韓草誌 (J. Korean Grassl. Sci.) 27(2) : 129~136, 2007

The Oxidative Stress Induction and Response of Antioxidative Enzymes in the Large Patch-Infected Zoysiagrass

Ⅰ. Oxidative stress induction

Dae Hyun Kim, Bok Rye Lee, Ming Li and Tae Hwan Kim*

라지 팻치에 감염된 잔디의 산화적 스트레스 발현과 항산화효소의 활력의 변화

Ⅰ. 산화적 스트레스 발현

김대현 · 이복례 · 이 명 · 김태환*

요 약

한국형 잔디에 있어 병원성(라지 팻치) 감염이 산화적 스트레스의 발현에 미치는 영향을 규명하기 위해 라지 팻치에 감염된 잔디의 생육, 뿌리 건사율, 과산화수소 농도 및 지질과산화 정도를 감염이 되지 않은 대조구와 비교하였다. 처리 후 6일 동안 이틀간격으로 잎과 뿌리 시료에 대해 각각 분석하였다. 라지 팻치에 감염된 잔디 뿌리의 건사율은 대조구에 비해 약 30% 증가 하였다. 건물 함량은 4일째까지는 유의적인 차이가 없었으며 6일차에서만 라지 팻치 감염에 따라 잎에서 14% 뿌리에서 20% 각각 감소하였다. 과산화수소의 농도는 라지 팻치에 감염된 잎에서 초기 2일 동안 약 28%의 높은 증가를 하였다가 이후 서서히 감소하여 6일차에는 대조구에 비교하여 11% 낮은 수준이었다. 뿌리에서는 감염에 따른 과산화수소의 증가가 뚜렷하여 6일차에는 대조구에 비해 약 1.7배 높았다. 지질과산화 정도는 잎의 경우, 라지 팻치 감염에 따라 초기 4일간 지속적으로 증가하다가 이후 정체하였으며 뿌리에서는 초기 2일간의 증가가 뚜렷하였다. 이러한 결과들은 잔디에 있어 라지 팻치 감염은 산화적 스트레스를 유도하며, 잎과 뿌리 조직 간에 산화적 스트레스 발현정도 및 역동성은 차이가 있음을 보여준다.

(**Key words** : H_2O_2, Large patch, Lipid peroxidation, Oxidative stress, Zoysiagrass)

Ⅰ. INTRODUCTION

Large patch of soil pathogen microorganism is a serious disease that affects the growth of zoysiagrass. This turf disease causes to decline turf quality by stress in plant growth. When plants are subjected to adverse conditions such as environmental and/or pathogenesis-related stresses, the scavenging system may lose its function and the balance between producing and quenching reactive oxygen species (ROS) can be disturbed, resulting in oxidative damage (Asada and Takahashi, 1987; Inzé and Van Montagu, 1995; Lopez-Huertas et al., 1999). Environ-

"이 논문은 2005년도 전남대학교 학술연구비 지원에 의하여 연구되었음"
(This study was financially supported by Chonnam National University)
전남대학교 (Chonnam National University, Gwangju 500-757, Korea)
Corresponding author: Tae-Hwan Kim, Department of Animal Science & Institute of Agriculture Science and Technology, College of Agriculture & Life Science, Chonnam National University, Gwangju 500-757, Korea
Tel : +82-62-530-2126, Fax : +82-62-530-2129, E-mail : grassl@chonnam.ac.kr

mental stresses such as drought, high temperature, chilling, high light, and air pollutants (Mittler and Zilinskas, 1994; Noctor and Foyer, 1998) cause ROS generation in plants. ROS including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), and hydroxy radical ($^{\bullet}OH$) are generated in cytoplasm, chloroplast (Asada and Takahashi, 1987), mitochondria (Jimenez et al., 1998), peroxisomes (Del Rio et al., 1992; Lopez-Huertas et al., 1999), and apoplast (Hernández et al., 2001). The uncontrolled accumulation of ROS generates oxidative stress and may cause damage to the biomolecules, membrane lipid peroxidation, inactivation of SH-containing enzymes, and RNA and DNA damage (Smirnoff, 1993; Menconi et al., 1995).

Typical symptoms of oxidative damage to cellular components, such lipid peroxidation of membranes and amino acid residue oxidation of proteins, may be checked because these have been observed in the biological materials exposed to the condition identified with an excessive production of active oxygen (McKersie and Leshem, 1994). Measurement of the level thiobarbituric acid (TBA) reactive substances in plant tissues is widely used as an index of lipid peroxidation (Smirnoff, 1993; Inzé and Van Montagu, 1995; Zhang and Kirkham, 1996; Fu and Huang, 2001). Oxidative stress as indicated by lipid peroxidation can occur when the scavenging of active oxygen species is overwhelmed by the production. Lipid peroxidation has been known to be related with cell damages provoked by various stresses (Hernández et al., 2001). Polyunsaturated fatty acids are the main membrane lipid components susceptible to peroxidation and degradation. ROS can react with polyunsaturated fatty acids, forming conjugated dienes or trienes, lipid peroxy radicals and lipid hydroperoxides (Smirnoff, 1993). Lipid peroxidation can also be initiated enzymatically through a sequential action of lipoxygenases, a ubiquitous plant enzyme which catalyses the hydroperoxidation of polyunsaturated fatty acids (Axelrod et al., 1981). This enzyme incorporates molecular oxygen into linoleic and linolenic acids, to form lipid hydroperoxides. Evidence suggests that various stresses cause the oxidative damage through enhanced generation of ROS and an inefficient response of antioxidant defense system in plants (Smirnoff, 1993; Zhang and Kirkhan, 1996).

The present study was designed to determine the extent of pathogen-induced oxidative stress by comparing plant growth, H_2O_2 generation, and lipid peroxidation between healthy (control) and pathogen-infected zoysiagrass plants.

II. MATERIALS AND METHOD

1. Plant culture and experiment procedure

Sods of zoysiagrass (*Zoysia japonica*) were taken from healthy fairway for control or from the sites where the symptom of large patch infection already appeared for pathogen-infected treatment at Muan CC, Chonnam, Korea. They were transplanted to 3 L pot containing a mixture of sand and fritted clay. During 2 weeks of adaptation, 10 mL (average of 1 x 10^5 zoospores per milliliter) of *Rhizoctonia spp.* cultured in the medium for was inoculated every 3 days as a pathogen inoculum. Sampling for each treatment was begun from the end of adaptation (day 0), and continued every 2 days for a period of 6 days. Harvested plants were separated into leaves + stolon and roots. Tissue samples were immediately frozen in liquid nitrogen. Freeze-dried samples were finely ground and stored under vacuum for further analysis.

2. Root mortality

Root mortality was measured using the modified method of Knievel (1973). The fresh roots (500 mg) were incubated with 10 mL of 0.6% 2,3,5-triphenyltetrazolium chloride in 0.05 M phosphate buffer, pH 7.4, for 24 h in the

dark at 30℃. Roots were then rinsed twice with deionized water. Formazan was extracted twice from the roots with 95% ethanol at 70℃ for 4 h. Combined extracts were adjusted to a final volume of 50 mL with 95% ethanol. Absorbance was read at 490nm. A standard curve was made using different proportions of living roots and killed roots to calculate root mortality. Root mortality was expressed as percentage dead root dry weight (DW) of total root DW.

3. Chemical analysis

H_2O_2 was extracted by homogenizing about 200 mg DW with 3 mL of phosphate buffer (50 mM, pH 6.8). The homogenate was centrifuged at 6,000 g for 25 min. To determine H_2O_2 levels, 3 mL of extracted solution was mixed with 1 mL of 0.1 % titanium chloride (Aldrich) in 20% (v/v) H_2SO_4 and mixture was then centrifuged at 6,000 g for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm as described by Lin and Kao (2002). H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1}cm^{-1}$.

The lipid peroxidation level was determined in term of malondialdehyde (MDA) content. In order to correct for interference generated by thiobarbituric acid (TBA)-sugar complexes (Du and Bramlage, 1992), TBA-MDA levels was estimated by correcting for compounds other than MDA which absorbance at 532 nm by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA. Plant tissue samples were homogenized with 80 % (v/v) ethanol, followed by centrifugation at 3,000 g for 10 min. A 1 mL aliquot of appropriately diluted sample was added to a test tube with 1 mL of either (i) −TBA solution comprised of 20% (w/v) trichloroacetic acid and 0.01 % butylated hydroxytoluene (ii) +TBA soution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95 ℃ in a block heater for 25 min, cooled, and centrifuged at 3,000 g for 10 min. Absorbances were read at 440 nm, 532 nm, and 600 nm. Malondialdehyde equivalents were calculated in the following manner:

1) $[(Abs532_{+TBA})-(Abs600_{+TBA}) - (Abs532_{-TBA}-Abs600_{-TBA})] = A$
2) $[(Abs440_{+TBA}-Abs600_{+TBA})\ 0.0571] = B$
3) MDA equivalents $(nM\ mL^{-1}) = (A-B/157000)10^6$

III. RESULTS

1. Root mortality

The changes in root mortality during 6 days are presented in Table 1. At day 0, the root mortality of infected plot was already significantly higher than that of control, and then continuously increased to 62% at day 6. However, no significant change occurred in non-infected (control) plants.

2. Dry mass

The changes in dry mass in leaves and roots of pathogen-infected or control plants are presented at Fig. 1. Leaf dry mass of control plants slowly increased from 2.33 mg DM hole

Table 1. Root mortality in pathogen-infected or healthy (control) zoysiagrass during 6 days of measurement. Each value is the mean ± S.E. for n=3

Treatment	Days after treatment			
	0	2	4	6
Root mortality (%)				
Control	29.88 ± 4.37	30.73 ± 2.78	33.29 ± 0.42	34.54 ± 1.79
Pathogen-infected	45.33 ± 2.61	55.18 ± 3.39	60.78 ± 0.59	62.36 ± 2.04

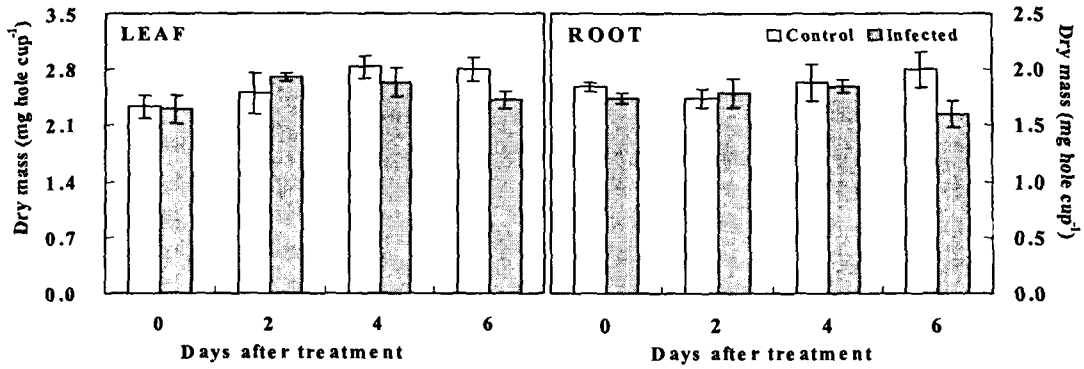

Fig. 1. Changes of dry matter in leaves and roots of pathogen-infected or healthy (control) zoysiagrass during 6 days of measurement. Each value is the mean ± S.E. for n=3.

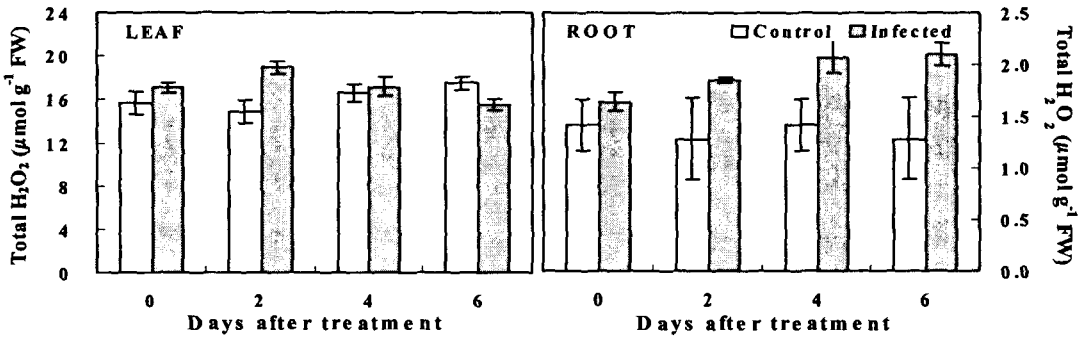

Fig. 2. Changes of H_2O_2 concentration in leaves and roots of the pathogen-infected or healthy (control) zoysiagrass during 6 days of measurement. Each value is the mean ± S.E. for n=3.

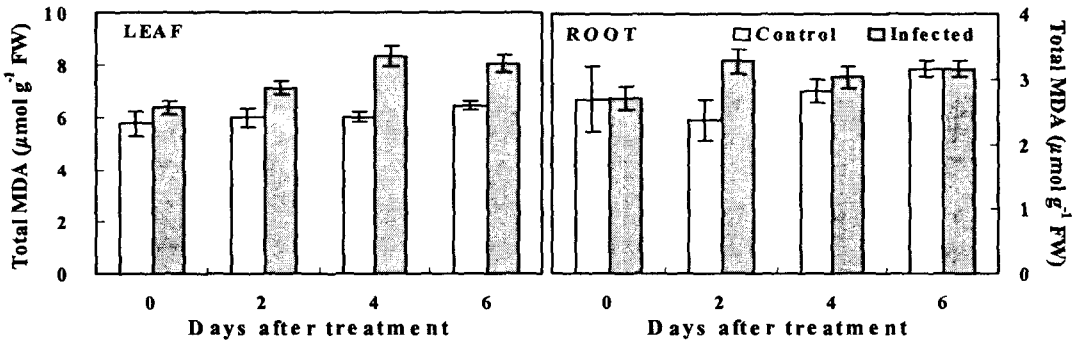

Fig. 3. Lipid peroxidation determined by malondialdehyde concentration in leaves and roots of the pathogen-infected or healthy (control) zoysiagrass during 6 days of measurement. Each value is the mean ± S.E. for n=3.

cup^{-1} at day 0 to 2.80 mg DM hole cup^{-1} at day 6, while that of the infected plants was less varied. The decrease (−14%) at day 6 caused by pathogen-infection was found to be significant ($p \leq 0.05$). Root dry mass was also not significantly changed by pathogen-infection

until day 4, and then significantly decreased by 20% compared with the control at day 6.

3. Hydrogen peroxide concentration and lipid peroxidation

The changes in H_2O_2 concentration in healthy (control) and pathogen-infected plants during 6 days of measurement are presented at Fig. 2. H_2O_2 concentration in the control was less changed within the range of 14.8~17.5 μmol g^{-1} FW for leaves and 1.28~1.41 μmol g^{-1} FW for roots (Fig. 2). The H_2O_2 concentration in pathogen-infected leaves rapidly increased by 28% within the first 2 days. It then slightly decreased, so that it arrived a similar or even lower level when compared with the controls. In the pathogen-infected roots, H_2O_2 concentration gradually increased throughout the experimental period, and reached at 1.64-fold higher level than control root at day 6. Pathogen-infection effects on the extent of lipid peroxidation are presented Fig 3. Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid method (Du and Bramlage, 1992). The MDA concentration in the leaves of control plant was nearly constant within the range of 5.75~6.46 μmol g^{-1} FW. However, the concentration in pathogen-infected leaves continually increased until day 4 and then maintained at the same level. In roots, a significant increase (+39%) in MDA concentration by pathogen-infection was observed only at day 2.

Ⅳ. DISCUSSION

Large patch infection provoked the stress-related and detrimental symptoms in zoysiagrass during the experiment, as evidenced by about 2-fold increase in root mortality in pathogen-infected plants at day 6 (Table 1). Decline of root activities could adversely affect shoot growth by limiting nutrient supply from roots to shoots (Kramer, 1983). In this study, root mortality highly increased in pathogen-infected plants, but not exceeded 60% at the end of experiment (day 6). This indicates that these plants are not completely dead by acquired stress resistance, although plants receive a bad influence from pathogen-infection during vegetative growth period. Leaf and root dry mass was not significantly for the first 4 days. A significant ($p \leq 0.05$) decrease in dry mass was recorded from day 6 (Fig. 1). These suggest that resistance mechanisms are possibly being in the process for the early period of pathogen-induced stress and that growth inhibition was driven from the cumulative level of stress rather than the instant intensity at given sampling time (Kim et al., 2004). The growth inhibition has been found to be associated with the loss of leaf capacity to maintain osmotic potential gradients (Chazen and Neumann, 1994) and the limited water availability for cell extension (Frensch, 1997).

In the present study, for the H_2O_2 concentration, a rapid increase (within 2 days) in leaves and a continuos increase in roots caused by pathogen-infection were observed (Fig. 2). Significant inverse relationships between H_2O_2 concentration and dry weight have been widely reported under environmental stressed-condition (Chazen and Neumann, 1994; Katerji et al., 1997; Lee et al., 2007). The inhibition of plant growth under stressed-condition has often been related to the decrease in the plastic extensibility of the growing cell walls (Fry, 1986; Neumann et al., 1994). In our previous work with the drought-stressed white clover, the increase in H_2O_2 concentration, which closely related to leaf growth inhibition, coincided with the increase of the activity of cell wall peroxidases (POD) (Lee et al., 2007). It has been postulated that the action of POD located in the cell walls would be to confer rigidity to the cell walls and to inhibit cell expansion involved in growth (Carpita and Gilbeaut, 1993; Neumann et al., 1994; Lin and Kao, 2002). Considering the facts that H_2O_2 is a necessary substrate for cell wall

stiffening process catalyzed by peroxidase (Schopfer, 1994; Hohl et al., 1995) and causes a rapid cross-linking of cell wall polymers (Bradley et al., 1992; Schopfer, 1994), pathogen infection-induced inhibition in shoot growth (Fig. 1) is possibly due to cell wall stiffening process related the formation of cross-linking among cell wall polymers. In Recent, Lin and Kao (2002) have shown that increasing H_2O_2 concentration of exogenous application progressively increased endogenous H_2O_2 levels in roots of rice seedlings and decreased fresh weight and dry weight. Moreover, H_2O_2 accumulation caused by stress, as strong oxidant, can initiate localized oxidative damage leading to disruption of metabolic function and loss of cellular integrity at sites where it accumulates (Foyer et al., 1997). On the other hand, H_2O_2 is considered to act as signal for activation of the PR-proteins such as peroxidases, chitinase and ß-1,3-glucanase (Moloi et al., 2006) that are involved in stress tolerance (Kwon et al., 2007).

The level of lipid peroxidation has been used as an indicator of free radical damage to cell membranes under stress conditions. Malondialdehyde (MDA) is a final product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane (Smirnoff, 1993; Liu and Huang, 2000; Fu and Huang, 2001). As expected, pathogen-infection increased MDA level in both leaves and roots (Fig. 3). Much higher increase of MDA level in pathogen-infected plants indicates that an acceleration of membrane lipid peroxidation would be induced by pathogen-infection. Such increases have been found in other species under drought (Zhang and Kirkham, 1994; Fu and Huang, 2001; Lee et al., 2007), heat (Liu and Huang, 2000) and Freezing (Moloi et al., 2006). Cell membrane stability was shown to be affected by lipid peroxidation caused by active oxygen species under various stress conditions (Dhindsa et al., 1981). Moreover, the increase in MDA level in drought-stressed plants was closely related to the increase in H_2O_2 concentration (Lee et al., 2007). Thus, the present data indicate that pathogen-infected plants are less able to scavenge active oxygen, and are suffered higher level of membrane damage. These suggest that H_2O_2 accumulation and MDA level might be served as useful indicators of stress intensity caused by pathogen- infection in zoysiagrass.

V. ABSTRACT

To investigate the effect of large patch infection on oxidative stress induction, growth, H_2O_2 concentration and lipid peroxidation were compared between pathogen-infected and healthy (control) zoysiagrass. The sampling for leaves and roots were carried out every 2 days for a period of 6 days. Pathogen-infection increased root mortality by 30% compared to control. Dry mass was not significantly affected by pathogen-infection until day 4, but significant decreases in both leaves (−14%) and roots (−20%) were observed at day 6. The H_2O_2 concentration in pathogen-infected leaves rapidly increased within the first 2 days (+28%) and then slightly decreased. The increase of H_2O_2 in pathogen-infected roots was distinct, showing 1.7-fold higher level than control at day 6. The extent of lipid peroxidation caused by pathogen-infection continuously increased for the first 4 days. This was then stagnated until day 6. In roots, the significant increase of lipid peroxidation was observed only at day 2. These results indicate that large patch-infection induces oxidative stress, and that the oxidative stress responsive pattern was plant organ specific.

VI. REFERENCES

1. Asada, K. and M. Takahashi. 1987. Production and scavenging of active oxygen in photosynthesis. In: Photoinhibition (Eds. Kyle, D.J., Osmond, C.B. and O. Arntzen). Elsevier, Amsterdam. pp. 227-289.
2. Axelrod, B., T.M. Cheesbrough and S.

Laakso. 1981. Lipoxygenase from soybeans. Methods Enzymol. 71:441-451.

3. Bradley, D.J., P. Kjellbom and C.J. Lamb. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline rich plant cell wall protein: A novel, rapid defense response. Cell 70:21-23.
4. Carpita, N.C. and D.M. Gilbeaut. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3:1-30.
5. Chazen, O. and P.M. Neumann. 1994. Hydraulic signals from roots and rapid cell-wall hardening in growing maize (*Zea maize* L.) leaves are primary responses to polyethylene glycol-induced water deficit. Plant Physiol. 104:1385-1392.
6. Del Rio, L.A., L.M. Sandalio, J.M. Palma, P. Bueno and F.J. Corpas. 1992. Metabolism of oxygen radicals in peroxisomes and cellular implication. Free Radic. Biol. Med. 13:557-580.
7. Dhindsa, R.S., P.P. Dhindsa and T.A. Thorpe. 1981. Leaf senescence: correlated with increased level of membrane permeability and lipid peroxidation, and decresed levels of superoxide dismutase and catalase. J. Exp. Bot. 32:93-101
8. Du, Z. and W.J. Bramlage. 1992. Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. J Agri. Food Chem. 40:1566-1570.
9. Foyer, C.H., H. Lopez-Delgado, J.F. Dat and I.M. Scott. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. Physiol. Plant. 100:241-254.
10. Frensch, J. 1997. Primary responses of root and leaf elongation to water deficits in the atmosphere and soil solution. J. Exp. Bot. 48:985-999.
11. Fry, S.C. 1986. Cross-linking of matrix polymers in the growing cells of angiosperms. Annu. Rev. Plant Physiol. 37:165-186.
12. Fu, J. and B. Huang. 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Envrion. Exp. Bot. 45:104-114.
13. Hohl M, H. Greiner and P. Schopfer. 1995. The cryptic growth response of maize coleoptile and its relationship to H_2O_2-dependent cell wall stiffening. Physiol. Plant. 94:491-498.
14. Hernández, J.A., M.A. Ferrer, A. Jimenez, A.R. Barcelo and F. Sevilla. 2001. Antioxidant systems and O_2 /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.
15. Inzé, D. and M. Van Montagu. 1995. Oxidative stress in plants. Curr. Opin. Biorech. 6:153-158.
16. Jimenez, A., J.A. Hernandez, G. Pastori, L.A. del Rio and F. Sevilla. 1998. Role of the ascorbate, glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. Plant Physiol. 118:1327-1335.
17. Katerji, N., J.W. van Hoorn, A. Hamdy, M. Mastrorilli and E. Mou Karzel. 1997. Osmotic adjustment of sugar beets in response to soil salinity and its influence on stomatal conductance, growth and yields. Agri. Water Manage. 34:57-69.
18. Kim, T.H., B.R. Lee, W.J. Jung, K.Y. Kim, J.C. Avice and A. Ourry. 2004. *De novo* protein synthesis in relation to ammonia and proline accumulation in water stressed white clover. Funct. Plant Biol. 31:847-855.
19. Knievel, D.P. 1973. Procedure for estimating ratio of living to dead root dry matter in root core sample. Crop Sci. 13:124-126.

20. Kramer, P.J. 1983. Water stress research: progress and problems. Curr. Top. Plant Biochem. Physiol. 2:129-144.
21. Kwon, Y.R., S.H. Kim, M.S. Jung, M.S. Kim, J.E. Oh, H.W. Ju, K.I. Kim, E. Vierling, H. Lee and S.W. Hong. 2007. Arabidopsis hot2 encodes an endochitinase-like protein that is essential for tolerance to heat, salt and drought stresses. Plant J. 49:184-193.
22. Lee, B.R., K.Y. Kim, W.J. Jung, J.C. Avice, A. Ourry and T.H. Kim. 2007. Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (*Trifolium repens* L.). J. Exp. Bot. 58:1271-1279.
23. Lin, C.L. and C.H. Kao. 2002. Osmotic stress-induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. Plant Growth Regul. 37:177-184.
24. Liu, X. and B. Huang. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass. Crop Sci. 40:503-510.
25. Lopez-Huertas, E., F.J. Corpas, L.M. Sandalio and L.A. del Rio. 1999. Characterization of membrane polypeptides from pea leaf peroxisomes involved in superoxide radical generation. Biochem. J. 337:531-536.
26. Mckersie, B.D. and Y.Y. Leshem. 1994. Stress and stress coping in cultivated plants. Kluwer Academic Publishers, Dordrecht. pp. 349-367.
27. Menconi, M., C.L.M. Sgherri, C. Pinzino and F. Navari-Izzo. 1995. Activated oxygen production and detoxification in wheat plants subjected to a water deficit programme. J. Exp. Bot. 46:1123-1130.
28. Mittler, R. and B.A. Zilinskas. 1994. Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. Plant J. 5:397-405.
29. Moloi, M.J., J. Amie and A.J. Westhuizen. 2006. The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. J. Plant Physiol. 163:1118-1125.
30. Neumann, P.M., H. Azaizeh and D. Leon. 1994. Hardening of root cell walls: Growth inhibitory responses to salinity stress. Plant Cell Environ. 17:303-309.
31. Noctor, G. and C. Foyer. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Mol. Biol. 49:249-279.
32. Schopfer, P. 1994 Histochemical demonstrations and localization of H_2O_2 in organs of higher plants by tissue printing on nitrocellulose paper. Plant Physiol. 104:1269-1275.
33. Smirnoff, N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125:27-58.
34. Zhang, J. and M.B. Kirkham. 1996. Lipid peroxidation in sorghum and sunflower seedlings as affected by ascorbic acid, benzoic acid and propyl gallate. J. Plant Physiol. 149:489-493.