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Full Length Research Paper

Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes

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This study investigates the current spoilage fruit fungi and their plant cell wall degrading enzymes of various fresh postharvest fruits sold in Jeddah city and share in establishment of a fungal profile of fruits. Ten fruit spoilage fungi were isolated and identified as follows *Fusarium oxysporum* (banana and grape), *Aspergillus japonicus* (pokhara and apricot), *Aspergillus oryzae* (orange), *Aspergillus awamori* (lemon), *Aspergillus phoenicis* (tomato), *Aspergillus tubingensis* (peach), *Aspergillus niger* (apple), *Aspergillus flavus* (mango), *Aspergillus foetidus* (kiwi) and *Rhizopus stolonifer* (date). The plant cell wall degrading enzymes xylanase, polygalacturonase, cellulase and α-amylase were screened in the cell-free broth of all tested fungi cultured on their fruit peels and potato dextrose broth (PDB) as media. Xylanase and polygalacturonase had the highest level contents as compared to the cellulase and α-amylase. In conclusion, *Aspergillus* spp. are widespread and the fungal polygalacturonases and xylanses are the main enzymes responsible for the spoilage of fruits.

Key words: *Aspergillus, Fusarium*, *Rhizopus*, fruits, xylanase, polygalacturonase.

INTRODUCTION

It has been known that fruits constitute commercially and nutritionally important indispensable food commodity. Fruits play a vital role in human nutrition by supplying the necessary growth factors such as vitamins and essential minerals in human daily diet and that can help to keep a good and normal health. Fruits are widely distributed in nature. One of the limiting factors that influence the fruits economic value is the relatively short shelf-life period caused by pathogens attacked. It is estimated that about 20-25% of the harvested fruits are decayed by pathogens during post-harvest handling even in developed countries (Droby, 2006; Zhu, 2006). In developing countries, post-harvest losses are often more severe due to inadequate storage and transportation facilities. Fungal fruits infection may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions, or after purchasing by the consumer. Fruits contain high levels of sugars and nutrients element and their low pH values make them particularly desirable to fungal decayed (Singh and Sharma, 2007).

Generally, spoiling fungi are considered toxigenic or pathogenic. Toxigenic fungi have been isolated from spoiling fruits (Stinson et al., 1981). During refrigeration some moulds may produce mycotoxins (Tournas and Stack, 2001). Pathogenic fungi, on the other hand, could cause infections or allergies (Monso, 2004). *Aspergillus* spp. are known to produce several toxic metabolites, such as malformins, naphthopyrones (Frisvad and Samson, 1991; Pitt and Hocking, 1997) and they can produce Ochratoxins (OTA), a mycotoxin which is a very important toxin worldwide because of the hazard it poses to human and animal health (Peraica et al., 1999; Petzinger and Weidenbach, 2002) thus extra care should be taken during personnel handling of these fruits; such as harvesting, cleaning, sorting, packaging, transport and storage.

The primary cell wall of fruit is composed of approximately 10% proteins and 90% polysaccharides, which can be divided into three groups: cellulose,

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Abbreviation: PDB, potato dextrose broth.

hemicellulose and pectin (McNeil et al., 1984; Nathalie, 2006). Numerous cell wall degrading enzymes can be secreted by pathogens to breach and use the plant cell walls as nutrient sources that reduced post-harvest life and finally lead to develop inedible, undesirable quality and soft rot spoilage. A remarkable array of polysaccharide degrading enzymes including exo- and endo-polygalacturonases, pectin methylesterases, pectin lyases and pectate lyases, acetyl esterases, xylanases and a variety of endo-glucanases that cleave cellulose, xyloglucan and other glucans (Lebeda et al., 2001; Gordon et al., 2002; Raviyan et al., 2005; Netsanet et al., 2009). Pectinases are the first enzymes to be secreted by fungal pathogens when they attack plant cell walls (Idnurm and Holett, 2001). Pectin degrading enzymes weaken the plant cell wall and expose other polymers to degradation by hemicellulases and cellulases. They are the first cell wall degrading enzymes that are secreted by pathogens and are important virulence factors (Boccara and Chatain, 1989; Tomassini et al., 2009).

The purpose of the current investigation was to isolate and identify some fruit spoilage fungi and screening their plant cell wall degrading enzymes, pectinases, xylanases, cellulases and α-amylases of various fruits sold in Jeddah city.

MATERIALS AND METHODS

Fruit materials

Twelve types of various fruits, banana, orange, lemon, tomato, peach, apple, grape, date, mango, pokhara, apricot and kiwi were purchased from markets (some fruits are local and others are imported) in Jeddah Province in their individual packages weighing approximately 3 kilos each.

Isolation of fruit spoilage fungi

Several methods were carried out individually for fungi isolation, by incubation of the whole fruits at 28°C, incubation of intact fruits after injuring their surfaces at 28°C and washing off the surfaces of intact fruits. The washing off method give the maximum growth of fungi compared to the other methods. Therefore, we choose the wash off method for isolation of fungi. The fruits were washed with sterile water then sub-culturing the fungi washed off water. The sub-culturing was carried out by using a sterile fresh medium of potato dextrose agar (PDA) and incubated at 28°C until fungal proliferation on medium surface. The isolation of pure fungal colony in culture medium was performed by using slants of a sterile fresh medium of PDA and incubated at 28°C for 5-7 days. The isolated fungi were maintained at 4°C.

Identification of the isolated fungi

The pure isolated fungi were identified according to the most documented keys in fungal identification (Domsch et al., 1993; Klich, 2002; Samson and Varga, 2007). The fungal isolates were subjected to certain morphological studies by an Image Analysis System using Soft-Imaging GmbH software (analysis Pro ver.3.0) as well as using the newly introduced RCMB Database Management System for *Aspergilli* identification at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. The gross morphology viz. the rate of growth, colony diameter, colony texture, colony color and reverse pigmentation as well as the measurements of the diagnostic structures that characterized the species were taken.

Production of cell wall degrading enzymes

Cell wall degrading enzymes as pectinases, xylanases, cellulases and amylases from the isolated fungi were produced using their spoilage fruits as culture media in stationary or agitation phases. Fungi were inoculated under aseptic conditions in 250 ml Erlenmeyer flasks contained 5% fruit peels. The inoculated flasks were incubated at 28°C with shaking on a rotary incubator shaker at 150 rpm for 5 days. Stationary phase was performed without shaking. The same procedure was carried out in presence of potato dextrose broth (PDB) as a control instead of fruit peels. The cell-free broth was recovered by filtration using a polyamide tissue. The cell-free broth was subjected to dialysis against 20 mM Tris-HCl buffer, pH 7.2 over night. The dialyzate was centrifuged at 10,000 rpm for 12 min and the supernatant was designed as crude extract.

Enzyme assays

Polygalacturonase (EC 3.2.1.15), cellulase (EC 3.2.1.21), xylanase (EC 3.2.1.8) and α-amylase (EC 3.2.1.1) activities were assayed by determining the liberated reducing end products using galacturonic acid, glucose, xylose and maltose as standards, respectively (Miller, 1959). The reaction mixture (0.5 ml) contained 1% substrate, 0.05 M sodium acetate buffer pH 5.5 and a suitable amount of crude extract. Assays were carried out at 37°C for 1 h. Then 0.5 ml dinitrosalicylic acid reagent was added to each tube. The tubes were heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. Substrates used were polygalacturonic acid, CM-cellulose, xylane and starch for polygalacturonase, cellulase, xylanase and α-amylase, respectively. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of reducing sugar per h under standard assay conditions.

Statistical analysis

Each value of enzyme activity represents the mean of three runs ±S.E.

RESULTS AND DISCUSSION

Screening of spoilage fungi in some fruits

Ten fruit spoilage fungi were isolated and identified as follows *Fusarium oxysporum* (banana and grape), *Aspergillus japonicus* (pokhara and apricot), *Aspergillus oryzae* (orange), *Aspergillus awamori* (lemon), *Aspergillus phoenicis* (tomato), *Aspergillus tubingensis* (peach), *Aspergillus niger* (apple), *Aspergillus flavus* (mango), *Aspergillus foetidus* (kiwi) and *Rhizopus stolonifer* (date). *Aspergillus* spp. were widespread among all examined spoilage fruits. Several fruit spoilage fungi from different region has been isolated and identified. *Fusarium* spp. was isolated from internal root and corm tissue from twenty-one banana cultivars in Central America (Pocasangre et al., 2000). Although, *F. oxysporum* was isolated from roots of declining grapevines of cv Semillon (*Vitis vinifera* L.) in the Hunter Valley, New South Wales. The pathogenicity of the fungus was confirmed by infecting grapevines growing in the glasshouse with material obtained from roots of field-grown vines (Highet and Nair 1995). *F. oxysporum* MTCC 1755 was also obtained from waste apple pomace (Chatanta et al., 2008). *A. niger* is a fungus commonly found on grapes (Chulze, 2006), apples (Oelofse, 2006) and tomatoes (Yildz and Baysal, 2006). Bali et al. (2008) reported that black mold *A. niger* were caused post harvest spoilage in sweet orange and acid lime at field. Okereke et al. (2010) indicated that the fungi species isolated from the infected mangoes were *A. niger, Alternaria* sp. *Botryodiolodia theobromae* and *Colletotrichum gloeosporioides. Fusarium* sp, *A. flavus* and *Phoma* sp. were also isolated but could not prove pathogenicity when inoculated into healthy mango fruits.

A. niger was responsible for brown round shaped spots showing a depression. Penetration of the fruits was through injuries and weakened areas of the mango flesh. Although, *A. niger* var. Tieghem (IMI No. 29005) was isolated from a spoiled ripe mango. Artificial infection studies showed that the fruits were susceptible to infection at all stages of ripeness (Palejwala et al., 1987). In post-harvest conditioned mango get infected by several fungal diseases like Rhizopus rot, Anthracnose, stem end rot, *A. niger* rot, Penicillium rot, *Aspergillus fumigatus*, *A. flavus* rot etc. (Dasgupta and Bhatt, 1946). The *A. flavus was* investigated by incubating inoculated mango fruits at different temperature, and at 35℃ and 100% R.H *A. flavus* rot severity was maximum (Gadgile and Chavan, 2010). However, eighty-three percent of the citrus fruit samples showed fungal growth at levels ranging from 25 to 100% of tested fruits and *Fusarium* spp. were the most common fungi in citrus fruits (Tournas and Katsoudas, 2005). Studies on the fungi associated with tomato rot showed seven fungi associated with fruit rot of tomato including *Fusarium equiseti, A. flavus* and *A. niger*, they were all pathogenic on tomato fruits (Oladiran and Iwu, 1993). *A. flavus* and *A. fumigatus* caused tomato spoilage were also investigated by Adisa (1993). Peach and orange had been studied for fungal decay in storage and its relation to shop (local storage places) and a number of *Aspergillus* spp., *A. nigei, A. nidulans, A. variecolour, A. fumigatus, A. Candidus* had been isolated (Sinha, 1946).

Screening of plant cell wall degrading enzymes

Generally, spoilage fungi exploit the fruit using extracelluar lytic enzymes that degrade the cell wall of fruit to release water and other intercellular constituents for using as nutrients for their growth. Therefore, the isolated fungi *F. oxysporum, A. oryzae, A. awamori*, *A. phoenicis, A. tubingensis*, *A. niger*, *A. flavus*, *A. japonicus, A. foetidus* and *R. stolonifer* were cultured on their spoilage fruits banana, orange, lemon, tomato and peach, apple, grape, date, mango, pokhara, apricot and kiwi peels in comparison with PDB medium. Xylanase, polygalacturonase, cellulase and α-amylase were detected in the cell-free broth of all tested fungi. Xylanase and polygalacturonase had highest level contents as compared to the cellulase and α-amylase (Tables 1, 2, 3, 4). This is consistent with several papers which suggested that polygalacturonases and xylanases are important pathogenecity factors for spoilage fungi (Dimatteo et al., 2006). Recently, Niturea et al. (2008) reported that both acidic and alkaline conditions the organism produced significant levels of inducible xylanase and amylase enzymes and the production of cellulase was lower compared with other enzymes. The secretion of pectin degrading enzymes during infection to the plants has been reported from various plant pathogenic fungi such as *F. oxysporum, Botrytis cinerea, Sclerotinia sclerotiorum* (tenHave et al., 2001; de las Heras et al., 2003; Li et al., 2004).

In the present study, xylanase had highest level in *A. tubingensis* (2786 ± 55 units/100 ml), *A. awamori* (1713 ± 85 units/100 ml) grown on peach and lemon with agitation, respectively, and *F. oxysporum* (3535 ± 176 units/100 ml), *A. niger* (1289 ± 77 units/100 ml) grown on banana and apple with stationary, respectively. Low level of xylanase activity (> 500 units/ 100 ml and < 1000 units/100 ml) was detected for some tested fungi grown on fruit peels and PDB with agitation and stationary phases (Tables 1, 2, 3 and 4). Comparing the polygalacturo-nase, the highest level of activity was detected in the *A. japonicus* (7433 ± 327 units/100 ml), *R. stolonifer* (4547 ± 227 units/100 ml), *A. niger* (4197 ± 209 units/100 ml) grown on PDB with stationary and *A. oryzae* (3124 ± 62 units/100 ml) and *A. niger* (4416 ± 44 units/100 ml) grown on orange peel and PDB with agitation, respectively. Several tested fungi grown on fruit peels and PDB with agitation and stationary phases had moderate (> 500 units/ 100 ml and < 2000 units/100 ml) and low (< 500 units/ 100 ml) polygalacturonase activity levels. The levels of xylanase activity were very low in all tested fungi grown on PDB as compared to fruit peels. Previous studies reported that the same tested fungi have been produced from several plant cell wall degrading enzymes. Genus *Fusarium* was able to secrete several cell wall degrading enzymes such as cellulase, xylanase, α-amylase and pectinase (Di Pietro et al., 2003). *F. oxysporum* produced high level of xylanase (Simoes et al., 2009). Tissues infected by *F. oxysporum* produced the highest pectolytic enzyme activity among the fungi studied (Bahkali et al., 1997). Filamentous fungi, *Aspergillus* spp. are widely distributed among the spoilage fruit fungi and also secreted several plant cell wall degrading enzymes. Induction of

Table 1. Cell wall degrading enzymes from spoilage fungi cultured on fruit peels with agitation.

Fruit peels	Fungi	Units / 100 ml			
		Xylanase	PGase	Cellulase	Amylase
Banana	*Fusarium oxysporum*	116 ± 3	948 ± 18	25 ± 2	20 ± 1
Orange	*Aspergillus oryzae*	176 ± 12	3124 ± 62	30 ± 2	69 ± 3
Lemon	*Aspergillus awamori*	1713 ± 85	912 ± 27	45 ± 3	203 ± 4
Tomato	*Aspergillus phoenicis*	618 ± 61	503 ± 5	149 ± 4	20 ± 1
Peach	*Aspergillus tubingensis*	2786 ± 55	856 ± 17	70 ± 2	39 ± 1
Apple	*Aspergillus niger*	305 ± 15	1052 ± 31	121 ± 12	25 ± 2
Grape	*Fusarium oxysporum*	221 ± 17	401 ± 20	55 ± 1	32 ± 2
Date	*Rhizopus stolonifer*	172 ± 13	189 ± 11	67 ± 3	43 ± 2
Mango	*Aspergillus flavus*	123 ± 6	1075 ± 32	87 ± 5	45 ± 3
Pokhara	*Aspergillus japonicus*	121 ± 7	467 ± 23	55 ± 4	18 ± 1
Apricot	*Aspergillus japonicus*	373 ± 12	322 ± 19	23 ± 1	48 ± 2
Kiwi	*Aspergillus foetidus*	111 ± 7	415 ± 29	44 ± 2	22 ± 1

Each value represents the mean of three runs ±S.E.

Table 2. Cell wall degrading enzymes from spoilage fungi cultured on fruit peels with stationary.

Fruit peels	Fungi	Units / 100 ml			
		Xylanase	PGase	Cellulase	Amylase
Banana	*Fusarium oxysporum*	3535 ± 176	122 ± 2	15 ± 1	14 ± 1
Orange	*Aspergillus oryzae*	360 ± 10	914 ± 82	20 ± 1	37 ± 2
Lemon	*Aspergillus awamori*	438 ± 17	1267 ± 88	48 ± 3	18 ± 1
Tomato	*Aspergillus phoenicis*	751 ± 7	566 ± 28	22 ± 1	17 ± 1
Peach	*Aspergillus tubingensis*	112 ± 5	179 ± 12	115 ± 6	20 ± 2
Apple	*Aspergillus niger*	1289 ± 77	63 ± 1	20 ± 1	33 ± 2
Grape	*Fusarium oxysporum*	126 ± 6	65 ± 3	24 ± 1	22 ± 1
Date	*Rhizopus stolonifer*	337 ± 16	281 ± 22	58 ± 2	67 ± 3
Mango	*Aspergillus flavus*	186 ± 3	409 ± 36	48 ± 1	60 ± 3
Pokhara	*Aspergillus japonicus*	157 ± 6	33 ± 1	35 ± 1	53 ± 4
Apricot	*Aspergillus japonicus*	127 ± 3	27 ± 1	146 ± 8	23 ± 1
Kiwi	*Aspergillus foetidus*	143 ± 11	544 ± 10	21 ± 1	44 ± 1

Each value represents the mean of three runs ±S.E.

polygalacturonases from *A. oryzae* by pectin was significantly higher than when rinds of citrus fruits were used as inducer (Malvessi and da Silveira, 2004). *A. oryzae* produced xylanase and polygalacturonase in solid-state and submerged cultures (Oda et al., 2006). *A. awamori* showed high extracellular endoxylanase (100 units/ml) and β-xylosidase activities (3.5 units/ml) when grown on milled sugar cane bagasse as the principal carbon source (Lemos and Nei, 2002). *A. tubingensis* produced xylanase (Bakri et al., 2010) and polygalacturonase (Kester et al., 1996) when synthetic media used as substrates under submerged culture cultivation. Also, induction of xylanolytic activity was examined in *A. phoenicis* grown on synthetic medium (Rizzatti et al., 2008).

Conclusion

This study detected the profile of spoilage fungi which caused pathogenecity of some local and imported fruits in Jeddah city, in addition to the fungal enzymes which responsible for the fruit spoilage. The characterization of these enzymes especially inhibitor studies, to combat these fungi, will be detected in the future studies.

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Table 3. Cell wall degrading enzymes from spoilage fungi cultured on PDB with agitation.

Fruit	Fungi	Units / 100 ml			
		Xylanase	**PGase**	**Cellulase**	**Amylase**
Banana	*Fusarium oxysporum*	219 ± 6	2403 ± 48	47 ± 1	180 ± 10
Orange	*Aspergillus oryzae*	142 ± 7	1408 ± 56	75 ± 5	15 ± 1
Lemon	*Aspergillus awamori*	258 ± 5	58 ± 2	54 ± 4	57 ± 3
Tomato	*Aspergillus phoenicis*	182 ± 14	1313 ± 39	23 ± 2	26 ± 1
Peach	*Aspergillus tubingensis*	411 ± 4	948 ± 28	14 ± 1	92 ± 2
Apple	*Aspergillus niger*	258 ± 12	4416 ± 44	22 ± 2	22 ± 1
Grape	*Fusarium oxysporum*	380 ± 11	36 ± 1	37 ± 3	52 ± 2
Date	*Rhizopus stolonifer*	223 ± 4	167 ± 8	26 ± 2	13 ± 1
Mango	*Aspergillus flavus*	76 ± 1	445 ± 22	26 ± 1	77 ± 6
Pokhara	*Aspergillus japonicus*	619 ± 18	1467 ± 44	24 ± 1	111 ± 11
Apricot	*Aspergillus japonicus*	197 ± 13	3043 ± 91	33 ± 2	114 ± 7
Kiwi	*Aspergillus foetidus*	192 ± 3	145 ± 2	21 ± 1	119 ± 7

Each value represents the mean of three runs ±S.E.

Table 4. Cell wall degrading enzymes from spoilage fungi cultured on PDB with stationary.

Fruit	Fungi	Units / 100 ml			
		Xylanase	**PGase**	**Cellulase**	**Amylase**
Banana	*Fusarium oxysporum*	101 ± 7	1068 ± 32	179 ± 17	150 ± 7
Orange	*Aspergillus oryzae*	111 ± 5	248 ± 7	1002 ± 20	335 ± 23
Lemon	*Aspergillus awamori*	451 ± 27	298 ± 26	121 ± 1	61 ± 6
Tomato	*Aspergillus phoenicis*	41 ± 3	857 ± 17	115 ± 5	24 ± 1
Peach	*Aspergillus tubingensis*	50 ± 1	3503 ± 35	19 ± 1	99 ± 3
Apple	*Aspergillus niger*	116 ± 6	4197 ± 209	71 ± 4	80 ± 2
Grape	*Fusarium oxysporum*	55 ± 1	467 ± 23	31 ± 2	62 ± 1
Date	*Rhizopus stolonifer*	157 ± 7	4547 ± 227	87 ± 6	121 ± 6
Mango	*Aspergillus flavus*	147 ± 10	1211 ± 84	33 ± 3	106 ± 8
Pokhara	*Aspergillus japonicus*	309 ± 27	2218 ± 22	24 ± 2	44 ± 1
Apricot	*Aspergillus japonicus*	292 ± 11	7443 ± 372	91 ± 4	150 ± 6
Kiwi	*Aspergillus foetidus*	109 ± 5	2427 ± 145	55 ± 4	49 ± 4

Each value represents the mean of three runs ±S.E.

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