Root System Plasticity to Water Stress Tolerance in a Food Legume, Mungbean (*Vigna radiata* L. Wilczek)

Submitted for the award of

Doctor of Philosophy

By

Debashree Sengupta

(08LPPH07)

Supervisor: Professor Attipalli R. Reddy

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500046, India

December 2012

Introduction

Sustainable agricultural crop production in an ever declining arable land area is a major challenge in front of our current agricultural system. The major contributors of declining crop yield are various abiotic stress factors including drought, salinity, heavy metals and high/low temperatures. Among various abiotic stress factors, drought has become one of the most critical factors affecting crop productivity (Roy et al. 2011). Moreover, in the current scenario of global climate change, there are predictions for long lasting droughts across the globe in the near future (IPCC 2007). As limitation of soil water availability to plants usually leads to a considerable loss in grain yields of major crops, a comprehensive knowledge of drought response of important crop plants is extremely crucial to ensure the survival of agricultural crops and sustainable food production under the present and future adverse climatic conditions (Al-Kaisi and Bronner 2009, Mittler and Blumwald 2010). Plant adaptation to drought is the result of a diverse array of inter-connected physiological, biochemical and molecular mechanisms. However, drought is considered to be a root borne stress during water-deficit conditions as the declining water-potential of the soil is primarily perceived by the plant roots. Thus, analysis of root responses under water-deficit conditions is extremely important for an overall understanding of drought response mechanisms of crop plants. Earlier reports have demonstrated the stress defense mechanisms operative inside the plant roots during abiotic stress conditions including the dynamic modulation of root architecture under drought conditions (Sharp et al. 2004, Asch et al. 2005, Malamy 2005).

Among plant defense responses against drought-induced oxidative stress, most important is enhancement in its antioxidant pool (both enzymatic as well as non-enzymatic), as drought primarily disbalances the cellular redox homeostasis, resulting in enhanced generation of cell damaging reactive oxygen species (ROS). Thus, a major portion of stress biology research was dedicated towards unraveling antioxidative defense responses of plants under various abiotic stress factors including drought. However, most of the reports emphasized on foliar responses and only a few reports were available on roots (Haberer et al. 2008, Selote and Khanna-Chopra 2010). Further, it is evident from previous reports that photosynthesis, which is the major metabolic process in plants, is directly and/or indirectly interlinked to the corresponding root metabolisms under drought stress (Zhao et al. 2001, Flexas and Medrano 2002, Wilkinson and Davies 2002). Therefore, any change in photosynthetic responses including stomatal conductance, photosynthetic electron transport, chlorophyll *a* fluorescence or rate of CO_2 assimilation under drought stress could be directly or indirectly correlated to root-level alterations.

Food legumes such as *Vigna radiata* (L.) Wilczek (Mungbean) have been major source of protein in human diet. Moreover, *V. radiata* is also an agriculturally important rotation crop, as it can fix atmospheric nitrogen in association with symbiotic soil bacteria (*Rhizobia*) and thus enhances soil fertility. However, the

crop is highly susceptible to various environmental cues, especially drought, as it is usually grown in marginal lands under rainfed conditions leading to considerable yield loss (Lawn and Ahn 1985). To understand various responses of food legumes under drought, plant responses including physiological, biochemical and growth under different abiotic stress conditions have been extensively studied (Sumithra et al. 2006, Saleh et al. 2007, Jaleel et al. 2009, Manavalan et al. 2009). Mungbean is a warm season crop and adequate rainfall is highly essential, especially from flowering to late pod fill stage to in order to ensure good yields. It is reported that due to rapid expansion of drought-prone areas worldwide, mungbean production is highly affected as among other environmental restraints, limitation of water supply primarily influences its yield (Ranawake et al. 2011). Also, rain-fed system plants experience cycles of water-deficit rather than continuous drought. Thus, to have a proper understanding of stress response mechanisms of rain-fed crops such as mungbean, analyses should be focused at different levels of stress intensity as well as stress recovery rather than at a single stress treatment period. In our study, we target to have an in-depth analysis on root system plasticity of mungbean along with its photosynthetic performance in response to progressive drought stress and recovery.

Objectives

1. Analysis of progressive drought-induced changes in the photosynthetic performance of *Vigna radiata* L.Wilczek.
2. Characterization of dynamic root protein modulation in response to progressive drought and recovery through comparative proteomic approaches.
3. Functional analyses of drought-induced ROS detoxifying NADPH-aldehyde reductase (VrALR) to understand its protective role in *V. radiata* roots.
4. Molecular characterization of key enzymes associated with proline and glutathione biosynthesis in *V. radiata* under progressive drought stress and recovery.

Methodology

Plant material and stress treatment procedure

Seeds of *V. radiata* (L.) Wilczek cv. Vamban-2 was obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore, India and germinated after surface sterilization with 0.1 % sodium hypochlorite in 12 L cement pots filled with mixture of red soil and sand inside glasshouse. The pots were arranged in a completely randomized block design (CRBD) with eight replications. Seedlings were grown

for 30 days at pot water holding capacity (PC) of 80-90 %. After 30 days, plants were subjected to two different watering treatments: well-watered (control) and water-stressed (drought). Control plants (D0) were maintained at PC of 80-90 % throughout the experiment and the stressed plants were subjected to progressive water stress by withholding of water for a period of three days (D3) and six days (D6) and then re-watered for the next six days for recovery (6R). The photosynthetic photon flux density (PPFD) inside the glasshouse ranged from 900-1200 µmolm^{-2} s^{-1} , air temperature 23 ± 1 ºC (early morning) to 34 ± 4 ºC (early afternoon) and relative humidity 36 ± 5% - 48 ± 2%.

Plant water status and root growth measurements

Leaf water status was determined by measuring the leaf relative water content (RWC) according to Castillo (1996). Primary root length was measured using a cm scale and numbers of lateral roots and root nodules/ plant were manually counted (n= 15). Leaf and root moisture contents (LMC and RMC %) were calculated as: fw-dw)/ fw where fw is fresh weight and dw is dry weight. Nodule dry weight/plant and root dry weight were taken after completely drying the tissue samples inside hot air oven.

Photosynthetic gas exchange measurements

Leaf gas exchange parameters were measured using a portable infra red gas analyzer (IRGA) (LC Pro+, ADC Bioscientific Ltd. U.K) equipped with a broad leaf chamber. The gas analyzer was used to measure the net photosynthetic CO_2 assimilation rate (P_n), stomatal conductance (g_s) and transpiration rates (E) and the instantaneous water use efficiency was calculated as P_n/E.

Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence measurements were taken with a portable handy PEA (Plant Efficiency Analyzer-2126) (Hansatech Instruments Ltd., kings Lynn Norfolk, UK). Leaves were dark adapted for 30 min and the fluorescence intensities were recorded after illuminating with a saturating light intensity of 3000 µmol m^{-2} s^{-1}. All fluorescence data were analyzed using the software Biolyzer versions vl 30-31 and SigmaPlot 11.0 was used for constructing the graphs.

Chlorophyll estimation

Leaf discs of 1 cm^2 area were used for pigments' extraction according to dimethyl sulpoxide (DMSO) method described by Hiscox and Israelstam (1979) and chlorophyll contents were calculated according to Lichtenthaler (1987) equations.

Quantification and in-situ localization of H_2O_2 and estimation of malondialdehyde (MDA) equivalents in V. radiata roots

For in-situ localization of H_2O_2, dihydrofluorescein diacetate (H_2DCFDA) stained roots were observed with a confocal microscope (Leica TCS SP2 AOBS Microscope, Germany) with a filter set no. 10, excitation 475 nm, emission 520 nm). Hydrogen peroxide concentration was estimated according to Velikova et al. (2000). Lipid peroxidation was estimated by determining the malondialdehyde (MDA) contents according to Fu and Huang (2001).

Proline and ascorbic acid (ASA) estimations and guaiacol peroxidase (GPOX) assay

Free proline was estimated according to Bates et al. (1973). Ascorbic acid content was determined according to Mukherjee and Choudhuri (1983). GPOX assay was done according to Zhang and Kirkham (1994). Enzyme activity was calculated as µmol guaiacol ozidized min^{-1} mg^{-1} protein using extinction coefficient of guaiacol as 26.6 $mM^{-1}cm^{-1}$.

Determination of oxidized and reduced homoglutathione

Oxidized and reduced glutathione contents were measured using fluorometric method according to Hissin and Hilf (1976) with slight modifications.

Protein extraction and two-dimensional electrophoresis (2-DE)

Root proteins were extracted as described by Sarvanan and Rose (2004) with minor modifications. Proteins were separated in the first dimension using 18 cm linear IPG strips of pH range 4 to 7 and for the second dimension, 12% SDS-PAGE was used. Protein estimation was done according to Bradford (1976).

Staining and image analysis

Gels were stained according to Wang et al. (2007) and images were acquired with calibrated densitometric scanner (GE Healthcare). Spot analysis (normalization, spot matching, expression analyses, and statistics) was performed using Image Master 2-D Platinum version 6 image analysis software (GE, Healthcare).

In gel digestion and mass spectrometry (MS)

In gel trypsin digestion of selected spots was done according to Shevchenko et al. (1996) and MALDI-TOF analysis was conducted with a MALDI- TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Germany).

Protein identification

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing biotools software (Bruker Daltonics, Germany).

Cloning, protein expression and purification of NADPH-aldehyde reductase (VrALR) from V. radiata roots

Total RNA was isolated using column based Plant Spectrum RNA isolation kit (Sigma-Aldrich) and the first strand of cDNA was synthesized using Revert Aid^{TM} First Strand cDNA synthesis kit (Fermentas life Sciences, St Leon-Rot, Germany). Primers were designed based on the already available sequence of *V. radiata* aldehyde reductase (Accession no. gi| 5852203|gb|AAD53967.1). VrALR protein was expressed in *E.coli* BL21 cells using 1 mM IPTG. Purification of the recombinant protein was carried out by passing the supernatant over CL-Agarose column from Genei^{TM}GST-Fusion protein purification kit (Bangalore Genei, Bangalore, India) and the GST tag was removed through thrombin digestion using Thrombin CleanCleave kit (Sigma-Aldrich).

Substrate specificity and enzyme kinetics of VrALR

VrALR enzyme activity was measured according to Guillén et al. (1998) using benzaldehyde, cinnamaldehyde and p-nitrobenzaldehyde as substrates. Substrate and cofactor (NADPH) concentrations were varied from 100–500 µM and the initial reaction velocities were used for determination of K_m values. Assuming Michaelis-Menten type performance of the enzyme, other kinetics parameters (V_{max} , K_{cat} and K_{cat}/K_m) were calculated. Aldehyde reducing activity in crude root extracts was performed by the method described by Negm (1986).

Oxidative stress protective role of VrALR in bacterial and yeast system

Bacterial cultures expressing only GST and GST+ VrALR were supplemented with H_2O_2 and p-nitrobenzaldehyde (p-NB) during induction with IPTG and the growth pattern was observed by measuring OD_{600} periodically at 1 h intervals till stationary phase. Vector p424 and the plasmid construct p424+

VrALR were used for transformation of yeast (*Saccharaomyces cerevisiae*) strain W3O3-1-A using frozen EZ-yeast transformation II™ kit (Zymo Research, http://www.zymoresearch.com). Spotting was done on SD-Trp plates supplemented with H_2O_2 and *p*-nitrobenzaldehyde (p-NB).

Real time RT-PCR analysis of VrALR expression

Primers were designed based on the obtained *VrALR* cDNA sequence to amplify a 158 bp product. Real time qPCR analysis was done on Eppendorf Realplex MasterCycler (Eppendorf, Hamburg, Germany) using the KAPA SYBR FAST (Mastermix (2X) Universal) (KAPA Biosystems, Woburn, USA) real time PCR kit.

Deducing the full length cDNA sequence of γ-glutamyl cysteine synthetase (VrγECS) gene

Primers were designed based on the conserved region of γECS from available sequences of *Phaseolus vulgaris*, *Medicago truncatula* and a clone of *Vigna unguiculata* available at NCBI GenBank (http://www.ncbi.nlm.nih.gov). Total RNA was used to synthesize the first strand of cDNA using Revert Aid™ First strand cDNA synthesis kit (Fermentas Life Sciences, St Leon-Rot, Germany). Rapid amplification of cDNA ends (RACE) PCR was performed using 5'/3' RACE kit (Roche Applied Sciences, Germany).

Real time RT-PCR analysis of VrγECS expression and* VrγECS *enzyme activity

Primers were designed based on the obtained *VrγECS* partial equence to amplify a 120 bp product. Real time qPCR analysis was done on Eppendorf Realplex MasterCycler (Eppendorf, Germany) using the KAPA SYBR FAST (Mastermix (2X) Universal) (KAPA Biosystems) real time PCR kit following the manufacturer's instructions. VrγECS enzyme activity was assayed according to Rüeggsegger and Brunold (1992). Protein content of the extracts was measured according to Bradford (1976) with BSA as the standard.

Real time RT-PCR analysis of Pyrroline 5-carboxylate synthetase (P5CS) gene

Amplified 1.48 Kb PCR product of *VrP5CS* was directly sequenced and internal primers were designed to amplify a 148 bp product for real time qPCR analysis using KAPA SYBR FAST (Mastermix (2X) Universal) (KAPA Biosystems, Woburn, USA) real time PCR kit in an Eppendorf Realplex MasterCycler (Eppendorf, Hamburg, Germany).

VrP5CS enzyme assay

The VrP5CS enzyme activity was determined based on the rate of consumption of NADPH, during the ATP and NADPH-dependent reduction of glutamate to γ-glutamic semialdehyde (GSA) as described by Garcia-Rios et al. (1997).

Statistical analysis

All data on physiological (LRWC, LMC, RMC, leaf gas exchange and chl *a* fluorescence) and biochemical (H_2O_2, MDA, enzyme activities, qPCR analyses) measurements were represented as mean ± standard deviations (n = 5 for LRWC, LMC,RMC, H_2O_2 and MDA while n = 3 for qPCR analysis). One way ANOVA and Tukey test was used to determine the significance of the differences between mean values of control and stressed plants. All statistical analyses were performed using the statistical package Sigma Plot 11.0.

Results and Discussion

Chapter 1: Analysis of progressive drought-induced changes in the photosynthetic performance of *Vigna radiata* L. Wilczek

Progressive drought caused a gradual and significant decline in the net CO_2 assimilation rate (P_n) in vegetatively mature *V. radiata* with a subsequent decline in the stomatal conductance (g_s) and transpiration rate (E). However, the observed decline in stomatal conductance and reduced CO_2 assimilation rates were a part of the adaptive mechanism of drought stressed *Vigna*, rather than permanent damage as the plants recovered significantly upon re-watering. It is known that decline of P_n could be either due to stomatal or non-stomatal limitations. In *V. radiata*, decline in P_n and g_s were not accompanied by a corresponding acute decline in the sub-stomatal CO_2 concentrations (Ci) which indicates involvement of non-stomatal factors along with stomatal limitations. For further insight on photosynthetic physiology of *V. radiata* under water deficit conditions, we analyzed the drought-induced effect on PSII efficiency by analyzing the modulations in chl *a* fluorescence. Drought differentially affected the various PSII reactions and the electron flux. However, drought induced damage on the PSII quantum yield and the stability of PSII complex was insignificant and no alternate electron donors were utilized. As roots are known to be the initial perceivers of drought stress signal rather than leaves, we analyzed oxidative stress responses in *V. radiata* roots under progressive drought and recovery. Progressive drought stress induced a gradual accumulation of H_2O_2 and lipid peroxidation in *V. radiata*

roots which declined upon re-watering. Ascorbic acid content and peroxidase activity in *V. radiata* roots showed a positive correlation with the level of drought stress intensity. However, glutathione content remained more or less stable except during D3. Root proline accumulation and its rapid catabolism upon re-watering indicate effective adaptive mechanism in *V. radiata.*

Chapter 2: Characterization of dynamic root protein modulations in response to progressive drought and recovery through comparative proteomics approach

Plant growth and survival depends upon its root growth pattern in response to soil water limitations. Drought stress is known to cause abscission of most lateral roots in dry regions of soil and induces secondary lateral roots in regions of soil containing higher soil water content (Smucker 1993). Our data on *V. radiata*, depicts abscission of the longer lateral roots while short-roots were observed in the drought-stressed population which indicate that *V. radiata* can regulate its root morphology towards maximum water uptake strategies depending on the availability of water in the soil. Majority of the current understanding of drought responses of legumes were based on foliar proteins only and a focused analysis involving root protein expression patterns at different levels of stress intensity and recovery period have not been reported. For a systematic analysis of the root protein expression patterns during progressive drought stress and recovery, we used comparative proteomics approach which has emerged as a promising tool for global analyses of protein expression levels in the recent past (Cánovas et al. 2004). Our proteomics-based study showed that *V. radiata* could express different sets of proteins at different stages of drought stress. Root proteins involved in root architecture, energy metabolism, ROS detoxification and cell signaling were differentially regulated during progressive drought stress. Expression patterns of proteins belonging to different functional groups were highly correlated and regulated by the soil water status in terms of medium and high stress intensity. Our data suggest that *V. radiata* could tolerate medium level drought stress intensity but gets susceptible under subsequent enhancement of drought treatment.

Chapter 3: Functional analyses of drought-induced ROS detoxifying NADPH-aldehyde reductase (VrALR) to understand its protective role in *V. radiata* roots

Drought stress-induced reactive oxygen species cause oxidation of lipids resulting in unstable lipid peroxides which degrade to form highly cytotoxic aldehydic products. Aldehyde reductases (ALRs), belonging to aldo-keto reductase superfamily, play important role in detoxifying the cytotoxic by-products of lipid peroxidation. Available literature indicates that ALRs could act as potential targets for generating stress tolerance in crop plants (Rodrigues et al. 2006, Wen et al. 2012). Through proteomics,

we observed a significant up-regulation of aldehyde reductase protein in roots of *V. radiata* (VrALR) during short term water deficit. Hence, in this objective we characterize the functional role of this enzyme in *V. radiata* under progressive drought. We cloned, expressed and purified the VrALR protein. The aldehyde reducing activity of the purified protein showed highest catalytic efficiency with p-nitrobenzaldehde and least with cinnamaldehyde. For analyzing the detoxification potential of VrALR, we expressed the protein in bacterial and yeast system. VrALR expression has successfully complemented the endogenous defense mechanism of *E.coli* BL21 and yeast mutant W3O3-1A against oxidative stress conditions indicating the effective detoxification of the H_2O_2 and p-NB-induced toxicity. However, in comparison with the bacterial system, yeast has shown lower detoxifying capacity. For analyzing the protective role of endogeneous ALR, we observed the VrALR mRNA expression pattern and the corresponding enzyme activity. We found that *V. radiata* effectively regulates the ALR gene in response to short term water defict by up-regulating its mRNA expression and consecutively the protein content as well as the aldehyde reducing activity. But higher oxidizing conditions inhibited the gene expression and thus the protein content. The continued enhancement of aldehyde reducing activity could be due to some other stable proteins in the root extract with aldehyde reducing enzymatic properties.

Chapter 4: Molecular characterization of key enzymes associated with proline and glutathione biosynthesis in *V. radiata* roots under progressive drought stress and recovery

In glutathione biosynthesis, the rate limiting enzyme is γ-glutamyl cysteine synthetase (γECS) which serves as the control point during abiotic stresses when the demand for GSH enhances. γECS activity is usually influenced by multiple regulatory factors which include older concept of feedback regulation by glutathione and also the recent concept of redox regulation by H_2O_2 and disulphide bridges. In this objective, we focused on the cloning and sequence analysis *VrγECS* and a subsequent insight into the regulation of this enzyme including mRNA expression levels in *V. radiata* under progressive drought stress and recovery. We observed that declining root moisture content during progressive drought has no direct impact on *VrγECS* gene expression and under progressive drought stress; homoglutathione (hGSH) biosynthesis in roots of *V. radiata* was directly dependent on the activity of VrγECS enzyme. Regression analysis with H_2O_2 concentrations showed that low concentrations of H_2O_2 could probably regulate VrγECS enzyme activity in vivo. The multifaceted protective role of proline under drought stress is well documented (Szabados and Savouré 2010). In the present study, *V. radiata* significantly enhanced proline biosynthesis under water-deficit conditions which was also degraded rapidly upon re-watering. To understand the expression pattern of proline in roots of *V. radiata*, we also analyzed the mRNA expression patterns and the corresponding enzyme activity levels of pyrroline-5-carboxylate synthetase

(VrP5CS) gene, the key regulatory enzyme of proline biosynthetic pathway. Elevated proline biosynthesis in *V. radiata* roots was highly correlated with a significant increase in mRNA expression and catalytic activity of VrP5CS. Pattern of proline accumulation, *VrP5CS* mRNA expression as well as enzyme activity showed negative correlations with declining root moisture content which were possibly acting as stress signals for roots to enhance biosynthesis of this protective osmolyte, proline.

Major conclusions

1. Stomatal closure and reduced CO_2 assimilation rates in *V. radiata* were part of the adaptive mechanism of the plant under drought, rather than permanent damage as the plants recovered significantly upon re-watering.

2. Drought differentially affected PSII reactions and the electron flux. However, drought-induced damage on the PSII quantum yield and the stability of PSII complex was insignificant.

3. *V. radiata* was able to tolerate medium level drought stress, but gets susceptible under subsequent progression of drought.

4. VrALR provides protection against H_2O_2 and p-NB induced- oxidative stress in yeast and bacteria. *V. radiata* effectively regulates the *VrALR* gene in response to short term water deficit by up-regulating its mRNA expression and consecutively the protein content as well as the aldehyde reducing activity.

5. VrγECS activity did not correlate with the corresponding gene expression levels under progressive drought and low concentrations of H_2O_2 could probably regulate VrγECS enzyme activity in vivo.

6. Proline accumulation in roots and its rapid catabolism upon re-watering indicate effective adaptive mechanism in *V. radiata* under drought stressed regimes.

Literature cited

Al-Kaisi MM, Broner I (2009) *Publication number 4.715, Colorado State University Extension publication,CO, USA.*

Asch F, Dingkuhn M, Sow A, Audebert A (2005) *Field Crops Research* **93**:223-236

Bates LS, Walderen RP, Teare ID (1973) Plant and Soil **39**:205-207.

Bradford MM (1976) *Analytical Biochemistry* **72**:248-254

Cánovas FM, Gaudot ED, Recorbet G, Jorrin J, Mock HP, Rossingol M (2004) *Proteomics* **4**:285-298

Castillo FJ (1996) *Oecologia* **107**:469-477

Flexas J, Medrano H (2002) *Annals of Botany* **89**:183-189

Fu J, Huang B (2001) *Environmental and Experimental Botany* **45**:105-114

Garcia-Rios M, Fujita T, Christopher LP, Locy RD, Clithero JM, Bressan RA, Csonka LN (1997) *Proceedings of National Academy of Science USA* **94**:8249-8254

Guillén P, Guis M, Martinez-Reina G, Colrat S, Dalmayrac S, Deswarte S, Bouzayen M, Roustan JP, Fallot J, Pech JC, Latché A (1998) *Plant Journal* **16**:335-343

Haberer K, Herbinger K, Alexou M, Rennenberg H, Tausz M (2008) *Tree Physiology* **28**: 713-719

Hiscox JD, Israelstam GF (1979) *Canadian Journal of Botany* **57**:1332-1334.

Hissin PJ, Hilf R (1976) *Analytical Biochemistry* **74**:214-226

Intergovernmental Panel on Climate Change (IPCC) (2007) McCarthy JJ, Canziani OF, Leary NA, Dokken DJ, White KS eds. *Impacts, Adaptations and Vulnerability. Contribution of Working Group II to the Third Assesment Report of the Intergovernmental Panel on Climate Change*. Camridge, UK and New York, USA, Cambridge University Press.

Jaleel CA, Jayakumar K, Chang-Xing Z, Azooz MM (2009) *Plant Omics Journal* **2**:120-126

Lawn RJ, Ahn CS (1985) 584–623. In: *R.J. Summerfield and E.H. Roberts (eds.), Grain legume crops. William Collins Sons & Co. Ltd, London*

Lichtenthaler HK (1987) *Methods in Enzymology* **148**:350-382.

Malamy JE (2005) *Plant Cell and Environment* **28**:67-77

Manavalan LP, Guttikonda SK, Tran LSP, Nguyen HT (2009) *Plant Cell and Physiology* **50**:1260-1276

Mittler R, Blumwald E (2010). *Annual Review of Plant Biology* **61:**443-462

Mukherjee SP, Choudhury MA. (1983) *Physiologia Plantarum* **58**:166-170

Negm FB (1986) *Plant Physiology* **80**:972-977

Ranawake AL, Dahanayaka N, Amarasingha UGS, Rodrigo WDRJ, Rodrigo UTD (2011) *Tropical Agricultural Research and Extension* 14(4):4p.

Rodrigues SM, Andrade MO, Gomes APS, DaMatta FM, Baracat-Pereira MC, Fontes EPB (2006) *Journal of Experimental Botany* **57**:1909-1918

Roy A, Rushton PJ, Rohila JS (2011*) Critical reviews in Plant Sciences* **30**:471-490.

Rüeggsegger A, Brunold C (1992) *Plant Physiology* **99**:428-433

Saleh AAH, Abdel-Kader DZ, El Elish AM (2007) *American Journal of Plant Physiology* **2**:344-355

Sarvanan RS, Rose JKC (2004) *Proteomics* **4**:2522-2532

Selote DS, Khanna-Chopra R (2010) *Protoplasma* **245**:153-163

Sharp RE, Poroyko V, Hejlek LG, Spollen WG, Springer GK, Bohnert HJ, Nguyen HT (2004) *Journal of Experimental Botany* **55**:2343-2351

Shevchenko A, Wilm A, Vorm O, Mann M (1996) *Analytical Chemistry* **68:** 850-858

Smucker AJM (1993) Annual Review on Phytopathology **31**:191-216

Sumithra K, Jutur PP, Carmel BD, Reddy AR (2006) *Plant Growth Regulation* **50**:11-22

Szabados L, Savouré A (2010) *Trends in Plant Sciences* **15**:89-97

Velikova V, Yordanov I, Edreva A Plant Sci 2000; 151:59-66

Wang X, Li X, Li Y (2007) *Biotechnology Letters* **29**:1599-1603

Wen Y, Wang X, Xiao S, Wang Y (2012) *Planta DOI 10.1007/s00425-012-1624-z*

Wilkinson S, Davies W.J. (2002) *Plant Cell and Environment* **25**:195-210

Zhang J, Kirkham MP (1994) *Plant Cell and Physiol*ogy **35**:785-791

Zhao ZG, Chen GC, Zhang CL (2001) *Australian Journal of Plant Physiology* **28**:1055-1061