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Mycotoxin-producing *Penicillium* species Involved in Apple Blue Mold

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Mycotoxin production and pathogenicity of *Penicillium* spp. involved in apple blue mold were investigated. Twenty four isolates representing nine species recovered from blue moldy apple samples were used. Mycotoxin production of these isolates was assayed using HPLC. Statistical analysis of pathogenicity test was undertaken and LSD was used to compare means. Mycotoxin assay revealed that all isolates were capable of producing patulin in their culture media with the highest production (19.70 ppm) from *Penicillium griseofulvum* isolate No. 14. In respect of other mycotoxins, *Penicillium puberulum* isolate No. 17 was the highest producer of citrinin (21.63 ppm), while *Penicillium verrucosum* isolate No. 22 was the highest producer of penicillic acid (8 ppm). Pathogenicity test revealed that all *Penicillium* isolates were pathogenic, exhibiting variable disease severity toward infested apple fruits. This study showed that all tested *Penicillium* isolates were virulent for apple fruits and *in vitro* toxigenic, capable of producing patulin, the characteristic mycotoxin of *Penicillium* species.

Key words: Apples, *Penicillium*, HPLC, Mycotoxins.

Mycotoxins that frequently occur in foodstuff, under variable conditions are associated with chronic health risks, such as allergies, cancer induction, digestive blood and immune suppression[1, 2]. *Penicillium* mycotoxins are among the toxic secondary metabolites of fungi that may be detrimental to humans and/or animals health[3, 4]. Agricultural commodities are vulnerable to mycotoxin contamination under adverse range of conditions. Contamination of apple and apple based products with *Penicillium* mycotoxins is also well known[5,6].

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Penicillium fungi are common contaminants, known as an apple blue mold agents worldwide. They cause a soft rot of transit, marketed and storage apples, resulting in destruction of the whole fruit in 5-7 days at ambient temperature[7]. The quality and maintenance of apple fruits are greatly affected by blue mold, the very common postharvest fungal disease, which may cause more than 70% of decay in stored apples (*Malus domestica* Borkh.). Apple blue mold caused mainly by *Penicillium expansum* but more than 50 *Penicillium* species found to be involved in such disease[8-10].

Apple molding *Penicillia* are responsible for *in vitro* and *in situ* production of various kinds of potent mycotoxins[11, 12]. These fungi are

widespread, attack different food commodities particularly in the storage and often produce variety of toxic secondary metabolites. Harmful mycotoxins and carcinogenic compounds such as citrinin, patulin, penicillic acid, roqoefortine and other secondary metabolites, which affect fruit value and harm the customers, could be produce by *Penicillium* fungi [13].

This study was aimed to investigate the mycotoxin productivity and pathogenicity of *Penicillium* species involved in postharvest apple blue mold.

MATERIALS AND METHODS

Penicillium isolates

Isolates used in this study were recovered from apple fruit samples, exhibiting typical blue mold symptoms collected from different locations (markets) in Riyadh, Saudi Arabia. Twenty four isolates representing nine *Penicillium* species were investigated. All tested isolates were identified by Assiut University Mycological Centre (AUMC), Egypt. Macro- and micromorphological features of *Penicillium* cultures were used for fungal identification as described by Pitt[14].

Mycotoxin assay

Mycotoxin productivity of 24 *Penicillium* isolates representing 9 species was studied using HPLC (PerkinElmer® Brownlee™ validated C18, 250 mm, equipped with UV detector. The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min). Isolates were aseptically cultured in 100 ml flasks of malt extract broth in triplicates and incubated at 27±2°C for 10 days. Cultures were blended for 2 min using a high speed homogenizer and filtered using glass filter paper. Patulin was extracted from homogenized filtrate using acetonitrile: water (5:95 v:v) solution. The solvent was then evaporated at 35°C under vacuum. The dried residues containing Patulin were dissolved in 1 ml of acetonitrile: water (5:95 v:v) solution. Extract was then passed through a 0.45 ìm micro-filter prior to HPLC analysis. The method described by Christian[15] was used to determine patulin.

Citrinin was extracted from homogenized filtrate using dichloromethane with the addition of phosphoric acid, and extract was then cleaned up on polyamide columns. HPLC analysis of citrinin employed the method described by Franco *et al.*[16]. The mobile phase consisted of methanol/acetonitrile/water (3:3:4 v:v:v), the pH value of the mixture was 2.5, and the flow rate was the 0.15 ml/min.

Penicillic acid was extracted with an acidic acetonitrile solution and cleaned using bonded phase cyano column. Hexane/propanol/acetic acid mobile phase was used in HPLC analysis of penicillic acid[17].

Pathogenicity

Apple fruits were surface sterilized by soaking in 70% ethanol for 3 min and wounded four times halfway between the calyx and the stem end, by removing plugs of 5mm diameter and 3mm depth from the surface. Fuji, green and red apple fruits were artificially inoculated in triplicates according to Pianzzola *et al.*[18]. A loopfull of dry spores (about 10^7or 10000000 conidia on a 4-mm-diameter loop) was transferred from a 4 to 7-day-old culture to each of the four wounds on a fruit surface. Inoculated fruits were immediately enclosed in individual plastic bags and incubated on the laboratory bench at 18–23°C. The diameter of the decayed area was measured after 7 and 12 days.

Statistical analysis

Analysis of variance (ANOVA) was performed with the MSTAT-C statistical package, Michigan State Univ., USA). Least significant difference (LSD) and Duncan's multiple test were used to compare means.

RESULTS

Mycotoxin assay

Mycotoxin assay revealed that *Penicillium* isolates were varied in the kind and concentration of mycotoxins produced. All isolates were capable of producing detectable amounts of patulin (ranged from 0.50-19.70 ppm) in the culture media, with the highest production (19.70 ppm) from *P. griseofulvum* isolate No. 14. Meanwhile, some isolates were completely failed to produce any citrinin or penicillic acid in the culture media. On the other hand, *P. puberulum* isolate No. 17 was the highest producer of citrinin (21.63 ppm), while *P. verrucosum* isolate No. 24 was the highest producer of penicillic acid (8 ppm) (Table 1).

Pathogenicity

ANOVA (Table 2) revealed that *Penicillium* isolates, apple cultivars and their interaction were highly significant sources of variation in disease severity on apple fruits. Due to the significance of *Penicillium* isolates x apple cultivars interaction; *Penicillium* isolates exhibited different virulence's against apple cultivars as well as within the same cultivar. *Penicillium* isolates were the first in importance as a source of variation in disease severity on apple fruits, while isolates x cultivars interaction, was the second important topic (Fig. 1A).

Table (3) shows the pathogenicity of 24 *Penicillium* isolates on Fuji, green and red apple cultivars. Due to the significance of *Penicillium* isolates x apple cultivars interaction; LSD for general mean was unconsidered. Pathogenicity test

Table 1. Mycotoxin productivity of *Penicillium* isolates involved in apple blue mold

S. No	*Penicillium* isolates	Mycotoxin (PPM)		
		Citrinin	Patulin	Penicillic acid
1.	*P. asperosporum*	5.20	6.60	0.00
2.	*P. aurantiogriseum*	2.41	17.20	0.00
3.	*P. canescens*	1.37	4.60	0.30
4.	*P. citrinum*	0.87	2.80	0.00
5.	*P. citrinum*	0.00	14.2	0.00
6.	*P. citrinum*	0.00	1.40	0.00
7.	*P. citrinum*	0.00	4.50	0.00
8.	*P. citrinum*	0.60	0.50	1.20
9.	*P. expansum*	0.00	1.60	0.00
10.	*P. expansum*	0.00	2.00	0.30
11.	*P. expansum*	1.80	5.50	0.90
12.	*P. expansum*	11.00	7.30	1.20
13.	*P. expansum*	0.00	1.90	0.00
14.	*P. griseofulvum*	8.87	19.70	0.00
15.	*P. griseofulvum*	0.00	1.90	0.20
16.	*P. griseofulvum*	0.00	5.00	1.20
17.	*P. puberulum*	21.63	8.90	3.60
18.	*P. puberulum*	0.59	3.60	0.80
19.	*P. puberulum*	4.50	5.70	0.90
20.	*P. restrictum*	0.00	2.70	0.00
21.	*P. verrucosum*	0.00	1.50	3.40
22.	*P. verrucosum*	1.09	4.10	8.00
23.	*P. verrucosum*	00.00	3.20	1.50
24.	*P. verrucosum*	00.00	1.90	0.00

Table 2. Analysis of variance of effects of *Penicillium* isolates, apple cultivar and their interaction on fruit infection

Source of variation	D.F	M.S	F. value	P>F
Replication	2	30.198	12.366	0.000
Isolate (I)	23	249.262	102.071	0.000
Cultivar (C)	2	1456.135	596.276	0.000
I x C	46	79.879	23.710	0.000
Error	142	2.442		

D.F.= Degrees of freedom M.S.= Mean square

indicated that isolate No. 2 and 13 were highly pathogenic on green apple with non-significant difference, while they were highly pathogenic on Fuji apple and significantly different. In addition the same isolates exhibited moderate pathogenicity on red apple but with non-significant difference (Table 3).

On the other hand isolates No. 3, 10, 12, and 24 were pathogenic and insignificantly different on green apple, while they were also pathogenic and significantly different on Fuji apple. The same isolates were pathogenic to red apple and significantly different from each other except for isolates No. 10 and 24 were insignificantly different.

ANOVA (Table 4) revealed that *Penicillium* species and apple cultivars were highly significant sources of variation in disease

Table 3. Pathogenic behavior of 24 *Penicillium* isolates on 3 apple fruit cultivars

S. No.	*Penicillium* Isolates	Spot diameter (mm)			
		Fuji apple	Green apple	Red apple	Mean
1.	*P. asperosporum*	28.67	23.00	22.67	24.78
2.	*P. aurantiogriseum*	24.67	22.50	23.00	23.39
3.	*P. canescens*	24.33	25.00	21.50	22.94
4.	*P. citrinum*	25.67	15.00	19.00	19.89
5.	*P. citrinum*	18.67	19.00	16.50	18.06
6.	*P. cltrinum*	18.00	14.00	10.33	14.11
7.	*P. citrinum*	37.33	35.00	15.00	29.11
8.	*P. citrlnum*	18.00	21.33	12.67	17.33
9.	*P. expansum*	16.33	19.33	12.00	15.89
10.	*P. expansum*	31.33	25.33	15.67	24.11
11.	*P. expansum*	15.33	16.00	9.00	13.44
12.	*P. expansum*	21.00	26.00	12.33	18.44
13.	*P. expansum*	27.67	14.00	10.00	17.44
14.	*P. griseofulvum*	10.67	19.33	17.00	15.67
15.	*P. griseofulvum*	24.33	15.00	13.00	17.56
16.	*P. griseofulvum*	17.33	15.50	17.00	16.61
17.	*P. puberulum*	20.00	19.00	16.33	18.44
18.	*P. puberulum*	19.33	20.00	10.33	16.56
19.	*P. puberulum*	16.67	24.50	22.33	21.17
20.	*P. restrictum*	47.33	23.00	20.00	30.11
21.	*P. verrucosum*	26.33	18.00	10.33	18.22
22.	*P. verrucosum*	41.00	35.67	18.33	31.67
23.	*P. verrucosum*	16.67	17.33	7.00	13.67
24.	*P. verrucosum*	37.67	25.00	18.00	26.89
	Mean	24.35	20.91	15.43	

LDS for cutover of apple isoletes of *Penicillium* interaction (P<0.05) =2.52 (P<0.01)=3.33

Table 4. Analysis of variance of effects of Penicillium pecies, apple cultivar and their interaction on fruit infection

Source of variation	D.F	M.S	F. value	P>F
Replication	2	18.11	0.37	N.S
Species (S)	2	345.11	7.13	0.003
Cultivation (C)	4	111.72	2.31	0.082
S X C	8	35.33		N.s
Error	28	48.39		

Table 5. Effect of apple cultivar, *Penicillium* species and their interaction on fruit infection

S. No.	*Penicillium* Isolates	Spot diameter (mm)			
		Fuji apple	Green apple	Red apple	Mean
1.	*P. citrinum*	24.67	22.67	13.93	20.42ab
2.	*P. expansum*	20.97	20.20	12.23	17.80b
3.	*P. griseofulvium*	17.43	16.60	15.77	16.60b
4.	*P. puberulum*	21.80	18.67	15.33	18.60ab
5.	*P. verrucosum*	35.01	26.23	15.54	25.59a
	Mean	20.87a	20.17a	18.57b	

General means value with the same letter were insignificant

severity on apple fruits, while their interaction was insignificant. Apple cultivars were the first in importance as a source of variation in disease severity on apple fruits, while *Penicillium* species were the second important topic (Fig. 1B).

In respect of the effect of *Penicillium* species on apple blue mold infection, pathogenicity of species however didn't influenced by tested cultivars (Table 5). It was shown that *P. verrucosum* was the highest virulent species, while *P. griseofulvum* was the least one.

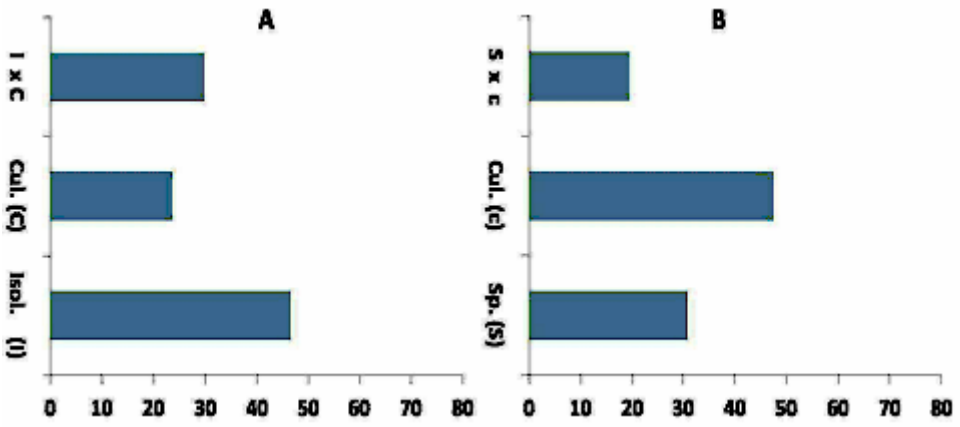

Fig. 1. Relative contributions of A: isolates, cultivar and their interaction; B: species, cultivar and their interaction; on apple fruit infection

DISCUSSION

Results of this study indicated that all examined isolates were capable of producing patulin, the *Penicillium* characterizing mycotoxin, in the culture media. Harmful mycotoxins and carcinogenic compounds; citrinin, patulin and penicillic acid, that affect fruit value and harm the health of the customers were found to be produced by tested isolates. The ability of several *Penicillium* species involved in postharvest decay of fruits to produce such mycotoxins has previously been demonstrated[13, 19]. Differences in isolate productivities of citrinin and penicillic acid that revealed in the present study, have also been documented. In a Canadian study on mycotoxin productivity of *P. expansum,* involved in apple blue mold; 91% of 24 tested isolates were citrinin producers and 83% were patulin producers[20]. Differences between and within tested fungi in mycotoxin productivity could be attributed to different genetic basis[21, 22].

Obtained results also demonstrated that all *Penicillium* isolates were capable of producing

typical blue mold symptoms in apple fruits. This result generally agreed with the published literature, where wound-invading *Penicillium* species were the most common and destructive post-harvest pathogens responsible for apple blue mold[18,23]. Various species of those had frequently been isolated from blue moldy fruits in different countries[8,10]. Variation in disease severity resulted from different *Penicillium* isolates had also been documented[9]. This might be attributed to fruit anatomical characteristics, which may allow the pathogen to enter across the natural openings. Apple fruit cultivars that showed a greater number of open lenticels were more susceptible to *P. expansum*[8]. Variation in disease severity observed as varied lesion diameters on apple fruits may also refers to isolate aggressiveness; that might be due to isolate ability to produce extracellular pectinolytic enzymes[24, 25].

CONCLUSION

It could be concluded that all tested *Penicillium* isolates were virulent for apple fruits and *in vitro* toxigenic, capable of producing patulin, the characteristic mycotoxin of *Penicillium* species. Probable production of harmful mycotoxins and carcinogenic compounds such as citrinin, patulin and penicillic acid, well affect fruit value and harm the health of the customers. Rigorous quarantine and healthy storage conditions should be undertaken to minimize fungal contamination of imported apple fruits and prevent further hazard to human health.

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