J. Agric. Chem. and Biotechn., Mansoura Univ. Vol. 3 (9): 391 - 397, 2012

EFFECT OF SOME ORGANIC ACIDS ON FUNGAL GROWTH AND THEIR TOXINS PRODUCTION

Hassan, R. A.*; M. I. Sand* and Sh. M. El-Kadi**

*** Agric. Chem. Dept., Fac. of Agric. Mans. Univ., Mansoura, Egypt .**

****Agric. Microbiol. Dept., Fac. of Agric. Damietta Univ., Damietta, Egypt .**

ABSTRACT

Many of food products and agricultural crops are contaminated with mycotoxins. Aflatoxins are the secondary metabolites of certain fungi such as Aspergillus flavus which have the ability to be toxigenic, carcinogenic and mutagenic agents. Hence many chemical compounds have been used in feed to prevent the fungal growth and aflatoxin formation. Organic acids have been used to prevent the growth reproduction of harmful fungi and secreting of aflatoxins. The effect of eight organic acids as antifungal agents on the growth of four fungi were studied. Acetic acid (10%) showed the highest inhibition effect on A. flavus growth being 45.21% while tartaric acid (5%) and citric acid (5%) gave the lowest inhibition effect of 0.42%. Formic, acetic and propionic acids had the highest inhibition effect on A. flavus growth. All Different organic acids under present study reduced aflatoxin B_1 (AB_1) secretion. The highest inhibition (50%) was observed for Rhizopus nigricans in the presence of formic acid (10%)

Keywords : Aflatoxin B_1, organic acids, antifungal, Aspergillus flavus, Penicillium purpurogenum, Fusarium oxysporum and Rhizopus nigricans.

INTRODUCTION

Mycotoxins are produced by fungi under laboratory conditions or naturally in various agricultural products (Massey *et al* 1995). Since 1960, mycotoxins have been receiving worldwide attention. Nowadays, several groups of mycotoxins are known such as ergot, aflatoxins, ochratoxins, citrinin, patulin and fumonisines (Mohanamba, 2002). The toxigenic *A. flavus* and *A. parasiticus* producing aflatoxins were isolated from different Egyptian commodities including corn and barley (Kheiralla, 1994). The occurrence of aflaoxin and its toxic effects on feed ingredients, livestock and poultry fees were investigated by Mohanamba *et al.* (2002). They found that poultry feed samples were contaminated with *A. flavus, A. fumigatus, A. niger, Penicillium sp.* and *Mucor sp.* They also found that aflaoxin B_1 could detect in 96 samples from 294 poultry feed samples.

Members of *Fusarium, Aspergillus* and *Penicillium* species were noted to be the major fungal populations in feed and corn. *Fusarium moniliforme* were the predominant fungi in feed that had been stored for about a year. The predominant naturally occurring fungi observed in the moldy corn belonging mainly to *Penicillium purpurogenum, Aspergillus glaucus* and *A. candidus.* (Dalie *et al.* 2010). Thin layer chromatography and ELISA were compared for the determination of aflatoxins, ochratoxin and Zearalenone by Milanez *et al.*, (1997). They found that the ELISA method was simpler to use, fast, showing higher concentrations than the thin layer chromatography method. Organic acids are known in the feed industry as an effective and affordable tool to control mold growth. However, there are little researches available on the

relative efficacy of organic acids (Higgins and Brinkhaus, 1999). Organic acids generally recognized as safe agents for the preservation of foods, diffuse through the membrane of the target organisms in their hydrophobic undissociated form and then reduce cytoplasmic pH and stop metabolic activities. Another hypothesis, organic acids act on the plasmic membrane by neutralizing its electrochemical potential and increasing its permeability, and caused the death of susceptible organisms (Dalie *et al.*, 2010).

The objectives of this study were to: (1) investigate the effect of organic acids of the fungal growth. (2) compare the potency of selected organic acids as an antifungal compounds on the secretion of (AB_1).

MATERIALS AND METHODS

Fungal strains used:

Three local fungal isolates, namely *Aspergillus flavus*, *Penicillium purpurogenum* and *Rhizopus nigricans* were used in this study obtained from Microbiol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The fourth one (*Fusarium oxysporum*) was obtained from plant pathology Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The fungal isolates were maintained on potato dextrose agar medium (PDA) slants at 5°C till use (Oxoid, 1982). Fungal isolates were subcultured on new slants of PDA and incubated at 30°C for 10 days before use.

Fungal spores suspensions and inoculum size used

Spores appeared on PDA slant after 10 days were scraped using 5 mL of 0.8% NaCl sterilized saline solution and then suspended in 45 mL of the same solution. Spores were measured directly by according to the of method Pintado *et al.*, (1997) using a Hematocytometer (model Buerker measured MOM). The spores suspensions stocks were stored at 4°C and adjusted to approximately $1xl0^6$ spores/ml before use. The inoculum size used was 1 ml ($1x10^6$ spores/ml) for inoculating the cultivation medium for all experiments (Karunaratne and Bullerman 1990).

Antifungal organic acids:

Eight organic acids were used as antifungal agents, namely propionic acid (99%, BDH) acetic acid glacial (99.8%, Almasria for Chemicals) formic acid (85%, BDH) lactic acid (Chemicals Ltd Poole England), tartaric acid (99%, ADWIC) citric acid anhydrous (99%, ADWIC), oxalic acid (99.5%, ADWIC), and malic acid (99%, LOBA Chemie, India). These acids were added at the time of inoculation to reduce the fungal growth and mycotoxins production. Potato dextrose broth (PDB) was used as a basal medium to evaluate the spores germination and growth of the tested fungi by surface culture technique. Forty ml of PDB were put into a 250 ml Erlenmeyer flask. All organic acids were dissolved in water at levels of 25 and 50%, 10 ml of each was added to the basal medium (PDB) to make a final concentrations (5% and 10%) of each organic acid as an antifungal agents using a milipore filter (0.2 µm, Flow Pore D, Germany). The inhibition percentage was calculated by the difference in growth (mycelial dry weight) in the absence or presence of the antifungal agent after 8 days of incubation at 30°C (Lin and Chen, 1995). All experiments were conducted in three replicates and values

of final pH were determined in the culture filtrate using a pH meter (model Hanna pH 211 microprocessor pH meter).

Determination of mycelial dry weight (MDW):

Mycelial mat resulted in surface culture was filtered through double-layered Whatman filter paper No. 1 and washed twice with distilled water, dried in an electrical oven (Nemmert, W. Germany) at 80°C to a constant weight (g/l) (Ansari and Shrivastava, 1991). Mycelial mat was determined using an electronic balance (type BL-32OH Shimadzu corporation, Japan). After filtration, the supernatant was collected and used for mycotoxins determination.

Purification and determination of aflatoxin B1:

Culture filtrates were extracted three times with 100 ml of chloroform. The extract was filtered through anhydrous sodium sulfate and evaporated under vacuum at 40°C. The residue was dissolved in 5 ml methanol 70%. Aflatoxin B_1 was determined by ELISA technique according to the A.O.A.C (2000). For the qualitative determination of aflatoxins compounds, the silica plates were developed in solvent system of toluene : ethyl acetate : formic acid (6:3:1, v/v/v) and scanned in a vitatron LTD 100 densitometer equipped with a mercury lamp (excitation at 366 nm and emission at 460 nm). The recorded areas of the spots were compared with standards of the respective compounds. All samples and standard were analyzed in duplicate. The standard of Aflatoxin B_1 was purchased from Sigma Chemical Co., (USA).

RESULTS AND DISCUSSION

Results presented in Table 1 show that, the effect of organic acids levels on *Aspergilus flavus* growth. The highest acidity appeared for oxalic acid that had the lowest initial pH value (0.14) at a concentration of 10%. The lowest acidity recorded for propionic acid that had the highest pH value of 2.71, at a concentration of 5%. Higgins and Brinkhaus (1999) reported that, the mechanism of inhibition molds growth by organic acids is generally not considered a pH phenomenon. However, it is well known that growth and morphology of molds is influenced by the pH of media. Acetic acid (10%) has the highest inhibition effect on *A. flavus* growth being 45.21% and the final pH was 3.25, but tartaric acid and citric acid gave the same lowest inhibition effect at 5% concentration (0.42%) and final pH was 3.12 and 3.24, respectively. Dalie *et al.* (2010) reported that, with a high dissociation constant, acetic acid was described as being more effective than lactic acid and had the best inhibitor of *A. flavus* growth.

Table 2. show that, the lowest value of *P. purpurogenum* MDW was 5.92 g/l when acetic acid (10%) was used. The inhibition effect and the final pH were 40.92% and 3.31, respectively. The highest value of MDW was 9.38 g/l when tartaric acid (5%) was used. Little correlation was observed between the final pH of the organic acids and its relative efficacy. For example, when the final pH value of oxalic acid (10%) was 1.75, inhibition growth was 32.74%. Similar results were obtained by Higgins and Brinkhaus, (1999) who reported that, the final pH values of cultivation broth for lactic acid and acetic

acid were 3.35 and 3.81, respectively, these tow acids displayed little efficacy in the growth controlling of *Aspergillus*, *Fusarium* and *Penicillium* species.

Table 1. Effect of organic acids levels on *Aspergilus flavus* growth.

Organic acids	5%				10%			
	Initial pH	Final pH	MDW (g/l)	Inhibition %	Initial pH	Final pH	MDW (g/l)	Inhibition %
Propionic	2.71	3.96	7.06	26.46%	2.45	3.49	5.72	40.42%
Acetic	2.56	3.76	6.92	27.92%	2.28	3.25	5.26	45.21%
Formic	1.75	3.15	7.50	21.88%	1.53	2.56	6.26	34.79%
Lactic	2.09	3.43	9.16	4.58%	1.83	2.62	8.58	10.63%
Tartaric	1.48	3.12	9.56	0.42%	1.29	2.40	8.00	16.67%
Citric	1.18	3.24	9.56	0.42%	1.12	2.60	7.90	17.71%
Oxalic	0.43	1.96	8.86	7.71%	0.14	1.58	8.78	8.54%
Malic	1.91	2.31	9.46	1.46%	1.72	2.62	9.06	5.63%
The Initial pH, final pH and MDW of the control were 5.0, 3.87 and 9.60 g/l, respectively								

Table 2. Effect of organic acids levels on *Penicillium purpurogenum* growth.

Organic acids	5%				10%			
	Initial pH	Final pH	MDW (g/l)	Inhibition %	Initial pH	Final pH	MDW (g/l)	Inhibition %
Propionic	2.71	4.25	8.10	19.16%	2.45	3.49	6.84	31.74%
Acetic	2.56	4.03	7.66	23.55%	2.28	3.31	5.92	40.92%
Formic	1.75	3.00	9.08	9.38%	1.53	2.39	8.32	16.97%
Lactic	2.09	3.27	9.36	6.59%	1.83	2.68	8.22	17.96%
Tartaric	1.48	3.63	9.38	6.39%	1.29	2.44	8.28	17.37%
Citric	1.18	2.89	9.16	8.58%	1.12	2.56	8.00	20.16%
Oxalic	0.43	2.18	8.50	15.17%	0.14	1.75	6.74	32.74%
Malic	1.91	3.12	8.50	15.17%	1.72	2.68	7.94	20.76%
The Initial pH, final pH and MDW of the control were 5.0, 4.24 and 10.02 g/l, respectively.								

Formic acid (10%) had a strong effect on *R. nigricans* growth and the lowest value of MDW was 7.52 g/l (Table 3). The highest value of inhibition effect and the final pH were 28.65% and 2.59, respectively. The highest value of MDW was 9.98 g/l when malic acid (5%) was used.

Table 3. Effect of organic acids levels on *Rhizopus nigricans* growth.

Organic acids	5%				10%			
	Initial pH	Final pH	MDW (g/l)	Inhibition %	Initial pH	Final pH	MDW (g/l)	Inhibition %
Propionic	2.71	3.65	8.66	17.84%	2.45	3.96	7.74	26.57%
Acetic	2.56	3.47	8.72	17.27%	2.28	3.17	7.76	26.38%
Formic	1.75	2.91	8.54	18.98%	1.53	2.59	7.52	28.65%
Lactic	2.09	2.79	8.94	15.18%	1.83	2.40	7.78	26.19%
Tartaric	1.48	2.80	8.84	16.13%	1.29	2.41	8.06	23.53%
Citric	1.18	2.64	8.64	18.03%	1.12	2.58	7.96	24.48%
Oxalic	0.43	2.03	9.86	6.45%	0.14	1.58	9.46	10.25%
Malic	1.91	2.95	9.98	5.31%	1.72	2.81	9.32	11.58%
The Initial pH, final pH and MDW of the control were 5.0, 3.89 and 10.54 g/l, respectively.								

Lactic acid (10%) has the highest inhibition effect on *F. oxysporum* growth being 34.45% and the lowest value of MDW was 7.04 g/l (Table 4).

Tartaric acid (5%) revealed the lowest value of inhibition and the final pH of 1.68% and 3.89, respectively. The highest value of MDW was 10.56 g/l when the same acid was used. Lind *et al.*, (2005) studied the effect of propionic, acetic and lactic acid on *A. fumigatus, A. nidulans, P. commune, P. roqueforti and F. sporotrichioides* and they found that, the moulds were inhibited at concentrations between 4 and 30 mM of propionic and acetic acid, while a concentration of 160 mM or more of lactic acid was required for total inhibition. At pH 5, all fungi were inhibited at 60 mM or less of propionic acid, 120 mM or less of acetic acid, but lactic acid concentrations above 500 mM were required for inhibition of most species.

Table 4. The effect of organic acids levels on *Fusarium oxysporum* growth.

Organic acids	5%				10%			
	Initial pH	Final pH	MDW (g/l)	Inhibition %	Initial pH	Final pH	MDW (g/l)	Inhibition %
Propionic	2.71	3.85	8.86	17.51%	2.45	3.17	8.16	24.02%
Acetic	2.56	3.16	8.20	23.65%	2.28	3.23	9.78	8.94%
Formic	1.75	3.08	9.76	9.13%	1.53	2.45	8.70	18.99%
Lactic	2.09	3.73	9.14	14.90%	1.83	2.49	7.04	34.45%
Tartaric	1.48	3.89	10.56	1.68%	1.29	2.27	10.52	2.05%
Citric	1.18	3.64	10.16	5.40%	1.12	2.71	8.50	20.86%
Oxalic	0.43	3.45	10.02	6.70%	0.14	1.44	9.98	7.08%
Malic	1.91	3.85	10.22	4.84%	1.72	2.56	9.32	13.22%
The Initial pH, final pH and MDW of the control were 5.0, 3.98 and 10.74 g/l, respectively.								

Effect of organic acids on aflatoxin B_1 secretion :

Four tests were chosen to determined aflatoxin B_1 (AB_1) production based on the highest inhibition effect on fungal growth. The different organic acids under study reduced aflatoxin B_1 (AB_1) secretion. The highest inhibition (50%) was observed for *R. nigricans* in the presence of formic acid (10%) (Table 5). Acetic acid in 10% level inhibited the toxin secretion from *A. flavus* and *P. purpurogenum* to become 25% and 40%, respectively. Lactic acid (10%) gave 35% inhibition for toxin production in the presence of *F. oxysporum*. Ghosh and Häggblom, (1985) reported that, propionic or butyric acid was added at sublethal doses (1–20%) to a growth medium for supporting growth of *A. flavus* and subsequent aflatoxin production. Gourama and Bullerman, (1995) reported that, the production of aflatoxin B1 by *F. graminarium* was after 1 week of incubation being 0.05 µg/g of substrate. But aflatoxin B1 produced by *Alternaria alternata was* detected after 48 h.

Table 5. The effect of organic acids on aflatoxin B_1 production.

Tested fungi	Aflatoxin B_1 production (ppb)		Inhibition (%)
	treated	control	
A. flavus (treated with acetic acid 10%)	8	12	25%
P. purpurogenum (treated with acetic acid 10%)	6	10	40%
R. nigricans (treated with formic acid 10%)	4	8	50%
F. oxysporum (treated with lactic acid 10%)	6.5	10	35%
The incubation time was 8 days at 30°C Prmicible limits was 20 ppb			

REFERENCES

A.O.A.C (2000). Association of Official Analytical Chemists. Methods of Analysis 17th Ed., Natural Toxins. Washington. USA. 11 (17): 7.

Ansari, A. A. and A. K. Shrivastava (1991). The effect of eucalyptus oil on growth and aflatoxin production by *Aspergillus falvus*. Lett. Appl. Microbiol., 13: 75-77.

Dalie, D.K.D.; A.M. Deschamps and F. R. Forget (2010). Lactic acid bacteria – potential for control of mould growth and mycotoxins: A review. Food Control. 21: 370–380.

Ghosh, J. and P. Häggblom (1985). Effect of sublethal concentrations of propionic or butyric acid on growth and aflatoxin production by *Aspergillus flavus*. 2(6): 323–330.

Gourama, H. and L.B. Bullerman (1995). Relationship between aflatoxin production and mold growth as measured by ergosterol and plate count. Lebensm. Wiss. Technol., 28: 185-189.

Higgins, C. and F. Brinkhaus (1999). Efficacy of several organic acids against molds. J. Appl. poultry Res., 8: 480-487.

Karunaratne, A and L.B. Bullerman (1990). Interactive effects of spore load and temperature on aflatoxin production. J. food protection. 53: 277-229.

Kheiralla, Z. (1994). Mycotoxins of corn grains. Egypt. J. Microbiol., 29 (2): 149-157.

Lin, CD. and T.C. Chen (1995). Relative antifungal efficacies of phosphoric acid and other compounds on fungi isolated from poultry feed. Animal Feed Science and Technology. 54: 217-226.

Lind, H.; H. Jonsson and J. Schnqrer (2005). Antifungal effect of dairy propionibacteria—contribution of organic acids. Inter. J. Food Microbiol., 98: 157– 165.

Massey, T. E.; R. K. Stewart; J. M. Daniels and L. Ling (1995). Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B1 carcinogenicity. Proc. the Society for Experimental Biology and Medicine, 208: 213-227.

Milanez, T. V.; M. B. Atui, and F.A. Lazzari (1997). Comparison between immuno assay and layer chromatography for determination of aflatoxins, ochratoxin A and Zearalenone in corn and corn meal. Revista Instituto Adolfo Lutz, 57: 65-71.

Mohanamba, T.; S. M. Habibi; P. R. Sastry; K. R. Rajeswari and M. R. Rao (2002). *Aspergillus flavus* contamination of feeds and its potential to produce aflatoxin. Indian Veterinary J., 97 : 76-77.

Pintado, J.; M. P. González and M. A. Murado (1997). Interactions between pretreatment and nutrient concentrations of mussel processing for citric acid production. Enzy. Microb. Technol., 20: 544-549.

Oxoid (1982). "Oxoid Manual", 5th Ed. Oxoid LTD, England. 246-247.

تأثير بعض الأحماض العضوية على نمو بعض الفطريات وإنتاج سمومها

رمضان أحمد حسن* ، مصطفى إبراهيم سند* وشريف محمد القاضى**
* قسم الكيمياء الزراعية – كلية الزراعة – جامعة المنصورة – المنصورة – مصر
** قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة دمياط – دمياط – مصر

السموم الفطرية تلوث العديد من المحاصيل الزراعية والمنتجات الغذائية. وتعتبر الأفلاتوكسينات نواتج تمثيل ثانوية لبعض الفطريات مثل فطر *Aspergillus flavus* وغيرها من الفطريات السامة حيث تعتبر هذه المركبات مواد سامة ومسببة للسرطان. ولقد استخدم العديد من المواد الكيميائية لمنع نمو الفطريات وإنتاج سمومها فى مواد العلف، وتعتبر الأحماض العضوية أحد تلك المواد . حيث تم إختبار تأثير ثمانية أحماض عضوية كمواد مانعة لنمو الفطريات وإنتاج سمومها. وكانت أعلى نسبة تثبيط ٤٥,٢١% فى حالة استخدام حمض الخليك (١٠%) على فطر *A. flavus* بينما أظهر حمض الطرطريك (٥%) وحمض الستريك (٥%) أقل تأثير تثبيطى على الفطريات بنسبه مئوية قدرها (٠,٤٢%). ولقد وجد أن هناك علاقة بين التركيب الكيميائى للأحماض العضوية ونوع الفطر التى تقوم بتثبيطه فمثلا الأحماض (فورميك – أسيتيك – بروبيونيك) وجد أنها تشترك فى تأثيرها على فطر *Rhizopus nigricans* بأعلى نسبة تثبيط.

قام بتحكيم البحث

أ.د / سامى طلعت ابو طالب	كلية الزراعة – جامعة المنصورة
أ.د / عبد العزيز على صقر	كلية الزراعة – جامعة المنوفيه