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Effect of Salt Stress on Amino Acids, Organic Acids and Ultra-structure of *Aspergillus flavus* and *Penicillium roquefortii*

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ABSTRACT

Aspergillus flavus Link ex Gray 1821 and *Penicillium roquefortii* Link Fr. Thom were isolated from a salt marsh soil at Sinai, Egypt. Both fungi were cultured on Czapek's yeast extract medium amended with 4 and 8% (w/v) sodium chloride. The amino acids profile of *A. flavus* and *P. roquefortii* indicated that both aspertate and glutamate families of amino acids are well operated. Interestingly, high quantity of glutamic acid, were detected in the presence of NaCl in the growth medium of *A. flavus*. Moreover, tryptophan, alanine, carrnosin, γ–amino-N-butyric acid, ß–amino isobutyric acid, phenyl alanine, cystin, methionin and proline were variably increased as well. Alternatively in *P. roquefortii*, glutamic acid and hydroxy proline were decreased. Several other amino acids were detected in irregular pattern. Nevertheless, the fungal responses towards NaCl stress seemed to vary with the concentration of NaCl. With regard to organic acids content in the fungi, irregular and varied patterns were obtained. In *A. flavus,* phethalic, malic, stearic, citric acids and to some extent linoleic acids were increased. Ultrastructural studies revealed that the thickness of cell wall decreased, while thickness of plasma membrane increased in the studied fungi. The size of the nucleus and mitochondria decreased, while the numbers of mitochondria increased. Conidia of the treated fungi showed more echinulation and increase in their size.

Key Words: Salt stress; Amino acids; Organic acids; Ultrastructure; Fungi

INTRODUCTION

Fungal cell adaptation to high saline environment is a promising biological process (Park & Garnder, 1998). Osmotic stress is caused by high concentration of either salts or non-ionic solutes in the surrounding medium (Da Costa, 1989; Blomberg & Alder, 1992). The majority of halotolerant fungal species include either *Aspergillus*, its teleomorph *Eurotium* or *Penicillium* species (Moubasher *et al*., 1990; Hashem, 1993; Carlile *et al*., 2001). Recently, different species of black yeasts have been isolated from hypersaline waters of solar salterns. These yeasts were described as a new group of eukaryotic halophiles (Petrovic *et al.,* 2002; Gunde-Cimerman *et al.,* 2000 & 2005). The salt marsh microbial community is exposed to varying salinity regimes depending on the time of day and season and to the rate of evaporation (Torzilli, 1997). Studying the mechanisms of physiological cell responses enables us to understand how organisms may thrive in a particular environment (Dix & Webster, 1995). Indeed, few microorganisms have mechanisms that enable them to grow well in low water activity or high salinity. These mechanisms have received a lot of attention of several investigators (Gadd *et al*., 1984; Beever & Laracy, 1986; Blomberg & Alder, 1993; Alder, 1996; Park & Garnder, 1998). The accumulation of osmoprotective compounds such as polyols (glycerol); sugars (trehalose & manitol); amino acids (glutamine & proline); amino acids derivatives (Peptides; N-acetylated amino acid) and some organic acids in fungi is a common response to salt stress (Jennings, 1984; Casonka, 1989; Casonka & Hanson, 1991; Fougere & Streeter, 1991; Luxo *et al*., 1993). Moreover, there is evidence that protein kinases and induction of genes are involved in salt responses in different organisms (Cyert, 2003; Bahn *et al*., 2005; Hernandez-Lopez *et al*., 2006). Hernandez-Lopez *et al*. (2006) suggested that salt stress is regulated differently, through un-covered regulators and molecular circuits.

Osmotic adjustment of fungal cells may take place by exclusion of Na^+ or by decreasing the cell volume, leading to increase the concentration of osmotic solute (Han & Prade, 2002; Kogej *et al*., 2005). Hocking (1986) demonstrated that increased salinity in the growth media of three non-xerophilic fungi caused decrease in major fatty acids. In the cells of *Candida albicans*, more than 1% NaCl decreases un-saturated fatty acids and increased saturated fatty acids (Combs *et al*., 1968). Furtades *et al*. (1982) found that the contents of free fatty acids of *P. caeicdum* increased with increasing of NaCl concentration. On the other hand, Mert and Ekmekci (1987) reported that total organic acids of *A. flavus* decreased with NaCl concentration more than 2%. Parekh and Chatpar (1989) found that the mitochondrial fractions of *A. sydowii* have a more functioning system under high salt stress. Khattab *et al*. (1996) reported an increase in mitochondrial size and thickness of plasma membrane of *A. flavus* and *A. parasiticus* as a result of increasing NaCl concentration in the growth medium. Similar results were obtained by

Hosono (1992) and Hefnawy (2001). The present study was aimed at investigating the influence of salt stress on fungal ultra-structure to clearly understand modes of tolerance towards salt (NaCl) stress.

MATERIALS AND METHODS

Cultures. Cultures of the fungal species used in this investigation (*A. flavus* Link ex Gray 1821 & *P. roquefortii* Link Fr. Thom) were isolated from salt marsh soil in north Sinai, Egypt.

Isolation and identification. Czapek's agar medium supplemented with 0.5% (w/v) yeast extract was used for identification. To this medium, a combination of rose-Bengal (1/15000) (w/v) and chloramphenicol (50 ppm) were added to suppress bacterial growth. Under sterile conditions of laminar flow cabinet, soil sample were added to solid media as 0.2 g soil per petridish and the dishes were incubated at 27ºC for 7 days. Identification was carried out using the cover slip technique (Kawato & Shinoba, 1959) and a software for image analysis (SIS version 2.11, 1996) at the Regional Center for Mycology and Biotechnology at Al- Azhar University Cairo, Egypt, according to the current manuals (Raper & Fennel, 1977; Domsch *et al*., 1980).

Growth medium. Czapek's yeast autolyzate (CYA) broth medium amended with 8% or 4% (gL^{-1}) pure NaCl was used. The control medium was NaCl free. According to Klich and Pitt (1988) the medium was prepared in the following composition (gL^{-1}): K_2HPO_4, 1.0; yeast extract, 5.0; sucrose, 30.0 and 10 mL Czapek's concentrate composed of (gL^{-1}): $NaNO_3$, 0.9; KCl, 0.59; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01.

Culture conditions. Sets of six 250 mL conical flasks were used for each concentration. Fifty mL sterile medium were employed in each flask and inoculated with fungal spore suspension using 7 days old cultures for each fungus. The flasks were incubated at 27ºC for 7 days.

Preparation of cell-free extract for amino acid analysis. Mycelia were harvested by filtration using a Buchner funnel. The mycelia were then washed thoroughly with distilled water. The harvested mycelia were then ground with clean glass using 70% (v/v) ethyl alcohol. The obtained slurry was centrifuged at 6000 rpm for 20 min. The supernatant was decanted and used for analysis. Amino acid composition was carried out by using amino acid auto analyzer (LC 3000 Eppendrof Biotronik). The experiment was carried out in duplicate.

Preparation of cell-free extract for organic acids analysis. The harvested mycelia were ground with sterile glass using chloroform-methanol (2:1 v/v). After separation, the chloroform-methanol layer was evaporated under vacuum using a rotary evaporator to near dryness. The residue was dissolved in (v/v) 2 mL methanol and kept under freezing. Two mL of each extract were injected to a GC/MS.

Analysis conditions and identification of organic acids. Cell free extract were separated on Shimadzu GCMS-QP 5050A Gas Chromatograph equipped with a DB1 capillary column (30 m x 0.53 mm) international diameter, film thickness 1.5 µm (JW Scientific, USA) and mass detector. The operation conditions were as follow: injector and detector temperature, 280ºC; helium carrier gas with a flow rate of 2 mL min^{-1}; injection volume 2 µL; column temperature program 115ºC (1 min - 200ºC (1 min) at 7.5ºCmin^{-1}- 240ºC (2 min) at 5ºCmin^{-1} - 260ºC (4 min) at 3ºC min^{-1} with ionization voltage of 70.

Organic acids identification was carried out by matching the obtained mass spectra of the compounds with those of the stored Wiley Mass Spectra database using the class 5000 software (Shimodzu GCMS- QP 5050A).

Electron microscopy studies. Electron microscopy studies were carried out at the Regional Center for Mycology and Biotechnol, Al-Azhar University, Cairo, Egypt. To prepare fungal material for microscopy, *A. flavus* and *P. roquefortii* were cultivated on cellophane membrane placed on CYA agar plates supplemented with different NaCl concentrations (8% & 4%) (w/v). Hyphal tips were cut under the light microscope from the colony margin and fixed for 24 h in primary fixative (5% gluteraldehyde). The specimens were washed three times with phosphate buffer (pH 7.2). Then the buffer was removed and the samples covered with an aqueous solution of 1% osmium tetraoxide for 2 h. After this the osmium solution was removed and the samples dehydrated by passage through a series of ethanol concentration ranging from 50% to 96%.

Embedding. The absolute alcohol was removed and propylene oxide was added to the sample for 1 h. The samples were put in propylene oxide and Epon 812 resin (2:1) after that put in pure resin for overnight, then placed in an oven at 60ºC for 48 h. Small blocks were sectioned (50 nm) using ultra microtome. The sections were stained by uranylacetate-lead citrate 500A and subsequently examined with the transmission electron microscope (C Joel Jem-1200 EX II. Acc. Voltage 120 KV. MAG- medium).

RESULTS

Effect of salinity on the free amino acids content. Both amino acids composition and percentages seem to be affected by the presence of NaCl in both the organisms (Table I). In case of *A. flavus* some of unusual amino acids disappeared as a result of applying high NaCl concentration, such as α- amino-adipic acid and α- aminobutyric acid. Moreover, glycine, which may act as osmolyte amino acid, and γ- amino-n-butric acid, were increased. Some amino acids were detected in the presence of NaCl in growth medium such as taurine and 3-methyl histidine in addition, to alanine as a compatible solute. Glutamic acid was increased and proline was slightly increased. On the other hand amino acids of *P. roquefortii* were more influenced than *A. flavus* as a result of presence of NaCl in the growth medium. The concentration of proline increased, while hydroxyproline disappeared in the presence of NaCl in the

Table I. Effect of sodium chloride concentrations on the percentage and composition of free amino acids of *A. flavus* and *P. roqufortii*

Amino acid (%)	*A. flavus*			Amino acid (%)	*P. roqufortii*		
	Control	4% NaCl	8% NaCl		Control	4% NaCl	8% NaCl
Phosphoserine	7.33	5.16	7.26	Phosphoserine	1.08	1.80	2.31
Phosphoethanol amine	1.98	2.13	0.69	Phosphoethanol amine	2.22	1.8	2.72
Aspartic acid	6.12	3.71	5.84	Aspartic acid	5.72	4.80	4.88
Threonine	3.14	3.32	1.29	Hdroxy proline	10.89	0	0
Serine	4.83	2.93	2.85	Theronine	2.91	4.40	1.87
Glutamic acid	7.99	14.10	24.7	Serine	4.51	2.56	3.78
α- amino adipic acid	0.54	4.50	0	Glutamic acid	9.14	4.01	6.86
Proline	4.05	4.2	4.31	α - amino adipic acid	1.35	4.80	3.09
Glycine	2.26	2.04	0	Proline	1.91	2.27	2.9
Citrulline	5.56	1.21	3.68	Glycine	1.89	1.22	1.22
α - amino butyric acid	0.42	2.88	0	Citruline	7.10	5.42	6.71
Valine	1.33	3.27	0.70	α - amino butyric acid	0.44	2.22	1.51
Cystine	1.98	4.70	3.03	Valine	1.16	2.51	1.11
Methionine	2.14	4.16	2.30	Cystine	1.85	3.64	1.45
Leucine	2.12	2.66	2.96	Methionine	1.74	1.7	5.60
Tyrosine	2.68	2.05	2.21	Leucine	1.87	2.93	2.47
Phenyl alanine	0.98	2.61	1.09	Phenylalanine	1.03	3.41	1.14
β- amino iso butyric acid	0.93	2.88	4.05	β - amino iso butyric acid	3.72	2.18	3.64
γ- amino N butyric acid	0.48	2.88	4.94	γ - amino N butyric acid	1.47	1.32	3.53
Histidine	0.46	2.33	0.90	Histidine	3.73	3.43	1.51
Carnosine	4.61	6.31	13.6	3-methyl-histidine	2.94	2.20	2.76
Ornithine	2.15	3.69	0.51	Tryptophan	0.50	8.37	2.25
Lysine	9.45	3.68	3.90	Carnosine	5.53	5.58	8.48
Tryptophan	0.9	5.69	5.00	Ornithine	2.03	0.24	0.37
Arginine	10.20	1.9	2.39	Lysine	7.48	1.75	4.82
Taurine	0	2.51	0.77	Arginine	9.13	3.42	9.70
Alanine	0	2.48	0.91	Taurine	0	5.51	0
3-metlyl histidine	0	0	2.44	Isolucine	0	2.56	0
				Tyrosine	0.51	4.55	2.36

Table II. Effect of sodium chloride concentration on the organic acids content of *A. flavus* and *P. roqufortii*

Organic acid (%)	*A. flavus*			Organic acid (%)	*P. roqufortii*		
	Control	4% NaCl	8% NaCl		Control	4% NaCl	8% NaCl
Malic	1.23	16.05	16.0	Malic	3.4	3.3	9.82
Acetic	4.0	1.21	2.82	Succinic	8.04	27.95	0
Adipic	6.54	4.71	1.99	Acetic	7.2	6.9	7.2
Citric	7.74	8.10	31.65	Adipic	8.8	1.4	1.54
Myristic	3.85	3.11	2.57	Citric	8.02	8.00	42.91
Palmitic	7.19	13.13	5.64	Myristic	8.58	1.08	4.2
Phthalic	8.66	6.14	27.03	Palmitic	12.71	28.0	4.13
Stearic	1.13	4.64	4.49	Phthalic	25.2	0.22	2.0
Linoleic	6.42	2.23	0	Stearic	10.32	0	0
				Linoleic	5.39	0	0

culture medium. Other compatible amino acids such as glutamic acids and glycine were decreased. Unusual amino acid such as α- amino adipic acid, α- amino butyric acid and γ- amino- n- butyric acid were increased.

Eftect of sodium chloride on organic acids. The NaCl had a great effect on organic acids composition (Table II). In *A. flavus*, malic acid, citric acid and stearic acid were increased, while acetic acid, adipic acid and myristic acid decreased. Linoleic acid disappeared with high concentration of NaCl. Regarding *P. roquefortii*, it was found to be more affected than *A. flavus*. Stearic acid and linoleic acid were not detected and several of other organic acids were severely decreased; adipic acid, myristic acid and phthalic acid whereas malic and citric acids were highly increased.

Effect of sodium chloride on ultra-structure. Electron micrographs of treated hyphal cells showed that the cell wall thickness decreased, while thickness of cell membrane increased compared to untreated hyphae in both *A. flavus* and *P. roquefortii.* In case of *P. roquefortii* severe disorder of cell wall was recorded (Fig. 1). The cell membrane was withdrawn away from the cell wall as a result of shrinkage of the protoplast. Mitochondrial size was slightly decreased and their number increased in both species; some of these were ruptured in *A. flavus* treated with 10% NaCl (Fig. 2). The treated cells showed enlarged, round and numerous electron dense globules; probably lipid droplets, which were scattered at the peripheral regions of the protoplast in response to presence of 10 or 8% NaCl. Generally, the stored lipids increased in relation to salt stress. Several numerous large vacuoles appeared in the cells of treated

Fig. 1. L.S. of *A. flavus* hyphae showing ultra-structure: cell wall (CW), cell membrane (CM), nucleus (N), vacuole (V) and lipid droplets (LD). **(A) Un-treated mycelium (Magnification 10000X). (B, C) Treated mycelium with 10% NaCl (Magnification 20000X & 30000X, respectively)**

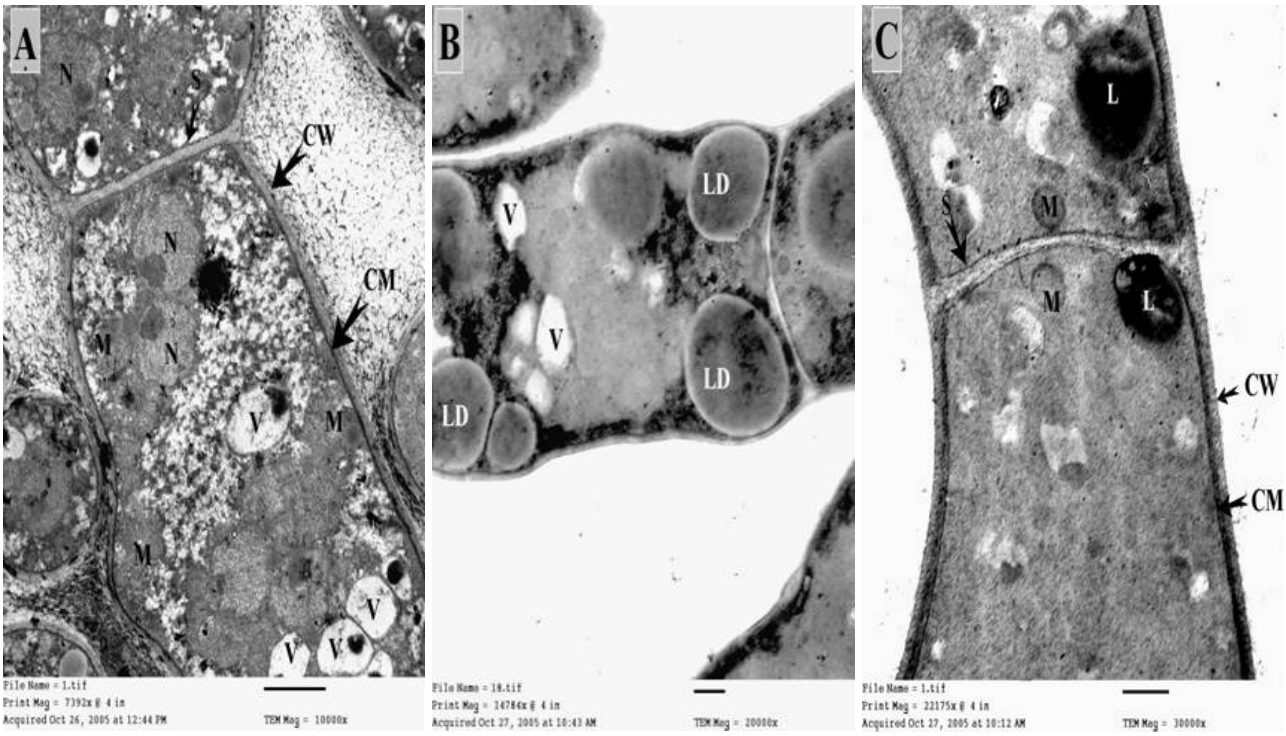
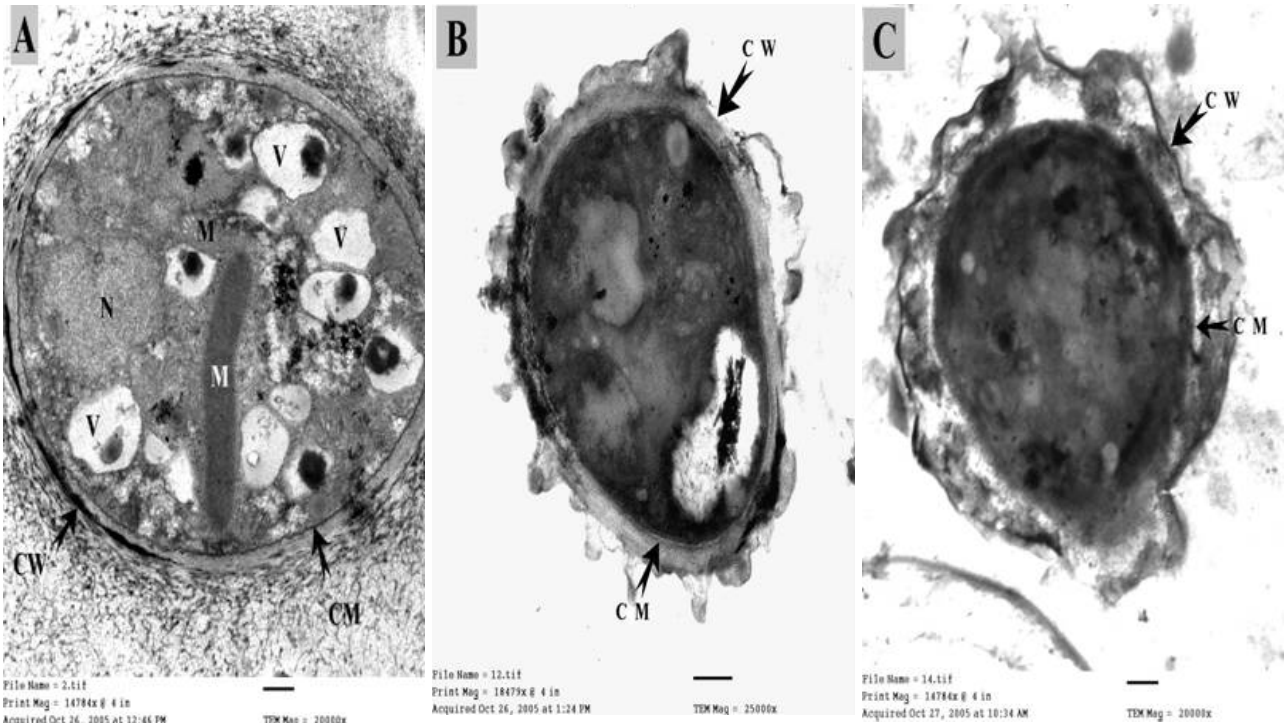
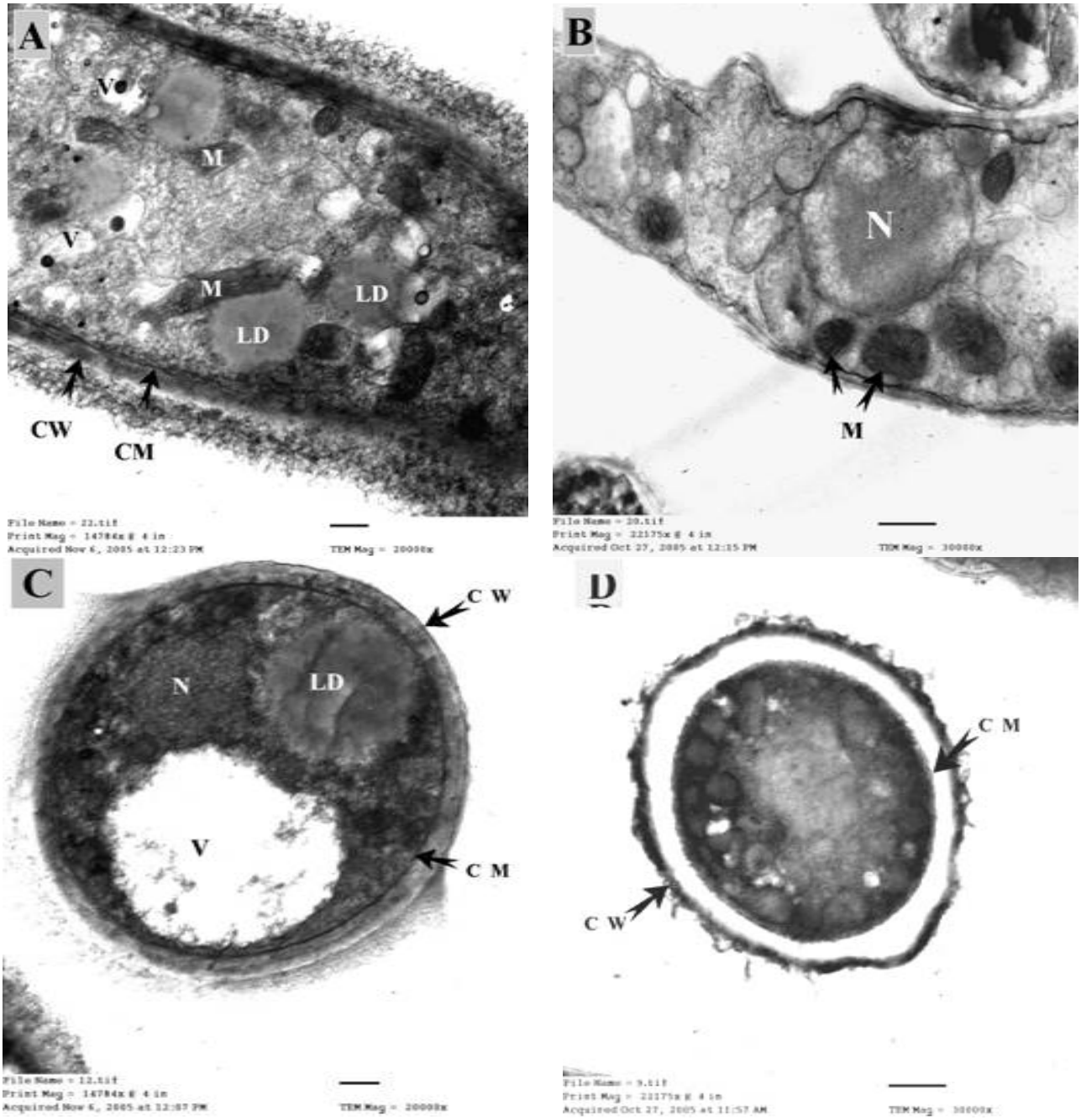


Fig. 2. L.S. of *A. flavus* hyphae showing ultra-structure: cell wall (CW), cell membrane (CM), nucleus (N), vacuole (V) and mitochondria (M). **(A) Un-treated mycelium (Magnification 20000X). (B) Untreated conidium (Magnification 25000X). (C) Treated conidium with 10% NaCl (Magnification 20000X)**



organisms. Great shrinkage of nucleus size appeared to be half of the normal size in case of *A. flavus.* Conidia of the treated *A. flavus* and *P. roquefortii* showed more echinulation and increase in their cell wall (Fig. 3).

Fig. 3. L.S. of *P. roquefortii* hyphae showing ultrasructure: cell wall (CW), cell membrane (CM), nucleus (N), vacuole (V) and mitochondria (M). **(A) Untreated mycelium (Magnification 20000X). (B) Treated mycelium with 8% NaCl (Magnification 30000X). (C) Untreated conidium (Magnification 20000X). (D) Treated conidium with 8% NaCl (Magnification 25000X).**



DISCUSSION

The understanding of the basic processes of salt tolerance and salt adaptation has important applications. Salt tolerant fungi have developed very efficient mechanisms to meet these demands (Alder, 1996; Hernandez-Lopez *et al*., 2006). It has been reported that some of amino acids, fatty acids and organic acids act as compatible solutes to adapt microorganisms to osmotic down shock. Moreover, intracellular pools of compatible solutes can be raised by increased synthesis or uptake from the medium (Casonka, 1989; Koo *et al*., 1991). The data showed that when NaCl was added to the medium, alteration of amino acid

composition and percentage was observed especially at higher concentration. The amino acids; proline and glutamic acid were increased in addition to taurine and 3- methyl histidine (Table I). These unusual amino acids provide a direct protection against osmotic stress and also act as chemical mediators, affecting the synthesis of other osmolytes (Talibart *et al*., 1994). Unusual amino acid γ-amino-N-butric acid and β-aminobutric acid were increased in the presence of NaCl in the growth media of *A. flavus.* Any decrease in amino acid composition in case of *P. roquefortii* may be due to higher rates of proteolysis or due to amino acid catabolism. In both *A. flavus* and *P. roquefortii* phenyl alanine and tyrosine were increased.

It is known that these amino acids are the precursor of many alkaloids (Smith, 1995). Unfortunately, little information has been published on the role of lipid composition and organic acid on the mechanisms that enable the maintenance of positive turgor potential (Hocking, 1993). One possible mechanism is the change of composition of fatty acids and hence permeability of cell membrane to balance the solute concentration in the external environment (De Kruff *et al*., 1973; Ohno *et al*., 1979). Among the studied species there was an osmolyte preference for organic acid. In *A. Flavus,* citric acid and myristic acid were highly increased with disappearance of linoleic acid at high NaCl concentration. In case of *P. roquefortii*, citric acid was highly increased, while stearic and linoleic acids were not detected (Table II). The accumulation or decreasing in organic acids depended on salt concentration and the treated organism. However, the organic osmolytes were important for the response of microorganism to hyper-osmotic conditions (Casonka & Hanson, 1991; Glaasker *et al*., 1996).

Regarding the ultrastructure of the studied species, decrease in the cell wall thickness and distortion and envagination in their surface especially in case of *P. roquefortii*. In both fungi, thickness of the cell membrane increased (Fig. 1 & 2). The increase of cell membrane thickness may be due to an increase in phospholipids content, fatty acids and sterol (Hosono, 1992; Khattab *et al*., 1996). More likely, the distortion in cell wall shape may leads to an adverse effect on function as a protector against high osmotic pressure. Consequently, its function to control membrane permeability seemed to be badly affected especially in case of *P. roquefortii*, which grow poorly above 8% (w/v) NaCl (Fig. 2). The changes in mitochondrial shape and volume under salt stress may be due to osmotic pressure changes. The increase in their number may be an enhancement of the respiratory activities of the treated organisms (Fig. 3). The results are consistent with those of Parekh and Chatpar (1989) and Kelavkar *et al.* (1993). The decrease in nuclear size was also observed as a result of salt stress. Presumably due to a drastic influence on all other physiological functions especially the differentiation processes of the newly formed conidia. Eventually, the process of conidiogenesis required energy for the activity of several enzymes. Stress conditions may cause energetic loss and inhibiting the enzymatic activities (Hassum & Nielsen, 1998).

In conclusion, unusual amino acids may play an important role under salt stress. Moreover, no conclusive pattern of organic acids content in both fungi under NaCl stress was obtained. Similar changes of several cellular organelles were obviously noticed in fungi. Generally, *A. flavus* was more tolerable to NaCl stress than *P. roquefortii*.

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