Pak. J. Bot., 44(1): 425-428, 2012.

EFFECT OF *EPHEDRA ALATA* ON NUCLEIC ACIDS AND NITROGEN METABOLISM OF SEEDBORNE *ASPERGILLUS FLAVUS*

AL-QARAWI[1*], A. A.; ABD ALLAH[1], E. F. AND ABEER HASHEM[2]

[1]*Plant Production Department, Faculty of Food and Agriculture Sciences, King Saud University, P.O. Box. 2460 Riyadh 11451, Saudi Arabia.*
[2]*Botany and Microbiology Department, Faculty of Science, King Saud University, P. O. Box. 2460 Riyadh 11451, Saudi Arabia.*

Abstract

The antifungal mechanism of *Ephedra alata* against aflatoxigenic seedbrone *Aspergillus flavus* was studied. The sensitivity of *A. flavus* to *E. alata* was investigated via studying the alteration in some biochemical compositions of mold mycelia. It has been observed that *E. alata* caused significant inhibitory alterations on synthesis of nucleic acids (DNA and RNA), and non-soluble nitrogen fractions (protein-N_2 and total N_2). The alteration in free amino acids of the experimental mold due to *E. alata* indicated significant increase in glutamic acid, proline, serine, leucine, and phenyl alanine. The results recorded here, clearly indicated the possibility of using the alteration in free amino acids in mycelial growth of *A. flavus* as sensitive monitor for the possible suggested mechanism of *E. alata*.

Introduction

During the past three decades, crop production has been greatly influenced by the dramatic increase in the use of fungicides as protection factors for crops against phytopathogenic fungi. The continuous uses of fungicides lead to an environmental disaster which is harmful to wildlife and to no-target as well as other beneficial organisms (Davidse, 1973; El-Hissy *et al*., 1995; Chen *et al*., 2008). For the above mentioned reasons and more, an increased effort must be carried out to develop alternative non-toxic means to control plant pathogenic fungi especially mycotoxigenic. Among these approaches, using biofungicides from plant sources such as range plants. Many investigations used such plants in the traditional medicine focused their antimicrobial potential (Abourashed *et al*., 2003; Cottiglia *et al*., 2005; Bagheri *et al*., 2009; Alqarawi *et al*., 2011). Ephedra is one of the most widely distributed range plants in Saudi Arabia (Abourashed *et al*., 2003). It has been used as feed-stuff for many grazing animals due to their acceptable aroma (Hussain & Durrani, 2009). Additionally, antifungal potential of Ephedra has been reported against aflatoxigenic molds (Bagheri *et al*., 2009; Alqarawi *et al*., 2011; Alqarawi & Abd_Allah 2012). Nevertheless, the antifungal mechanism of Ephedra against the growth and metabolic activities of aflatoxigenic molds still poorly understood. In our previous investigations, we have reported that *E. alata* caused significant inhibition in conidial production, conidial germination and germ tube elongation of seedbrone aflatoxigenic *A. flavus* (Alqarawi *et al*., 2011). In the same context, significant alterations in lipids metabolism of *A. flavus* towards catabolism has been reported by *E. alata* (Alqarawi & Abd_Allah, 2012). The objectives of this investigation were to study the antifungal mechanism of range plant *E. alata* against aflatoxigenic seedbrone mold (*A. flavus*) as model via investigation the alterations in nucleic acids, nitrogen fractions and free amino acids composition in its mycelia as sensitive monitor for the in vitro resistance profiles towards the antifungal potential of *E. alata*.

Materials and Methods

The organism: The experimental mold was aflatoxigenic seedbrone isolate similar to *Aspergillus flavus* Link, which was used in our previous study (Alqarawi *et al*., 2011).

The range plant: *Ephedra alata* Decne (fresh aerial parts) were collected from Wildlife Research and development station at Thumama, Riyadh, Saudi Arabia.

Preparation of plant extract: The aqueous ethanol extract of *E. alata* has been done according to Alqarawi *et al*., (2011) and expressed as weight (w) of air dry plant materials as gram per volume (v) of mold culture growth medium as ml (w/v).

General culture conditions: The experimental mold (*A. flavus*) was grown using glucose-ammonium nitrate salt broth medium (Brain *et al*., 1961) in 250 ml capacity Erlenmyer conical flasks, each contain 100 ml culture medium. After autoclaving, the culture flasks were supplemented with different concentrations (0.5, 1.0 and 2.0%, [w/v]) of plant (*E. alata*) extract as described in details by Alqarawi *et al*., (2011). Control flasks were used as reference. Discs (0.5 mm diameter) of seven days old culture (*A. flavus*) were used for inoculation. The flasks were incubated at static state (28^{o}C±1) for 10 days in dark. At the end of incubation period, the cultures were filtered through filter paper (Whatman no 1) followed with washing carefully using distilled water. The mycelial growth dried at 80^{o}C up to two successive weights were obtained and used for biochemical analysis.

Biochemical analysis

a. Nucleic acids: Nucleic acids (DNA & RNA) were extracted according to Shiboko *et al*., (1967). The quantitative estimations of DNA and RNA were carried out according to Burton (1968) and Ashwell (1957), respectively.

*Corresponding author E-mail: alqarawi@ksu.edu.sa

b. Nitrogen fractions: Soluble nitrogen fractions were extracted according to the method adopted by Said & El-Shishiny (1944). The methods described by Chaney & Marbach (1962) used for determination of ammonia-nitrogen spectrophotometrically. Standard curve of ammonium chloride (10-70 µg) was used as reference. Protein-nitrogen was estimated according to the method described by Lowry *et al*., (1951). Total soluble and total nitrogen contents were determined using the conventional micro-kjeldahl method (Allen, 1953). Crude protein calculated mathematically (crude protein= total nitrogen content X 6.25) according to Conklin-Brittain *et al*., (1999).

c. Free amino acids: Free amino acids were extracted from dry mycelial growth using ethyl alcohol (80%, v/v) according to Malik & Singh (1980). The qualitative and quantitative determination of amino acids was carried out using LKB 415 alpha plus amino acid analyzer according to Christias *et al*., (1975). Standard amino acids were used as reference.

Statistical analysis: In each experiment, the data were statistically analyzed and means were compared using the protected least significant difference values according to Daniel (1987).

Results and Discussion

The antifungal potential of plants belonging to Ephedra has been reported previously *in vitro* (Bagheri *et al*., 2009) and *in vivo* (Alqarawi *et al*., 2011) and attributed to presence of cis-3,4-methanoproline (Caveney *et al*., 2001), citronellol (Rosato *et al*., 2007) and heptadecane (Bagheri *et al*., 2009) which were recorded as antimicrobial substances and had been found in Ephedra. The antifungal mechanism of *Ephedra alata* against some metabolic activities of *Aspergillus flavus* indicated clearly to the inhibitory effect of plant (*E. alata*) extract on synthesis of nucleic acids (DNA and RNA) and increase the number of nuclei per germ tube in directly proportional with increase contamination of plant extract (Table 1). The mechanism of *E. alata* observed here is agree with other mechanisms of chemical fungicides (Richmond & Phillips, 1975); mycotoxins (Torralba *et al*., 1998) and antifungal from plant sources (Helal *et al*., 2007) and can be ascribed to an interference with mitosis (Davidse, 1973) hence arrested mitotic activity (Richmond & Phillips, 1975; Ezzat *et al*., 2005). In the context, it was observed that, RNA synthesis was less inhibited than DNA (Table 1). Such mechanism striking resemblance with that reported by Bogle *et al*., (1994) and Ghannoum & Rice (1999).

Table 1. Effect of different concentrations of *E. alata* extract (w/v) on number of nuclei /germ tube and nucleic acids (DNA and RNA) of *A. flavus*.

Treatments concentrations of *E. alata* extract (w/v)	Number of nuclei /germ-tube	Nucleic acids (mg/g dry weight)	
		DNA	RNA
Control	3.125 ± 0.295	13.907 ± 0.378	29.997 ± 0.439
0.5 % (w/v)	4.625 ± 0.323	5.562 ± 1.644	21.897 ± 1.120
1.0 % (w/v)	6.375 ± 0.375	2.457 ± 0.243	16.475 ± 0.852
2.0 % (w/v)	8.375 ± 0.263	0.820 ± 0.076	10.852 ± 0.354
LSD at: 0.05	0.918	2.629	2.337

± : Standard Error = Standard deviation / √n

The results demonstrated significantly that *E. alata* caused an increase in ammonia-nitrogen and total soluble nitrogen (soluble nitrogen fractions) with association by significant decrease in protein-nitrogen, total nitrogen and crude protein (non-soluble nitrogen fraction) as compared with those of control *A. flavus* (Table 2). These results were in agreement with analogous reports by Moharram *et al*., (1994) and El-Hissy *et al*., (1995) who reported that chemicals antifungal caused significant decrease in peptide (protein)-nitrogen and total nitrogen contents. The further increase in ammonia content of *A. flavus* indicates that *E. alata* stimulated protein-hydrolytic enzymes such as protease. This was in agreement with the finding of Sonawane & Chavan, (2005). In the same context, it was reported that plant extract of neem (*Azadirachta indica*) caused stimulatory effect on protease activity of *Macrophomina phaseolina* (Dubey *et al*., 2009).

Table 2. Effect of different concentrations of *E. alata* extract (w/v) on nitrogen fractions (mg/g dry weight) of *A. flavus*.

Treatments Concentrations of *E. alata* extract (w/v)	Nitrogen fractions content (mg/g dry weight)				
	Amino-N	Protein-N	TSN	TN	CP
Control	0.6200 ± 0.0288	29.1400 ± 0.6781	7.1833 ± 0.2082	58.7900 ± 0.6141	367.4376 ± 3.8385
0.5 % (w/v)	1.3800 ± 0.0550	23.5266 ± 0.7217	12.7666 ± 0.1581	43.8166 ± 0.4734	273.8546 ± 2.9592
1.0 % (w/v)	1.6733 ± 0.0260	19.0133 ± 0.3976	15.0733 ± 0.1393	31.1666 ± 0.1602	194.7920 ± 1.0013
2.0 % (w/v)	0.4400 ± 0.0152	16.7933 ± 0.4420	4.8966 ± 0.0920	20.8600 ± 0.1289	130.3753 ± 0.8059
LSD at: 0.05	0.1127	1.8836	0.506	1.3083	8.1764

TSN= Total soluble nitrogen; TN= Total nitrogen; CP= Crude protein

± : Standard Error = Standard deviation/√n

The results revealed the presence of 13 free amino acids namely aspartic acid, tyrosine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, phenyl alanine and tryptophane in mycelial control *A. flavus* (Table 3). Ezzat & Sarhan (1991) reported parallel report. The alteration in amino acids composition of molds has been used as sensitive monitor for mold resistance against abiotic stress of fungicides (Perea & Patterson, 2002; El-Mehalawy *et al*., 2008; AbdEl-Ghany *et al*., 2009). In the same context, our data shown that, employment of *E. alata* at 0.5% (w/v) caused significant increase in glutamic acid, proline, serine, leucine, and phenyl alanine, however aspartic acid, tyrosine, glycine, alanine, methionine, isoleucine and tryptophane has been decreased as compared with amino acids of control *A. flavus*. A concentration 0.2% (w/v) of *E. alata* caused disappearance of tyrosine, serine, alanine, valine, isoleucine, leucine and phenyl alanine likewise appearance of histidine, lycine, threonine and arginine with clear decrease in total free amino acids content. The accumulation of glutamate amino acids (glutamic acid and proline) in mycelial growth of *A. flavus* due to *E. alata* means activation of their biosynthesis from glutamate via both glutamate kinase followed by γ-semialdehyde dehydrogenase (for proline) and glutamine synthetase (for glutamic acid), respectively (Albert *et al*., 2002). On the other hand, the consumption of such glutamate amino acids decreased due to *E. alata* to support more energy (Adenosine Tri-phosphate, ATP) required for mold resistance against antifungal potential of *E. alata* (Yamaguchi & Fujimura, 2005; Chen *et al*., 2008). In the same connection, proline amino acid was shown to minimize cellular damage by enhancing the stability of proteins and biological membrane (Csonka, 1989) as suggested mechanism of *E. alata*.

In conclusion, the present data in our current investigation demonstrated that nucleic acids and protein (involve amino acids) metabolism in *A. flavus* directly influenced by antifungal potential of *E. alata*, and such aspects of mold physiology can be employ as sensitive monitor for mold resistance against antifungal from plant origin. Our investigation will extend to study the antifungal potential of *E. alata* on ultrastructure of *A. flavus* as model for seedbrone aflatoxigenic fungi has been inhabited by *E. alata*.

Table 3. Effect of different concentrations of *E. alata* extract (w/v) on cellular free amino acids (mg/g dry weight) profile of *A. flavus*.

Treatments	Amino acids profile of *A. flavus* (mg/g dry weight)																	
	aspartic acid	Tyrosine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Valine	Methionine	Isoleucine	Leucine	Threonine	Phenylalanine	Histidine	Lysine	Arginine	Tryptophan	TFAA
Control	0.960 ±0.01	0.140 ±0.01	0.423 ±0.02	0.683 ±0.02	0.520 ±0.01	0.683 ±0.02	0.443 ±0.02	0.346 ±0.01	0.616 ±0.01	0.143 ±0.01	0.573 ±0.02	ND	0.393 ±0.01	ND	ND	ND	0.750 ±0.01	6.673
0.5 % (w/v)	0.723 ±0.04	0.086 ±0.01	0.686 ±0.02	0.940 ±0.01	0.970 ±0.05	0.453 ±0.01	0.206 ±0.01	0.906 ±0.04	0.506 ±0.03	ND	0.803 ±0.04	ND	0.970 ±0.03	ND	ND	0.183 ±0.01	0.636 ±0.02	8.068
1.0% (w/v)	0.580 ±0.02	ND	0.850 ±0.11	0.316 ±0.02	1.226 ±0.03	0.316 ±0.02	ND	ND	0.403 ±0.01	ND	ND	0.493 ±0.01	ND	0.526 ±0.03	0.290 ±0.01	0.253 ±0.02	0.450 ±0.02	5.503
2.0% (w/v)	0.273 ±0.02	ND	ND	0.140 ±0.01	1.376 ±0.02	0.140 ±0.01	ND	ND	0.293 ±0.01	ND	ND	0.663 ±0.03	ND	0.643 ±0.02	0.756 ±0.01	0.393 ±0.01	0.290 ±0.01	5.077
LSD at 0.5	0.091	0.031	0.054	0.059	0.112	0.059	0.037	0.076	0.063	0.021	0.078	0.06	0.049	0.06	0.025	0.045	0.056	

TFAA: Total free amino acids
ND= Not detected under the experimental conditions
± : Standard Error = Standard deviation/√n

Acknowledgment

This research was supported by King Saud University, Deanship of Scientific Research, College of Food & Agriculture Sciences, Research Center.

References

AbdEl-Ghany, T.M., M. AbdEl-Mongy and M.M. Afify. 2009. Dynamic changes in amino acids and volatile metabolites of soil fungus *Aspergillus flavus* as a result of chloropyrifose methyl application. *J. Appl. Sci. Res*., 5: 1-8.

Abourashed, E.A., A.T. El-Alfy, I.A. Khan and L. Walker. 2003. *Ephedra* in perspective-a current review. *Phytother. Res.*, 17: 703-712.

Albert, G.M., W.F. John and P.S. Michael. 2002. Biosynthesis and metabolism of amino acids. In: *Microbial Physiology* 4th Edition, by Wiley-Liss, Inc. ISBN: 0-471-39483-1, pp. 736.

(http://docencia.izt.uam.mx/hgm/bq_fisiol_microbiana/documentos/pdf/Moat_microbial_physiol/cap_15.pdf).

Allen, M.B. 1953. *Experiments in soil bacteriology*. 1st Ed. Burgess Pub. Co., USA.

Alqarawi, A.A. and E.F. Abd-Allah. 2012. Effect of *Ephedra alata* on Lipids Metabolism of *Aspergillus flavus*.

Bangladesh J. Bot, (Accepted).

Alqarawi, A.A., E.F. Abd_Allah and Abeer Hashem. 2011. *Ephedra alata* as biologically-based strategy inhibit aflatoxigenic seedborne mold. *African J. Microbiol. Res.*, 5: 2297-2303.

Ashwell, G. 1957. *Methods in Enzymology*. 3: 73-105 Interscince Publishers, Inc. NY.

Bagheri, G., M. Bigdeli, G.M. Shams and A.M. Razzaghi. 2009. Inhibitory effects of *Ephedra* major host on *Aspergillus parasiticus* growth and aflatoxin production. *Mycopathologia*, 168: 249-255.

Bogle, R.G., S.C. Soo, G.S.T.J. Whitley, A.P. Johnstone and P. Vallance. 1994. Effect of anti-fungal imidazoles on mRNA levels and enzyme activity of inducible nitric oxide synthase. *Br. J. Pharmacol.*, 111: 1257-1261.

Brain, P.W., A.W. Dowkins, J.F. Grove, D.L. Hemming and G.L.F. Norris. 1961. Phytotoxic compounds produced by *Fusarium equiseti*. *J. Exp. Bot.*, 12: 1-12.

Burton, K. 1968. Determination of DNA concentration with diphenylamine. *Methods in Enzymology* 12(Pt. A): 163-166.

Caveney, S., D.A. Charlet, H. Freitag, M. Maier-Stolte and A. Starratt. 2001. New observations on the secondary chemistry of world *Ephedra* (Ephedraceae). *American J. Bot.*, 88: 1199-1208.

Chaney, A.L. and E.P. Marbach 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.*, 8:130-132.

Chen, Y., L. Hengkui, C. Changjun and Z. Mingguo. 2008. Sensitivity of *Fusarium graminearum* to fungicide JS399-19: *In vitro* determination of baseline sensitivity and the risk of developing fungicide resistance. *Phytoparasitica*, 36: 326-337.

Christias, C., C. Couvaraki, S.G. Georgopoulos, B. Macris and V. Vomvoyanni. 1975. Protein content and amino acid composition of certain fungi evaluated for microbial protein production. *Appl. Microbiol.*, 29: 250-254.

Conklin-Brittain, N.L., E.S. Dierenfeld, R.W. Wrangham, M. Norconk and S.C. Silver. 1999. Chemical protein analysis: a comparison of Kjeldahl crude protein and total ninhydrin protein from wild, tropical vegetation. *J. Chem. Ecol.*, 25: 2601-2622.

Cottiglia, F., L. Bonsignore, L. Casu and D. Deidda. 2005. Phenolic constituents from *Ephedra nebrodensis*. *Natl. Prod. Res.*, 19: 117-23.

Csonka, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.*, 53: 121-147.

Daniel, W.W. 1987. Biostatistics: *A foundation for Analysis in the Health Science*. 4th ed., John Wiley and Sons, New York, NY. pp. 292-293.

Davidse, L.C. 1973. Antimitotic activity of methyl benzimidazol-2-yl carbamate (MBC) in *Aspergillus nidulans*. *Pest. Biochem & Physiol.*, 3(3): 317-325.

Dubey, R.C., H. Kumar and R.R. Pandey. 2009. Fungitoxic Effect of Neem Extracts on Growth and Sclerotial Survival of *Macrophomina phaseolina in vitro*. *J. Ameri. Sci.*, 5:17-24.

El-Hissy, F.T., M.A.El-Nagdy, H.M. El-Sharouny, and G.A. AbdElaah, 1995. Effect of the fungicide chlorothalonil (Bravo) on some metabolic activities of aquatic fungi. *Folia Microbiol.*, 40(3), 341-344.

El-Mehalawy, A.A., H.M. Gebreel, H.M. Rifaat, I.M. El-Kholy and A.A. Humid. 2008. Effect of antifungal compounds produced by certain bacteria on physiological activities of human and Plant- pathogenic fungi. *J. Appl. Sci. Res.*, 4: 425-432.

Ezzat, S.M. and M.M. Sarhan. 1991. Effect of different concentrations of sodium chloride on the growth and the biochemical activities of *Aspergillus flavus* Link. *Egypt. J. Microbiol.*, 26: 133-146.

Ezzat, S.M., M.M. Sarhan and A.A. Ismaiel. 2005. Morphogenic and ultrastructure aberrations induced by cytochalasin B in some microorganisms. *Egypt. J. Biotechnol.*, 19: 106-121.

Ghannoum, M.A. and L.B. Rice. 1999. Antifungal Agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.*, 12(4): 501-517.

Helal, G.A., M.M. Sarhan, A.N.K. Abu Shahla, E.K. Abou El-Khair. 2007. Effects of *Cymbopogon citratus* L. essential oil on the growth, morphogenesis and aflatoxin production of *Aspergillus flavus* ML2- strain. *J. Basic Microbiol.*, 47: 5-15.

Hussain, F. and M.J. Durrani. 2009. Seasonal vailability, palatability, and animal preferences of range plants in Harboi arid range land, Kalat, Pakistan. *Pak. J. Bot.*, 41(2): 539-445.

Lowry, O.H., N.J. Rosenbough, A.L. Farr and R.J. Randall. 1951. Protein measurement with folin reagent. *J. Biol. Chem.*, 193: 269-275.

Malik, C.P. and M.B. Singh. 1980. Extraction and estimation of amino acids and keto acids. In: *Plant Enzymology and Histo-Enzymology*. (Eds.): CP. Malik, MB. Singh, Kalyani Publishers, New Delhi-Lud Hana, India. pp. 286.

Moharram, T.M.M., M.S.A. Safwat and M.M. Farghaly. 1994. Effect of inoculation rates and phosphorus fertilization on nitrogen fixation in soybean. *Afr. Crop Sci. J.*, 2: 125-129.

Perea, S. and T.F. Patterson. 2002. Antifungal resistance in pathogenic fungi. *Clin. Infec. Dis.*, 35:1073-1080.

Richmond, D.V. and A. Phillips. 1975. The effect of benomyl and carbendazim on mitosis in hyphae of *Botrytis cinerea* Pers. ex Fr. and roots of *Allium cepa* L. *Pesticide Bioch. and Physiol.*., 5: 367-379.

Rosato, A., C. Vitali and N. De Laurentis. 2007. Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. *Phytomedicine*, 14: 727-32.

Said, H. and E.D.H. EL Shishiny. 1944. Researches on plant metabolism. III. The effect of disc thickness on the respiration and the various nitrogen fractions of cut discs of radish roots immersed in water and in sugar solutions. *Plant Physiol.*, 19: 660-670.

Shiboko, S., P. Kiovistoinen, C. A. Tramyek, A. R. Newhali and L. Friedman. 1967. A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid and glycogen from a single rat liver homogenate from a subcellular fraction. *Anal. Biochem.*, 19: 514-528.

Sonawane, V.V. and A.M. Chavan. 2005. Effect of antibiotics and fungicides on protease production by some seed-borne fungi of Pea. *Dr. BAMU J. Sci.*, 33: 85-87.

Torralba, S., M. Raudaskoski, A.M. Pedregosa and F. Laborda. 1998. Effect of cytochalasin A on apical growth, actin cytoskeleton organization and enzyme secretion in *Aspergillus nidulans*. *Microbiol.*, 144: 45-53.

Yamaguchi, I. and M. Fujimura. 2005. Recent topics on action mechanisms of fungicides. *J. Pestic. Sci.*, 30: 67-74.

(Received for publication 20 March 2011)