Egypt. J.Microbiol. **45**, pp. 45 - 61 (2010)

Protective Strategies Induced by *Azotobacter* sp. Strain and its Role after Exposure to H_2O_2 in Improving *Hibiscus sabdarriffa* Performance

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THE EFFECT of hydrogen peroxide (H_2O_2) solutions, applied to pure cultures of *Azotobacter* at concentrations (0.0, 0.05, 0.1 and 1%), was determined by measuring survival, protein content, antioxidant enzymes, H_2O_2 content and malondialdehyde content after 15 and 30 min. The impact of the treated bacteria to affect seed vigour of *Hibiscus sabdarriffa* was then evaluated. Survival of *Azotobacter* cells was not affected significantly at H_2O_2 concentrations of 0.05 % and 0.1 % after 15 min (11 % and 34 % reduction, respectively) compared to the control. With 30 min exposure, the protein content was reduced 2 fold compared to 15 min exposure time. However, for superoxide dismutase (SOD), ascorbat peroxidase (APX) and catalase (CAT), no significant differences were observed at low concentrations of H_2O_2, but only at the highest concentration (1%). In contrast, peroxidases (PODs) showed slightly increased activity at low concentrations and significant reduction at higher concentrations. Also, H_2O_2 content and malondialdehyde (MDA) content were increased in treated *Azotobacter* cells. The native isolates of *Azotobacter* sp. strain greatly increased the vigour index and germination rate of *H. sabdarriffa* up to 90% as compared to only 50% in the untreated control. We believe the response of *Azotobacter*, treated with H_2O_2 was probably related to the different protective effects of antioxidant enzymes in this strain. The ability of the bacterium to respond and survive at exposure to H_2O_2 during logarithmic growth could be important during colonization of the root surface, when the cells are presumably entering into an actively growing phase.

Keywords: Antioxidant enzymes, *Azotobacter*, *Hibiscus sabdarriffa*, Hydrogen peroxide.

All aerobically grown microorganisms encounter reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) (Yan *et al*., 2006). ROS can be generated as a consequence of normal cellular metabolic processes such as respiration or the β- oxidation of fatty acids. Alternatively, ROS can be produced through the metabolism of foreign chemicals or the host immune system (Jamieson *et al*., 1994). ROS are highly toxic to bacterial cells through their detrimental effects on many biological macromolecules including DNA, proteins, and membrane (Fridovich, 1978). Consequently, bacteria have evolved several ways to protect themselves

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from oxidative damage through the evolution of enzymatic and non- enzymatic systems that either directly detoxify or repair damage caused by ROS. Also, bacteria have protective strategies that involve stress-induced adaptive and cross-protective response (Yan *et al*., 2006).

Superoxide dismutases protect the cell by converting superoxide to hydrogen peroxide, and the expression of two such enzymes has been described for *A. vinelandii* (Qurollo *et al*., 2001). Hydrogen peroxide is then removed from the cell by either dismutation by a catalase (Equation 1) or reduction by a peroxidase (Equation 2):

$$2\ H_2O_2 + E \longrightarrow 2\ H_2O + O_2 + E \quad (1)$$

$$H_2O_2 + 2\ AH_2 + E \longrightarrow 2\ H_2O + 2\cdot AH + E \quad (2)$$

where E is the enzyme, AH_2 is a hydrogen donor, and ·AH is the radical form of the donor. Thus, hydrogen peroxide degradation by catalase activity is independent of the metabolic state of the bacterium, whereas degradation by peroxidase activity is metabolically dependent since the electron donor must be regenerated (Sandercock & Page, 2008).

Exposure to a nonlethal concentration of one oxidant can induce protection against subsequent exposure to lethal concentrations of the same oxidant (an adaptive response) or untreated agents (a cross-protective response) (Mongkolsuk *et al*., 1997). Previous studies have confirmed that these responses are highly conserved in many bacteria, including *Escherichia coli* (Demple, 1996), *Saccharomyces cerevisiae* (Flattery *et al*., 1993), and *Candida albicans* (Jamieson *et al*., 1996). As a model prokaryote, *Bacillus subtilis* has been extensively studied and shown to possess an adaptation mechanism against H_2O_2 (Antelmann *et al*., 1996). Many previous investigations on adaptive response of microorganisms to oxidative stress are focused on stress inducible genes and proteins, but not usually on the activities of particular enzymes (Zheng *et al*., 2001 and Gasch & Werner, 2002). Although H_2O_2 effect can be observed by measurement of mRNA or total cell protein levels, the final step in cell adaptive response is the activity of the respective enzymes.

Catalase, peroxidase, and superoxide dismutase are used by aerobes in all kingdoms to degrade reactive oxygen intermediates (ROIs), such as O_2^- and H_2O_2, produced during respiration (Sandercock & Page, 2008). Bacterial growth on root surfaces would expose the cells to plant enzymes that produce the superoxide anion, O_2^-, and hydrogen peroxide (H_2O_2). Root surface peroxidases generate H_2O_2 and O_2 during the oxidation of NAD (P)H (Albert *et al*., 1986). Indoleacetic acid oxidase on the root surface liberates O_2^- during the oxidation of indoleacetic acid (Mottley & Mason, 1986). The reduction of Fe^{3+} to Fe^{2+} at the root surface is reported to involve O_2 production (Cakmak *et al*., 1987).

Azotobacter spp. have a very high respiratory rate, and their ability to fix N_2 in O_2 tensions at and above air saturation levels has intrigued researchers for many years (Gallon, 1992). One mechanism *Azotobacter* sp. use to protect nitrogenase from O_2 damage is termed conformational protection (Robson

& Postgate, 1980), and involves the association of FeSII protein with nitrogenase during periods of oxygen stress (Moshiri *et al*., 1994). Roselle plant (*Hibiscus sabdarriffa*) is one of the important and popular medicinal plants (Chang *et al*., 2006).

In order to expand our understanding of cell adaptive response to oxidative stress, it is important to use distinct experimental approaches for detection of gene expression and to measure the activity of protective enzymes. The aim of the present work was to study the effect of hydrogen peroxide on cell survival and activity of antioxidant enzymes in *Azotobacter* sp. strain and the cell defense induced against hydrogen peroxide in the exponential phase of growth. We also investigated the role of *Azotobacter* sp. strain -as a native rhizosphere organism- on *Hibiscus sabdarriffa* seedling germination and vigour.

Materials and Methods

Soil sampling and isolation of Azotobacter

Three soil samples (0-10 cm depth) were taken from inside the South Valley University campus at Qena, Egypt. Each sample was a composite of several cores to yield a total mass of 3- 4 kg. Samples were extracted by suspending a known weight of soil in a known volume of water and stirring continuously for 30 min (Case & Johnson, 1984). The homogenate was filtered through Whatman no. 1 filter paper. Serial dilutions were made up and used for isolation of *Azotobacter* sp. strain, by using Ashby medium supplemented with sucrose or mannitol (20 g/l) (Rodina, 1965 and Kurdish *et al*., 2008).

Hydrogen peroxide and Azotobacter survival assay

Bacteria used for hydrogen peroxide treatment were grown in shake flask cultures to the desired growth phase at 28 °C. They were removed from the culture medium by centrifugation at 5,000 *g* for 10 min at room temperature and washed thoroughly with distilled water before being resuspended in 50 mM potassium phosphate buffer (pH 7.0). The resuspended cells were treated with different concentrations of H_2O_2 (0.0, 0.05, 0.1, 0.5 and 1%) in phosphate buffer for 30 min. An aliquot was taken at 15 min intervals, for protein determination as described below. Growth rates are defined as protein content recovered after the treatment.

Cells were also homogenized in a glass tissue homogenizer. Aliquots of the homogenates were sonicated for one minute to release cell constituents, using an ultrasonicator (Dr. Hilscher, Germany) adjusted at maximum power (Sayed & Wheeler, 1999). Protein content was determined using the Bradford method (Bradford, 1976) by measuring the optical density at 595 nm on a "SPECTRONIC® GENESYS™ 2PC" Spectrophotometer, Spectronic Instruments, USA.

All experiments were independently repeated three times, and data in figures and table are expressed as averages ± standard deviations.

Impact of Azotobacter seed treatment on the germination and seedling vigour of Hibiscus sabdarriffa

To find out the effect of these isolated diazotrophic *Azotobacter* on the germination and vigour index of *H. sabdarriffa*, 50 sterilized seeds were placed in a sterile petri plate and inoculated with 10 ml broth culture of H_2O_2 treated *Azotobacter* for 2 hr and then shade- dried. The H_2O_2 concentrations were 0.0, 0.05, 0.1, 0.5 and 1% and the initial *Azotobacter* populations were 10^6 cells/ml. The inoculated seeds were tested for the germination rate using the paper towel method (ISTA, 1976). The germination percentage was calculated at 8 days after sowing (DAS) to 10 DAS. The morphological parameters, *i.e.* shoot length and root length were measured on 15 DAS. The vigour index (*VI*) of the seedlings was estimated as suggested by Abdul-Baki & Anderson (1973).

$$VI=RL+SL\times GP,$$

where *RL* is root length (cm), *SL* is shoot length (cm) and *GP* is germination percentage.

Enzyme extraction

Cell free extracts of H_2O_2- treated *Azotobacter* cells were used for enzyme analysis. Sample preparation was described by Mukherjee & Choudhuri (1983). A sample (0.5 gm) was frozen in liquid nitrogen and finely ground by pestle and mortar. The frozen powder was added to 10 ml of 100 mM phosphate buffer (KH_2PO_4 / K_2HPO_4), pH 7.0, containing 0.1 mM Na_2EDTA and 0.1 gm of polyvinylpyrrolidone. The homogenate was filtered through cheese cloth and centrifuged at 15,000 rpm at 4^{o}C for 10 min. The supernatant was re-centrifuged at 18,000 rpm at 4^{o}C for 10 min. The resulted supernatant was collected and stored at 4^{o}C for enzyme assay.

Determination of antioxidant enzyme activities

A. Superoxide dismutase (SOD) activity (EC 1.15.1.1) was measured according to Dhindsa *et al*. (1981). Three ml of mixture contained 13 mM methionine, 0.025 mM ρ- nitro blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium bicarbonate and 0.5ml enzyme extract, reaction was started by adding 0.002 mM riboflavin and placing the tubes below two 15W fluorescent lamps for 15 min. The reaction was stopped by switching off light and covering the tubes with black cloth. The tubes without enzyme developed maximal color, a non-irradiated complete reaction mixture served as blank. Absorbance was measured at 560 nm; one unit of SOD was defined as the amount of enzyme causing half the maximum inhibition of NBT to blue formazan.

B. Catalase (CAT) activity (EC 1.11.1.6) was assayed in a 3 ml reaction solution composed of 50 mM phosphate buffer (pH 7.0), 30% (w/v) H_2O_2 and 0.5 ml of plant extract (Aebi, 1984). The activity of catalase was estimated by the decrease of absorbency at 240 nm as a consequence of H_2O_2 consumption compared to free enzyme extract sample (blank) (Havir & Mellate, 1987).

C. Peroxidases (PODs) activity (EC 1.11.1.7) was determined using guaiacol, reaction solution containing 10 mM (KH_2PO_4/K_2HPO_4, pH 7.0), 10 mM H_2O_2, 20 mM guaiacol and 0.5 ml crude extract in 3 ml (Maehly & Chance, 1954). The increase in absorbance due to formation of tetraguaiacol was monitored at 470 nm (Klapheck *et al*., 1990).

D. Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined from the decrease in absorbency of ascorbate at 290 nm as ascorbic acid is oxidized (Asada & Chen, 1992). Ascorbate peroxidase was assayed in 3 ml of the extract containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.5 mM H_2O_2, the reaction was started with addition of H_2O_2.

Determination of lipid peroxidation

The level of lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced due to peroxidation of unsaturated fatty acids (Kukreja *et al*., 2005). A fresh bacterial sample (0.5 gm) was homogenized in 10 ml of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 rpm at 4^{o}C for 10 min. To 2.0 ml aliquot of the supernatant, 4.0 ml of 0.5% thiobarbituric acid in 20% TCA was added. The mixture was heated at 95^{o}C for 30 min, quickly cooled in an ice bath, and then centrifuged at 10,000 rpm for 10 min. The absorbency of supernatant was recorded at 532 nm. The value for non-specific absorbency at 600 nm was subtracted. The MDA content was calculated using its extinction coefficient of 155 n mol cm^{-1} and expressed as n mol (MDA) gm^{-1} fresh matter.

H_2O_2 determination

H_2O_2 level was colorimetically measured as described by Jana & Choudhuri (1981), H_2O_2 was extracted by homogenizing with phosphate buffer (50 mmol L^{-1}, pH 6.8) including 1 mmol L^{-1} hydroxylamine. The homogenate was centrifuged at 6000 g for 25 min to determine H_2O_2 level, extracted solution was mixed with 0.1% titanium chloride (Aldrich) in 20% (v/v) and the mixture was centrifuged at 6000 g for 15 min. The intensity of yellow color of supernatant was measured at 410 nm. H_2O_2 level was calculated by using the extension coefficient 0.25 µ $mol^{-1}cm^{-1}$.

Statistical analysis

The data of all experiments were subjected to analysis by the least significant differences test (L.S.D) using PC-STATE program version 1A, coded by Rao, M.; Blane, K. and Zannenberg, M., University of Georgia.

Results

Hydrogen peroxide and Azotobacter survival assay

The effect of hydrogen peroxide on growth of *Azotobacter* sp. strain was determined by measuring protein content of treated *Azotobacter* sp. after 15 and 30 min under stirring conditions at 28 ˚C (Fig. 1). Significant differences were observed for protein content between untreated bacteria (control) and H_2O_2-treated bacteria. The maximum protein content was recorded for control. The effect was observed to be time dependent as survival of treated *Azotobacter* cells

was not affected significantly at H_2O_2 concentrations of 0.05 and 0.1 % after 15 min, in which growth rate was reduced up to 11 and 34 %, respectively compared to control. After 30 min exposure, the protein content was reduced 2 fold compared to 15 min exposure, and protein content was reduced significantly (P= 0.01) with levels being decreased by 45, 170, 267 and 695% compared to the control for H_2O_2 concentrations of (0.05, 0.1, 0.5 and 1%), respectively.

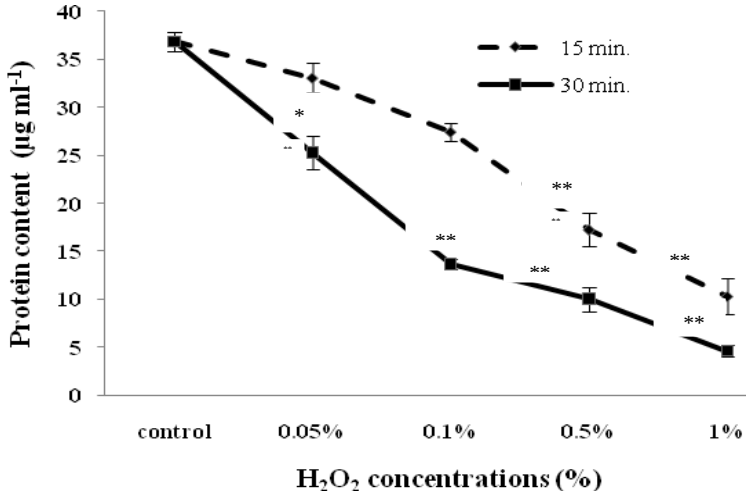

Fig. 1. Growth of *Azotobacter* sp. on broth media supplemented with different concentrations of H_2O_2 under stirring condition. Growth was determined as protein content (µgml^{-1}) ± values (n=4).

*Significant differences (*P*= 0.05) and ** highly significant differences (*P*= 0.01) as compared with control.

Impact of Azotobacter seed treatment on the germination and seedling vigour of H. sabdarriffa

Significant differences have been observed in germination rate, root length, shoot length, and vigour index between untreated seeds and treated seeds of *H. sabdarriffa*. The maximum germination percentage of 90% was recorded for seeds inoculated with *Azotobacter* treated with 0.1% H_2O_2 followed by 85% for *Azotobacter* only. The native isolates of *Azotobacter* greatly increased the germination rate of *H. sabdarriffa* up to 90%, as against 50% for the untreated control (Table 1). Similar trend has been observed for the increase in vigour index of *H. sabdarriffa* for H_2O_2- *Azotobacter* seed treatment. In general the seed treatment with native isolates of *Azotobacter* increased the germination percentage, root length, shoot length and vigour index of the *H. sabdarriffa* compared to control seedlings.

TABLE 1. Effect of *Azotobacter* seed treatment on seedling parameters of *Hibiscus sabdarriffa* under different concentration of H_2O_2.

Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Vigour index
Untreated control	50	3.37± 1.2	8.40± 2.4	587.5±183
Azotobacter only	85	4± 1	9.87± 2.5	1179.3**±293
A+ 0.05 % H_2O_2	80	6.12± 2	9.87± 2.9	1280**±397
A+ 0.1 % H_2O_2	90	5.25± 0.86	9.75± 1.5	1350**±191
A + 0. 5 % H_2O_2	65	3.25± 0.64	9± 1.8	796.2±151.3
A + 1 % H_2O_2	80	4.75± 1.8	9.12± 1.4	1110**±191.5

*Significant differences (P= 0.05) and **Highly significant differences (P= 0.01) as compared with control (Means ± SD).

Activity of antioxidant enzymes, lipid peroxidation and H_2O_2 content

The data of antioxidant enzymes, lipid peroxidation and H_2O_2 content of the treated *Azotobacter* sp. are presented in Fig. 2, 3 and 4.

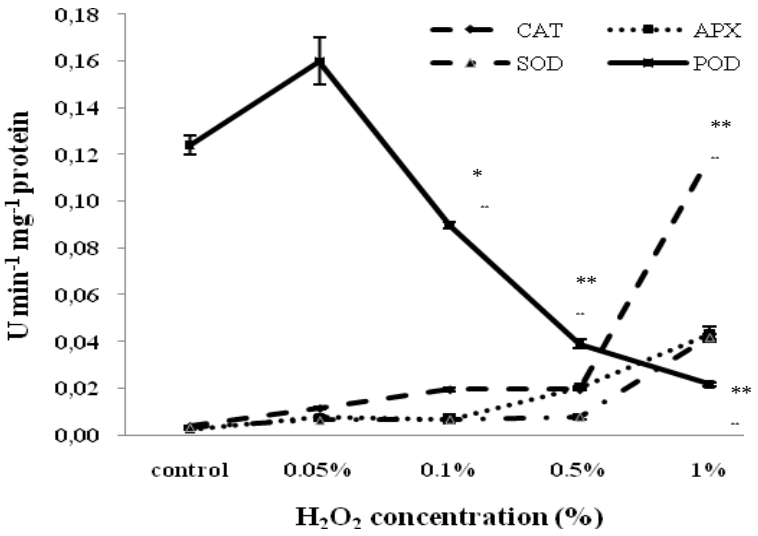

Fig. 2. Effects of different concentrations of H_2O_2 on antioxidant enzyme activities of *Azotobacter* sp. in broth media after 30 min stirring.

*Significant differences (P= 0.05) and ** highly significant differences (P= 0.01) as compared with control.

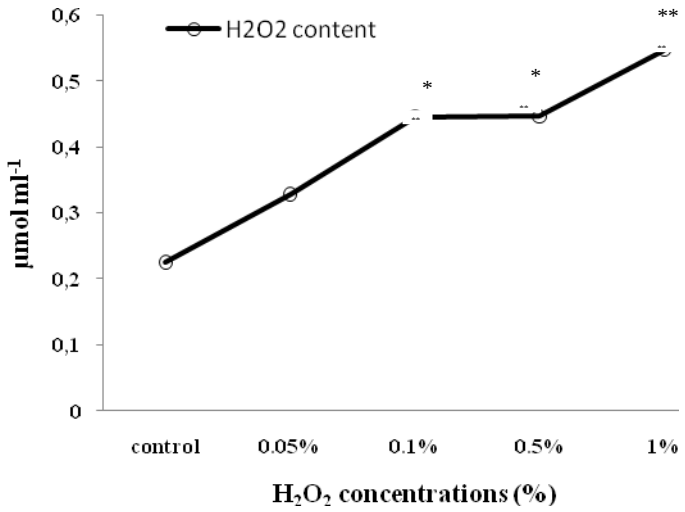

Fig. 3. Effects of different concentrations of H_2O_2 on H_2O_2 content of *Azotobacter sp*. grown in broth media after 30 min under stirring condition.

*Significant differences (P = 0.05) and ** highly significant differences (P = 0.01) as compared with control.

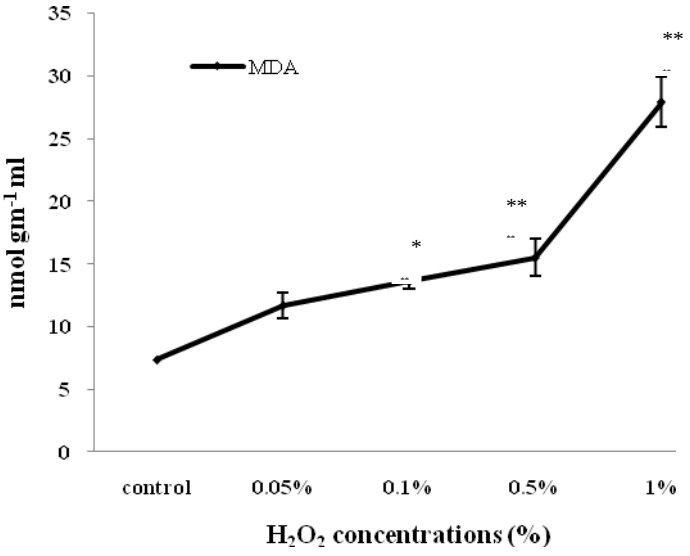

Fig. 4. Effects of different concentrations of H_2O_2 on MDA content of *Azotobacter sp.* grown in broth media after 30 min. under stirring condition.

*Significant differences (P= 0.05) and ** highly significant differences (P= 0.01) as compared with control.

Antioxidant enzymes (SOD, CAT, POD and APX)

Azotobacter cells exposed to H_2O_2 for 30 min changed CAT activity in a dose dependent manner (Fig. 2). The activity reached a maximum value that was -32 fold higher than control- during incubation with as little as 1% H_2O_2 and remained significantly higher than control and the treatments with H_2O_2 concentrations of 0.05, 0.1 and 1%. The CAT activity was increased by 3-fold in *Azotobacter* in response to cell treatment with 0.05% H_2O_2, and was sustained at constant levels when H_2O_2 concentrations of 0.1 and 0.5% H_2O_2 were supplied.

SOD and APX activities also depended on the dose of hydrogen peroxide in a manner similar to CAT (Fig. 2). The data presented showed that the highest activity of SOD (13-fold higher than control) and of APX (17-fold higher than control) in *Azotobacter* cells was observed at 1% H_2O_2. At lower concentrations the activity was not changed greatly at 0.05, 0.1 and 0.5% H_2O_2 concentration, being approximately 2- and 3-fold greater than for the control for SOD and APX, respectively.

POD activity, in contrast to the other antioxidant enzymes, showed a slight increase at the level of 0.05% H_2O_2 only. At higher concentrations, the activity was reduced up to 576%, 319% and 138% for H_2O_2 concentrations of 1, 0.5 and 0.1%, respectively, in comparison with the control (Fig. 2).

Lipid peroxidation and H_2O_2 content

Hydrogen peroxide exposure stimulated the accumulation of MDA and H_2O_2 content in *Azotobacter* treated cells. Compared to the control, there was a significant increase for both MDA and H_2O_2 content in treated *Azotobacter* (Fig. 3, 4). The data presented showed that the highest value of MDA (3.8-fold higher than control) and of H_2O_2 content (2.5-fold higher than control) in treated *Azotobacter* cells was observed at 1% H_2O_2. But at lower concentrations of 0.05, 0.1 and 0.5% H_2O_2, the accumulation for both MDA and H_2O_2 content was not changed greatly, being approximately 1.6, 1.8 and 2 fold greater rather than that of the control for MDA content (Fig. 4). H_2O_2 content was 1.4, 1.9 and 1.9-fold greater rather than that of the control for H_2O_2 content (Fig. 3).

Discussion

Oxidative stress seems to be one of the most common stresses experienced by microorganisms in their environment. Antioxidant mechanisms act to protect cells against oxidation and different strains of microorganisms have various sensitivity to oxidants (Semchyshyn *et al*., 2005 and Bayliak *et al*., 2006). The response to H_2O_2 treatment also varies with growth conditions and previous exposure to H_2O_2 (Katsuwon & Anderson, 1989). Direct exposure to H_2O_2 had more of a deleterious effect on the viability of log-phase cells, but stationary-phase cells were resistant (Imlay & Linn, 1988). Therefore, we were interested in determining the effect of hydrogen peroxide on survival of *Azotobacter* sp. and its role under these conditions for improving plant performance. As seen in Fig. 1,

Azotobacter survival decreased with increasing hydrogen peroxide concentration in all cases. But, after 15 min exposure time, there were no significant differences for *Azotobacter* protein content compared to the control at low H_2O_2 concentrations (0.05 and 0.1%). Katsuwon & Anderson (1989) observed that treatments of logarithmic-phase cells of *Pseudomonas putida* with low concentrations of H_2O_2 enhanced survival upon subsequent exposure to higher H_2O_2 concentrations. Our results coincide with the previous studies, however, the dose-response curve of *Pseudomonas putida* to direct H_2O_2 exposure differed from that of *Escherichia coli*, which had a biphasic response with a sensitive zone for treatments with H_2O_2 (Imlay & Linn, 1988). The ability of the bacterium to respond and survive exposure to H_2O_2 during logarithmic growth could be important during colonization of the root surface, when presumably the cells must enter into an actively growing phase.

Katsuwon & Anderson (1989) suggested that *Pesudomonas putida* must have mechanisms to negate the possible toxic effects of H_2O_2 and O_2^{-}. Production of catalase and superoxide dismutase (SOD), which will degrade hydrogen peroxide and superoxide anion, respectively, may be involved in these mechanisms. Adaption of the bacterium to external sources of H_2O_2 correlated with an enhanced level of catalase activity. Similar adaptions have been observed in *E. coli* and *Salmonella typhimurium* (Demple & Halbrook, 1983), with 10-fold increases in catalase activity (Imlay & Linn, 1987) and four- to five-fold increases, respectively (Christman *et al*., 1985), upon exposure to H_2O_2. Although catalase activity increased in *Pseudomonas putida* upon H_2O_2 exposure, there was no change in SOD level. Thus, even though catalase and SOD activities are connected with oxidative stress, these enzymes can be regulated independently in *Pseudomonas putida*.

Azotobacter response to oxidative stress is induced by different concentrations of hydrogen peroxide. The survival of *Azotobacter* under hydrogen peroxide exposure especially at low concentrations may be explained by the compensatory elevation of CAT, APX and SOD at higher concentrations activities in the cells treated with H_2O_2 (Fig. 2). We did not observe any PODs activation in treated *Azotobacter* at higher H_2O_2 concentrations (0.1, 0.5 and 1%), demonstrating lower availability under exposure to hydrogen peroxide.

A strong positive correlation between CAT and SOD activities was observed (Fig. 2). It is conceivable that CAT protects SOD against inactivation caused by oxidative stress and *vice versa*. A similar relationship between the two enzymes was found by Lushchak *et al*. (2005a, b). The above observations may have some physiological meaning. First of all, SOD produces hydrogen peroxide which serves as a substrate for catalase. In addition, it is well known that both enzymes prevent inactivation by hydrogen peroxide of SOD and by superoxide of catalase, defending each other against oxidation in the active centers (Demple, 1991 and Beyer *et al*., 1991). In contrast, H_2O_2 is known to be causative of Cu, Zn-SOD carbonylation and, in turn, of Cu, Zn-SOD inactivation (Costa *et al*.,

2002). This represents up to 90% of the total SOD activity in most microorganisms (Sturz *et al*., 2001). The slight decrease in SOD activity after treatment with low doses of hydrogen peroxide (Fig. 2) should be associated with Cu, Zn-SOD inactivation. Lower POD activity in cells exposed to 0.1-1% H_2O_2 (Fig. 2) could be explained by inactivation of peroxidases *in vivo* by high concentrations of substrate. Earlier *in vivo* and *in vitro* experiments demonstrated that PODs activity in *E. coli* did not depend linearly on hydrogen peroxide concentration (Semchyshyn *et al*., 2005). The results suggested enzyme inactivation by high peroxide concentrations. It is possible that CAT, APX and SOD are somehow involved in regulation of each other, Fig. 2, clearly shows no changes of SOD activity by increasing activity in both CAT and APX. There is also a strong positive correlation between catalase activity in the untreated control cells of *Azotobacter* investigated and their survival after exposure up to 0.5% H_2O_2. In addition, the low level of cell survival in PODs demonstrated strong positive correlation with unchanged SOD activity under hydrogen peroxide stress (Fig. 2).

H_2O_2 is a membrane permeable molecule that has been demonstrated to function as diffusible intercellular signal (Neill *et al*., 2002) and an elevation of H_2O_2 up to some levels seems to be sufficient for inducing antioxidant enzymes (Yoshimura *et al*., 2000). The level of lipid peroxidation at the amount of malondialdehyde (MDA) produced, as a result of peroxidation of unsaturated fatty acids, has been used as an indicator of cell membrane damage by free radicals under stress conditions (McCord, 2000 and Elkahoui *et al*., 2005).

On the basis of a previous report (Gonzalez & Demple, 1997) and our observations, which showed accumulations of both MDA and H_2O_2 content, it can be concluded that MDA and H_2O_2 content are closely related to the sensitivity of the H_2O_2 toxic effect that interacted with *Azotobacter* survival performance. It seems that the maximum intracellular H_2O_2 concentrations may be accumulating inside *Azotobacter* cells (Fig. 3 and 4). Exceeding that level may lead to SOD and even CAT inactivation. H_2O_2 at concentrations ranging between steady state intracellular level and this virtual maximum value causes adaptive activation of CAT and SOD and may reflect a complicated activation/inactivation balance in the cell that, in turn, strongly depends on intracellular levels of H_2O_2 and/or products of its metabolism. It is suggested these critical concentrations of the oxidant, which can be "sensed" by cells, depend on many circumstances; for example growth phase, conditions of *Azotobacter* cultivation, and strain specificity.

Previous work has demonstrated that treatment of seeds of several plant species with *A. vinelandii* suspension stimulated their germination and seedling development to different degrees (Kurdish *et al*., 2008). For example, the initial (undiluted) bacterial suspension had the most pronounced effect on these characteristics of beat cultivar *Bordo* seeds. With this treatment, the germination, sprouting, and seedling length increased by 19.2, 34.2, and 5.1%, respectively.

In our experiment *H. sabdarriffa* seeds were presoaked for 2 hr with native isolates of *Azotobacter* and the obtained results showed significant increase in the germination percentage, root length, shoot length and vigour index of the *H. sabdarriffa*. The occurrence *Azotobacter* in and around the root system of cereals and the beneficial effect upon inoculation have been well established (Karthikeyan *et al*., 2007). In the present study, the increased seedling parameters in *H. sabdarriffa* may be due to the production of growth hormones (auxins, gibberellins and cytokinins) by these heterotrophic, nitrogen-fixing bacteria. Similar results were confirmed by Salem & Abou Alhamed (2009) where *Azotobacter*, inoculated onto *Lupinus termis* seedlings increased significantly seedling performance including shoot height, root length, total plant dry mass and total nitrogen. Similarly, Lakshmanan *et al*. (2005) reported that in some medicinal plants, such as Senna and Ashwagandha inoculation with *Azotobacter* significantly increased germination percentage, root length, shoot length and dry weight of seedlings. The earlier research determined that the increase in plant growth due to inoculation with *Azotobacter* was not caused by nitrogen fixation, but by bacterial production of plant hormones (Brown & Burlingham, 1968).

In conclusion, we found that isolated *Azotobacter* sp. demonstrated different sensitivities towards hydrogen peroxide. This was attributed to the *Azotobacter* having different antioxidant potentials. We also observed a strong positive correlation between CAT and SOD activities in *Azotobacter* cells treated with different concentrations of hydrogen peroxide. The treatments of *Hibiscus* plant seeds, with *Azotobacter* suspensions, have different positive effects on the seed's germination and plant seed vigour. This indicates a sensitivity of seeds to the biologically active substances accumulated in culture liquid of these bacteria.

Acknowledgements: We are grateful to Dr. Abou Alhamed, M. F. (South Valley University) lecturer of Plant Physiology and also, we are thankful to Mr. M. Sayed (South Valley University) Demonstrator of Bacteriology, for their technical assistance during this work.

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(*Received* 21/2/2010;
accepted 13/7/2010)

الإستراتيجيات الوقائية المستحثة من بكتريا *الأزوتوبكتر* بعد التعرض لفوق أكسيد الهيدروجين ودورها تحت تأثير هذه الظروف فى تحسين قوة انبات بذور الكركدية

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يهدف هذا البحث إلى دراسة تأثير تركيزات مختلفة من فوق أكسيد الهيدروجين على نمو ونشاط الأنزيمات المضادة للأكسدة فى بكتريا *الأزوتوبكتر* وذلك بعد التعرض إلى ١٥ و ٣٠ دقيقة . وتم أيضاً دراسة تأثير نقع بذور الكركدية فى مستخلص بكتريا *الأزوتوبكتر* تحت تأثير هذه الظروف لدراسة تأثيرها على معدل وقوة الأنبات للبذور .

وقد لوحظ أن تأثير فوق أكسيد الهيدروجين يعتمد على الوقت المعرض له البكتريا وأن نمو البكتريا لم يتأثر معنوياً عند التركيزات المنخفضة (٠،٠١ و ٠،٠٥٪) وذلك بعد التعرض إلى ١٥ دقيقة (١١ و ٣٤ ٪ نسبة الإنخفاض فى البروتين مقارنة بالكنترول).

وفى حالة التعرض إلى ٣٠ دقيقة لوحظ أن نسبة النمو قد انخفضت للضعف مقارنة بالتعرض إلى ١٥ دقيقة. وعلى الجانب الأخر لوحظ أن نشاط انزيمات مضادات الأكسدة وهى السوبر اكسيد ديسموتيز و الكاتاليز والأسكوربيت بيروكسيديز لم تسجل أى فروق معنويه عند التركيزات المنخفضة من فوق أكسيد الهيدروجين وكانت الفروق المعنوية فقط عند أعلى تركيز مستخدم وهو ١٪. وعلى النقيض من ذلك سجل انزيم البيروكسيديز ارتفاع طفيف فى نشاطه عند التركيز المنخفض ثم انخفاض فى التركيزات العالية .

كما لوحظ تراكم فى كميات كلا من فوق أكسيد الهيدروجين والمالوندای الدهيد بداخل الخلايا البكترية. وعلى الجانب الأخر لوحظت زيادة معنوية فى قوة انبات بذور الكركديه المنقوعة لمده ساعتين مقارنة بالبذور غير المعاملة.

ونستخلص من ذلك أن استجابة بكتريا *الأزوتوبكتر* لتأثير فوق أكسيد الهيدروجين لها علاقة بالدور الدفاعى لانزيمات مضادات الأكسدة فى هذه السلالة. كما لوحظ ان هناك علاقة بين هذه الأنزيمات فى حماية البكتريا من التأثير الضار لتركيزات فوق أكسيد الهيدروجين وكان ذلك له دور هام فى عملية التصاق البكتريا بأسطح البذور للنبات تحت هذه الظروف والتى ساعدت على زيادة قوة الأنبات لبذور الكركدية المختبرة.