cultivation of fungi

The effect of different ecological and

nutritional factors on production of cellulase enzymes by *Alternaria citri* and *Cochliobolus spicifer* (for C_1) and *Alternaria alternata* and *A. citri* (for C_x) were studied; since these species were found to be highly active in cellulase production.

The previous fungal isolates were grown on medium containing (g/L); $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; $FeCl_3$, 1.0 mg; $ZnSO_4.7H_2O$, 10.0 mg; $MnSO_4.H_2O$, 0.4 mg; thiamine, 100 mg; biotin, 10 mg; and cellulose powder 10 g (Deacon 1985). Fifty ml of medium were dispensed into each 250 ml Erlenmeyer flask and each was inoculated with 2 agar mycelial discs (10 mm diameter) of the mould obtained from 7 days old cultures growing on solid basal medium.

Effect of incubation periods

Alternaria alternata, A. citri and *Cochliobolus spicifer* were grown on the basal medium of Deacon (1985) with cellulose or CMC as inductive substrates. The inoculated flasks were incubated at 28°C for 14 days and harvested at 48 hours intervals. Culture fluids were filtered and centrifuged at 5000 rpm for 10 minutes. The clear supernatants were assayed for enzyme activity.

Effect of temperature

The inoculated flasks were incubated at 15, 20, 25, 30, 35 and 40°C for 6 days for C_1 and 8 days for C_x enzyme. At the end of the incubation period, the cultures were filtered and centrifuged at 5000 rpm for 10 minutes and the clear supernatants were assayed for enzyme activity.

Effect of pH value

Alternaria alternata, A. citri and *Cochliobolus spicifer* were grown on the basal medium of Deacon (1985). The initial pH of the medium was adjusted by 0.1 N NaOH or 0.1 N HCl to different values ranging from 2 to 12.

After inoculation with moulds, cultures were incubated at 30°C for 6 days for C_1 and 8 days for C_x enzyme. At the end of the incubation period, cultures were filtered and centrifuged at 5000 rpm for 10 minutes. The clear supernatants were assayed for cellulase activity.

Effect of different carbon sources

The basal medium of Deacon (1985) with pH 6 was supplemented with 1% of one of the following carbon sources: CMC, cellulose powder, starch, glucose, fructose, maltose and lactose. The inoculated flasks were incubated at 30°C for 6 and 8 days for C_1 and C_x enzymes respectively. After the incubation period, cultures were filtered and centrifuged at 5000 rpm for 10 minutes. The clear filtrates were used to detect cellulase activity.

Effect of different nitrogen sources

To determine the effect of nitrogen source on cellulase, sodium nitrate (5 g/l) in the basal medium were replaced by the same amount of various nitrogen compounds such as: Ammonium sulphate, ammonium nitrate, calcium nitrate, magnesium nitrate, potassium nitrate, sodium nitrite, peptone and yeast extract in addition to sodium nitrate as a control.

Cultures were incubated at 30°C for 6 days (for C_1) and 8 days (for C_x). Cultures were filtered, centrifuged at 5000 rpm for 10 minutes and the clear filtrates were used for detection of cellulase activity.

Assay for cellulase activity (C_1 and C_x enzymes)

The method described by Nelson (1944) and modified by Naguib (1964) was employed as follows: One ml of each 1% cellulose powder (for C_1-cellulase) or CMC (for C_X-cellulase) was added separately to 1 ml of acetate buffer (pH=6) and 1 ml of each culture filtrate. The mixtures were incubated for 30 minutes at 28°C. Similar reaction mixtures using distilled water with reagents were used as a blank. 3 ml of Nelson's solution were added and the reaction mixtures were shaken and placed in a boiling water bath for 15 minutes. After cooling, 3 ml of the arsenomolybdate solution was added, mixed and diluted to l0 ml with distilled water. The mixture was centrifuged to remove any turbidity. The amount of reducing sugars produced was estimated by determining the optical density (absorption spectrum) at 700 nm wave length with a spectrophotometer (Spectronic ® GeneSys ™ 2 PC). A standard curve was plotted using aqueous solutions of D-glucose with concentrations from 10-100 μg/ml.

Statistical analysis of results

Statistical analysis of data was carried out by one way analysis of variance and the means were separated by Tukey's honest significant difference test using Biostat 2008 statistical analysis program (Copyright © 2001-2009 Analystsoft).

Results and Discussion

Leaf surface fungi of broad bean

Forty five species and two varieties belonging to 23 genera were collected from phyllosphere (21 genera and 42 species +1 variety) and phylloplane (20 genera and 35 species+1 variety) of broad bean diseased leaves on plates of glucose-Czapek's, dichloran-chloramphenicol-malt extract and dichloran-chloramphenicol-peptone agar at 28°C. The most prevalent genera on the three types of media were *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mycosphaerella* and *Penicillium*. From the above genera the most common species were *Alternaria alternata, Aspergillus flavus, A. fumigatus, A. niger, Cladosporium cladosporioides, Fusarium merismoides, Mycosphaerella tassiana* and *Penicillium chrysogenum* (Tables 1,2). These fungal genera and species were isolated with different numbers and frequencies from leaf surface of several plants in many parts of the world including Egypt (Lee and Hyde 2002; Osono 2002, 2006; Akimitsu et al. 2003; El-Naggar and Abdel-Hafez 2003; Sadaka and Ponge 2003; Gunasekera 2004; Hemida 2004; Murace and Cellitin 2005; Rekosz-Burlaga and Garbolinska 2006; Osono and Taked 2007; Evueh and Ogbebor 2008; and Mukhtar et al. 2010b). El-Said et al. (2006) reported that *Alternaria alternata*, *A. petroselini*, *A. citri*, *Aspergillus niger*, *A. flavus* and *Cladosporium cladosporioides* were the most common species on the leaf surface of broad bean cultivated in Upper Egypt.

Recently, Kayini and Pandey (2010) isolated 38 epiphytic and endophytic phyllosphere fungi from living leaves of *Alnus nepalensis*, *Castanopsis hystrix* and *Schima walichii* in a subtropical forest of north east India. *Alternaria alternata*, *Cladosporium cladosporioides*, *Fusarium oxysporum* and *Pestalotiopsis* sp. were the dominant colonizers of three forest tree leaves. Mukhtar et al. (2010a) reported

that 46 fungi were isolated as endo- and epiphytic fungal isolates from phyllosphere of four weeds. From these fungi *Alternaria*, *Aspergillus*, *Drechslera*, *Penicillium* and *Cladosporium* were common.

Cellulase activity

Screening of fungi for their abilities to produce cellulase enzymes

Screening of eight phytopathogenic fungi of Dematiaceous hyphomycetes isolated from diseased leaves of broad bean for their abilities to produce both exo- and endo-β-1,4-glucanase enzymes on solid medium proved that all fungi able to utilize cellulose, but with various degrees. *Alternaria citri* showed high cellulolytic activity in both exo- and endo-β-1,4-glucanases. Four fungal species showed high activity in production of exo-β-1,4-glucanase enzyme only and these were *Alternaria raphani*, *A. tenuissima*, *Cochliobolus spicifer* and *Stachybotrys atra* var. *microspora*. *Alternaria alternata* exhibited the highest activity in production of endo-β-1,4-glucanase enzyme.

However *Alternaria alternata* and *Ulocladium botrytis* exhibited moderate activity for exo-β-1,4-glucanase enzyme and *Alternaria raphani*, *Cochliobolus spicifer* and *Stachybotrys atra* var. *microspora* were found to be moderate activity for endo-β-1,4-glucanase enzyme. The remaining species were weak producers of exo- and endo-β-1,4-glucanase enzymes (Table 3). It is evident in literatures that different fungal genera and species exhibited variable capabilities in the degradation of cellulosic materials (Moharram et al. 2004, Baig 2005, El-Said et al. 2005, Gautam et al. 2010, Sherief et al. 2010, Amir Ijaz et al. 2011, Juwaied et al. 2011 and Saleem et al. 2012b). El-Said (2001) found that all fungal isolates recovered from banana leaves and screened for their abilities to produce exo- and endo-β-1,4-glucanase enzymes proved to be active to utilize cellulose with different degrees. Eight isolates showed high cellulolytic activity in both exo- and endo-β-1,4-glucanase and these were *Acremonium strictum*, *Chaetomium globosum*, *Gibberella fujikuroi*, *G. zeae*, *Nectria haematococca*, *Setosphaeria rostrata*, *Stachybotrys chartarum* and *Trichoderma pseudokoningii*. Recently, Saleem et al. (2012b) found that among forty four fungal isolates recovered from broad bean and screened for exo- and endo-β-1,4-glucanase enzymes, six isolates showed high cellulase activity for exo-β-1,4-glucanase enzyme while five isolates were high activity for endo-β-1,4-glucanase enzyme. However twenty one isolates were found to be moderate cellulase activity for both exo- and endo-β-1,4-glucanase enzymes. The remaining isolates were low producers of cellulose

Effect of environmental and nutritional parameters on cellulase production

Alternaria citri and *Cochliobolus spicifer* were in the top of phytopathogenic fungi in producing exo-β-1,4-glucanase, while *Alternaria alternata* and *A. citri* were the most active fungi in producing endo-β-1,4-glucanase. So they were chosen for detection of the most suitable environmental and nutritional conditions for production of cellulases.

Effect of incubation periods

Maximum production of exo- and endo-β-1,4-glucanases by tested fungi were recorded after 6 and 8 days of incubation respectively. The other incubation periods exhibited lower amounts of cellulase enzymes (Figs. 2,4).

Fig.1 Leaves of broad bean infected with phytopathogenic fungi in the field

Fig. 2 Effect of incubation period, temperature and pH value on exo-β-1,4-glucanase

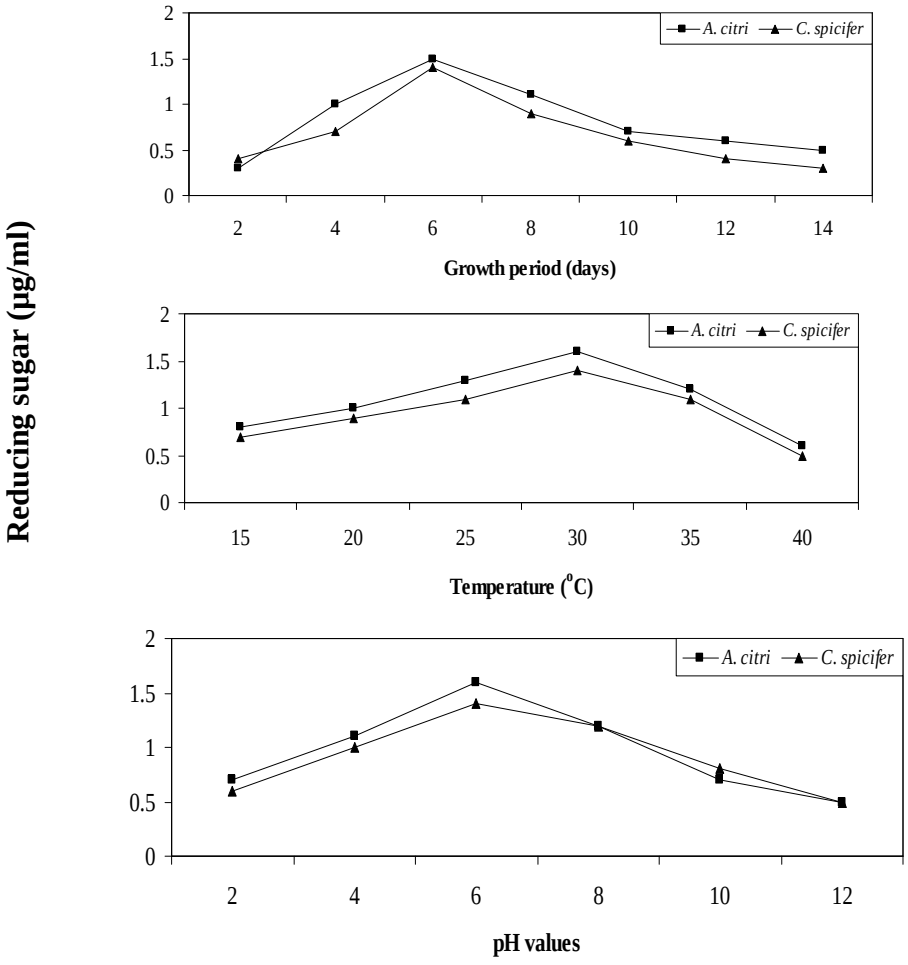

Fig.3 Effect of carbon and nitrogen sources on exo-β-1,4-glucanase production by *Alternaria citri* and *Cochliobolus spicifer*.

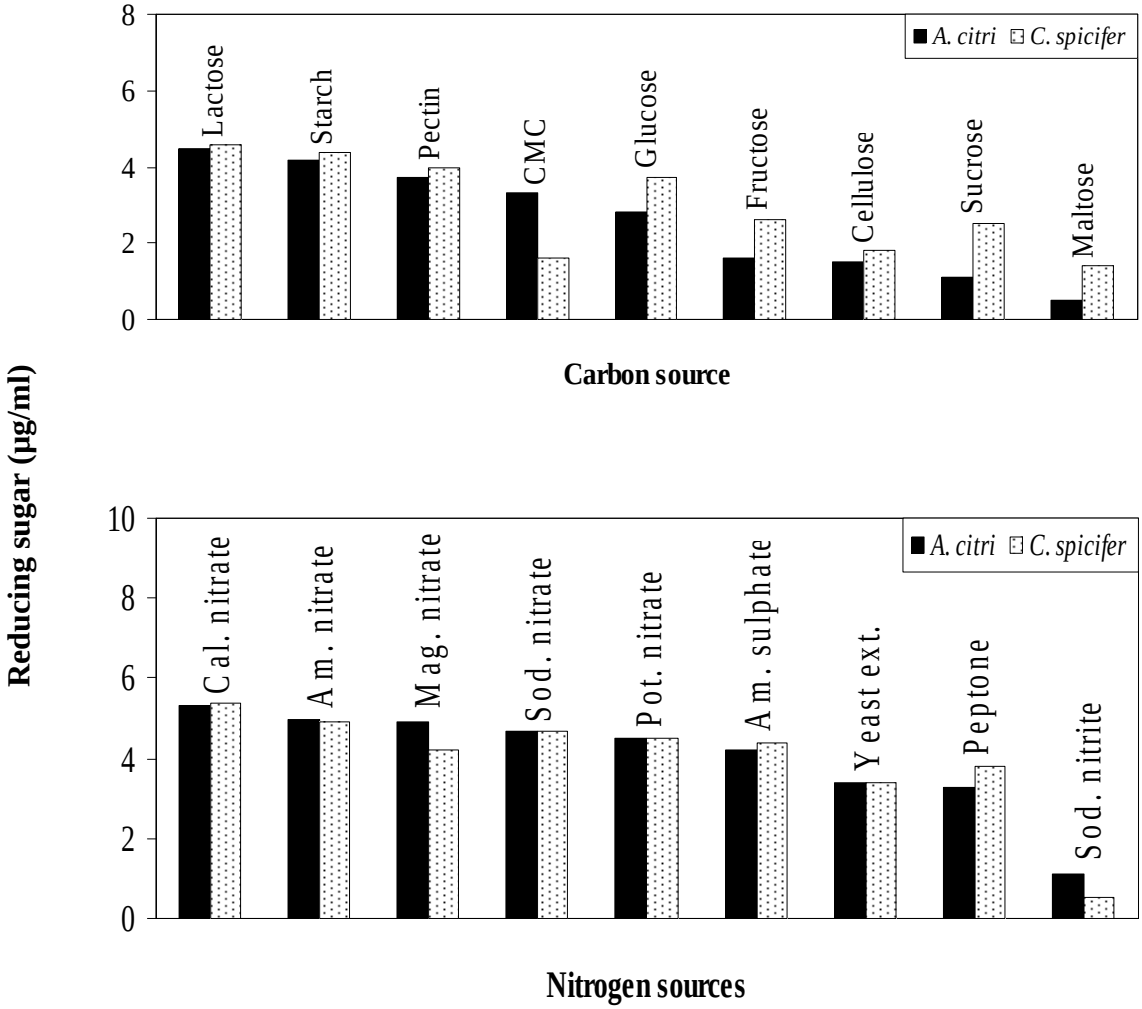

Fig. 4 Effect of incubation periods, temperature and pH value on endo-β-1,4-glucanase production by *Alternaria alternata and A. citri*

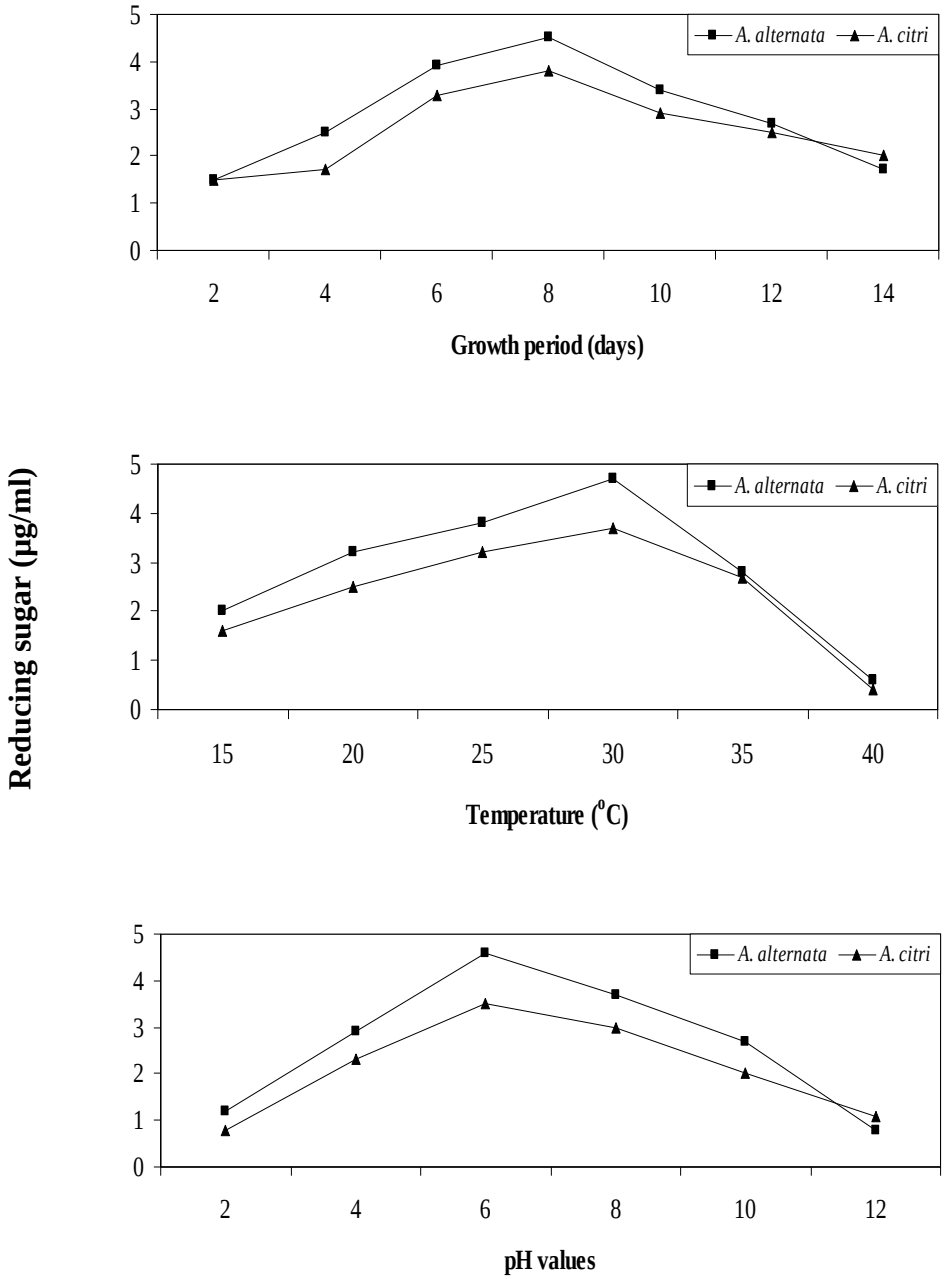

Table.1 Average total counts (calculated per g fresh weight leaves in every sample), number of cases of isolation (NCI, out of 50) and occurrence remarks (OR) of fungal genera and species recovered from phyllosphere of *Vicia faba* plant on glucose-Capek's, dichloran-chloramphenicol-malt extract (DCMA) and dichloran-chloramphenicol-peptone agar (DCPA) at 28°C.

Genera & species	Glucose-Czapeks		DCMA		DCPA	
	ATC±SD	NCI &OR	ATC±SD	NCI &OR	ATC±SD	NCI &OR
Acremonium strictum	18900±543	6 L	—	—	15862.5±141	4 R
Alternaria	46237.5±249	43 H	51975±672	46 H	52312.5±1065	45 H
A. alternata	41512.5±597	43 H	46575±613	44 H	4900±1208	44 H
A. citri	1687.5±192	6 L	1125±50	5 R	—	—
A. raphani	3037.5±360	7 L	2250±379	6 L	1350±92	5 R
A. tenuissima	—	—	2025±145	6 L	1462.5±192	4 R
Aspergillus	100575±3522	43 H	24525±555	24 M	53437.5±925	42 H
A. flavus	24075±1029	37 H	2025±215	5 R	29025±616	30 H
A. fumigatus	23850±1113	25 H	4050±92	5 R	15637.5±878	17 M
A. giganteus	13162.5±604	11 L	3150±159	2 R	3037.5±296	4 R
A. melleus	1012.5±141	4 R	—	—	—	—
A. niger	33637.5±781	31 H	14512.5±249	18 M	7312.5±360	12 L
A. parasiticus	—	—	—	—	337.5±108	2 R
A. terreus	2587.5±232	6 L	—	—	—	—
A. ustus	—	—	—	—	1462.5±108	8 L
A. versicolor	2250±243	4 R	787.5±232	3 R	1575±112	5 R
Cladosporium	84037.5± 348	39 H	102825±1045	41 H	53437.5±1468	32 H
C. cladosporioides	59962.5±834	31 H	73237.5±1370	32 H	34762.5±1287	21 M
C. sphaerospermum	24075±584	12 L	29587.5±818	13 M	18675±455	11 L
Cochliobolus spicifer	5400±159	9 L	13162.5±612	12 L	6300±205	5 R
Cunninghamella echinulata	—	—	—	—	225±65	1 R
Curvularia lunata	900±159	4 R	787.5±232	3 R	450±130	1 R
Drechslera papendorfii	—	—	450±130	2 R	—	—
Emericella nidulans	1575±145	6 L	—	—	450±92	1 R
Epicoccum purpurascens	1237.5±249	4 R	8550±290	20 M	1012.5±56	3 R
Fusarium	4500±400	16 M	900±225	3 R	13387.5±383	24 M
F. dimerum	—	—	—	—	337.5±108	3 R
F. merismoides	2362.5±108	9 L	900±225	3 R	10800±318	19 M
F. oxysporum	2137.5±360	8 L	—	—	2250±205	6 L
Gibberella fujikuroi	—	—	—	—	337.5±108	2 R
Mucor hiemalis	6862.5±310	16 M	675±215	2 R	—	—
Mycosphaerella tassiana	13725±1041	14 M	29137.5±538	15 M	1687.5±108	3 R
Penicillium	15862.5±514	24 M	1012.5±195	3 R	3600±275	9 L
P. aurantiogriseum	1350±59	2 R	—	—	—	—
P. brevi-compactum	3600±130	8 L	—	—	—	—
P. chrysogenum	5175±268	7 L	675±195	2 R	1912.5±56	5 R
P. citrinum	900±379	2 R	—	—	—	—
P. corylophilum	3600±477	4 R	337.5±108	1 R	1575±215	4 R
P. funiculosum	1237.5±249	4 R	—	—	—	—
P. steckii	—	—	—	—	112.5±56	1 R
Scopulariopsis brevicaulis	—	—	—	—	3037.5±108	12 L
Setosphaeria rostrata	562.5±141	4 R	675±65	4 R	337.5±108	2 R
Stachybotrys	1800±205	5 R	2137.5±192	6 L	—	—
S. atra var. *microspora*	—	—	225±112	1 R	—	—
S. parvispora	1800±205	5 R	1912.5±213	5 R	—	—
Sterile mycelia	1125±65	3 R	1125±165	5 R	787.5±108	4 R
Trichoderma hamatum	—	—	787.5±141	2 R	—	—
Trichothecium roseum	—	—	—	—	225±65	2 R
Ulocladium botrytis	900±92	4 R	337.5±56	3 R	—	—
Average total count	304425		239062.5		211837.5	
Number of genera (21)	15		16		16	
Number of species (42+1 variety)	31		24+ 1 variety		29	

Table.2 Total counts (calculated per 48 segments), number of cases of isolation (NCI, out of 50), occurrence remark (OR) and relative importance values (RIV) of fungal genera and species recovered from phylloplane of *Vicia faba* on glucose-Capek's, dichloran-chloramphenicol-malt extract (DCMA) and dichloran-chloramphenicol-peptone agar (DCPA) at 28°C.

Genera & Species	Glucose-Czapeks			DCMA			DCPA		
	TC	NCI & OR	RIV	TC	NCI & OR	RIV	TC	NCI & OR	RIV
Acremonium strictum	12	2 R	4.9	—	—	—	50	5 R	14.8
Alternaria	574	49 H	143.4	596	46 H	144.8	575	49 H	153.2
A. alternata	528	48 H	137.7	556	46 H	141.3	552	49 H	150.9
A. citri	11	6 L	12.8	17	7 L	15.5	—	—	—
A. raphani	18	9 L	19.4	12	7 L	15.0	7	5 R	10.6
A. tenuissima	17	9 L	19.3	11	6 L	12.9	16	9 L	19.5
Aspergillus	325	44 H	113.7	78	28 H	62.9	155	37 H	88.8
A. flavus	155	33 H	78.2	28	12 L	26.5	61	17 M	39.8
A. flavus var. *columnaris*	—	—	—	—	—	—	50	12 L	28.8
A. fumigatus	44	12 L	27.4	15	7 L	15.3	14	8 L	17.3
A. giganteus	57	11 L	26.5	—	—	—	13	4 R	9.2
A. niger	69	21 M	47.4	35	12 L	27.1	17	10 L	21.6
Chaetomium globosum	1	1 R	2.0	3	1 R	2.2	—	—	—
Cladosporium	59	15 M	34.6	83	24 M	55.3	17	7 L	15.6
C. cladosporioides	42	10 L	23.3	49	15 M	34.3	9	4 R	8.8
C. sphaerospermum	17	5 R	11.3	34	11 L	25.0	8	4 R	8.7
Cochliobolus spicifer	70	8 L	21.5	152	21 M	55.5	—	—	—
Curvularia lunata	6	3 R	6.4	11	7 L	14.9	—	—	—
Epicoccum purpurascens	22	7 L	15.7	132	22 M	55.7	5	2 R	4.4
Fusarium	32	15 M	32.5	3	2 R	4.2	184	39 H	95.6
F. dimerum	—	—	—	—	—	—	17	5 R	11.6
F. merismoides	27	13 M	28.1	3	2 R	4.2	135	31 H	74.9
F. oxysporum	5	3 R	6.4	—	—	—	32	9 L	21.0
Gibberella fujikuroi	10	4 R	8.8	—	—	—	11	6 L	13.0
Mucor	66	24 M	53.2	—	—	—	—	—	—
M. circinelloides	6	2 R	4.4	—	—	—	—	—	—
M. hiemalis	60	22 M	48.7	—	—	—	—	—	—
Mycosphaerella tassiana	—	—	—	43	13 M	29.8	—	—	—
Myrothecium verrucaria	2	1 R	2.1	—	—	—	—	—	—
Penicillium	54	13 M	30.7	—	—	—	—	—	—
P. chrysogenum	11	4 R	8.8	—	—	—	—	—	—
P. citrinum	22	5 R	11.7	—	—	—	—	—	—
P. corylophilum	19	3 R	7.5	—	—	—	—	—	—
P. steckii	2	1 R	2.1	—	—	—	—	—	—
Scopulariopsis brevicaulis	—	—	—	—	—	—	25	12 L	26.4
Setosphaeria rostrata	3	3 R	6.2	8	6 L	12.7	3	1 R	2.3
Stachybotrys parvispora	4	2 R	4.3	4	3R	6.3	—	—	—
Sterile mycelia	7	4 R	8.5	7	5 R	10.6	5	3 R	6.4
Trichoderma	8	4 R	8.6	3	2 R	4.2	7	2 R	4.6
T. hamatum	—	—	—	3	2 R	4.2	—	—	—
T. viride	8	4 R	8.6	—	—	—	7	2 R	4.6
Trichothecium roseum	3	1 R	2.2	3	1 R	2.2	5	3 R	6.4
Ulocladium botrytis	4	3 R	6.3	2	2 R	4.1	—	—	—
Total count	1264			1127			1042		
Number of genera (20)	18			14			11		
Number of species (35+1 variety)	30			20			19+1 variety		

Table.3 Screening of some phytopathogenic fungi for their abilities to produce cellulase enzymes

Fungi	Cellulases	
	C_1	C_x
Alternaria alternata	22 M	24 H
citri	26 H	23 H
raphani	24 H	21 M
tenuissima	23 H	19 W
Cochliobolus spicifer	25 H	22 M
Curvularia lunata	20 W	17 W
Stachybotrys atra var. *microspora*	24 H	22 M
Ulocladium botrytis	22 M	18 W

Enzyme activity: High activity, H: from 23-26 mm; moderate activity, M= 21-22 mm; weak activity, W= less than 21 mm

El-Said (2001) reported that the exo- and endo-β-1,4-glucanases activity of *Chaetomium globosum* reached the highest value after 6 and 8 days of incubation, respectively. Gautam et al. (2010) found that cellulase production of *Trichoderma viride* reached the maximum value after 6 day of incubation.

Effect of temperature

Temperature is one of the most important factors that influenced the secretion of fungal enzymes. The most suitable temperature for both exo- and endo-β-1,4-glucanase enzymes by tested fungi was 30°C. Considerable amounts of these enzymes were also recorded at 25°C. The other incubation temperatures decreased the production of cellulase enzymes by tested fungi (Figs. 2,4). Some articles showed that 30°C was the optimum temperature for cellulase production by several fungal genera and species including *Fusarium oxysporum* (El-Said *et al*. 2006), *Chaetomium globosum* (El-Said and Saleem 2008) and *Aspergillus niger* (Ja'afaru and Fagade 2010 and Ilyas et al. 2011). However, 25-28°C was the optimum for cellulase produced by *Chaetomium globosum* (El-Said 2001) and *Trichoderma harzianum* (Ahmed *et al*. 2009). Recently, Amir Ijaz et al. (2011) reported that the optimum temperature for cellulase produced by *Alternaria alternata* by solid state fermentation was 35°C.

Effect of pH value

The hydrogen ion concentration (pH) of the medium is greatly affected the production of hydrolytic enzymes. Maximum exo- and endo-β-1,4-glucanase enzymes produced by *Alternaria alternata, A. citri* and *Cochliobolus spicifer* were achieved at pH 6. Considerable amounts of cellulase enzymes were recorded at pH 8. The other pH values decreased the production of cellulases by tested fungi (Figs. 2,4). These results are in agreement with those obtained with *Trichoderma viride* (Abdel-Hafez et al. 1995), *Fusarium oxysporum* (El-Said et al. 2006), *Chaetomium globosum* (El-Said and Saleem 2008) and *A. fumigatus* (Sherief et al. 2010). Baig

(2005) observed that cellulase production of *Trichoderma lignorum* reached the maximum value at pH 5.6-5.8. Ahmed *et al*. (2009) and Gautam et al. (2010) reported that, the most suitable pH for cellulases production by *Trichoderm harzianum* and *T. viride* was 5.5. Amir Ijaz et al. (2011) found that pH 6 was the optimum for cellulase production by *Alternaria alternata* by solid state fermentation.

Effect of carbon sources

Lactose was the most favourable carbon source for exo-β-1,4-glucanase by *Alternaria citri* and *Cochlibolus spicifer* but carboxymethylcellulose was the best for endo-β-1,4-glucanase by *Alternaria alternata* and *A. citri*. Considerable amounts of

exo- and endo-β-1,4-glucanase enzymes was also achieved in the culture medium supplemented with starch, pectin (for C_1) and cellulose (for C_x) as carbon sources. However, the remaining carbon sources decreased the enzyme production by tested fungi (Figs. 3,5). Moharram et al. (1995) reported that, the maximum production of CMCase was achieved in the culture containing lactose or wheat bran as carbon source. Fang et al. (2008) showed that, the addition of lactose improved cellulase activity by *Acremonium cellulolyticus*. Baig (2005) found that, CMC, paper dust as well as banana biomass were the best substrates for cellulase production by *Trichoderma lignorum*. El-Said and Saleem (2008) reported that maltose was the most suitable carbon source for cellulase production by *Chaetomium globosum*. Recently, Ja'afaru and Fagade (2010) found that, the highest cellulase synthesis by *Aspergillus niger* was achieved when sugarcane bagasse followed by wheat bran were used as carbon sources. Juwaied et al. (2011) reported that sugar cane waste was the best carbon source enhanced cellulase production in *Aspergillus niger* and *Trichoderma viride*.

Effect of nitrogen sources

The highest yields of exo-β-1,4-glucanase enzyme by *A. citri* and *C. spicifer* was achieved in the presence of calcium nitrate as nitrogen source. While the maximum amount of endo-β-1,4-glucanase enzyme of *A. alternata* and *A. citri* were produced in the presence of sodium or calcium nitrate as nitrogen source (Figs. 3,5). The production of exo- and endo-β-1,4-glucanase enzymes was varied among different nitrogen sources used.

In this respect, ammonium nitrate followed by calcium nitrate were the most favourable nitrogen sources for cellulase production by *Chaetomium globosum* (El-Said and Saleem 2008), while sodium nitrate was found to be the best for cellulase production by *Aspergillus fumigatus* (Sherief et al. 2010). Ammonium dihydrogen phosphate was optimum for cellulase produced by *Aspergillus niger* (Ja'afaru and Fagade 2010), malt extract for cellulase produced by *Trichoderma viride* (Gautam et al. 2010). Ammonium sulphate was optimum for cellullase produced by *A. niger* (Ilyas et al. 2011).

Usually the infection of plant shoot system comes from the leaf surface fungi. Production of hydrolytic enzymes by pathogen is the first step of pathogenicity. This step is necessary for infection and penetration of host plant cell. Study of the ability of phytopathogenic microorganisms to produce cellulase enzymes as well as its environmental and nutritional factors

enable to understand the host pathogen interaction and would contribute in control of plant diseases and protection of plant crops.

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