B -18

CELLULAR DISTRIBUTIONS OF CHLORIDE AND HYDROGEN PEROXIDE IN MESOPHYLL AND BUNDLE SHEATH CELLS OF MAIZE EXPOSED TO SALINITY STRESS

Rusdi Hasan[1] and Hiroshi Miyake[2]

[1]Department of Biology Education, Muhammadiyah University of Bengkulu;
[2]Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Abstract

Cellular localization of chloride(Cl) and hydrogen peroxide (H_2O_2) in the leaf blades of maize plants exposed to 3% NaCl were investigated. This study was aimed to investigate whetherdifferential sensitivity to salinity between mesophyll cells (MC) and bundle sheath cells (BSC) in maize associated with differential accumulation of Cl and H_2O_2 in MC and BSC. Cl distribution was examined with X-ray microanalysis, whereas H_2O_2 was analyzed histochemically and cytochemically using 3,3-diamino-benzidine(DAB) and Cerium Chloride ($CeCl_{30}$.The present study shows that Cl accumulation in MC and BSC of the plants treated with NaCl was relatively comparable. However, salinity-induced H_2O_2 formation in MC chloroplasts was higher compared with that in BSC chloroplasts. In addition, H_2O_2 accumulation was also detected in apoplast of MC and BSC with the greatest accumulation being detected in the cell walls of MC facing to the intercellular space. These results suggest that differential sensitivity between MC and BSC chloroplasts to salinity is not caused by differential accumulationof Cl between the cells. Apoplastic accumulation of H_2O_2 may rather reflect changes in oxidative balance generated by ROS scavenger in the cells that affects the homeostasis of the whole cell.

Key words: C_4 Plant, Chloroplast, H_2O_2, Maize, Salinity, SEM, X-ray microanalysis

INTRODUCTION

Salinity is one of the major abiotic stresses in arid and semiarid region (Rengasamy et al., 2003). Over 6% (800 million ha) of the world's total land area, about 2% (32 million ha) of 1,500 million ha dryland agriculture and 20% (45 million ha) of230 million ha irrigated lands are affected by salinity (FAO, 2000). The stress is caused by excessive uptake of toxic ions from soil solution. Although the mechanisms of salt stress are still complicated, it is considered that excessive accumulation of salt ions, mainly Na^+ and Cl^- in the plant tissues is a major factor to the damage caused by salinity (Flowers and Hajibagheri, 2001).

High salt content in the plant tissues influences physiological and biochemical processes of the plants through osmotic and ionic stresses (Munns, 2005). Osmotic stress occurs due to Na and Cl uptake, which leads to a deficit of water in the plant tissues. Ionic stress occurs due to high concentrations of toxic ions such as Na^+ and Cl^- reduces uptake of other mineral nutrients

such as Ca and K, which causes metabolic disturbances (Hasegawa et al, 2000).

One of the biochemical changes that occur when the plants exposed to salinity is the increase of ROS production such as O_2^-, H_2O_2 and hydroxyl $^{\cdot}OH$ (Zhu, 2001; Bolkhina et al., 2003). The major sites of ROS production in the plant cells during abiotic stress are chloroplasts, mitochondria and microbodies (del Rio et al., 2002). Under a normal condition, plants produce a relatively low concentration of ROS which are not harmful and used for signaling cascade such as in defense and acclimatory processes. However, high concentration of ROS can seriously disrupt normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Neill et al., 2002).

Capability of the plants to limit the uptake of toxic ions is one of the factors which determine sensitivity of the plants to salinity. Accumulation of toxic ions in the plant tissue of salt tolerant species is usually lower than in the salt sensitive species when the plants are exposed to salinity (Garthwaite et al., 2005). In addition, the older leaf blades accumulate higher concentration of toxic ions and more sensitive to salinity than the younger leaf blades (Garthwaite et al., 2005). In maize, an NADP-malic enzyme (NADP-ME) type C_4 plants that possesses deferential of MC and BSC chloroplasts due to the varies of abundant in chloroplasts genes transcript as leaves develop (Sharpe et al., 2011), the MC were relatively more sensitive to salinity than chloroplasts in BSC (Hasan et al., 2005). Some investigators revealed that the damage of chloroplasts in the plants exposed to salinity is caused by oxidative stress that is mediated by ROS (Hernandez et al., 2001; Yamane et al., 2004a). However, there is no information about the cellular accumulation of toxic ions especially Na and Cl in MC and BSC and its relation to differential sensitivity of MC and BSC chloroplasts to salinity in maize plants. Therefore, the present study investigated the cellular localization of Cl and H_2O_2 in MC and BSC in maize plants exposed to salinity. Our results showed that the content of Cl in MC and BSC was relatively comparable, while H_2O_2 in MC was higher than in BSC.

MATERIALS AND METHODS

1. Plant materials

Maize *(Zea mays* L. 'Golden Bantam') plants were grown as described previously (Hasan et al., 2005). The salt treatment was started when the second leaf blades (coleoptile was numbered as leaf zero) of the plants were fully developed. The plants were daily supplied with 50 ml of dw (control) or 3% NaCl solutions. Five days after the start of NaCl treatment at which the visible symptoms were shown in the plants treated with 2 and 3% NaCl, the samples were taken to analyze Cl and H_2O_2 localization in the leaf tissue.

2. X-ray microanalysis

Cellular distribution of Cl was analyzed in the second leaf blades of control and 3% NaCl-*treated* plants. Samples were cut and immediately frozen in liquid nitrogen subsequently transferred to a freezing device (OKA Science Co.) overnight. The temperature was started from about of -75 ^{0}C and gradually increased until the temperature of 25 ^{0}C (room temperature) was reached.Then, the freeze-driedsamples were taken from the freezing device and sliced free hand transversely with a razor blade. The sections were mounted on a stub and coated with gold in a vacuum sputter coater. The coated specimens were analyzed in a Hitachi-4500 scanning electron microscope (SEM) fitted with an energy-dispersive X-ray microanalyzer (Horiba). Counts per second of chloride ion were measured in a transverse section of leaf blades and root from control and NaCl-treated plants.

3. Histochemical detection of H_2O_2

H_2O_2 was visually detected in the leaves of plants by using 3,3-diamino-benzidine (DAB) as substrate (Thordal-Christensen, 1997). Fully developed second leaf blades were excised from the plant at the base of leaves with a razor blade, placed in distilled water for 1 h to avoid wounding stress then placed in 1 mg/ml solution of DAB (pH 3.8) for 8 h in light at 25 ^{O}C, and then exposed to distilled water or 200 mM NaCl. After these treatments, the leave blades were decolorized by immersion of leaves in boiling ethanol (96%) for 10 min. Then, samples were transferred to a new cool ethanol (96%). After cooling, the leaves were extracted at room temperature with fresh ethanol and photographed. H_2O_2 was visualized as a reddish-brown coloration of the leaf blades.

4. Cytochemical localization of H_2O_2

Cytochemical detection of H_2O_2 was conducted by $CeCl_3$ staining. Small portions (1x2 mm) of the middle part of the second leaf of control and the plants treated with 3% NaCl for 5 h were incubated in freshly prepared 5 mM $CeCl_3$ in in 50 mM 3-(N-morpholino) propanesulfonic acid (Mops) at pH 7.2 for 1 h (Bestwick et al. 1997). The samples were then fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2), post-fixed in 2% osmium tetroxide in the same buffer, dehydrated with graded acetone series and propylene oxide, embedded in Spurr's resin and polymerized at 70 ^{O}C for 24 h (Hasan et al. 2005). Sections were cut (80-90 nm) with a diamond knife on an Ultracut-N microtome (Reichert-Nissei), mounted on a 200 mesh grid and stained with uranyl acetate and lead citrate solutions. Thereafter, sections were examined with a transmission electron microscope (Hitachi H-7500, Tokyo) at an accelerating voltage of 100 kV. The localization of H_2O_2 was detected as electron dense of cerium perhydroxide precipitates.

RESULTS AND DISCUSSION

1. X-ray microanalysis

Transverse sections of the leaf blades were scanned by X-ray microanalysis. Data from both line and map scanning show that Cl accumulation was greatly higher in the plants treated with NaCl compared to control (Fig. 1 and 2). Comparison of Cl accumulation between MC and BSC from map scanning data of leaf sections shows that Cl was distributed almost homogenous and comparable throughout the cell in both MC and BSC (Figs. 1A,B). Data from line scanning of leaf sections show that Cl distribution at the area of cell where organelles are located was relatively comparable in both MC and BSC (Fig. 2).

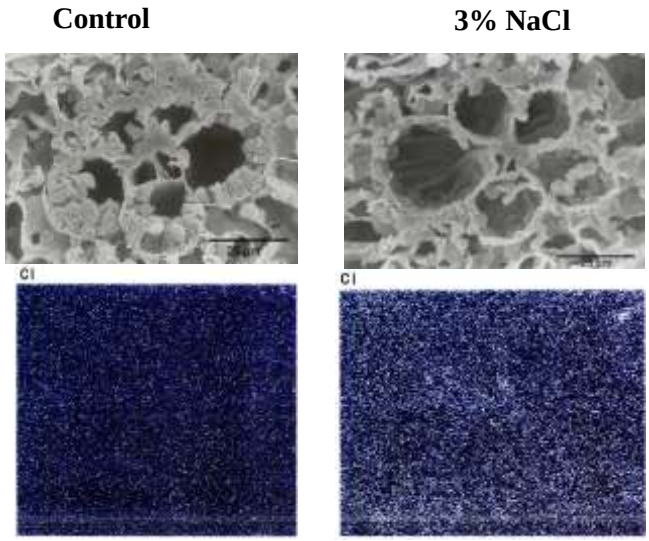

Figure 1. SEM images of transverse sections of leaf blade in maizewith map scanning of Cl distribution oncontrol (left side) and 3% NaCl treated plants (right side)

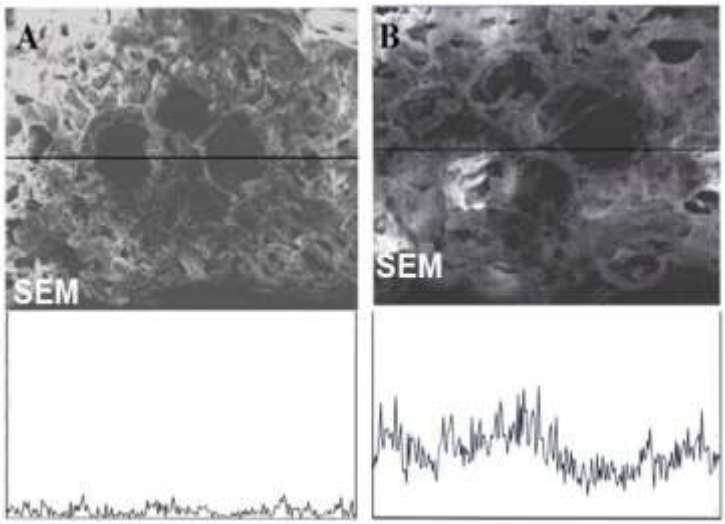

Figure. 2 Cellular distribution of Cl from line scanning on transverse sections of leaf blades in maize. A. Control. B. 3% NaCl-treated plant.

2. H_2O_2 localization

Figure 3 shows the accumulation of H_2O_2 in the leaves of maize plants exposed to NaCl. Localization of H_2O_2 was indicated by dark brown polymerization as reaction with DAB in the presence of peroxidase (Thordal-Chrisrtensen et al., 1997). In the leaf blades of control plants almost no visible accumulation of H_2O_2 was observed. In contrast, in the leaf blades of NaCl-treated plants, accumulation of H_2O_2 was clearly observed. H_2O_2 appeared mainly in major veins throughout the leaf blades (Fig. 3B).

Fig. 3
Histochemical detectionof H_2O_2 with DAB staining inmaize leaves. The detached leaves were treated withdistilled water (A) and 200 mM NaCl (B).

Cellular localization of H_2O_2 in MC and BSC of the leaves exposed to NaCl was shown in figure 4. H_2O_2 was detected as electron dense cerium perhydroxyde precipitates resulted from the reaction of H_2O_2 with $CeCl_3$. In control plants, H_2O_2 accumulation were not detectable in chloroplasts of either MC or BSC (Figs. 4A,B). In NaCl-treated plants, H_2O_2 accumulation was detected in chloroplasts of both MC and BSC. The accumulation of H_2O_2 was relatively higher in MC chloroplasts (Fig. 4C) than in BSC chloroplasts (Fig. 4D). However, the accumulation of H_2O_2 in other organelles such as mitochondria and peroxisome in both MC and BSC was lower than in chloroplasts. The accumulation of H_2O_2 was also clearly detected in apoplasts in which relatively higher compared with the other cell compartments. The greatest accumulation of H_2O_2 was observed in the cell walls of MC facing intercellular space (Figurers not shown).

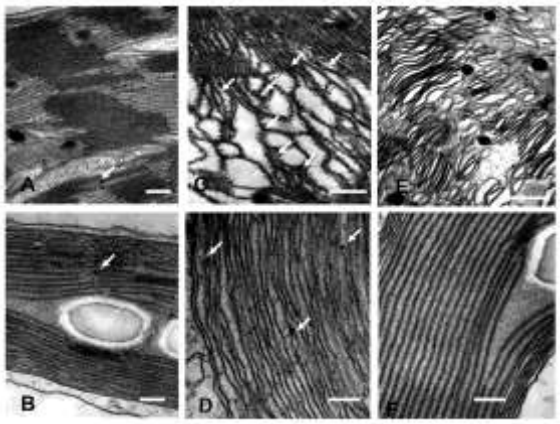

Fig. 4
Cytochemical detection of H_2O_2 localization (arrows) with $CeCl_3$ staining in the chloroplasts of MC and BSC. A. Mesophyll chloroplast of control plant. B. Bundle sheath chloroplast of control plant. C. Mesophyll chloroplast of 3% NaCl-treated plant. D. Bundle sheath chloroplast of 3% NaCl-treated plant. E,F. Mesophyll and bundle sheath chloroplasts of 3% NaCl-treated plant without CeCl3 staining. Bars = 250 nm.

DISCUSSION

Salinity stress has a broad physiological effect influencing many metabolic processes in the plant development (Zhu, 2001; Yokoi et al., 2002). In tissue or organ levels, previous studies have shown that the sensitivity of plants to salinity is closely related to the level of salt ions accumulated in the tissues or organs (Munn et al., 2002; Garthwaite., 2005). The present study showed that MC and BSC of the plants treated with NaCl accumulated higher Cl than that

of control. However, the content of Cl in MC and BSC of NaCl-treated plants was relatively comparable (Figs. 1 and 2). It has been reported that MC chloroplasts are more sensitive than BSC chloroplasts in response to salinity (Hasan et al., 2005). The present results revealed that differential sensitivity between MC and BSC chloroplasts were not caused by differential accumulation of Cl in those cells. Less sensitivity of BSC chloroplasts than MC chloroplasts to salinity in maize plants may not due to the function of BSC which has higher capability than MC to limit Cl influx. It has been documented that excessive accumulation of salt ions mainly Na and Cl causes ionic imbalance and metabolic damages of the cells (Hasegawa et al., 2000). In addition, MC and BSC in maize have different biochemical properties such as the content of antioxidants and photosynthetic enzymes (von Caemmerer and Furbank, 2003). Therefore, it is suggested that physiological processes following the accumulation of salt ions are different between MC and BSC.

ROS generation such as O_2^-, H_2O_2 and $^{\cdot}OH$ are a major characteristic of biochemical changes of the plants in response to environmental stresses including salt stress (Alscher et al., 1997; Mittler, 2002; Neill et al., 2002). ROS in high concentration is highly toxic and can alter normal cellular metabolism through oxidative damage to lipids, proteins and nucleic acids (Alscher et al., 1997; Imlay, 2003). In the present study, H_2O_2 was detected histochemically with DAB and cytochemically with $CeCl_3$, the methods which have been widely used to detect H_2O_2 generated in plant tissues in response to either biotic or abiotic stresses (Bestwick et al., 1997; Thordal-Christensen et al., 1997; Pellinen, 1999). Histochemical detection using DAB showed that NaCl induces H_2O_2 generation and can be detected visually (Fig. 3). Ultrastructural observation on cellular localization of H_2O_2 with $CeCl_3$ revealed that H_2O_2 in MC chloroplasts was higher than in BSC chloroplasts (Figs. 4C,D), while those cells accumulated comparable Cl (Figs. 1 and 2). Differential accumulation of H_2O_2 in MC and BSC chloroplasts may be one of the factors which made MC chloroplasts more sensitive and show severe damage by salinity than BSC chloroplasts (Hasan et al, 2005). Although the mechanism of ROS generation under salinity in plants is still not clear, several reports from studies on salinity stress have suggested that high concentration of ROS is induced by excessive accumulation of salt ions. Mitsuya et al. (2003) proposed that chloroplast damages induced by salinity is not caused by high accumulation salt ions directly but through oxidative stress mediated by overproduction of ROS. Blokhina et al. (2003) proposed that the extent of oxidative stress in the plants exposed to salinity is determined by the amounts of ROS.

The detection of higher H_2O_2 in MC than in BSC has also been reported in maize plants exposed to the unfavorable low temperature condition (Pastori et al., 2000). These results may be associated with the fact that MC and BSC chloroplasts in maize are different, both structurally and physiologically. MC chloroplasts have well-developed grana and both PSI and PSII activities. In contrast, BSC chloroplasts have reduced grana and a very low activity of PSII and are limited for non-cyclic electron flow. Therefore MC chloroplasts may have higher potential to generate $H_2O_2$and the other ROS under unfavorable condition such as salinity stress. It has been suggested that generation of ROS is caused by light-dependent processes associated with photosynthesis and photorespiration (Foyer and Noctor, 2003). It the chloroplasts, ROS can be formed either at the reducing site of PSI in the non-cyclic electron transport or at the acceptor site of PSII as the result of passing electrons from quinone to O_2 (Polle, 1996).

Another factor possibly caused differential H_2O_2 generation in MC and BSC chloroplasts in response to salinity is ROS scavenger system belonging to MC and BSC in maize leaves. It has been documented that antioxidants are differentially distributed between MC and BSC in maize leaves. Glutathione reductase and dehydroascorbate reductase were almost exclusively localized in MC, whereas ascorbate, ascorbate peroxidase, and superoxide dismutase were largely absent from MC. Catalase, reduced glutathione, and monodehydroascorbate reductase were found to be approximately equally distributed between

MC and BSC (Pastori et al., 2000). Although the mechanism is still not well understood, it has been documented that low temperature causes the changes of distribution of these antioxidants between MC and BSC chloroplasts. However, the distribution of antioxidants in the leaves of C_4 plants exposed to salinity has not been documented and needed further investigation.

The localization of H_2O_2 in mitochondria and peroxisomes were not detected with $CeCl_3$ staining in this study. Foyer and Noctor (2003) reported that H_2O_2 generation in the mitochondria of photosynthetic tissues is very low (<216 $nmol.m^{-2}.s^{-1}$), whereas in the chloroplast is about 4030 $nmol.m^{-2}.s^{-1}$. It has been reported that H_2O_2 concentration in intact mitochondria purified from pea leaf was not apparently altered by salinity (Hernandez et al., 2001). Peroxisome is thought to produce H_2O_2 at high rates through several reactions, including oxidation of long chain fatty acids and glycolate oxidase. However to overcome this condition peroxisome has a very high antioxidant capacity, notably including catalase but also APX and other enzymes of the ascorbate glutathione system (Jimenez et al. 1997).

Salinity induces overproduction of H_2O_2 in the apoplasts. The greatest accumulation of H_2O_2 was observed in the cell walls of MC facing to the large intercellular space. Similar localization of H_2O_2has also been documented in the cell of plants in response to air pollutant, wounding and pathogen attack (Pellinen et al., 1999; Orozco-Cardenaz et al., 2001). Some sources of H_2O_2 production in the apoplast such as plasma membrane bound-NADPH oxidase, cell wall peroxidase, extracellular peroxidase and amine oxidase are involved in the production of apoplastic H_2O_2 induced by biotic and abiotic stresses (Vranova et al., 2002; Mittler et al., 2002). It is considered that H_2O_2 have dual role in physiological processes; as signaling factor and oxidative stress (Alcher et al., 2002; Foyer and Noctor, 2003). H_2O_2 has half-life about of 1 ms, relatively longer than other ROS such as O_2^- (2-4 µs) and $^{\cdot}OH$ (<1 µs) and can diffuse for a considerable distance from its production site to other cell compartments (Vranova et al., 2002). In the present study, high accumulation of H_2O_2 in apoplasts of MC and BSC in maize plants exposed to salinity may be due to the fact that apoplasts have a relatively low ROS scavenger system compared with other cell compartments (Neill et al., 2002). This response may not be connected with defense systems to localize and restrict the area of tissue damage such as those reported in hypersensitive response or pathogen attack (Orozco-Cardenaz et al., 2001), but may rather reflect changes in oxidative balance in the cells that affects the homeostasis of the whole cell (Pellinen et al., 1999; Hu et al., 2005).

CONCLUSION

The present study suggested that differential sensitivity of MC and BSC chloroplasts in maize plants exposed to salinity may be associated with differential H_2O_2 generation which induces oxidative damage preferably in MC chloroplasts. Less sensitivity of BSC chloroplasts to salinity compared to MC chloroplasts may not be due to a mechanism which controls salt ions to enter MC and BSC.

REFERENCES

Bestwick, C.B., Brown, I.R., Bennett, M.H.R. and Mansfield, J.W. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola. Plant Cell* 9 : 209-221.

Blokhina, O., Virolainen, E. and Fagerstedt, K.V. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals Bot. 91 : 179-194.

del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., Gomez, M. and Barroso, J.B. 2002.

Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *J. Exp. Bot*. 53 : 1255-1272.

Flowers, T.J., and Hajibagheri, M.A. 2001. Salinity tolerance in *Hordeum vulgare*: ion concentrations in root cells of cultivars differing in salt tolerance. *Plant and Soil*231: 1-9.

Foyer, C.H. and Noctor, G. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant*. 119 : 355-364.

Garthwaite, A.G., von Bothmer, R. and Colmer, T.D. 2005. Salt tolerance in wild Hordeum species is associated with restricted entry of $Na^+$and Cl^-into the shoots. *J. Exp. Bot*. 56 : 2365-2378.

Hasan, R., Ohnuki, Y., Kawasaki, M. Taniguchi, M. and Miyake, H. 2005. Differential sensitivity of chloroplasts in mesophyll and bundle sheath cells in maize, an NADP-malic enzyme-type C_4 plant, to salinity stress. *Plant Prod. Sci*. 8 : 569-579.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. 2000. Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. *Plant Mol. Biol*. 51 : 463-499.

Hernandez, J.A., Ferrer, M.A., Jimenez, A., Ros Barcelo, A. and Sevilla, F. 2001. Antioxidant systems and $O_2^{\cdot-}/H_2O_2$ production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiol.*127 : 817-831.

Hu, X.,Jiang, M., Zhang, A. and Lu, J. 2005. Abscisic acid-induced apoplastic H_2O_2 accumulation up-regulates the activities of chloroplastic and cytosolic antioxidant enzymes in maize leaves. Planta 223: 57-68.

Imlay, J.A., 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol*. 57 : 395-418.

Jimenez, A., Hernandez, J.A.,del Rio, L.A. and Sevilla, F. 1997. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol*. 114 : 275-284.

Mitsuya, S., Kawasaki, M., Taniguchi, M. and Miyake, H. 2003. Light dependency of salinity-induced chloroplast degradation. *Plant Prod. Sci*. 6 : 219-223.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. 7 : 405-410.

Munns, R. 2005. Genes and salt tolerance: bringing them together.*New Phytol.*167 : 645-663.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D. and Hancock, J.T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot*. 53 : 1237-1247.

Orozco-Cardenas, M.L., Narvaez-Vasquez, J. and Ryan, C.A. 2001. Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* 13 : 179-191.

Pastori, G., Foyer, C.H. and Mullineaux, P. 2000. Low temperature-induced changes in the

distribution of H_2O_2 and antioxidants between the bundle sheth and mesophyll cells of maize leaves. *J. Exp. Bot*. 51 : 107-113.

Pellinen, R., Palva, T. and Kangasjarvi, J. 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant J*. 20 : 349–356.

Polle, A. 1996. Mehler reaction: Friend or foe in photosynthesis? *Bot. Acta* 109 : 84-89.

Rengasamy, P., Chittleborough, D. and Helyar, K. 2003.Root-zone constraints and plant-based solutions for dryland salinity. *Plant and Soil*257 : 249-260.

Sharpe, R.M., Mahajan, A., Takacs, E.M., Stern, D.B. and Cahoon, A.B.2011. Developmental and cell type characterization of bundle sheathand mesophyll chloroplast transcript abundance in maize. *Curr. Genet*. 57:89–102

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J*. 11 : 1187-1194.

von Caemmerer, S. and Furbank, R.T. 2003. The C_4 pathway: an efficient CO_2 pump. Photosynth. Res. 77 : 191-207.

Vranova, E., Inze, D. and Van Breusegem, F. 2002. Signal transduction during oxidative stress. *J. Exp. Bot.* 53 : 1227-1136.

Yamane, K., Rahman, M.S., Kawasaki, M., Taniguchi, M. and Miyake, H. 2004. Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.). Plant Prod. Sci. 7 : 292-300.

Yokoi, S., Bressan, R. A. and Hasegawa, P.M. 2002. Salt stress tolerance of plants. *JIRCAS Working Report* 25-33.

Zhu, J.K. 2001. Plant salt tolerant. *Trends Plant Sci*. 6 : 66-71.