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Response of the Higher Basidiomycetic *Ganoderma resinaceum* to Sodium Chloride Stress

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Ganoderma resinaceum **tolerated sodium chloride salt stress within a range of 0 mM till 300 mM. It responded to salt stress with fluctuation in proline formation at different NaCl concentrations. However, the mycelial dry weight, total protein contents and exopolysaccharides did not changed considerably. Increasing sodium chloride concentration led to morphological alteration in fungal mycelia with disappearance of fungal cell wall, plasmolysis, and vacuolation as indicated with electron microscopic examination of the fungal growth.**

KEYWORDS: Exopolysaccharides, *Ganoderma resinaceum*, Proline, Sodium chloride

Extreme environments lead to survival of some microorganisms and excluding of the others. Salt stress to the living cells appears in disturbance of their osmotic homeostasis. Bois *et al*. (2006) stated that cells under salt stress initially accumulate salts as free osmotica, then specific ion toxic effect appears once a certain threshold level of Na and/or Cl or other salt accumulation has been reached. In the fungus *Dendryphiella salina*, Wethered *et al*. (1985) reported increased levels of polyols when the fungus was grown in saline media. Actually, an excess of Na and/or Cl ions may alter membrane integrity, enzymatic activity, and protein and nucleic acid metabolism (Niu *et al*., 1995). Hyperosmotic stress in plants and fungi is associated with inhibition of cell wall extension and cellular expansion, leading to reduction of growth (Niu *et al*., 1995; Hasegawa *et el*., 2000).

A living organism is considered resistant by avoidance to a certain physiological changes or stress if it could be able to get ride of it completely or even partially by interfering with the intracellular permeation of that toxicant. On the other hand, it is resistant by tolerance if that organism allows penetration of the toxicant and uses this process to alleviate the external effects (Segner and Braunbeck, 1998). Mannitol, arabitol and glycerol (polyols), and non-reducing saccharides, such as a trehalose, are the main soluble carbohydrates found in basidiomycetes and ascomycetes fungi classes (Lewis and Smith, 1967). Mannitol, glycerol and trehalose and some other metabolites, such as the proline amino acid, have been observed to increase in fungi under osmotic stress (Shen *et al*., 1999) and freezing (Tibbett *et al*., 2002). It was established that trehalose in fungi is used as a reserve carbohydrate. Moreover, it also serves as a protectant against cell damage induced by nutrient limitation, heat shock, oxidative stress and reduced osmotic potential (Arguelles, 2000), also it favors stabilization of membranes and protein (Wiemken, 1990). The main objective of this study was to investigate the effect of sodium chloride at different concentrations on *Ganoderma resinaceum* vegetative growth, carbohydrate production, and cellular protein and hypha cellular components. The main goal is to investigate whether the sodium chloride stress could increase *G. resinaceum* exopolysaccharides or not, where it has many industrial and medicinal applications in general. Also to take insight to the morphological changes of the fungus under sodium chloride stress. For our information this is the first study to speculate the response of higher fungi to sodium chloride stress.

Materials and Methods

The fresh fruiting bodies *G. resinaceum* was collected from different parts in Shabsheer El Hassa, Gaharbia Governorate.

Hyphae isolation of *G. resinaceum*. Fresh collected *G. resinaceum* fruiting bodies were used to produce mycelia by cutting out the fresh fruiting body longitudinally using sterilized knife (by flame and ethyl alcohol) then cutting transversely and with sterilized forceps picking up the fresh parts carrying the basidiospores of the hymenia which was inoculated in sterilized malt agar medium and inoculated at 28°C for 9 days (Mustafa, 2002).

Sodium chloride stress. The GYP medium containing glucose 40 g, yeast extract 10 g, peptone 5 g as designed

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by Wang *et al*. (2001) was used to study the effect of sodium chloride different concentrations (0.0, 35, 75, 140, and 300 mM) on *Ganoderma resinaceum* growth, proline and EPS production. The medium is consisting of glucose 40 g, yeast extract 10 g, and peptone 5 g made up to 1L with distilled water at pH 5 under static conditions at 26°C for 12 days.

***Ganoderma resinaceum* expolysaccharides; ethanol precipitation.** The culture filtrate (100 *ml*) from static flasks of different cultures at different concentrations of sodium chloride cultures were centrifuged at 10,000 rpm (SIGA laboratory centrifuge-16,500rpm-3K18-rotar Nr./ 2156-H) for 20 min and the resulting supernatant was filtered (0.45 μm, Millipore). The supernatant (protein) was removed using trichloroacitic acid (TCA) 5% according to Klalil (2002), and stored at freezer overnight. The resulting culture filtrate was mixed with 4 times its volume of absolute ethanol, stirred vigorously and kept overnight at 4°C, then the precipitated EPS was obtained by centrifugation at 10,000 rpm for 20 min and the supernatant was discarded (Bae *et al*., 2000).

Determination of exopolysaccharides (EPS) concentration. It has been determined by phenol-sulfuric acid method. Simple sugars, oligosaccharides, polysaccharides and their derivatives including the methyl esters with free or potentially free reducing groups, give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the color is stable. The total sugar determination was achieved by mixing 1 *ml* sugar solution with 1 *ml* 5% phenol in water and 5 *ml* of concentrated sulfuric acid. The tubes were allowed to stand for 10 minutes at 30°C (Dubois *et al*., 1956). The characteristic yellow-orange color was measured spectrophotometrically at 490 nm for hexoses using glucose as standard.

Mycelia dry weight determination. The dry weight of mycelia were measured after separation from the culuture filtrate with repeated washing of the mycelial pellets with distilled water and dried at 70°C overnight to a constant weight (Bae *et al*., 2000).

Protein concentration determination. Mycelial protein concentration of *G. resinaceum* under different sodium chloride stress was determined according to Bradford method (1976).

Proline determination. A liquot of 0.5 *ml* of the samples was mixed with 0.3 *ml* of a ninhydrine solution [0.125 g of ninhydrine dissolved in 5 *ml* of H_3PO_4 (6 M): glacial acetic acid, 2 : 3, v/v) and 0.2 *ml* glacial acetic acid (Paquin and Lechasseur, 1979). Each sample was thoroughly mixed and incubated at 100°C for 45 min. Samples were cooled prior to the addition of 800 μl of toluene. After 45 min, the optical sensitivity (OD) of the upper phase (toluene) was assessed by spectrophotometer at 515 nm. The proline content was calculated from the regression curve of OD obtained from standard solutions of pure proline, ranging from 0 to 20 μg.

Transmission electron microscope. A small portions of *G. resinaceum* grown at 0, 75 and 300 mM sodium chloride in GYP medium after 15 days of growth were fixed at room temperature in 20% (v/v) glutaraldehyde mixed with potassium phosphate buffer at pH 7.0 for 2 h, followed by post fixation in 2% (w/v) osmium tetroxide buffered in 5 mM sodium cacodylate at pH 6.5, for 40 min. After fixation, the material was washed overnight in the appropriate buffer, dehydrated at room temperature in acetone, and embedded overnight at 65°C in low viscosity epoxy resin (Spurr, 1969). At these conditions the material was polymerized; ultrathin sections were cut by glass knives of an ULKD ultramicrotome. Sections were collected each on stabilized copper grids, stained with lead citrate and examined in a GOL 100 cX electron microscope. This method was carried out according To Ellis and Griffith (1974) at Faculty of Science, Alexandria University, Central Lab., Electron Microscope Unit. Egypt.

Results and Discussion

Ganoderma resinaceum has been grown under different concentrations of sodium chloride (0, 35, 75, 140 and 300 mM) in order to investigate whether the sodium chloride stress on *G. resinaceum* led to increase its production from exopolysaccharides or not (Table 1). *G. resinaceum* has survived all tested concentrations of sodium chloride (0~ 300 mM). There was a gradual decrease for mycelial dry weight, total cellular protein, exopolysaccharides production and proline with increasing the sodium chloride stress when be compared with the control. Mycelial dry weight of *G. resinaceum* might be slightly increased at 35 mM and then decreased until 300 mM of NaCl, and these results were almost in accordance to those reported by Bois *et al*. (2006). During their study on three mycorrhizal fungi (*Laccaria bicolor*, *Hebeloma crustuliniforme*, and *Phialocephala* sp.). They found decrease in dry mass of *H. crustuliniforme* and Phialocephala and *L. bicolor* at 50 and 100 mM until 300 mM of NaCl. In response to the halostress created by the addition of different concentrations of NaCl in the media, the fungal isolates lowered their growth rates and altered their structure and lipid compositions (Mulder *et al*., 1989). The concentration of exopolysaccharide produced at 0 mM of NaCl was 0.167 ± 0.01 *ml*/*ml* which decreased five fold at 300 mM

Table 1. Effects of different concentrations of NaCl on dry weight, total protein, EPS (exopolysaccharides) production and proline contents of *Ganoderma resinaceum*

Sodium chloride concentration (mM)	Mycelial Dry. wt (g/100 *ml*)	Total protein (mg/*ml*)	EPS (mg/*ml*)	Proline μ moles/g FW
0 (Control)	0.90 ± 0.01*	0.880 ± 0.02	0.167 ± 0.01	20.10 ± 1.0
35	0.97 ± 0.03	0.879 ± 0.04	0.145 ± 0.02	11.86 ± 0.2
75	0.85 ± 0.03	0.750 ± 0.01	0.070 ± 0.01	12.93 ± 0.3
140	0.80 ± 0.01	0.727 ± 0.01	0.050 ± 0.01	10.13 ± 0.8
300	0.77 ± 0.01	0.727 ± 0.002	0.034 ± 0.01	13.60 ± 0.7

*Reading is the mean of three experimental reading ± SD.
FW is mycelial fresh weight.

NaCl to produce (0.034 ± 0.01 mg/*ml*). The proline content of *G. resinaceum* in culture growth medium at different concentrations of sodium chloride was fluctuated with the highest concentration at 300 mM NaCl in contrary to other fungal group. Whereas, Wethered and Jenning (1985) reported that marine fungi accumulated proline and other soluble amino-acids within their mycelia and these compounds increased with salinity. Also, in lower fungi endogenous proline increased with reduced water potential and increased salinity as reported by Luard (1982). Actually, protein synthesis involved more energy than production of carbohydrates (Niu and Wang, 1997). Total cellular protein of *G. resinaceum* at 0 and 35 mM sodium chloride was the same (0.879 mg/*ml*), however it decreased at 75 mM NaCl to produce 0.75 mg/*ml*). Furthermore, it became stable at 140 and 300 mM NaCl stress and forms 0.727 ± 0.01 mg/*ml*.

Morphological characteristics under sodium chloride stress. Three concentrations of sodium chloride have been chosen to investigate its effect on mycelial structure of *G. resinaceum* in morphology. Examining *G. resinaceum* mycelial growth under electron microscope at 85000 × indicated the presence of clamp connections with three layers in the cell wall (Fig. 1). Also, *G. resinaceum* cells appeared with no plasmolysis and no vacuolation). Mycelia of *G. resinaceum* at 75 mM sodium chloride appeared with mycelial construction and plasmolysed (Fig. 2). At 300 mM of sodium chloride; the mycelia appeared distorted with disappearance of cell wall layers

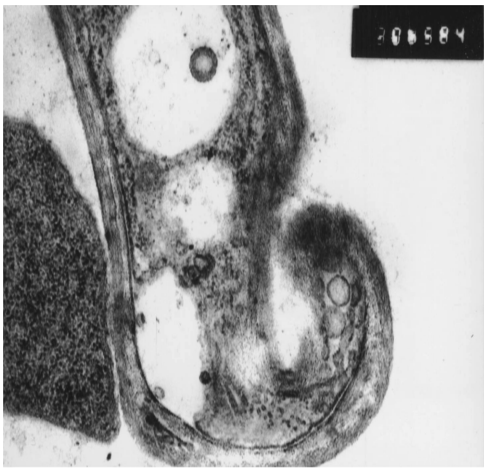

Fig. 1. *G. resinaceum* mycelium at 0 mg/*ml* sodium chloride (85,000 ×).

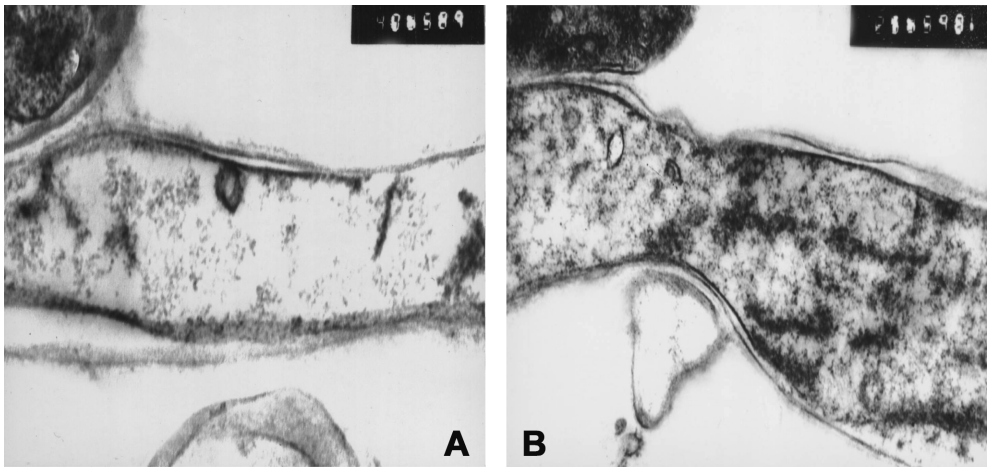

Fig. 2. *G. resinaceum* at 75 mM sodium chloride indicated cytoplasmic plasomolysis (A) at 89,000 × and mycelial construction (B) at 98,000 ×.

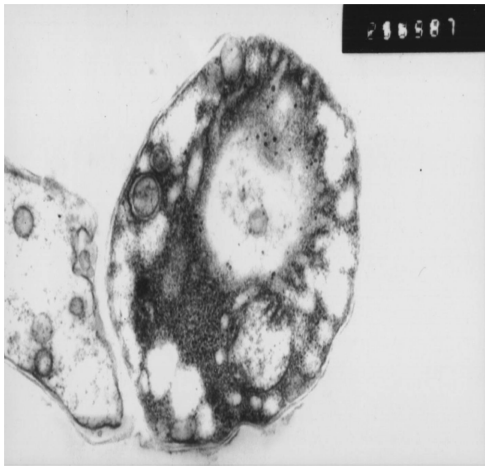

Fig. 3. *G. resinaceum* mycelia at 300 mM Sodium chloride (87000 ×) indicated complete cells distortion with the disappearance of cell wall layers. Cells have many vacuoles, and become to fuse with each other.

and many vacuoles (Fig 3). Also, the mycelial cells became to fuse with each other. Elliott (1972) has reported a similar result during his work on *Phytophthora cactorum* salt stress that showed short and contorted branches. Ali (2005) mentioned that increasing NaCl molarity accelerated the appearance time and degree of plasmolysis of *Saprolenia parasitica*. However, Mulder *et al*. (1989) studied the response of *Alternaria phragospora*, *A.chlamydospor*a and *Ulocladium chartarum* to the halostress created by the addition of different concentrations of NaCl in the media, the fungal isolates lowered their growth rates and their structure and lipid composition. The pattern of morphological change, which included the increase in septation and width as well as the production of chamydospores, was similar in all three isolates.

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