

Catalase Expression in Normal Metabolism and Under Stress in the Model Legume *Medicago truncatula*

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Summary

Legume N₂ fixation is particularly sensitive to adverse environmental conditions linked to enhanced production of reactive oxygen species (ROS). Successful defense against oxidative stress is crucial for survival, and the protective mechanisms and their roles are under thorough investigation. In this study we examined the well characterized in other species antioxidant system of catalase in the model legume *Medicago truncatula*. We determined the isoenzymic profile of CAT in leaves, stems and roots and analyzed the enzyme responses to osmotic and oxidative stress. Our results demonstrated that at least two CAT isozymes are expressed in different tissues of *M. truncatula*, which respond differently to stress conditions. We also examined the expression profiles of catalase in leaves, stems, and roots after stress treatments. Catalase induction in leaves was higher than that in roots. In roots the highest induction was observed in NaCl stressed plants while in leaves the highest induction was observed in iron-treated plants.

Introduction

Aerobic organisms gain significant energetic advantage by using molecular oxygen as the terminal oxidant in respiration. However, they can be severely damaged by the partially reduced oxygen species superoxide radical (O₂⁻), hydroxyl radical (*OH) and hydrogen peroxide (H₂O₂), which are produced through normal or aberrant metabolic processes, and are commonly known reactive oxygen species (ROS). To minimize the adverse effects of ROS aerobic organisms evolved antioxidant defenses including catalases (CATs),

among others. Many plants subjected to a variety of environmental stresses respond by increasing the levels of mRNA and/or enzyme activity of antioxidant defense enzymes (Scandalios et al, 1997). Successful defense against oxidative stress is crucial for survival, and the protective mechanisms and their roles are under thorough investigation. In several recent studies, attempts were made to enhance protection with constitutive overexpression of defense enzymes in transgenic plants (Alien, 1995). Results of these studies confirm the important role of antioxidant defense enzymes in protection from oxidative stress of various types and origins, and point to the significance of transgenic plants as tools for plant improvement.

Legume N₂ fixation is particularly sensitive to environmental perturbations, including defoliation, water deficit, continuous darkness, and nitrate fertilization (Vance et al., 1979; Witty et al., 1986; Layzell et al., 1990). In most types of stress, the initial decrease of nitrogenase activity is associated with a decline in the O₂ concentration reaching the infected cells and bacteroids (Witty et al., 1986; Carroll et al., 1987; Layzell et al., 1990; Escuredo et al., 1996). Prolongation of stress induces premature nodule senescence, which shares some features with natural senescence (Pfeiffer et al., 1983; Sarath et al., 1986). This stress-induced senescence has been linked to the enhanced production of oxidants and the lowering of antioxidant defenses (Escuredo et al., 1996; Gogorcena et al., 1997). Oxidants include inorganic (H₂O₂) and organic (lipid) peroxides as well as "catalytic iron," the fraction of iron in plant tissues capable of catalyzing the generation of hydroxyl radicals through Fenton reactions (Becana et al., 1998). The mitochondria of legume root nodules are critical to sustain the energy-intensive process of nitrogen fixation. They also generate ROS at high rates. Other subcellular compartments of leaves, such as chloroplasts, peroxisomes and mitochondria, are also potential generators of superoxide radical and H₂O₂, mainly as a consequence of electron transport and enzymatic reactions (Del Rio et al., 1992).

In this study, we examined the well characterized in other species antioxidant system of catalase, in *Medicago truncatula*. We determined the isoenzymic profile of CAT in leaves and roots, and analyzed the enzyme responses under stress. We also examined expression profiles of catalase (*Cat*) in the same tissues. Our results demonstrate that antioxidant responses are induced by different abiotic stresses at the gene expression and the enzyme activity levels, and show specificity for the tissues examined.

Materials and Methods

Plant material and Treatments: Seeds from the *Medicago truncatula* A17 line were sterilized by commercial sodium hypochlorite solution (5%), scarified and imbibed for 8h in sterile water. Subsequently seeds were sown individually in magenda boxes supplied with nutrient solution and grown in a growth chamber at 22° C, 16h light / 8h dark photoperiod, and 70% relative humidity. After one month plantlets were subjected to stress treatments.

For all treatments the nutrient solution was decanted and replaced with 250mM NaCl, 250mM Mannitol, 1mM Fe(III)-EDTA, or 100mM H₂O₂. Withholding irrigation for 1 week induced water deficit. For cold treatment the box was placed at 4° C in the dark.

Enzyme activity, protein determination, and isozyme analysis: Catalase activity and protein determination were performed as described (Baum JA. and Scandalios JG., 1981, Beers and Sizer, 1952). CAT isoforms were resolved by nondenaturing PAGE and stained for catalase activity in a 1% ferric chloride, 1% potassium ferricyanide solution.

Clones and primers: Catalase cDNA clones were selected from EST sequences deposited to the *Medicago truncatula* database at TIGR (<http://www.tigr.org/tdb/tgi/mtgi/>). Several clones, kindly provided by Dr. R. Dixon (The Samuel Roberts Noble Foundation, OK, USA) were sequenced. One clone corresponding to a full-length cDNA was used for primer design and testing. Actin-beta was used as a reference control and specific primers were designed according to the deposited sequence of the gene (TC44355) at the same web site and used in the real-time RT-PCR experiments to amplify the reference gene.

RNA isolation and gene expression analysis: Plant material was harvested, frozen in liquid nitrogen and stored at -80°C for analysis. Total RNA from plant material was extracted using the RNeasy Plant mini kit (Qiagen) with on column DNA digestion during RNA purification using the RNase-Free DNase set (Qiagen) according to the instructions. cDNA was prepared with reverse transcription using 1 µg total RNA, 0.5 µg oligo dT adaptor primer, 1mM dNTPs, 200 units M-MuLV reverse transcriptase (NEB) in a total volume of 25 µl at 37° C for 1h, followed by inactivation of RT at 70° C for 10 min and final storage at 4° C.

Catalase gene expression analysis was performed with real-time RT-PCR using an Opticon (MJ Research) real-time PCR system. PCR was performed in 1X PCR buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 0.4µM Cat-F and 0.4µM Cat-R primers, 1/40.000 dilution of a 10.000X Sybr-Green solution (Sigma), and 1 unit of the DyNAzyme II PCR polymerase (Finnzymes) having as template the cDNA synthesized in the RT reaction at 1/25 dilution in the PCR. Primer sequences were:

Cat-F: GCACCCGACAGGCAAGATAGATT,

Cat-R: CGAGTCACGTACATGGAGTTTA

amplifying a 314bp fragment of the catalase gene 3' end containing 149 bases from the 3' untranslated region. As a control for relative gene expression quantification, the actin-beta gene was used in a similar PCR using the primers

Actin-F: CCGGTGTCATGGTTGGTAT

Actin-R: GCAGGCACATTGAAGGTCT

The PCR program was incubation at 94° C for 2 min, followed by 3 5 cycles of incubation at 94° C for 20 sec, 54° C 30 sec, 72° C 20 sec, plate read at 0° C, and a final extension step at 72° C for 2 min. For identification of the

PCR products a melting curve was performed from 65° C to 90.1° C with read every 1° C and hold for 10 sec between reads. A control in real-time RT-PCR was included for each treatment using as template the RT reaction mixture without reverse transcriptase at the same dilution as the cDNA template. Relative quantification of catalase expression was performed using the Q-Gen software for estimation of the normalized gene expression (Muller et al. 2002).

Results

CAT isozyme profiles in different tissues: Isozyme analysis of *M. truncatula* catalase in leaves, roots, stems and pods revealed a slowly migrating band in PAGE stained for CAT activity (Figure 1A). In roots and stems of untreated plants CAT activity is very low and can hardly be detected on a gel loaded with equal amounts of protein. However, in root tissue treated with Fe and mannitol, CAT activity was highly increased and this CAT band was immediately detected (Figure 1B). In roots subjected to osmotic stress with NaCl and mannitol, a second fast band became clearly evident (Figure 1B). These data demonstrate that at least two CAT isozymes are expressed in different tissues of *M. truncatula*.

CAT responses to abiotic stress: Induction of antioxidant enzymatic defenses is often one of the first responses of plants subjected to abiotic stress. In order to determine the significance and the role of CATs and SODs in *M. truncatula* stress adaptation we examined their response to different types of induced stress. We estimated activity levels in response to osmotic stress induced by mannitol and NaCl (the later of which also causes ionic imbalance), to oxidative stress induced directly by H₂O₂ or by iron overload which can cause the formation of OH through the Fenton reaction, and stress caused by environmental parameters like drought and cold, all of which have been

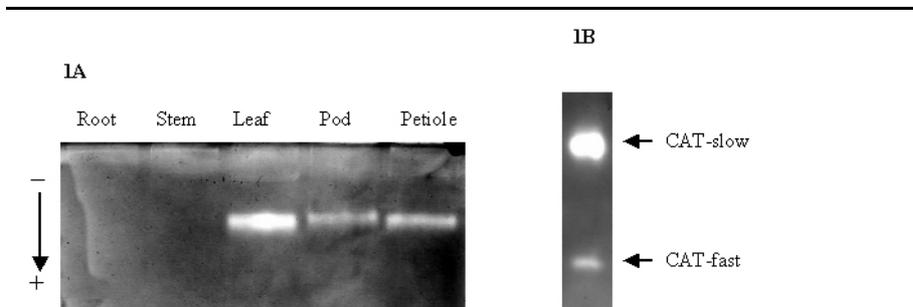


Figure 1. Zymogram analysis of catalase activity resolved in a 9% non-denaturing PAGE in different organs of *M. truncatula* plants. (1A) Protein extracts from different untreated tissues (40ng per lane). (1B) Protein extract (40µg) was prepared from root tissue treated with 250mM mannitol for 24h.

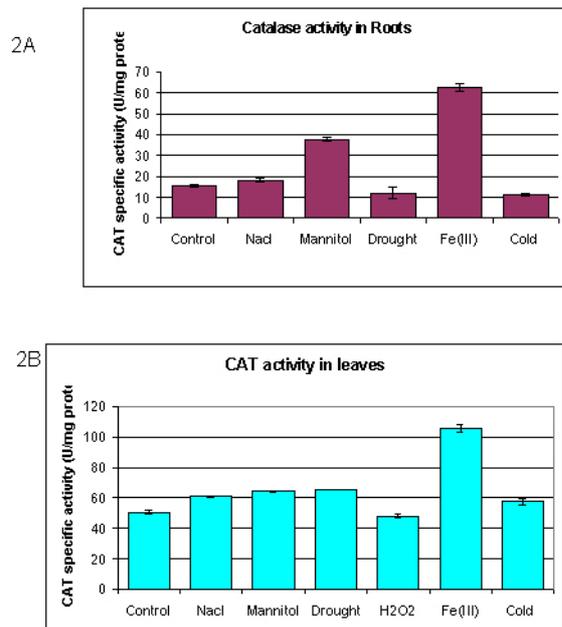


Figure 2. (2A) Catalase specific activity in roots of *M. truncatula* under various stress conditions. (2B) Catalase specific activity in leaves of *M. truncatula* under various stress conditions. Untreated plants (control), NaCl (250mM), mannitol (250mM), drought, H₂O₂ (100mM), Fe(III) (1mM) and cold (+4°C).

implicated in the production of ROS. Our material was root and leaf tissue of one month old plants.

Our data showed that CAT activity was strongly induced by Fe(III) in roots and leaves (Figure 2). In roots mannitol also caused a high CAT activity induction, while the rest of the treatments did not affect it (Figure 2 A). In leaves there was a moderate enhancement of CAT activity in treatments with mannitol, NaCl, drought and cold, while it was not affected by H₂O₂ (Figure 2B).

In conclusion our data revealed that iron overload caused the highest induction of enzymatic activity in the antioxidant gene CAT in *M. truncatula*. Mannitol caused induction of CAT activity, which was much higher in roots than in leaves. Other treatments caused slight induction only in roots.

Catalase genes in *M. truncatula*: In a first attempt to characterize the catalase gene family in *M. truncatula* we searched the truncatula EST database hosted by The Institute for Genomic Research (<http://www.tigr.org/tdb/tgi/mtgi/>). We identified two tentative consensus (TC) sequences for catalase (TC37674 and TC40117) and obtained several clones for each TC from the corresponding researchers. We fully sequenced three clones pro-

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1 GTGTTATTTA TATATGAGGC tGATAAGACT CCGGATTATC aGCACGAAT WGTTTTCTCA
64 ATTTCTTAT CTCTATCGAT TTCTTCTTCT CCACCGTCT CRTCCCTTCT CTCTACGATT
121 ACCCACCTG GATCGRTRCA AGCACCTGTC GTCCAGGCT TRCARTTCTC CTTTCTGAGG
181 TCCARTTCT GGGGCACTG TTTGRRTRC CACTCATCC CTAA CGGTG RATCCAGGG
241 TCCARTTCT TTGGAAGATT ATCATCTTGT GRRRAGGCT GC CCAATTTG ATAGGGAACG
301 GATCCCAAA CFTGTTGTCC ATGCCAGGGG AGCAAGTGCA AAGGGTTTCT TTGAACTCAC
361 ACATGATATT TCGCACTGCA CTTGTGCA GA TTTCTACGA GCTCCTGGAG TTCAAGCACCC
421 GGTCAATTGT CFTTCTGCA CTGTCTTCA TGAACGTGGT AGCCCTGAAA CTTGAGAGA
481 CCCTCGA GGT TTTGCTGTGA AATTTTACAC CAGAGAGGGT AACTTTGA CC TTGTTGAAA
541 CCACTTTCT GTCTTCTTGG TTCACGACGG GATGAAATTT CCAATAT GG TCCTGCTCT
601 TBAACCCAAC CCCCAGTCCC ACATTCAGGA GAAATGAGA ATCCTTGA CT TCTTTCTCA
661 CTTTCCGAAA AGCCTTCA CA TGTTCCTCTT CTRATTTGAT GATGAGGGTG TCCCAAGGA
721 TTAATGGCAC ATGGRGTGTT TTGGAATTA CACTATTTCC CTGATCA CA AGGCTGGGA
781 AGCAGTGTC GTGAAATTC ACTGGAGCC CACTGTGGT GTGAGTGTG TGTAGAGGA
841 AAGGGCCATT AAGGTGGGA GAGC CACCA CA CCGCATGCT ACTCAAGACC TGTTGATTC
901 AATTCCTGCT GGTAACTATC CTGAGTGGAA AcTGTGTTGTT CAAACATAG ATCCTGCTCA
961 CBAAGTAAA TTTGATTTTG ACCCACTT GA TGTACTTAG ACTTGGCCTG AGCAATTT
1021 ACCCCTTCA G CCGTAGGTC GCCTGGTCTT GACCAAGCA ATAGCAATTT TCTTTGCTGA
1081 GATGAA CA CTTGCAATTT GTCCTGCCAT TATTGTGCCT GGTGTATATT ACTCGGATGA
1141 TBAATGCTT CBAAC CAGGA TTTTCTCTA TGTCTATTCA CAGAGGCA CA GGCTTGGGG
1201 AACTATCTG CACTTCCAG CTRAGCTCC CAGTGTGCT CACCACCA CA ATCAACCAAG
1261 GGGATTCATG AATTTCTTCT ACGGGAT GA AAGGT AAT TACTTCCAG CAGGCATGA
1321 TCCTGTTGCT CATGCAAGAA AGGTTCCCAT TCTACTGCT AATTTCTCTG CATGCCCTGA
1381 AATGTGCAT ATTCCBAAGC ABAACCACTT CAGCA GCT GAGAGGCAAT ACCGATCTG
1441 GGCACCCAC AGGCBAAGTA GATTTCTCCG CAGTGGGT GATGCTTTAT CCGATCCAG
1501 GTCACCCAT GAATCCGCA GCATCTGGAT CTCTA CTGG TCTCAGGCTG ATCGTTCTCT
1561 TGAACBAAG ATTGCTCTC ATCTGACAT GAGGCTTAGC ATTTAAGGTT GTTGCBAAC
1621 ATTGAAATAT CGCCAGACT GCAGATCTG AAATTTGATG ATAAAGGATG TTTGTTGGA
1681 TATTTTBAAG GATCTTATT TTTCTGCA TRTTRTTCG TGAATGATR CA AATTTCTG
1741 GATGTGCTG AATCTTGGG CTCTTCAAT AATGTGCTT TGTATTTT ABAABAAAA
1801 AAAAAAAA AACACGAGGG GGGGCCGGT ACCCAATTSG CCCTRATGT AATCGATTA
1861 CAATTAATG CCGCTGCTT TTTTAAAGC T

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Figure 3. The catalase gene sequence of the EST with the full-length cDNA insert. The shaded light grey sequence corresponds to the amino acid coding cDNA and the shaded dark grey sequences with white letters are the primers used for gene expression analysis with real-time RT-PCR.

vided by Dr. R. Dixon (The Samuel Roberts Noble Foundation, OK, USA). One clone contained 1.895bp of insert comprised of the full length 1479bp cDNA with 127bp 5' and 285bp 3' untranslated regions (Figure 5). Comparison of this clone with the two TCs revealed that TC40117 corresponds to the 5'-end and TC37674 to the 3'-end region of the same gene represented by the sequenced clone. These TCs have been compiled from ESTs originated from libraries constructed from plants subjected to herbivory, infection, drought, elicitation, and phosphate starvation indicating that the gene is responding to a wide variety of stress conditions.

Catalase gene expression under stress: In order to investigate catalase gene responses under stress we analyzed the expression of the sequenced clone under the different treatments. We employed a real-time RT-PCR assay and estimated the mean normalized expression of the gene using as reference an actin-beta transcript, which serves as a control in a wide range of experimental systems. Initially, we confirmed that the primer pairs and the PCR conditions employed would amplify a single product of the expected size (Figure 6A). Subsequently we performed a quantitative real-time RT-PCR assay to estimate the threshold cycle (CT) values corresponding to the cycle point where the PCR amplification entered the exponential phase (Figure 4B).

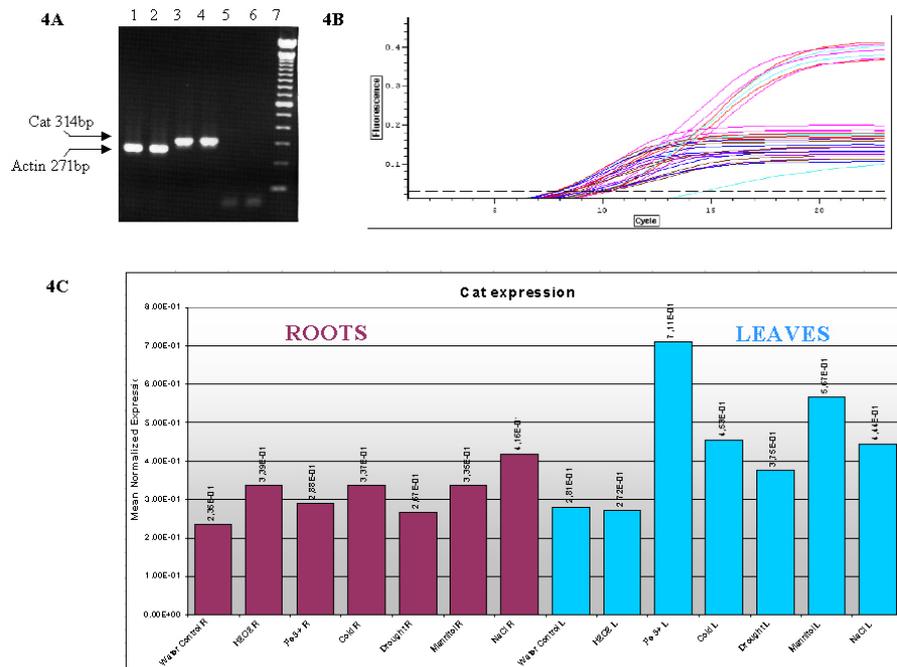


Figure 4. (4A) Confirmation of the PCR primers and conditions applied for the real-time RT-PCR. Lanes 1 and 2 are -with the actin primers, 3 and 4 with the catalase primers and template the RT product of two different reactions. Lanes 5 and 6 are negative controls for actin and catalase respectively and lane 7 is the 1kb MW marker. (4B) The real-time RT-PCR amplification curves of all the target and reference samples as plotted by the Opticon instrument. (4C) The mean normalized catalase gene expression of the different samples after analysis with the Q-Gen software. Each treatment is indicated on the X-axis followed by R for root samples and L for leaf samples.

Under the assumption that PCR amplification efficiencies were the same for both the target (catalase) and the reference (actin-beta) genes (which is supported also by the data presented in figure 4A) we estimated the normalized gene expression of catalase in the samples using the Q-Gen software (Muller et al. 2002). Data plotted in figure 4C revealed that catalase expression is induced under specific stress treatments. In general, catalase induction in leaves is higher than that in roots. In roots the highest induction was observed in NaCl stressed plants while H₂O₂, cold, and mannitol stress resulted in moderate increase and Fe and drought in slight elevation of the transcript level. In leaves the highest induction was observed in iron-treated plants while mannitol, cold and NaCl treatments resulted in significant induction, drought in some increase and H₂O₂ did not affect the catalase transcript level.

Discussion

Abiotic stresses that can be imposed by adverse environmental conditions are the factors most limiting to plant productivity. This is particularly true for the nitrogen fixation efficiency of the legume plant species. A common theme in many of the abiotic stresses is the increased production of ROS leading to oxidative stress. Thus, elucidation of the mechanisms by which plants perceive and respond to oxidative stress is critical for genetic manipulation of stress tolerance.

In this study we focused on the antioxidant enzyme CAT of *M.truncatula* in order to determine responses to oxidative stress. Our data indicated that CAT activity is resolved as one isozymic band in polyacrylamide gels in leaves, pods and petioles. Roots have lower CAT activity and CAT isozymes can be more readily detected under osmotic stress conditions. These conditions reveal a second CAT isozyme which resembles the isozyme of plant tissue origin induced only in nodules of faba bean plants (Bosabalidis and Tsaftaris 1995). A cDNA clone from a *M.truncatula* EST library was fully sequenced and revealed the full cDNA sequence of one catalase gene.

Stress in our experiments was imposed by high concentrations of NaCl and mannitol (osmotic), iron (hydroxyl radical production) and H₂O₂ (oxidative), cold and drought. A common feature (direct or indirect) of the above conditions is the increased production of ROS. Catalase responses were examined at the enzymatic activity and the gene expression level. In general, elevated CAT activity was not always accompanied with increased steady-state mRNA levels. This difference was more characteristic in root samples, where CAT activity was low. A better match of activity and mRNA level could be observed in leaf tissue, where the highest induction in both enzyme activity and gene expression were observed in iron treated plants.

The critical role of Fe in biological processes largely stems from its redox properties (Fe²⁺ / Fe³⁺), which allow its participation in electron transfer reactions at physiological pH. In cells, free Fe²⁺ is toxic because it is able to catalyze the decomposition of H₂O₂ to the extremely reactive hydroxyl radical. This is known as the Fenton reaction. The resulting Fe³⁺ can be reduced back to Fe²⁺ by the superoxide radical, regenerating Fe²⁺ and allowing the reaction to continue. The sum of these two reactions is known as the Haber-Weiss reaction. Catalase can play a critical role removing H₂O₂ thus not allowing the reaction to proceed, and this is the probable reason why catalase responses are so profound in the iron treated plants. It is also worth noting that H₂O₂ was not that potent to induce catalase responses. This could be due to the fast removal of H₂O₂ by catalase and other peroxidases in the roots, thus not allowing it to translocate and have an action. To this point could also be relevant that catalase expression was slightly enhanced by H₂O₂ in the roots but not affected in leaves where it probably never reached.

Overall, our data indicate that antioxidant defense of catalase in *M. truncatula* is highly regulated at both the enzyme activity and the gene expression levels. Experiments are underway to determine the composition

of the *Cat* gene family and the specific responses of each member of the family in abiotic and biotic stress. Continuing this study we plan to examine expression patterns and responses of several other antioxidant genes including superoxide dismutase, ascorbate peroxidase, alternative oxidase and glutathione S-transferase in *Medicago truncatula* as well as the responses of these genes during infection with symbiotic bacteria. Following, we will determine the effects of overexpression and silencing of these genes and the possibility to increase N₂ fixation efficiency in transgenic systems with enhanced antioxidant capacities. Our research goal is an attempt to link N₂-fixation efficiency and antioxidant defense capacity in legumes, especially under stress conditions, and provide the tools to exploit legume species with enhanced antioxidant activities in sustainable agricultural systems and regions with adverse environmental conditions.

References

- ALLEN R, Dissection of oxidative stress tolerance using transgenic plants, *Plant Physiol.*, 107, 1049-1054, 1995.
- BAUM JA., SCANDALIOS JG., Isolation and characterization of the cytosolic and mitochondrial superoxide dismutases of maize, *Arch. Biochem. Biophys.*, 206, 249-264, 1981.
- BEERS and SIZER, A spectrophotometric assay measuring the breakdown of hydrogen peroxide by catalase, *J. Biol. Chem.*, 195, 133-138, 1952.
- BECANA M, MORAN JF, ITURBE-ORMAETXE I, Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection, *Plant Soil*, 201, 137-147, 1998.
- BOSABALIDIS A., TSAFTARIS A., Differential structural features of *Vicia faba* root nodules infected with *Rhizobium leguminosarum* wild type and a symbiotically impaired exopolysaccharide mutant, *Cytobios*, 82, 209-217, 1995.
- CARROLL BJ, HANSEN AP, MCNEIL DL, ORESSHOFF PM, Effect of oxygen supply on nitrogenase activity of nitrate- and dark-stressed soybean (*Glycine max* [L.] Merr.) plants, *Aust J Plant Physiol.*, 14, 679-687, 1987.
- DALTON DA, JOYNER SL, BECANA M, ITURBE-ORMAETXE I, CHATFIELD JM, Enhanced antioxidant defenses in the peripheral cell layers of legume root nodules, *Plant Physiol*, 116, 37-43, 1998.
- DEL RIO LA, SANDALIO LM, PALMA JM, BUENO P, CORPAS FJ, Metabolism of oxygen radicals in peroxisomes and cellular implications, *Free Rad Biol Med*, 13, 557-580, 1992.
- ESCUREDO PR, MINCHIN FR, GOGORCENA Y, ITURBE-ORMAETXE I, KLUCAS RV, BECANA M, Involvement of activated oxygen in nitrate-induced senescence of pea root nodules, *Plant Physiol* 110, 1187-1195, 1996.
- GOGORCENA Y, GORDON AJ, ESCUREDO PR, MINCHIN FR, WITTY JF, MORAN JF, BECANA M, N₂ fixation, carbon metabolism, and oxidative damage in nodules of dark-stressed common bean plants, *Plant Physiol*, 113, 1193-1201, 1997.
- LAYZELL DB, HUNT S, PALMER GR, Mechanisms of nitrogenase inhibition

- in soybean nodules: pulse-modulated spectroscopy indicates that nitrogenase activity is limited by O₂. *Plant Physiol*, 92, 1101-1107, 1990.
- MULLER P, JANOVJAK H, MISEREZ A, AND DOBBIE Z, Processing of gene expression data generated by quantitative Real-time RT-PCR, *Biotechniques* 32, June 2002. SARATH G, PFEIFFER ME, SODHI CS, WAGNER FW, Bacteroids are stable during dark-induced senescence of soybean root nodules. *Plant Physiol* 82' 346-350, 1986.
- SCANDALIOS J.G., GUAN L., POLIDOROS A., Catalases in plants: gene structure, properties, regulation, and expression, *In* J.G. Scandalios (eds): *Oxidative stress and the molecular biology of antioxidant defenses*, Cold Spring Harbor Laboratory, New York, pp 343-406, 1997.
- VANCE, C.P., Legume symbiotic nitrogen fixation: agronomic aspects, *In* H.P. Spaink et al. (eds.): *The Rhizobiaceae*, Kluwer Academic Publishers, Dordrecht, pp. 509-530, 1998.
- WITTY JF, SKAT L, REVSBECH NP, Direct evidence for changes in the resistance of legume root nodules to O diffusion, *J Exp Bot*, 38, 1129-1140, 1987.