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Cellulolytic activity of fungi isolated from anise and cumin spices and potential of their oils as antifungal agents

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Mycological analysis of 100 samples of anise and cumin seeds collected from four governorates (Aswan, Qena, Sohag and Assiut) in Upper Egypt revealed the isolation of seventy-one fungal species belonging to 30 genera. Anise seeds yielded a wider spectrum of fungal species (51 species) than those of cumin (41 species) using diluted seed suspension cultured on glucose- and cellulose-Czapek`s agar. The most common fungal species isolated from anise and cumin seeds were *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus sydowii*, *Aspergillus terreus*, *Emericella nidulans*, *Mucor hiemalis*, *Penicillium chrysogenum*, *Stachybotrys pomiformis* and *Sterile mycelia*. The ability of 71 fungal species recovered from both anise and cumin seeds to produce cellulases (exo- and endo-β-1,4-glucanase) on solid media was studied. *A. flavus* and *Mucor circinelloides* were the most active cellulase producers, so they were chosen to determine the best favorable environmental and nutritional conditions for exo- and endo-β-1,4-glucanase production. Maximum production of exo- β-1,4-glucanase by *M. circinelloides* was recorded after 6 days of incubation at 30°C and initial pH 6 with incorporation of fructose and potassium or sodium nitrate as carbon and nitrogen sources, respectively. However, maximum yield of endo-β-1,4-glucanase by *A. flavus* was obtained after 6 days of incubation at 30°C and initial pH 6 with medium containing sucrose and sodium nitrate as carbon and nitrogen sources, respectively. *In vitro* inhibitory effect of essential oils extracted from anise and cumin seeds were tested against the mycelial growth of 71 fungal isolates. Cumin oil was highly effective causing complete inhibition of all tested fungal isolates. Anise oil completely inhibited 62% and showed varying degrees of activity towards 38% of total isolates.

Key words: Anise and cumin seed fungi, cellulase activity, antifungal effect of oils.

INTRODUCTION

Spices are aromatic or pungent vegetable substances used in minute quantities to enrich, alter or mask the flavor of food (Pereira et al., 2006; Al-Mofleh, 2010). They are used in fresh or dry form (Frankic et al., 2009). Today the market offers different extracts of certain aromatic plants, combinations of extracts of different plants, purified active components or combinations of purified active components and synthesized active molecules (Indresh, 2007). They include leaves, flowers, bulbs, fruits, stems and rhizomes (Rathore and Shekhawat, 2008; Iyer et al., 2009). Spices are among the most valuable items of trade food. They are used through the

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Middle East to the Eastern Mediterranean and Europe (Rathore and Shekhawat, 2008). Spices have also been used for preservation of food products as they have been reported to have antiseptic and disinfectant properties (Dewar et al., 2008; Hashem and Alamri, 2010). They prolong the storage life of foods by preventing rancidity and oxidation of lipids (Kelen and Tepe, 2008) or through antibacterial and antifungal activity (Kasetsart, 2006; Pundir and Jain, 2010; Tajkarimi et al., 2010; Ejele et al., 2012). Anise (*Pimpinella anisum* L.) and cumin (*Cuminum cyminum* L.) are annual herbs of Umbelliferae family and widely cultivated in the Mediterranean rim (Turkey, Egypt, Spain, etc.) and in Mexico and Chile (Hansel et al., 1999). Cumin seeds and distilled cumin are used as a stimulant, antispasmodic, carminative and antimicrobial agent (Romeilah et al., 2010). Study of seed borne fungi is very significant due to the production of toxic substances (mycotoxins) by associated fungi and the relationships of these toxins to diseases (mycotoxicoses) of animals, fowls and humans. Fungi represent one of the major factors which induce deterioration of agricultural commodities during storage. Numerous investigations have been carried out on the mycoflora associated with various types of spices by several researches (Garcia et al., 2001; Little et al., 2003; Rizzo et al., 2004; Mandeel, 2005; Bungo et al., 2006; Koci-Tanackov et al., 2007; Abou-Donia, 2008; Dimic et al., 2008; Sagoo et al., 2009; Moorthy et al., 2010).

Cellulose, a major polysaccharide constituent of plant cell walls, is β-1,4 linked linear polymer of 8000 to 12000 glucose units. Three major enzymes involved in the degradation of cellulose to glucose are endoglucanase (endo-1,4-D-glucanase, EG), cellobiohydrolase (exo-1,4-D-glucanase, CBH), and β-glucosidase (1,4-D-glucosidase, BG). EG acts in random fashion, cleaving linked bonds within the cellulose molecule; CBH removes cellobiose units from the nonreducing ends of the cellulose chain and BG degrades cellobiose and cellooligosaccharides to glucose (Bhat, 2000; Saha, 2004).

Researchers have strong interests in cellulases, because of their applications in industries processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Jamel et al., 2005; Zhou et al., 2008; Ahmed et al., 2009b). Several fungi such as members of *Aspergillus*, *Penicillium*, *Trichoderma*, *Chaetomium* and some other moulds of Mucors and dematiaceous hyphomycetes produced cellulolytic enzymes as reported by several researchers (Berlin et al., 2005; El-Said et al., 2005, 2006; Jorgensen et al., 2005; Amouri and Gargouri, 2006; Ahmed et al., 2009a, 2012; Abdel-Hafez et al., 2010; Ghori et al., 2011, 2012; Saleem et al., 2012).

Anise oil is the essential oil obtained by steam distillation from dry ripe fruits of anise or star anise. Essential oil of anise fruits contains from 80 to 95% *trans*-anethole as the main compound, followed by chavicol methyl ether (estragole), anisaldehyde and *cis*-anethole (Hansel et al., 1999). Anon (1993) and Shaath and Azzo (1993) reported that the main constituents of Egyptian cumin seed oil were cumin aldehyde, b-pinene, g-terpinene, r-mentha-1,3-dien-7-al, r-mentha-1,4-dien-7-al and p-cymene. Several studies have shown that essential oils of spices and herbs demonstrated antifungal effects (De et al., 2001; Elgayyar et al., 2001; Gulcin et al., 2003; Kosalec et al., 2005; Lopez et al., 2005; Rasooli et al., 2006; Gachkar et al., 2007; Chen et al., 2008; Skrinjar and Nemet 2009; Prasad et al., 2010). Ozkalp and Ozcan (2009) screened 16 water distilled of anise, basil, cumin, dill, aegean sage, fennel (sweet), laural, mint, oregano, pickling herb, rosemary, sage, savory, sea fennel, sumac and thyme (black) for their antifungal activity against *Aspergillus flavus*. Mycelial growth of *A. flavus* was inhibited at various degrees. The distilled waters of oregano, pickling herb, savory and thyme samples caused the highest inhibition percentage (100%) against *A. flavus*.

The most active inhibitors followed the sequence from high to low: cumin, laurel, fennel and anise. Sumac, sea fennel, sage, dill, aegean sage, mint and rosemary and basil had the least inhibitory effect on the mycelial growth of *A. flavus*. Yazdani et al. (2009) found that the extracts of anise seeds inhibited only dermatophyte species, while extracts of star anise fruits inhibited growth of all dermatophytes and saprophytes. This study was performed to determine the natural occurrence and distribution of glucophilic and cellulose decomposing fungi of anise and cumin spices in addition to the antifungal activity of their essential oils on 71 fungal species isolated from these spices.

MATERIALS AND METHODS

Collection of anise and cumin seed samples

A total of one hundred samples of anise and cumin seeds (50 samples each) were collected from different places covering four governorates (Aswan, Qena, Sohag and Assiut) in Upper Egypt. Each sample was put in a sterile polyethylene bag and transferred to the mycological laboratory and kept in a cool place (5°C) for fungal analysis.

Determination of seed-borne fungi

The dilution-plate method was used for the estimation of fungal flora associated with anise and cumin seeds as described by Christensen (1963). A known weight of seeds was suspended in 200 ml sterile distilled water inside 500 ml conical flasks. Preliminary trials showed that dilutions of 1:40 and 1:100 were suitable to obtain reasonable number of fungal colonies in agar cultures of anise and cumin seeds, respectively. Fifteen ml of melted glucose and cellulose-Czapek's agar media, cooled to 45°C, were poured over the seed suspension in petri plates which were swirled to distribute the suspension. Four replicates were prepared and the cultures were incubated at 28°C for 7 days. The developing fungi were examined, identified, and counted. The numbers were calculated as colonies per gram dry seeds.

Types of media used for isolation of fungi

Glucophilic fungi

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; agar, 15.0) was used for the isolation of glucophilic fungi. Rose Bengal (0.1 mg/ml) and chloramphenicol (0.5 mg/L) were used as bacteriostatic agent (Smith and Dawson, 1944; AL-Doory, 1980).

Cellulose-decomposing fungi

Cellulose-Czapek's agar medium was used for isolation of cellulose decomposing fungi, in which cellulose powder (20 g/L) replaced glucose with the incorporation of Rose Bengal (0.1 mg/ml) and chloramphenicol (0.5 mg/L) as bacteriostatic agents.

Screening of fungal isolates for cellulase production

Seventy-one fungal species belonging to 30 genera were screened for their abilities to produce exo- and endo-β-1,4-glucanase (C_I and C_x enzyme, respectively). Fungal isolates were cultured on Eggins and Pugh medium (1962) of the following composition (g/L): $(NH_4)_2SO_4$, 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 microcrystalline (Merck), 10; agar, 20. pH was adjusted to 5.4 using acetate buffer. 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-glucanase production and Prasad and Verma medium (1979) for endo-glucanase enzyme. The later 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 incubation at 28°C, the cultures were filtered and the filtrates were used to detect the activity of the enzymes as the following.

Detection of exo-β-1,4-glucanase (C_I enzyme)

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 h, then the plates were flooded with chloroiodide of zinc solution and the measurement of clear zone around the cavities gave indication of cellulolytic activity of the isolates.

Detection of endo-β-1,4-glucanase (C_x enzyme)

Ten milliliter cavities were cut in plates containing solid medium of Dingle et al. (1953) of the following composition (g/L): carboxymethyl cellulose (CMC), 10; agar, 17; pH 5.4. 0.1 ml culture filtrate obtained from 7 days old fungal cultures grown on Prasad and Verma (1979) medium was dropped in each cavity. After 24 h incubation at 28°C, plates were flooded with chloroiodide of zinc solution and the clear zones around cavities were measured.

Factors affecting cellulase production

The effect of different ecological and nutritional factors on production of exo- and endo 1,4-β-glucanase (C_I and C_x enzyme, respectively) by *Mucor circinelloides* for C_I enzyme and *A. flavus* for C_x enzyme were studied. These fungal species were found to be highly active producers of exo- and endo-β-1,4-glucanase, respectively. The previous 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 (Deacon, 1985). Fifty milliliter of the medium were dispensed into each 100 ml Erlenmeyer flask and inoculated with an agar mycelial disc (10 mm diameter) of the mold obtained from 7-day-old cultures growing on the solid basal medium. Experiments were done to indicate the best cultural conditions enhancing the highest yield of cellulolytic enzymes.

Effect of temperature and time course

M. circinelloides and *A. flavus* were grown on the basal medium of Deacon (1985). The inoculated flasks were incubated at 20, 30 and 40°C for 14 days and harvested at 48 h intervals. Cultures were filtered and centrifuged at 5000 rpm for 10 min. The clear supernatants were assayed for enzyme activity.

Effect of pH values

The initial pH of the medium was adjusted with 0.1 N NaOH or 0.1 N HCl to different values ranging from 2 to 12. After inoculation with *M. circinelloides* for C_I enzyme and *A. flavus* for C_x enzyme, cultures were incubated at 30°C for 6 days. At the end of the incubation period, cultures were filtered, centrifuged and the clear supernatants were assayed for cellulase activity.

Effect of different carbon sources

The basal medium (Deacon, 1985) with pH 6 (the best pH for exo- and endo-β-1, 4-glucanase production) was supplemented with 1% of one of the following carbon sources: dextrose, fructose, glucose, starch and sucrose, in addition to cellulose as control. Cultures were incubated at 30°C for 6 days followed by filtration and centrifugation. Clear filtrates were used to detect the cellulase activity.

Effect of various nitrogen sources

To determine the effect of nitrogen sources on cellulase production, the sodium nitrate (2 g/L) in the basal medium was replaced by the same amount of various nitrogen compounds such as; ammonium chloride, ammonium nitrate, ammonium sulphate, potassium nitrate, in addition to sodium nitrate as control. Cultures in flasks were incubated at 30°C for 6 days and the cultures were filtered, centrifuged and the clear filtrate was used for the detection of cellulase activity.

Assay of cellulase activity (C_I and C_x enzymes)

The method described by Nelson (1944) was employed as follows: each of the 50 mg of filter paper (Whatman No. 1) and 1 ml of 1% CMC were added separately to 1 ml of acetate buffer (pH 6) and 1 ml of each culture filtrate and incubated for 30 min at 25°C for assaying activities of C_I and C_x enzymes, respectively. Similar reaction mixtures using boiled inactive enzyme solution were also prepared as controls and water with reagents as a blank. Three milliliter of Nelson's solution were added and the reaction mixtures were shaken and placed in a boiling water bath for 15 min. After cooling, 3 ml of the arsenomolybdate solution was added, mixed

thoroughly and then diluted to 10 ml with distilled water. The whole mixtures were 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 model (Bausch and Lomb Spectronic 2000 colorimeter). A standard curve was plotted using aqueous solutions of D-glucose with concentrations from 10 to 90 µg/ml.

Antifungal activity of anise and cumin oils

Seventy-one species belonging to 30 genera obtained from anise and cumin seeds were cultivated on glucose-Czapek's agar medium for about 10 days at 28°C until they were well sporulated, then by a sterile cork borer (10 mm diameter) one disk was cut from the pure culture and inoculated into 100 ml Erlenmeyer flask containing 50 ml sterilized distillated water to make spore suspension. The modified agar-well diffusion method (Collins et al., 1995) was employed to determine the antifungal activity of the oils. Anise and cumin oils were obtained from Qus factory of oils. One milliliter of the spore suspension was poured into sterile plate and 20 ml of sterile Czapek's medium was poured into a sterile culture plate and allowed to set at room temperature for about 30 min, with a sterile cork borer, three bores (10 mm diameter) were punched on the plates, 0.5 ml of essential oil was poured into each cavity and the plates were incubated at 28°C for ten days. The inhibition zone in mm was measured.

Statistical analysis

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

RESULTS AND DISCUSSION

Isolation of fungi

Seventy-one fungal species belonging to 30 genera were recovered from 100 seed samples of anise and cumin. Anise seeds yielded a wider spectrum of fungal species (51 species) than those of cumin (41 species) using the diluted seed suspensions cultured on glucose- and cellulose-Czapek`s agar. The most common fungal species isolated from anise and cumin seeds were *Alternaria alternata*, *A. flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus sydowii*, *Aspergillus terreus*, *Emericella nidulans*, *Mucor hiemalis*, *Penicillium chrysogenum*, *Stachybotrys pomiformis* and *Sterile mycelia* (Tables 1 and 2).

The aforementioned fungal species were isolated from various types of spices with different numbers and frequencies by several workers (Garcia et al., 2001; Mandeel, 2005; Bugno et al., 2006; Kocic-Tanackov et al., 2007; Dimic et al., 2008; Abdullah et al., 2009; Hashem and Alamri, 2010). Recently, Abou-Donia (2008) collected 303 samples representing different types of spices and medicinal plants in Egypt. *Aspergillus*, *Fusarium* and *Penicillium* were the most common genera and were isolated from all samples.

Moorthy et al. (2010) studied the occurrence of mycopopulation in spices and herbal drugs of 16 different varieties (among them anise and cumin seeds). The most predominant fungal genera encountered were *Aspergillus*, *Curvularia*, *Geotrichum*, *Penicillium* and *Trichoderma*.

Screening of fungi for cellulase activity

Seventy-one fungal species were screened for their abilities to produce exo- and endo-1,4-β-glucanase (C_1 and C_x enzymes) on solid medium. The data proved that all isolates were active to utilize cellulose, but with variable degrees. Thirteen isolates (18.3% of total isolates) showed high cellulolytic activity for exo-β-1,4-glucanase and these were *Acremonium rutilum*, *Alternaria chlamydospora*, *Aspergillus candidus*, *A. fumigatus*, *Eurotium chevalieri*, *Fusarium merismoides*, *M. circinelloides*, *Penicillium aurantigriseum*, *Penicillium brevicompactum*, *Penicillium corylophilum*, *Phoma eupyrena*, *Trichoderma viride* and *Ulocladium alternariae.* The moderately cellulolytic isolates included 34 isolates (47.9% of total isolates); however, 24 isolates (33.8% of total isolates) were found to be weak cellulolytic activity. For endo-1,4-β-glucanase enzyme, twelve isolates (16.9% of total isolates) exhibited high enzymatic activity and these were *Alternaria raphani*, *Aspergillus clavatus*, *A. flavus*, *Drechslera erythrospila*, *M. circinelloides*, *Nectria haematococca*, *Penicillium aurantiogriseum*, *P. corylophilum*, *Penicillium duclauxii*, *Penicillium spinulosum*, *Syncephalastrum racemosum* and *T. viride*. However, 32 isolates (45.1% of total isolates) had moderate activity and 27 isolates (38% of total isolates) had week activity (Table 3).

Several fungal species were reported as cellulase producers, but with variable capabilities by several workers (Moharram et al., 2004; Berlin et al., 2005; El-Said, 2006; El-Said and Saleem, 2008; Abdel-Hafez et al., 2010). Recently, Saleem et al. (2012) screened forty four fungal isolates recovered from *Vicia faba* plant for their abilities to produce exo- and endo-β-1,4 glucanase. All fungal isolates tested had the ability to produce cellulases, but with variable degrees. For exo-β-1,4 glucanase, six isolates (13.7% of total isolates) showed high cellulase activity and twenty one isolates (47.7% of total isolates) were found to have moderate cellulase activity.

However, seventeen isolates (38.6% of total isolates) were low producers of cellulase. For endo-β-1,4 glucanase enzyme, five isolates (11.4% of total isolates) showed high cellulase activity. However, twenty one isolates (47.7% of total isolates) had moderate ability to produce cellulase and eighteen isolates (40.9% of total isolates) were low producers of cellulase.

A. flavus and *M. circinelloides* were the most active cellulase producers, so they were chosen to determine the best favorable environmental and nutritional

Table 1. Average total counts, number of cases of isolation (NCI, out of 50 sample), and occurrence remarks (OR) of fungal genera and species recovered from *P. anisum* seeds on glucose and cellulose Czapek's agar at 28°C.

Genera and species	Glucose		Cellulose	
	ATC ± SD	NCI and OR	ATC ± SD	NCI and OR
Acremonium	400 ± 97.6	5 R	300 ± 36.9	8 L
A. furcatum	-	-	10 ± 5	1 R
A. kiliense	370 ±94.2	3 R	280 ± 36.5	6 L
A. strictum	30 ± 5	2 R	10 ± 5	1 R
Alternaria	1070 ± 94.2	21 M	1300 ± 127.1	22 M
A. alternata	710 ± 78.0	19 M	960 ± 146.7	21 M
A. brassicicola	350 ± 40.3	2 R	260 ± 71.8	1 R
A. chlamydospora	10 ± 5	1 R	80 ± 27.0	1 R
Aspergillus	16990 ± 271.5	50 H	15700 ± 351.2	49 H
A. candidus	1120 ± 67.8	1 R	-	-
A. clavatus	10 ± 5	1 R	-	-
A. flavus	2550 ± 68.4	37 H	1880 ± 116.9	34 H
A. fumigatus	3050 ± 80.5	29 H	4250 ± 57.3	34 H
A. niger	8450 ± 89.9	50 H	8380 ± 152.8	49 H
A. ochraceus	540 ± 57.4	7 L	310 ± 22.1	7 L
A. sydowii	900 ± 44.3	13 M	1200 ± 88.3	17 M
A. terreus	280 ± 40.8	16 M	370 ± 40.3	16 M
A. ustus	-	-	30 ± 9.5	1 R
A. versicolor	90 ± 9.5	4 R	-	-
Botryotrichum piluliferum	20 ± 10	1 R	90 ± 18.9	4 R
Chaetomium	20 ± 5.7	2 R	110 ± 17.0	7 L
C. atrobrunneum	10 ± 5	1 R	10 ± 5	1 R
C. citrinum	-	-	10 ± 5	1 R
C. crispatum	-	-	20 ± 10	1 R
C. globosum	10 ± 5	1 R	60 ± 12.9	3R
Circinella muscae	10 ± 5	1 R	10 ± 5	1 R
Cladosporium	190 ± 45.7	4 R	110 ± 9.5	5 R
C. cladosporioides	20 ± 10	1 R	20 ± 5.7	2 R
C. sphaerospermum	170 ± 36.8	4 R	90 ± 5	3 R
Cochliobolus	210 ± 9.5	13 M	210 ± 20.6	13 M
C. lunatus	20 ± 5.7	2 R	30 ± 9.5	2 R
C. spicifer	190 ± 12.5	11 L	140 ± 26.4	9 L
C. tuberculatus	-	-	40 ± 8.1	3 R
Cunninghamella echinulata	20 ± 5.7	2 R	10 ± 5	1 R
Curvularia pallescens	-	-	10 ± 5	1 R
Drechslera	20 ± 5.7	1 R	10 ± 5	1 R
D. australiensis	-	-	10 ± 5	1 R
D. erythrospila	20 ± 5.7	1 R	-	-
Emericella nidulans	1750 ± 77.6	32 H	1720 ± 72.5	27 H

Table 1. Contd.

Epicoccum purpurascens	20 ± 5.7	2 R	10 ± 5	1 R
Eurotium chevalieri	90 ± 12.5	5 R	-	-
Fusarium	530 ± 43.4	5 R	70 ± 20.6	1 R
F. merismoides	530 ± 43.4	5 R	-	-
F. oxysporum	-	-	70 ± 20.6	1 R
Gibberella fujikuroi	300 ± 57.4	2 R	20 ± 10	1 R
Monographella nivalis	20 ± 10	1 R	-	-
Mucor	850 ± 56.1	20 M	610 ± 89.9	11 L
M. circinelloides	10 ± 5	1 R	70 ± 22.1	3 R
M. hiemalis	810 ± 51.8	17 M	540 ± 73.2	9 L
M. racemosus	30 ± 5	2 R	-	-
Myrothecium verrucaria	-	-	80 ± 16.3	3 R
Nectria haematococca	30 ± 9.5	2 R	210 ± 12.5	2 R
Penicillium	2250 ± 166.0	30 H	890 ± 128.3	14 M
P. aurantiogriseum	40 ± 8.1	4 R	320 ± 42.4	6 L
P. brevicompactum	10 ± 5	1 R	10 ± 5	1 R
P. chrysogenum	1780 ± 132.7	18 M	200 ± 21.6	3 R
P. citrinum	80 ± 8.1	3 R	40 ± 14.1	1 R
P. corylophilum	170 ± 33.0	6 L	290 ± 78.0	2 R
P. duclauxii	30 ± 5	3 R	20 ± 5.7	2 R
P. funiculosum	20 ± 10	1 R	10 ± 5	1 R
P. purpurogenum	70 ± 17.0	2 R	-	-
P. spinulosum	50 ± 12.5	2 R	-	-
Rhizopus stolonifer	700 ± 33.1	10 L	30 ± 15	2 R
Scopulariopsis	90± 9.5	6 L	60 ± 12.9	3 R
S. brevicaulis	40 ± 10	4 R	60 ± 12.9	3 R
S. sphaerospora	50 ± 9.5	3 R	-	-
Setosphaeria rostrata	30 ± 9.5	3 R	110 ± 27.5	5 R
Stachybotrys pomiformis	350 ± 12.5	16 M	2380 ± 185.2	27 H
Stemphylium solani	40 ± 14.1	1 R	60 ± 30	1 R
Sterile mycelia	690 ± 15	26 H	950 ± 101.4	27 H
Trichoderma	10 ± 5	1 R	30 ± 5	1 R
T. hamatum	-	-	20 ± 5.7	1 R
T. viride	10 ± 5	1 R	10 ± 5	1 R
Ulocladium	210 ± 20	11 L	310 ± 47.8	10 L
U. alternariae	30 ± 9.5	2 R	30 ± 15	1 R
U. botrytis	130 ± 12.5	6 L	100 ± 12.9	5 R
U. chartarum	50 ± 9.5	4 R	180 ± 23.8	5 R
Gross total count	26910 ± 467.9	-	26120 ± 630.1	-
Number of genera (28)	26	-	26	-
Number of species (62)	51	-	51	-

Table 2. Average total counts, number of cases of isolation (NCI, out of 50 samples), and occurrence remarks (OR) of fungal genera and species recovered from *C. cyminum* seeds on glucose- and cellulose-Czapeks agar at 28°C.

Genera and species	Glucose		Cellulose	
	ATC ± SD	NCI and OR	ATC ± SD	NCI and OR
Acremonium	225 ± 65.7	2 R	125 ± 62.5	1 R
A. furcatum	200 ± 70.7	2 R	-	-
A. kiliense	-	-	125 ± 62.5	1 R
A. strictum	25 ± 12.5	1 R	-	-
Alternaria	1100 ± 102.1	11 L	1525 ± 94.3	12 M
A. alternata	1025 ± 102.8	11 L	1425 ± 114.3	10 L
A. brassicicola	50 ± 25	2 R	100 ± 20.4	2 R
A. raphani	25 ± 12.5	1 R	-	-
Aspergillus	33375 ± 518.1	48 H	33075 ± 1279.7	48 H
A. candidus	25 ± 12.5	1 R	350 ± 32.2	1 R
A. flavus	6000 ± 113.6	30 H	7350 ± 261.8	30 H
A. fumigatus	15000 ± 408.2	32 H	12725 ± 568.0	35 H
A. niger	11125 ± 631.2	44 H	11550 ± 1395.4	42 H
A. ochraceus	50 ± 14.4	1 R	150 ± 14.4	5 R
A. sydowii	400 ± 88.9	9 L	50 ± 14.4	2 R
A. terreus	650 ± 47.8	11 L	875 ± 108.7	13 M
A. versicolor	125 ± 31.4	4 R	25 ± 12.5	1 R
Chaetomium	50 ± 14.4	2 R	425 ± 65.7	6 L
C. atrobrunneum	-	-	125 ± 37.5	1 R
C. citrinum	-	-	50 ± 25	2 R
C. globosum	50 ± 14.4	2 R	225 ± 82.6	3 R
C. uniporum	-	-	25 ± 12.5	1 R
Cladosporium	400 ± 79.0	5 R	1175 ± 77.3	7 L
C. cladosporioides	150 ± 32.2	3 R	325 ± 42.6	2 R
C. musae	225 ± 51.5	2 R	400 ± 117.2	1 R
C. sphaerospermum	25 ± 12.5	1 R	375 ± 31.4	4 R
C. variabile	-	-	75± 37.5	1 R
Cochliobolus	525 ± 23.9	6 L	350± 52.0	5 R
C. lunatus	25 ± 12.5	1 R	50± 25	1 R
C. spicifer	475 ± 37.5	6 L	300± 61.2	5 R
Cunninghamella echinulata	25 ± 12.5	1 R	-	-
Drechslera	50 ± 14.4	2 R	100± 50	1 R
D. australiensis	25 ± 12.5	1 R	100± 50	1 R
D. pedicellata	25 ± 12.5	1 R		
Emericella nidulans	2200 ± 61.2	23 M	2650± 165.2	20 M
Eurotium chevalieri	1475 ± 108.7	6 L	-	-
Fusarium semitectum	2075 ± 37.5	1 R	-	-
Mucor	1525 ± 170.0	6 L	700± 141.4	8 L
M. circinelloides	975 ± 137.5	2 R	400± 141.4	3 R

Table 2. Contd.

M. hiemalis	525 ± 42.6	3 R	300± 61.2	5 R
M. racemosus	25 ± 12.5 (25)	1 R	-	-
Myrothecium verrucaria	-	-	25± 12.5	1 R
Nectria haematococca	1150 ± 85.3	2 R	350± 43.3	3 R
Penicillium	825 ± 62.5	16 M	1800± 265.3	9 L
P. aurantiogriseum			425± 132.8	3 R
P. chrysogenum	325 ± 31.4	7 L	575± 108.7	4 R
P. citrinum	75 ± 23.9	2 R	-	-
P. corylophilum	225 ± 42.6	5 R	775± 171.2	2 R
P. duclauxii	200 ± 35.3	6 L	-	-
P. funiculosum	-	-	25± 12.5	1 R
Phoma eupyrena	1250 ± 125	2 R	1450± 62.9	3 R
Rhizopus stolonifer	925 ± 147.7	6 L	25± 12.5	1 R
Scopulariopsis brevicaulis	50 ± 25	1 R	-	-
Setosphaeria rostrata	-	-	25± 12.5	1 R
Stachybotrys pomiformis	300 ± 20.4	6 L	1300± 93.5	3 R
Stemphylium solani	450 ± 75	2 R	-	-
Sterile mycelia	2800 ± 167.1	31 H	875± 74.6	18 M
Syncephalastrum racemosum	25 ± 12.5	1 R	-	-
Trichoderma viride	100 ± 20.4	2 R	-	-
Ulocladium	1050 ± 52.0	12 M	1575± 181.8	9 L
U. alternariae	25 ± 12.5	1 R	-	-
U. botrytis	900 ± 28.8	10 L	600± 61.2	4 R
U. chartarum	125 ± 62.5	2 R	975± 124.7	6 L
Gross total count	51950 ± 712.2	-	47550 ± 1642.4	-
Number of genera 24	22	-	17	-
Number of species 50	41	-	34	-

conditions for exo- and endo-β-1,4-glucanase production. Maximum production of exo-β-1,4-glucanase by *M. circinelloides* was recorded after 6 days of incubation at 30°C and initial pH 6 in the medium with incorporation of fructose and potassium or sodium nitrate as carbon and nitrogen sources, respectively. However, the maximum yield of endo-β-1,4-glucanase by *A. flavus* was obtained after 6 days of incubation at 30°C and initial pH 6 with medium contained sucrose and sodium nitrate as carbon and nitrogen sources, respectively (Figures 1 and 2). Several investigations have been carried out on the effect of numbers of organic and inorganic nitrogen, carbon materials, pH values, temperatures and incubation periods on the production of cellulase enzyme complex by several fungi (Saha, 2004; Berlin et al., 2005; El-Said et al., 2005, 2006; Narasimha et al., 2006; El-Said and Saleem, 2008; Yang et al., 2008; Ahmed et al., 2009a; Abd El-Zaher and Fadel, 2010).

Inhibitory effect of anise and cumin essential oils on growth of fungi

The cup plate technique was used to study the inhibitory effect of anise and cumin essential oils on growth of 71 fungal species. Cumin oil was highly effective against all fungal isolates tested and completely inhibited the mycelial growth of all fungi when added to solid medium. Anise oil showed variable effects on fungal growth ranging from complete inhibition (62% of total isolates) in case of sensitive isolates to limit of inhibition or no inhibition (38%) in case of resistant isolates. Five isolates (7% of total isolates) were completely inhibited with anise oil and these were *D. erythrospila*, *Epicoccum purpurascens*, *Scopulariopsis brevicaulis*, *Setosphaeria rostrata* and *Stemphylium solani*. 22 isolates (31% of total isolates) were inhibited by anise oil, but with different effects and these were *A. clavatus*, *A. sydowii*,

Table 3. Cellulolytic activity (calculated as average diameter of clear zone in mm) of fungi and inhibitory effect of oils on different fungal isolates.

Fungal isolate	Cellulase		Inhibitory effect	
	Exo-ß-1,4-glucanase	Endo-ß-1,4-glucanase	Anise oil	Cumin oil
Acremonium				
A. furcatum	10 W	15 W	N.I	C.I
A. kiliense	24 M	27 M	N.I	C.I
A. rutilum	39 H	26 M	N.I	C.I
A. strictum	21 M	25 M	N.I	C.I
Alternaria				
A. alternata	24 M	16 W	N.I	C.I
A. brassicicola	24 M	16 W	N.I	C.I
A. chlamydospora	37 H	10 W	N.I	C.I
A. raphani	28 M	35 H	N.I	C.I
Aspergillus				
A. candidus	32 H	27 M	N.I	C.I
A. clavatus	25 M	35 H	14	C.I
A. flavus	22 M	38 H	N.I	C.I
A. fumigatus	30 H	25 M	N.I	C.I
A. niger	27 M	24 M	N.I	C.I
A. ochraceus	10 W	24 M	N.I	C.I
A. sydowii	26 M	27 M	30	C.I
A. terreus	20 M	23 M	N.I	C.I
A. ustus	26 M	29 M	17	C.I
A. versicolor	25 M	10 W	17	C.I
Botryotrichum piluliferum	10 W	25 M	20	C.I
Chaetomium				
C. anguipilium	10 W	14 W	N.I	C.I
C. atrobrunneum	15 W	14 W	N.I	C.I
C. citrinum	10 W	21 M	N.I	C.I
C. crispatum	29 M	15 W	N.I	C.I
C. globosum	20 M	17W	N.I	C.I
Circinella muscae	27 M	13 W	15	C.I
Cladosporium				
C. cladosporioides	10 W	16 W	N.I	C.I
C. musae	18 W	17 W	23	C.I
C. sphaerospermum	10 W	10 W	N.I	C.I
C. variabile	11 W	10 W	N.I	C.I
Cochliobolus				
C. lunatus	21 M	19 W	N.I	C.I
C. spicifer	24 M	28 M	N.I	C.I
Cunninghamella echinulata	24 M	17 W	N.I	C.I
Curvularia pallescens	24 M	23 M	N.I	C.I
Drechslera				
D. australiensis	12 W	12 W	N.I	C.I
D. erythrospila	22 M	30 H	C.I	C.I
D. pedicellata	10 W	23 M	N.I	C.I

Table 3. Contd.

Emericella nidulans	10 W	18 W	33	C.I
Epicoccum purpurascens	15 W	16 W	C.I	C.I
Eurotium chevalieri	31 H	27 M	20	C.I
Fusarium				
F. merismoides	36 H	26 M	N.I	C.I
F. oxysporum	23 M	25 M	22	C.I
F. semitectum	12 W	16 W	N.I	C.I
Gibberella fujikuroi	21 M	18 W	N.I	C.I
Monographella nivalis	17 W	10 W	N.I	C.I
Mucor				
M. circinelloides	45 H	30 H	N.I	C.I
M. hiemalis	25 M	21 M	N.I	C.I
M. racemosus	21 M	17 W	N.I	C.I
Myrothecium verrucaria	10 W	13 W	14	C.I
Nectria haematococca	19 W	30 H	16	C.I
Penicillium				
P. aurantiogriseum	30 H	32 H	23	C.I
P. brevicompactum	34 H	26 M	19	C.I
P. chrysogenum	29 M	22 M	N.I	C.I
P. citrinum	17 W	26 M	N.I	C.I
P. corylophilum	31 H	31 H	N.I	C.I
P. duclauxii	27 M	34 H	15	C.I
P. funiculosum	17 W	26 M	31	C.I
P. purpurogenum	20 M	28 M	N.I	C.I
P. spinulosum	20 M	30 H	17	C.I
Phoma eupyrena	38 H	20 M	N.I	C.I
Rhizopus stolonifer	24 M	21 M	N.I	C.I
Scopulariopsis				
S. brevicaulis	10 W	10 W	C.I	C.I
S. sphaerospora	17 W	28 M	N.I	C.I
Setosphaeria rostrata	26 M	27 M	C.I	C.I
Stachybotrys pomiformis	17 W	23 M	26	C.I
Stemphylium solani	28 M	20 M	C.I	C.I
Syncephalastrum racemosum	24 M	31 H	50	C.I
Trichoderma				
T. hamatum	14 W	28 M	36	C.I
T. viride	35 H	33 H	16	C.I
Ulocladium				
U. alternariae	40 H	16 W	N.I	C.I
U. botrytis	29 M	13 W	N.I	C.I
U. chartarum	29 M	20 M	18	C.I

High activity, H = 30 to 45 mm; Moderate activity, M= 20 to 29 mm; and Weak activity, W = <20 mm. C.I = Complete inhibition; N.I = No inhibition.

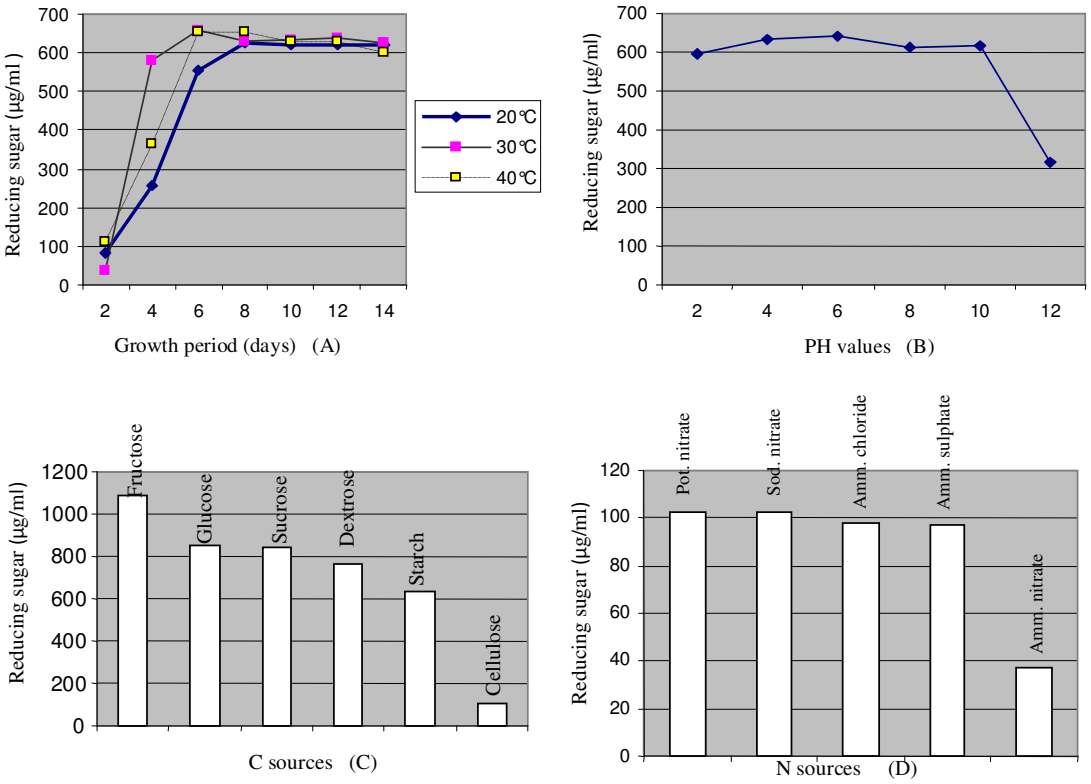

Figure 1. Effect of time course and temperature, pH values, carbon sources and nitrogen sources (A, B, C and D) on the production of exo-ß-1,4-glucanase (C_1) by *Mucor circinelloides*. Pot.: Potassium; Sod.: sodium; Amm.: ammonium.

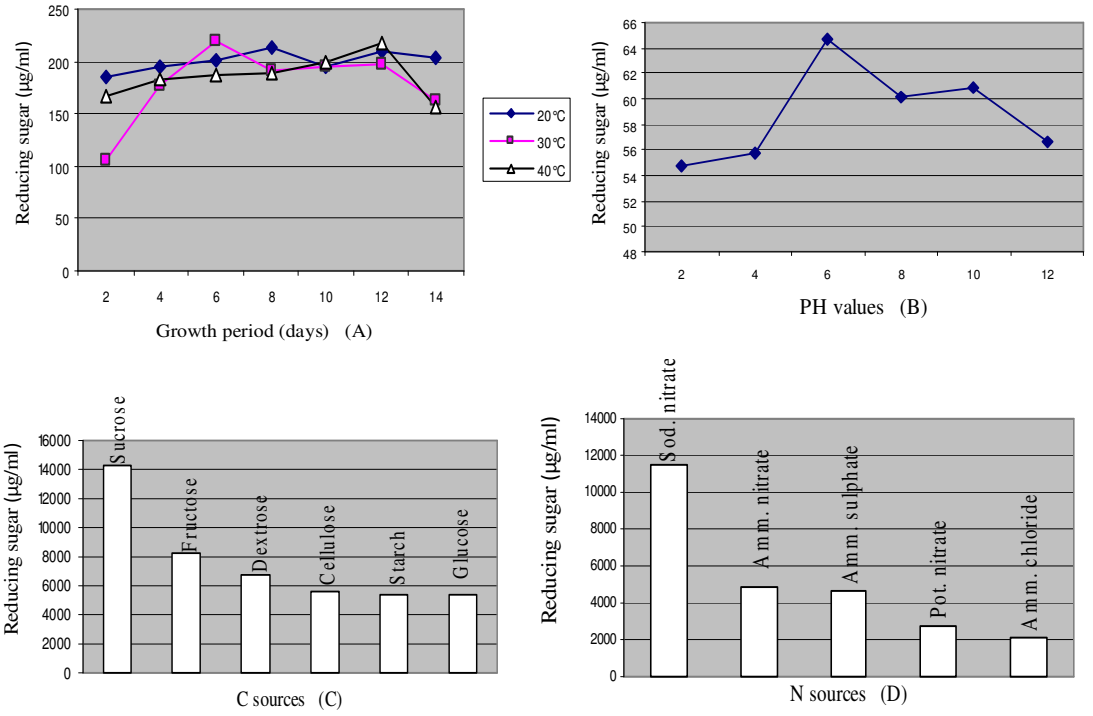

Figure 2. Effect of time course and temperature, pH values, carbon sources and nitrogen sources (A, B, C and D) on the production of endo-ß-1,4-glucanase (C_x) by *A. flavus*. Pot.: Potassium; Sod.: sodium; Amm.: ammonium.

Aspergillus ustus, *Avicularia versicolor*, *Botryotrichum piluliferum*, *Circinella muscae*, *Cladosporium musae*, *E. nidulans*, *Eurotium chevalieri*, *Fusarium oxysporum*, *Myrothecium verrucaria*, *N. haematococca*, *P. aurantiogriseum*, *P. brevicompactum*, *P. duclauxii*, *Penicillium funiculosum*, *P. spinulosum*, *S. pomiformis*, *S. racemosum*, *Trichoderma hamatum*, *T. viride* and *Ulocladium chartarum*. However, 44 isolates (62% of total isolates) were not inhibited by anise oil (Table 3). The inhibitory effect of essential oils of spices and herbs was recorded by some workers in different places of the world (Elgayyar et al., 2001; Angioni et al., 2004; Kosalec et al., 2005; Lopez et al., 2005; Wang et al., 2005; Chen et al., 2008; Skrinjar and Nemet, 2009; Prasad et al., 2010). Recently, Ozkalp and Ozcan (2009) screened 16 water distilled of anise, basil, cumin, dill, Aegean sage, fennel (sweet), laural, mint, oregano, pickling herb, rosemary, sage, savory, sea fennel, sumac and thyme (black) for their antifungal activity against *A. flavus*. Mycelial growth of *A. flavus* was inhibited at various degrees. The distilled waters of oregano, pickling herb, savory and thyme samples caused the highest inhibition percentage (100%) against *A. flavus*. The most active inhibitors followed the sequence from high to low: cumin, laurel, fennel and anise. Sumac, sea fennel, sage, dill, Aegean sage, mint and rosemary. Basil had the least inhibitory effect on the mycelial growth of *A. flavus*. Extracts of anise seeds inhibited only dermatophyte species, while extracts of star anise fruits inhibited growth of all dermatophytes and saprophytes (Yazdani et al., 2009).

Conclusion

Study of the occurrence and distribution of fungi in herbs would contribute in the knowledge of the most dominant fungi specially which have harmful effect on human health such as mycotoxin producing fungi, in addition to the biodegradation of these herbs by fungal enzymes such as cellulases under different environmental conditions and the role of their essential oils as antifungal agents for protection of herbs against fungal activity.

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