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Growth, Ochratoxin A Production and Some Metabolic Activities of *Aspergillus ochraceus* at Different NaCl Concentrations

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Abstract: The growth of *Aspergillus ochraceus* was decreased with increasing NaCl concentration. The decreasing ratio of ochratoxin A by NaCl ranged from 25 to 74% at 5 and 15% NaCl respectively. The total soluble protein increased to 37% at 15% NaCl. The total lipids decreased only at 15% NaCl. The myristic and palmitic acids represented the highest concentrations of saturated fatty acids while their concentrations decreased with increasing NaCl. Whereas, 10% NaCl increased the concentrations of the unsaturated fatty acids (oleic and linoleic) and decreased palmitoleic acid. On the other hand 15% NaCl increased linolenic and decreased palmitoleic and oleic acid concentrations. Most of the detected phospholipids increased with increasing NaCl concentrations in the growth medium up to 10%, whereas they decreased at 15% NaCl. Phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidic acid were the major phospholipids.The genomic DNA of the control and the five NaCl treated samples showed a common band at higher molecular weight.

Key words: *Aspergillus ochraceus*, genomic DNA, lipid, fatty acids, protein

Introduction

Aspergillus ochraceus (Whilhelm) is a widespread storage fungus that has been isolated from grains (Ramos *et al.*, 1998). Their growth and the associated toxin production are closely related to the degree of moisture to which they are exposed, which itself depends upon weather conditions at harvest and techniques for drying and storage. Ochractoxin A (OTA) is found as a frequent contaminant of a large variety of food and feed and beverage such as beer, coffee and wine (Creppy *et al.*, 1998). It is produced as a secondary metabolite of *A. ochraceus*. OTA is the sole cause of balkan endemic nephropathy, renal tumors and necrosis of kidney tissues (Diekrnan and Green, 1992).

Solute-tolerant micro-organisms demonstrate different structural and biochemical responses to alterations in solute concentrations. In the fungus *Dendryphiella salina*, Wethered *et al.* (1985) reported increased levels of polyols when the fungus was grown in saline media. The effects of NaCl concentrations on the growth, lipid content and fatty acid composition of different fungi have been studied (Mulder *et al.*, 1989; El-Mougith *et al.*, 1993; Torzilli, 1997 and Hefnawy *et al.*, 1997).

The production of ochratoxin by *Aspergillus* spp. has been studied (Sansing *et al.*, 1973; Halt, 1998 and Atalla *et al.*, 1999).

The objective of this study was to determine the effect of NaCl on the growth of *A. ochraceus*, OTA, lipids production and other metabolic activities.

Materials and Methods

Culture. Aspergillus ochraceus was isolated from infected Egyptian corn. The cultures were maintained on slant containing yeast extract sucrose medium (YES).

Growth studies: *A. ochraceus* was inoculated into autoclaved 250 ml Erlenmeyer flasks containing 50 ml of 4% sucrose and 2% YES media. The media was used also for toxin production (Sansing *et al.*, 1973). The varying saline concentrations of NaCl (5.0, 7.5, 10, 12.5 and 15%) were prepared. NaCl was not present in the control media. After 7 days incubation at 25°C without shaking, mycelial mats were harvested, washed and dried for determination of the total dry weights.

Radial growth: Triplicate plates for each concentration were incubated at 25°C. Colony diameter were determined daily for a period of 7 d. This provided another measure of the growth rate at different salinities.

Extraction and quantitative estimation of ochratoxin A: Ochratoxin A was extracted three times with 100 mL chloroform in separating funnel. Chloroform extracts were evaporated to dryness and 0.5 mL of chloroform was added to each sample, of which 50 μL was spotted on a thin layer chromatography (TLC) plate (20x20 cm^2) glass, coated with a 250μ layer of silica gel R250 adjacent to 50 μL of standard ochratoxin A (0.01 μg of ochratoxin A per microliter of ethyl alcohol). TLC plates were developed in unlined chromatography tanks containing a 2-cm layer of toluene-ethyl acetate-90% formic acid (5:4:1 v/v) (Scott and Hand, 1967). The quantity of ochratoxin A in the samples were determined by spectrophotometer at 254 nm.

Biological assay (Bacterial test): Egyptian isolates of gram positive *Bacillus subtilus* and gram negative *Escherichia coli* were used. The nutrient agar medium containing 5.0g NaCl, 5.0g peptone, 3g leaf extract and 20g agar/L dist. H_2O was used. A log. phase of each bacterial suspension was poured into the surface of agar plate. A disc of filter paper (Whatman No. 1, 1cm in diameter) was saturated by 10 μL of chloroform and put on the dish as control and two discs each one saturated by a dose of 10 μL of crude extract were put also on the same dish. The dishes were incubated for 24h at 37°C, after which the diameter of clear zone (no growth) was measured (Abou-Zeid, 2000).

Electrophoretic procedures: Native polyacrylamide gel electrophoresis (PAGE) for total soluble proteins was carried in 10% polyacrylamide gel slabs for control and colonies grown at different NaCl concentrations according to Laemmli (1970).

Agarose gel electrophoresis, 0.8% agarose was carried out for extraction and isolation of genomic DNA using CTAB buffer (Cetyltrimethyl ammonium bromide).

Lipid extraction and analysis.

Total lipids: Lipid extraction from fungal mycelium was carried out twice with 20 volumes of chloroform: methanol (2:1 v/v) in a warring blender for 2 min and washed with 0.2% of its volume 0.73% NaCl. The mixture was allowed to separate into 2 phases. The lower phase was taken and dried to constant weight for quantitative estimation of total lipids, according to the method described by Barnes and Blackstock (1973).

Fatty acids: Total fatty acids (free and bound) in oils were determined qualitatively and quantitatively according to A.O.A.C. (1975) with minor modifications. The method could be summarized as follows (1) saponification of the oil sample (1.0g) by 20% KOH in methanol, (2) separation of un saponifiable matter from the saponifiable (containing fatty acids) with petroleum ether (40-60°C), (3) acidification of the saponifiable matter with excess of 10% H_2SO_4 (4) extraction of fatty acids with petroleum ether (40-60°C), (5) methylation of fatty acids with diazomethane (CH_3N_2) in ether and (6) injection of 1 µL fatty acid methylester solution into Gas-liquid chromatography (G.L. C/pye µnicam/PU 4550/Packed, using the column SP-2310-55% cyanopropyl phenyl silicone 1.5mx4mm) which was adjusted to achart speed of 0.35/min (Principle Cent. Lab., Fac. Agric, Cairo Univ. Egypt). The degree of unsaturation was expressed as unsaturation index, defined by Kates and Hagen (1964) as:

Δ_mol^{-1} = 1x (% monoenes)/100 +2x (% dienes)/100 +3x (% trienes)/100.

Phospholipids determination: Phospholipids were extracted from the mycelial pellets using the method of Kates (1972). Phospholipids were separated by TLC according to the method of Nichols (1964). The spots of phospholipids were revealed by iodine vapour and outlined with a pencil. The chromatogram was then sprayed with a fine mist of cupric acetate (3% w/v) in sulphuric acid (8% w/v) and heated to approximately 170°C for 30 min. Phospholipid spots appeared dark brown. The content of each spot was determined according to the method of Miller (1985).

Extraction of DNA: Total genomic DNA was extracted from control and treated samples using CTAB method, as described by Doyle and Doyle (1990).

Statistics: All experiments were repeated at least three times and the results obtained were treated statistically for significance by calculating the standard error of the mean (SEM) (Swinscow, 1985).

Results

Microorganisms respond to environmental and physical agents in the surrounding atmosphere and their behaviour is affected by the variation in these factors.

Effect of NaCl on *A. ochraceus* growth: Slight growth was recorded on 15% NaCl media (2.88 cm) after a 7 days incubation period (Fig. 1A), while 5.7cm was recorded in control (0.0 NaCl). 5% NaCl has no effect on the process of germination. The biomass values for *A. ochraceus* are presented in Fig. (1B). A 35% biomass reduction was recorded at 12.5% NaCl, while the reduction in the biomass at 15% was 51.6%.

Effect of salinity on fungal protein: NaCl slightly increased the total soluble protein. The increasing ratio ranged from 6.6% in cases of 5% NaCl to 37% in 15% NaCl, when compared to control. (Table 1).

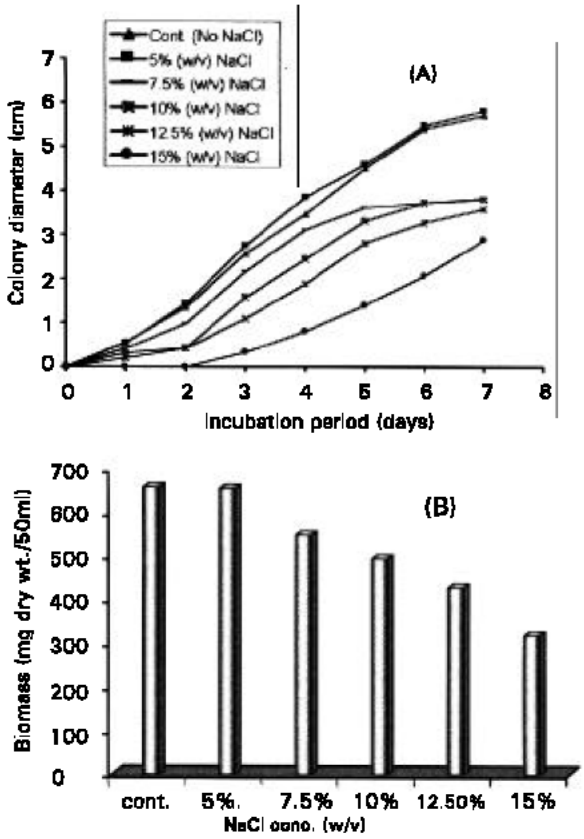

Fig. 1: Relative growth of *A. ochraceus* at different NaCl concentrations : (A) Linear growth, (B) Production of biomass (Dry weight) after 7 dats

Effect of salinity on ochratoxin A production :

As shown in Table 1, NaCl decreased the ochratoxin A production. The decreasing ratio ranged from 25% in case of 5% NaCl to 74% in 12.5 and 15% NaCl.

Biological assay of ochratoxin A: Toxicity test of ochratoxin A was proved using two Egyptian bacterial isolates, gram positive *Bacillus subtilis* and gram negative *Escherichia coli*. Inhibition zones of 0.15 cm and 0.13 cm were observed on crude ochratoxin A by *B. subtilis* and *E. coli* respectively.

Effect of NaCl on total lipids, fatty acids and phospholipids: As shown in Table 1, total lipids were increased with increasing salinity up to 7.5 (40%) and 10% (36.7%) then it decreased. With respect to fatty acid analysis, cultures at 0, 10 and 15% NaCl were chosen on the basis of the evidence obtained in the growth studies. As shown in Table 2 the highest concentrations of saturated fatty acids were found in myristic and palmitic, but their concentrations decreased with increasing NaCl. On the other hand 15% NaCl increased the concentrations of caproic and stearic acid as compared to control. Behinic acid was detected only at 15% NaCl. With respect to the unsaturated fatty acids, oleic, palmitoleic and linoleic were the highest concentrations obtained. 10% NaCl

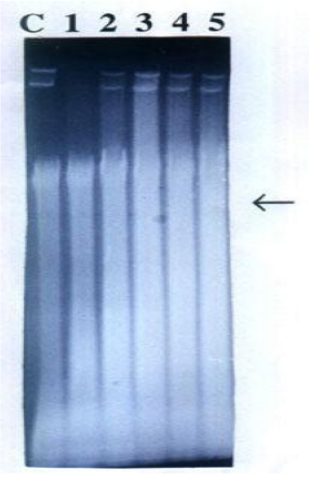

Fig. 2: Genomic DNA, (C) control and treated samples : (1) 5%, (2) 7.5% , (3) 10%) , (4) 12.5% and (5) 15% NaCl as revealed on 0.8% agarose gel electrophoresis. Arrow head is indicating the degraded (smeared) DNA.

Table 1: Effect of NaCl on total soluble proteins, ochratoxin A and total lipids of *A. ochraceus*.

NaCl conc. %	Total soluble protein (mg/mL)	Ochratoxin A (µg/50 ml)	Total lipids (mg/g fresh wt.)
0.0	7.31±0.2	484.6±4.8	30±1.2
5	7.79±0.1	363.9±3.2	33±0.9
7.5	9.21±0.3	166.8±4.2	42±2.1
10	9.63±0.07	128.9±1.3	41±1.2
12.5	9.8±0.11	125.32±1.4	30±3.1
15	10.01±0.12	125.643±1.2	25±2.3

Table 2: Constituent fatty acids of *A.ochraceus* grown at different salinities.

Fatty acids		NaCl (%)		
		0.0	10	15
Caproic	(6:0)	0.362	0.487	0.431
Caprylic	(8:0)	0.076	0.065	0.061
Capric	(10:0)	0.261	0.174	0.238
Lauric	(12:0)	1.283	0.924	1.044
Myristic	(14:0)	12.575	8.836	10.692
Palmitic	(16:0)	10.122	9.587	9.570
Stearic	(18:0)	1.691	1.415	2.449
Arachidic	(20:0)	0.688	0.439	0.538
Behinic	(22:0)	ND	ND	1.596
Palmitoleic	(16:1)	24.123	18.668	20.489
Oleic	(18:1)	25.953	27.258	24.725
Linoleic	(18:2)	21.909	31.239	24.762
Linolenic	(18:3)	0.465	0.555	2.552
Δ mol^{-1}		0.94	1.1006	0.9839

ND = Not detected

increased the concentration of linoleic and oleic acids and decreased palmitoleic acid. On the other hand 15% NaCl increased linolenic and decreased palmitoleic and oleic acid concentrations.

Their is a slight increase in the unsaturation index of the identified fatty acids in the presence of NaCl. This may indicate a little effect on plasma membrane fluidity of the *A. ochraceus* cells.

Phospholipids content in *A. ochraceus* in presence of different concentrations of NaCl: Phospholipids content in the mycelium of *A. ochraceus* (Table 3) increased with increasing NaCl up to 10% and decreased at 15%. Phosphatidylcoline, sphingolipid and cardiolipin were not detected in absence of NaCl whereas, they detected at higher concentrations of NaCl. At 10% NaCl Phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidic acid were increased approximately 2.6, 2.4

Table 3: Phospholipids composition of *A. ochraceus* grown in the presence of different concentrations of NaCl.

Phospholipids	NaCl (%w/v)			
	0	5	10	15
Phosphatidyl coline	ND	202±15	384±17	366±7
Phosphatidyl glycerol	374±9	550±14	970±9	753±14
Phosphatidyl ethanolamine	313±11	416±7	750±14	423±17
Phosphatidic acid	380±15	623±14	750±16	398±10
Sphingolipid	ND	ND	132±5	201±8
Cardiolipin	ND	ND	163±21	225±17

Data are expressed as nanomol of phospholipid per gram fresh weight mycelium ±SE of three determinations. ND = not detected.

and 2 times respectively higher than in the control mycelium. The relative rate of biosynthesis of phosphatidyl glycerol (PG) was higher than phosphatidyl ethanolamine (PE). PG at 0, 5, 10 and 15% NaCl approximately increased 1.2, 1.3, 1.3 and 1.8 times respectively over PE.

DNA extraction: Genomic DNA was extracted and isolated from control and treated samples using CTAB method and isolated DNA was electrophoresed on 0.8% agarose gel. The control and the five treated samples showed a common band of a high molecular weight. The DNA pattern on the gel revealed degradation of the DNA of samples treated with high concentrations of NaCl (10,12.5 and 15%). This was indicated by light (smeared) area on the gel (Fig. 2). While lower NaCl concentrations showed a relatively lower degradation.

Discussion

With increasing salinity a decrease in the growth rate of *A. ochraceus* was observed for the linear extension. Increasing salinity resulted in the expected gradual decrease in biomass production. This is in agreement with that of Mulder *et al.* (1989). They recorded a decreased growth of the tested fungi with increasing salinity. Also, Abou-Zeid (2000) reported that with increasing salinity a decrease in the growth rate of *Fusarium moniliforme* was observed. A 80% biomass reduction was recorded at 12.5% NaCl and no growth was observed at 15% NaCl.

Total soluble proteins were slightly increased with increasing NaCl. On the other hand the native PAGE (not shown) shows that when cells of *A. ochraceus* grown in different NaCl concentrations a number of new protein bands appeared only at higher concentrations, while other proteins band showed increased synthesis. Torzilli (1997) also reported that when the cells of *Aureobasidium pullulans* were grown at 4.5% NaCl a three of the 12 protein bands showed increased synthesis and he concluded that the stimulation in synthesis of these proteins by sub-lethal stress dosages correlated with the ability of cells to survive in salt marshes. Also Abou-Zeid (2000) concluded that a six new protein bands appeared only at 10 and 12.5% NaCl and the other seven of the 13 protein bands showed increased synthesis at the same concentrations.

NaCl decreased the ochratoxin A production, only 26% was observed in 12.5 and 15% NaCl. The toxicity test was proved by *B. subtilis* and *E. coli*.

It is evident from the present study that the reduction or stimulation of the total lipids and fatty acids were not directly related to the NaCl concentration. 15% NaCl increased the concentration of fatty acids capric, stearic, linoleic and linolenic and decreased palmitoleic and oleic. On the other

hand 10% NaCl increased the concentration of linoleic, oleic and linolenic acids. These results are in accord with those of Mulder *et al.* (1989), who concluded that the major fatty acid in all isolates cultured (*Alternaria phragmospora, Alternaria chlamydospora* and *Ulocladium chartarum*), was oleic acid (18:1) which decreased at 10% NaCl. A similar pattern was observed for palmitic acid (16.0). The concentration of linoleic acid was increased at 10% NaCl. El-Mougith *et al.* (1993) mentioned that total lipids, phospholipids, free fatty acids, free sterols and the unsaturated fatty acids in some salt tolerant fungi increased with increasing NaCl concentration, neutral lipids and unsaturated fatty acids decreased. Also Abou-Zeid (2000) reported decreased total lipids at 10 and 12.5% NaCl. Phospholipids content in *A. ochraceus* increased with increasing NaCl in the growth medium up to 10%. Phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidic acid increased with increasing NaCl. These results are in contrast with those of Mulder *et al.* (1989). They found that most of the phospholipids in dematiaceous hyphomycetes decreased at high salinity. Total phospholipids in *Penicillium corylophillum* and *Halobacterium halobium* were also increased in presence of NaCl in the media. PG and PE in both organisms showed the highest increase in presence of NaCl in the media (Hefnawy *et al.*, 1997).

Adams *et al.* (1987) found in *Vibrio costicola* that there is a relative increase in PG synthesis over that of PE following 2 or 3 fold shift-up in NaCl or sucrose in the media. The observations are quit similar as *A. ochraceus* and may indicate that fungal and bacterial cells may respond to salt stress by the same way with some changes in phospholipids composition.

With respect to the effect of NaCl on the genomic DNA, only one band was detected on 0.8 agarose gel for the control and the treated samples. This fact is in accordance with that of Abou-Zeid (2000). He reported that no effect of salinity treatments on fungal DNA structure and composition.

The response of a fungus to NaCl stress involves the functioning of many diverse capacities of the organism. In the light of our results we can concluded that NaCl reduced *A. ochraceus* growth. Also the salt increased the total soluble proteins and 15% NaCl reduced the total lipids and phospholipids. The higher NaCl concentrations alter the amounts of saturated and unsaturated fatty acids.

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