Response of Some Mitochondrial Enzymes to NaCl Induced Oxidative Stress in Strawberry *cv* Chandler and Wild type *Fragaria vesca L.*

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ABSTRACT

The activity of mitochondrial enzymes which coordinate the mitochondrial redox system (Succinate dehydrogenase, NADH dehydrogenase, NADH oxidase and ATPase) were investigated against salt exposure. The increased activity of all mitochondrial enzymes in leaves of strawberry were observed up 0.75 % NaCl concentration, which probably explains the survival of the strawberry plant at this concentration.. The higher concentration of NaCl, (above the 0.75 % NaCl concentrations) affected the growth towards reduction and finally caused death of strawberry plants at 3 % NaCl concentration. The efficient mitochondrial redox system was exhibited by chandler variety of strawberry at 0.75 % NaCl concentration. Specific activity of succinate dehydrogenase, NADH dehydrogenase and NADH oxidase showed the highest rate of redox reaction at 0.75 % NaCl concentration, and ATPase also showed the maximum oxidative phosphorylation at 0.75 % NaCl concentration. Mitochondrial enzymes studied to understand the response to NaCl induced stress was exhibited better performance at 0.75 % NaCl concentration in strawberry and this may probably suggest the redox system operated by mitochondria was efficient in the development of resistance against NaCl oxidative stress. So these mitochondrial enzymes may be used as tools for the improvement of resistance against the salinity stress by genetic engineering experiments i.e. transgenic plant development. The toxicity developed was appears to be probably due to the suppressed activity of mitochondrial enzymes and antioxidants.This also suggests that the minor saline soils may possibly adapt to the strawberry variety chandler as a cultivar at farmer level.

Keywords: Succinate dehydrogenase, mitochondrial ,phosphorylation

INTRODUCTION

Mitochondria consume oxygen during respiratory electron transport different sites of electron leakage and release of O_2^- and H_2O_2 in respiration have been proposed. It linked the cellular processes of carbon and nitrogen metabolism through the tricarboxylic acid cycle and the photo respiratory cycle. Salt stress lead to damage of specific mitochondrial targets through the direct action of lipid peroxidation product.

The known site of mitochondrial ROS (mtROS) product in the respiratory electron transport chain are complexes I and III, where superoxide anion (O_2^-) is formed and in turn is reduced by dissimulation to H_2O_2. H_2O_2, a compound of relatively low toxicity, can react with reduced Fe^{+2} and Cu^{+2} to produced highly toxic OH^- and, being unchanged, can also penetrate membranes and leave the mitochondrion **(1)**. The ubiquinone intermediate formed at complexes I and III is the principal electron donor to oxygen, although other complex I sites are potential donors. Thus, the overall reduction level of mitochondrial ubiquinone pool will be the primary determinate of mtROS output.

Plant mitochondria can modulate superoxide production from the mtETC by two mechanisms that act to keep the ubiquinone pool reduction level low. The first, AOX, is not inhibited by the proton gradient across the inner membrane and can function when the cytochrome pathway is impair (2). That AOX might act to maintain a basal ubiquinone pool reduction state was initially proposed by Purvis and Shewfelt (3) and is supported by studies with root treated with a cytochrome pathway inhibitor (4). Further operation on AOX diminishes mtROS production (5). Increased expression of alternative mitochondrial NAD(P)H dehydrogenase, which does not translocate protons, offer accompanies increased AOX expression (6). Use of these enzymes serves as an alternative to complex I and could further help decrease mtROS production. The second, uncoupling protein (UCP) also is found in the inner mitochondrial membrane. UCP uncouples by facilitating a proton leak across the membrane and consequently removes inhibition of the mtETC (7). Like AOX, UCP function is isolated mitochondria decreases ROS formation (8). On the other hand, ROS are actually required for UCP activity (9), a direct activator being 4-hydroxy-2-nanenol (HNE) **(10)**. This ROS requirement by UCP is in contrast to the effect of ROS on AOX. Exposure of AOX to experimental oxidative stress can derive the protein in to the inactive disulfide-linked form and HNE inhibits AOX (11). Therefore while both AOX and UCP may act to forestall mtROS production, only UCP may be able to operate when ROS level become increased.

General understanding of mitochondria is as organelles in plant cells that produce cellular ATP through an electron transport chain containing four respiratory complexes. Complex I, NADH dehydrogenase; Complex II, Succinate dehydrogenase; Complex III, bc_1; and Complex IV, cytochrome oxidase. Mitochondria produce carbon dioxide (CO_2) through the TCA-cycle as well as cellular biosynthetic substrates. By way of contrast, plant mitochondria exist in cells /organism that (a) contain chloroplasts, thus producing APT and synthesizing a large portion of their own respiratory substrates; (b) lack the ability to escape many environmental stresses; (c) produce a wealth of primary and secondary metabolites, some in response to specific

stresses, all of which require carbon skeletons; and (d) photorespire, to meet these novel demands and through little-under stood mechanism. Plant mitochondria have evolved to function in dramatic contrast to their non photosynthetic counterparts.

The purpose of the study was to examine the changes in the activity of mitochondrial enzymes in the exposed strawberry (*Fragaria ananassa* D. chandler) species to different levels of NaCl concentration in soil.

MATERIALS AND METHODS

The experiment was conducted in the Department of Biochemistry & Biochemical Engineering, Sam Higginbottom Institute of Agriculture, Technology & Sciences, Deemed to-be University, Allahabad to investigate the effect of NaCl stress on determine the response of some mitochondrial enzymes to NaCl induced oxidative stress in the leaves of .The five months old runners were transplanted in pots and maintained in green house condition. The runners were supplied with ½ Hoagland medium. Salt treatments (0.00, 0.25%. 0.50, 0.75, 1.00, and 1.50%, 2.0% & 3.0% NaCl) were given by dissolving sodium chloride in ½ Hoagland medium at four-leaf stage. Triplicate samples were used for the study. Pots irrigated only with the ½ Hoagland medium served as control.

Plant Sampling and Analysis

Leaves were sampled according to life-cycle of strawberry at pre-flowering, flowering and fruiting stages. Leaf samples were standardized by using only fully expanded leaves from the middle part of plants in each replicate, as they reflect most clearly from the nutritional and metabolic standpoint and the effects of salinity. The material was rinsed three times in distilled water and then blotted on filter paper. At each sampling, leaf matter was used fresh for analysis of mitochondrial enzymes

Spectrophotometric assay of hydrogen peroxide (H_2O_2 Content)

H_2O_2 Contentwas quantified by the modified method of Goth (1991) with ammonium molybdate forming a stable complex product with unreacted hydrogen peroxide (A405). Reaction mixture (2.2ml) contained 45mM potassium phosphate buffer (pH 7.4), 7mM hydrogen peroxide, and 0.2ml of the crude enzyme extract. Ammonium molybdate 1 ml of 32.4mM solution) was added after 4 min of the incubation to stop the reaction and the yellow complex of molybdate and hydrogen peroxide was measured at 405mM against blank 3.

NADH Oxidase (EC 1.6.5.3) activity

The reduced NADH showed maximum absorption at 340 nm in UV light. Decrease in concentration of NADH was followed by decrease in absorbance. When NADH was converted to NAD absorption was zero, and when NAD is converted to NADH The absorbance increases (12).

NADH Dehydrogenase (EC 1.6.5.3) activity

When NADH was added to mitochondria, the NADH gets oxidized to NAD^+ in the

presence of NADH dehydrogenase. This reduced NADH dehydrogenase is reoxidized by passing electrons to potassium ferricyanide, which was an artificial electron counter (13).

Succinate dehydrogenase (EC 1.3.5.1) activity

Succinate is an artificial electron donor, which provides electron to the electron transport chain by getting oxidized to fumarate in presence of mitochondrial succinate dehydrogenase. The electrons from the reduced $FADH_2$ are accepted by the reducible dye 2, 6-dichlorophenolindophenol, which is an artificial electron acceptor. This dye is blue in colour in its fully oxidized state and colorless in its reduced state (14).

ATPase (EC 3.6.1.34) activity

ATPase activity was high in intact mitochondria of the energy released due to oxidation of substrate in electron transport chain was coupled to phosphorylation of ADP. Uncoupled mitochondria show ATPase activity and hydrolyze.This inorganic phosphate reacts with ammonium molybdate to form phosphomolybdate which is reduced to phosphomolybdenum with ANSA reagents (15).

Estimation of total soluble Protein

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the Biuret reaction of the protein with the alkaline cupric tartrate were measured in the Lowry's method (16).

Statistical Analysis

Data from the experiment was subjected to completely randomized design (factorial) using Drysoft Statistical Analysis package**.**

RESULTS AND DISCUSSION

Effect of salinity on H_2O_2 Production –Oxidative stress magnitude

H_2O_2 generation increased significantly at all salinity levels, but the magnitude of increase was more profound in chandler. Between the cultivar and wild it was higher in, especially at 1.5% NaCl in chandler than other cultivars and wild berry. Regarding the effects of period, Long period of NaCl treatments (fruiting) caused higher levels of H_2O_2 than short period of NaCl treatments (Pre-flowering) in strawberry cultivars and wild species.

NaCl induced oxidative stress – Specific activity of NADH Oxidase

The specific activity of NADH Oxidase has shown up regulated activity from the 0.25 % to 0.75 % NaCl and follows the inhibitory levels of specific activity of NADH oxidase up to 1.5 % NaCl Fig 1). The highest value was recorded in plant treated with 0.75 % NaCl with the value of 3.219 Units mg^{-1} protein FW in fruiting stage in wild berry. In the present investigation, NADH-oxidase was decreased with the increase in salinity.

Table 1: Effect of NaCl on on hydrozen peroxide (H_2O_2) production in leaves of strawberry

	F. Vesca			Chandler		
NaCL	Pre-Flowering	Flowering	Fruiting	Pre-Flowering	Flowering	Fruiting
0.00%	11.29	25.213	40.01	33.88	43.19	52.885
0.25%	20.957	29.763	38.68	25.497	28.19	31.333
0.50%	9.533	14.023	18.925	20.623	19.49	17.74
0.75%	21.2	30.703	39.371	24.127	29.713	34.7
1.00%	43.823	45.433	47.033	52.953	53.923	54.96
1.50%	48.178	45.04	43.137	34.733	34.9	35.3

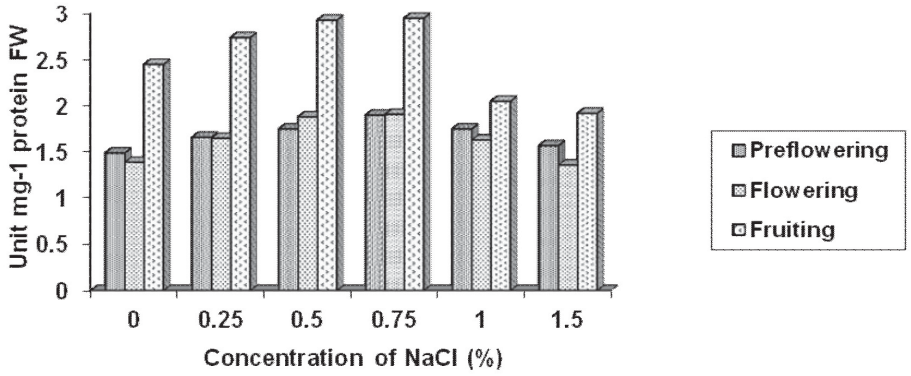

Fig.1: Effect of NaCl on specific activity of NADH oxidase in leaves of strawberry.

NaCl induced oxidative stress – Specific activity of NADH Dehydrogenase: The enzyme activity was maintained up to 0.50% NaCl, even after prolonged treatment of NaCl for fruiting stage in both, cultivar and wild type. In case of cultivar chandler it was increased up to 50% than control (Fig 2).According to the variance analysis of NADH Dehydrogenase ,the effect of periods and its interactions with cultivar and wild and NaCl were significant.

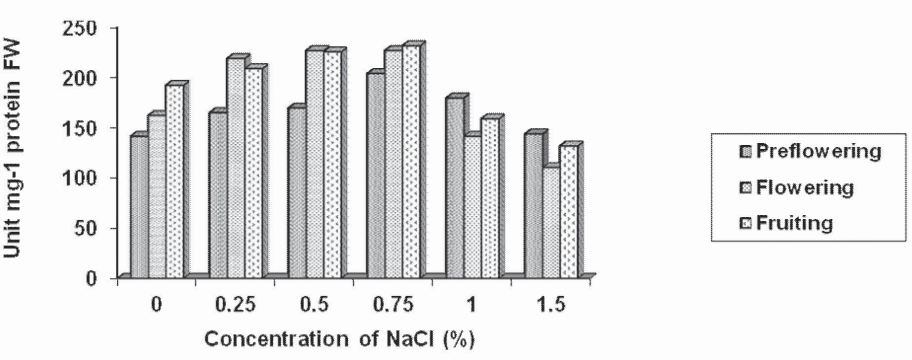

Fig. 2: Effect of NaCl on specific activity of NADH dehydrogenase in leaves of strawberry.

NaCl induced oxidative stress – Specific activity of Succinate Dehydrogenase: The Succinate Dehydrogenase activity was up regulated up to 1.5 % in both cultivar and wild berry with increasing salinity. However, it is important to note that the enzyme activity was still maintained after prolonged period of salinity and the rate of increase was more in chandler. According to the variance analysis of succinate dehydrogenase data the effect of periods and its interactions with cultivar and wild and NaCl were significant.(Fig 3)

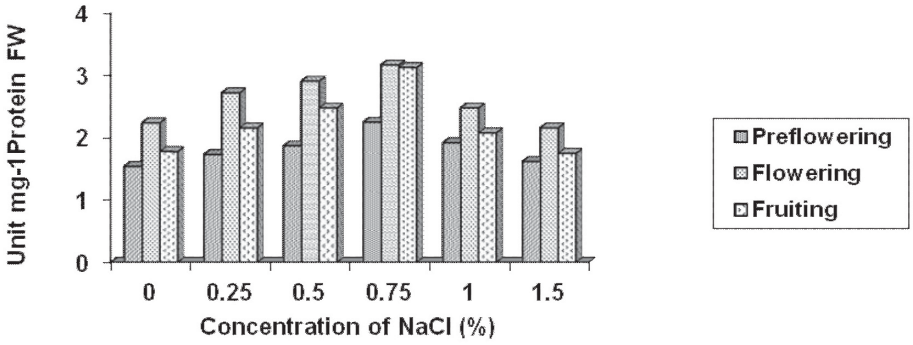

Fig. 3: Effect of NaCl on specific activity of succinate dehydrogenase in leaves of strawberry.

NaCl induced oxidative stress – ATP ase activity

ATPase activity in leaves of both wild (salt-tolerant cultivar) and chandler (salt-sensitive cultivar) increased as the crop grew older during experiments. Salt treatment decreased ATPase activity in both wild and cultivar progressively. ATPase activity inthe leaves of salt-treated plants decreased to 0.670, 0.030 (pre-flowering) 1.121 and 0.067(flowering)) and fruiting (0.831 and 0.107) respectively (Fig 4). ATPase activity was significantly higher in salt-tolerant cultivar than in salt-sensitive cultivar under NaCl stress.According to the variance analysis of ATP ase data the effect of periods and its interactions with cultivar and wild and NaCl were significant.

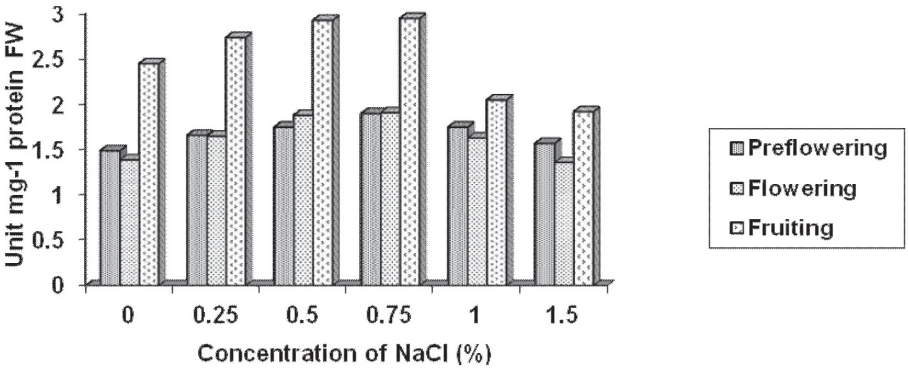

Fig. 4: Effect of NaCl on specific activity of **ATPase** in leaves of strawberry.

NaCl induced oxidative stress – Total soluble protein content

The total soluble protein content quantification was studied after giving different NaCl treatments to strawberry plants. The total soluble protein content quantification was recorded in the three significant stages of plant life cycle such as preflowering, flowering and fruiting. The total soluble protein content was respond to NaCl induced oxidative stresses of the observations were as follows

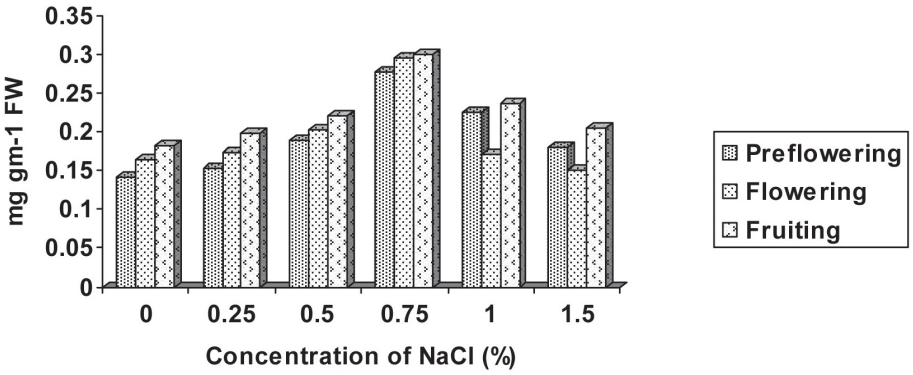

Fig. 5: Effect of NaCl on Soluble protein content in leaves of strawberry.

Effect of Salinity on mitochondrial enzymes The overall reduction level of mitochondrial ubiquinone pool will be the primary determinate of mt ROS output. After having conformed that the enzymatic and Non-enzymatic antioxidative responses, membrane stability, chandler and wild species *Fragaria vesca L.* were selected on the basis of biochemical response to salinity and used to study some of enzymes with reference to salinity the influence of NaCl induced salinity stress on the respiratory component of *Fragaria ananassa* D. chandler and wild species *Fragaria vesca L*. The purpose of investigation was to understand the response of these enzymes activity coupled with some antioxidant enzymes in saline environment.

Electron transport chain – NADH dehydrogenase and NADH oxidase

Use of NADH dehydrogenase enzyme serves as an alternative to complex I and could further help decreased mtROS production (6). . The production of ROS by NADH oxidase have extra cellular (EC) and intracellular ramifications. EC-ROS products were associated with direct oxidative cross-linked of cell wall components during defense, differentiation of plant vascular tissue and suberization in wounded potato tubers (17). ROS that have been shown to play a role in development were produced by NADH Oxidases that generate the superoxide radical (O_2^-), using NADH as electron donor (18).

Tricarboxylic acid cycle – Succinate dehydrogenase

Specific activity of succinate dehydrogenase showed the maximum rate of redox reaction at 0.75 %(3.17 ± 0.034) NaCl concentration, where the operation of TCA cycle enzymatic reactions may be coordinated effectively. The oxidation of succinate dehydrogenase results in the reduction of FAD, FAD finally given the $FADH_2$. The optimized succinate dehydrogenase undergoes re-oxidation during the electron transport at complex II reactions.

Oxidative phosphorylation – ATPase

Detailed analyses of the regulation of the ATPase activity and of the transcription and translation of ATPase subunits in response to salinity stress have been carried out for the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant). It had been shown that the activity of the ATPase increased in *M. crystallinum* under treatment with NaCl **(19)**.

The above parameters studied to understand the response of mitochondrial enzymes to NaCl induced stress has exhibited better performance at 0.75 % NaCl concentration in strawberry cultivar chandler. This may probably suggest the redox system operated by mitochondria at this concentration (0.75 % NaCl) was efficient in the development of resistance against NaCl oxidative stress. Unlike, in animal cells NAD (P) H oxidase responded to salinity with the increasing salinity, in order to combat the oxidative stress and trigger the inter-organellar signaling by initiating ROS based signaling cascades.

On the mitochondrial enzyme investigation, it was observed that as the mitochondria are involved in many fundamental processes underpinning plant growth, development and death. Due to their multiple roles: as the sites of the TCA cycle and oxidative phosphorylation, as harbourers of their own genomes, and as sensors of cell redox status, amongst others, mitochondria are in a unique position to act as sentinels of cell physiology to understand salinity response.

REFERENCES

1. Grene, R. 2002**.** Oxidative stress and acclimation mechanisms in plants, In CR Somerville, EM Myerowiz, eds. The Arabidopsis Book. *American Society of Plant Biologists*, Rockville.
2. Finnegan, P. M., Soole, K. L., Umbach, A. L. 2004**.** Alternative mitochondrial electron transport proteins in higher plants. In D. A. Day, A.H. Miller, J. Whelan, eds. Plant mitochondria: From gene to function. *Advances in photosynthesis and respiration*. Kluwer, Dordrecht, the Netherlands **17**:163-230.
3. Purvis, A. C., Shewfelt, R. L. 1993. Does the alternative pathway ameliorate chilling injury in sensitive plant tissue? *Plant Physiology* **88:** 712-718.
4. Millenaar, F. F., Benschop, J. J., Wagner, A. M., Lembers, H. 1998. The role of the alternative oxidase is stabilizing the in vivo reduction state of the ubiquinone pool and the activation state of the alternative oxidase: *Plant Physiology* **118:** 845-856.
5. Maxwell, D. P., Wang, Y., Mclntosh, L. 2002. The alternative oxidase lowers the mitochondrial reactive oxygen production in plant Cells. *Proceeding of National Academy of Science* **96:** 8271-8276.
6. Clifton, R., Lister, R., Parker, K. J., Sappl, P. G., Elhafez, D., Miller, A. H., Day, D. A., Whelon, J. 2005. Stress-induced Co-expression of alternative respiratory chain components in Arabidopsis thaliana. *Plant Molecular Biol*ogy **58:** 193-212
7. Hourton-Cabassa, C., Matos, A. R., Zachowski, A., Moreau, F. 2004. The plant uncoupling protein homologues: a new family of energy-dissipating proteins in plant mitochondria. *Plant Physioogy and Biochem*istry **42:** 283-290.
8. Casolo, V., Braidot, E., Chaindussi, E., Macrl, F., Vianello, A. 2000. The role of mild uncoupling and non-coupled respiration in the regulation of hydrogen peroxide generation by plant mitochondria. *FEBS Letter* **474:** 53-57.

9. Considine, M. J., Goodman, M., Echtay, K. S., Laloli, M., Whelan, J., Brand, M. D., Sweetlove, L. J. 2003. Superoxide stimulates a proton leak in potato mitochondria that is related to the activity of uncoupling protein. *Journal of Biological Chemistry* **278:** 22298-22302.
10. Smith, A. M. O., Ratcliffe, R. G., Sweetlove, L. J., 2004. Activation and function of mitochondrial uncoupling protein in plants. *J.ournal of Biological Chemistry* **279:** 51944-51952.
11. Winger, A. M., Miller, A. H. and Day, D. A. 2005. Sensitivity of plant mitochondrial terminal oxidase to the lipid peroxidation product 4-hydroxy-2-nonenol (HNE). *Biochemistry Journal* **387:** 865-870.
12. Duke, S. H., Ham, G. E. 1976. *Plant and Cell Physiology* **17:** 1037-1044.
13. Singer, T. P. 1974. Determination of the activity of succinate, NADH, choline, á-glycerophosphate dehydrogenase. *In methods of Biochemical Analysis (ed. D. Glick), John Wiley and Sons New York USA.* 123-175.
14. Singer, T. P., Oestreicher, G., Hogue, P. 1973**.** Regulation of succinate dehydrogenase in higher plants. Some characteristics of the membrane-bound enzyme. *Plant Physiology,* **52:** 616-621.
15. Fiske, C. H., Subbarow, Y. J. 1925.*Journal of Biological Chemistry* **66:** 375.
16. Lowry, O. H., Rosebrough, A. L., Randall, R. J. 1951. Protein measurement with folin phenol reagent, *J. Biol Chem.***193:** 265.
17. Apel, K., Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress and signal transduction. *Annual Review of Plant Biology* **55:** 373-399.
18. Catherine, G., Lian, D. 2006. Control of plant development by reactive oxygen species. *Plant Physiology.* **141:** 341-345.
19. Tsaintis, M. S., Bartholomew, D. M., Smith, J. A. C. 1996. Salt regulation of transcript levels for the c subunit of a leaf vacuolar H^+-ATPase in the halophyte *M. crystallium*. *The Plant Journal.* **9:** 729-736.