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Water relations, activities of antioxidants, ethylene evolution and membrane integrity of pigeonpea roots as affected by soil moisture

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Abstract

The plants of pigeonpea (*Cajanus cajan* L.) cv. H77-216 were subjected to moderate [soil moisture content (SMC) = 7.3 ± 0.5 %] and severe (SMC = 4.3 ± 0.5 %) drought by withholding the irrigation at vegetative stage (45 d after sowing). The control plants were maintained at SMC of 11.0 ± 0.5 %. Half of the stressed plants were re-irrigated and their recovery was studied after 2 d. Leaf water potential, osmotic potential, and relative water content of leaf and root decreased significantly while a sharp rise in proline and total soluble sugars contents were noticed. Drought induced a significant increase in 1-aminocyclopropane 1-carboxylic acid (ACC) content and ACC oxidase activity which caused a considerable increase in ethylene evolution. Malondialdehyde content and relative stress injury were increased under drought whereas reverse was true for ascorbic acid content. The membrane integrity of roots decreased during stress and recovered on rehydration. The specific activity of total superoxide dismutase, ascorbate peroxidase, glutathione reductase, and glutathione transferase decreased to 37 - 78 %, 17 - 62 %, 29 - 36 % and 57 - 79 % at moderate and severe drought, respectively. The increase in activity of catalase and peroxidase could not overcome the accumulation of H_2O_2 content in the roots.

Additional key words: ascorbic acid, lipid peroxidation, plant water status, proline, rehydration, total soluble sugars.

The major factor limiting pigeonpea growth and development in arid and semiarid region is the amount of soil moisture available to crop during the growing season. Leaf water potential (Ψ_w), osmotic potential (Ψ_s), relative water content (RWC) and accumulation of solutes are good indicators of plant water stress and are well associated with different plant functions (Nayyer and Walia 2003, Nayyer 2003/4, Hare *et al*. 1998). The reactive oxygen species (ROS) are responsible for various stress induced damage to macromolecules. To prevent damage, the roles of the protective enzymes, namely ascorbate-glutathione (ASC-GSH) cycle, in addition to superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) and metabolites with antioxidant properties like ascorbic acid, glutathione, α-tocopherol, *etc*., responsible for the quenching ROS are significant (Polle 2001, Sairam *et al*. 2002). The induction of stress in plants has also been correlated with ethylene evolution under environmental stresses (Abeles *et al*. 1992). Roots are known to appear the most sensitive organ to oxidative stress induced by drought (Morabito and Guerrier 2000). Under drought conditions production of ROS depends on species, intensity of stress, duration of stress and developmental stage. In order to expand the knowledge of stress physiology the present investigation was undertaken to study interrelationship between the stress-induced membrane dysfunction, ROS formation and ethylene evolution in the roots of this tropical legume under depleted soil moisture content and on subsequent rehydration.

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Abbreviations: AA - ascorbic acid; ACC - 1-aminocyclopropane-1-carboxylic acid; ASC - ascorbate; CAT - catalase; CD - critical difference; GR - glutathione reductase; GSH - glutathione; GTase - glutathione transferase; MDA - malondialdehyde; POX - peroxidase; ROS - reactive oxygen species; RWC - relative water content; SMC - soil moisture content; SOD - superoxide dismutase; TSS - total soluble sugars; Ψ_s - osmotic potential; Ψ_w - water potential.

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Pigeonpea (*Cajanus cajan* L. Mill) cv. H-77-216 was raised in earthenpots filled with 5.5 kg of dune sand in screenhouse. The seeds before sowing were surface sterilized and inoculated with effective *Rhizobium* culture (PH8666). The crop was supplied with nitrogen free nutrient solution at weekly interval. After thinning two plants were retained in each pot. At vegetative stage, (45 d after sowing) the drought was created by withholding irrigation and described as moderate [(soil moisture content, SMC = 7.3 ± 0.5 %)] and severe (SMC = 4.3 ± 0.5 %) stress. Another set of pots was given normal irrigation, served as control (SMC = 11.0 ± 0.5 %). Half of the pots containing severely stressed plants were sampled and other half were reirrigated and sampled after 2 d, described as recovery having the SMC = 10.7 ± 0.5 %.

Three replicates consist of three pots and each pot containing two plants was used for each observation under each treatment. The data was analysed statistically using complete randomized design and the significance was tested at 5 % level of critical difference (CD).

SMC of the mixed soil of the pots was determined by gravimetric method. The fourth fully expanded trifoliate leaf from the top was used to measure water potential (Ψ_w) with a pressure chamber (*Model-3005*, *Soil Moisture Equipment Corporation*, Santa Barbara, USA). The extracted sap of leaf and roots was used to determine the osmotic potential (Ψ_s) with a vapor pressure osmometer (*Model-5100*, *Wescor*, Logan, USA). The relative water content (RWC) of leaf and roots was calculated as described by Weatherley (1950). These measurements were made between 09:00 and 11:00 (local time) during a sunny day.

The proline content of roots was estimated according to the method of Bates *et al*. (1973). The total soluble sugars (TSS) of roots were determined with the method of Dubois *et al*. (1956). Ascorbic acid (AA) content of roots was measured using the method of Schopfer (1966). Free ACC content of fresh roots was assayed following the method of Miller and Pengelly (1984). The activity of ACC oxidase and ethylene evolution were measured by the method described by Fearn and La Rue (1991).

One g of roots were washed in chilled distilled water and homogenized with a chilled pestle and mortar in 5 cm^3 of extraction buffer (0.1 M phosphate buffer, pH 7.0), containing 10 mM KCl, 1 mM $MgCl_2$ and 10 mM EDTA and centrifuged at 10 000 *g* at 4 °C for 20 min. The supernatant was used for the following enzymes assay. The enzymatic protein was determined by the method of Lowry *et al*. (1951). The specific activity of catalase (CAT; EC 1.11.1.6), peroxidase (POX; EC 1.11.1.7), glutathione reductase (GR; EC 1.11.1.9), glutathione transferase (GTase; EC 2.5.1.18), ascorbate peroxidase activity (ASC-POX; EC 1.11.1.11) and superoxide dismutase (SOD; EC 1.15.1.1) were measured by the method of Aebi (1983), Shannon *et al*. (1966), Goldberg and Spooner (1983), Habig and Jakoby (1981), Nakano and Asada (1981) and Giannopolitis and Ries (1977), respectively. H_2O_2 content of roots was determined by a modified Patterson *et al*. (1984) method. Lipid peroxidation in roots was measured in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction (Heath and Packer 1968). The relative stress injury (RSI) was determined by recording the electrical conductivity (EC) of roots leachates in deionized water (Sairam *et al*. 2002).

The Ψ_w, Ψ_s and RWC of leaf decreased significantly from -0.35 to -1.70 MPa, -0.95 to -1.90 MPa, and 83.5 to 50.69 %, respectively, with the increase in drought (Table 1). Similarly, the Ψ_s and RWC of roots decreased from -0.44 to -1.69 MPa and from 89.14 to 65.01 % under depleted soil moisture. The stressed plants showed partial improvement in Ψ_w, Ψ_s and RWC upon re-irrigation. The roots showed 2 to 13 fold rise in proline content depending on the level of drought, however, upon re-irrigation a considerable decrease in proline content was noticed (Table 1). Similar to proline, the TSS content of roots also increased from 12 to 37 % under drought. The TSS content was declined with rewatering. The H_2O_2 and lipid peroxidation (MDA content) and RSI increased continuously from 22 to 63, 124 to 257, and 7 to 21 %, respectively with increasing the drought from moderate to severe (Table 1). However, on rehydration these values decreased but were still higher than the control. The AA content of roots decreased to 70.72 and 93.79 % as compared to control at moderate and severe drought, respectively (Table 1). When stressed plants were re-irrigated, the AA content increased.

Total SOD, ASC-POX, GR and GTase activities significantly declined from 38 to 78, 17 - 62, 29 - 36, and 57 - 81 % at moderate to severe drought, respectively (Table 1). On subsequent rehydration, a partial recovery was noticed, whereas ASC-POX activity slightly increased (5 - 11 %) as compared to control. A considerable increase in the activities of CAT and POX under drought was seen. However, upon re-irrigation, the activities of these enzymes declined but were still higher than the control.

The ACC content, ACC oxidase activity and ethylene evolution significantly increased from 20 - 37, 37 - 66, and 83 - 270 % as the drought increased to moderate and severe, respectively (Table 1). The ACC content, ACC oxidase activity and ethylene evolution decreased with subsequent rehydration, but were still higher than the control.

Drought is one of the most important factors which adversely affect various physiological and metabolic activities in plants. Withholding the irrigation caused significant decrease in Ψ_w of leaf and Ψ_s and RWC of leaf and roots. These results are in accordance with earlier reports (Nayyer and Walia 2003, Medici *et* al. 2003/4, Nayyer 2003/4). The proposed reason for decreasing Ψ_s is that plants adjust to drought conditions to maintain pressure potential (Wright *et al*. 1997). Decline in Ψ_s can be result of either simple passive concentration of solutes due to dehydration or net accumulation of, *e.g.*, proline and total soluble sugars (TSS) (Table 1). The proline and TSS contents sharply decreased due to utilization after rehydration. Osmolytes are also involved in preserving

Table 1. Changes in plant water status, membrane injury, specific activity of protective enzymes and ethylene production in pigeonpea under drought and on rehydration. C - control, MS - moderate stress, SS - severe stress, RMS - recovery after moderate stress, RSS - recovery after severe stress.

Parameters	C	MS	RMS	SS	RSS	CD at 5 %
Leaf Ψ_w [-MPa]	0.74	1.04	0.81	1.48	1.01	0.18
Leaf Ψ_s [-MPa]	0.95	1.29	1.08	1.90	1.18	0.22
Leaf RWC [%]	83.50	68.61	76.52	50.69	57.98	4.38
Root Ψ_s [-MPa]	0.44	0.80	0.47	1.69	0.92	0.15
Root RWC [%]	89.14	80.95	82.97	65.01	69.70	3.26
Root proline content [mg g^{-1} (d.m.)]	0.116	0.258	0.175	1.529	0.207	0.04
Root TSS content [mg g^{-1}(d.m.)]	13.90	16.59	14.28	19.04	16.61	1.99
Hydrogen peroxide [mmol g^{-1}(d.m.)]	0.934	1.145	1.080	1.523	1.058	0.175
Lipid peroxidation [nmol(MDA) g^{-1}(d.m.)]	107.43	241.38	127.78	384.03	225.67	20.16
Relative stress injury [%]	27.0	34.95	29.20	48.28	37.60	2.19
AA [µmol g^{-1}(d.m.)]	0.177	0.052	0.105	0.012	0.119	0.062
ASC-POX [nmol(ascorbate) mg^{-1}(protein) s^{-1}]	1.83	1.52	1.98	1.14	2.04	0.17
SOD [U mg^{-1}(protein) s^{-1}]	0.384	0.237	0.344	0.082	0.203	0.034
CAT [µmol(H_2O_2) mg^{-1}(protein) s^{-1}]	0.030	0.045	0.034	0.038	0.036	0.006
POX [U mg^{-1}(protein) s^{-1}]	68.33	82.83	74.83	147.50	125.16	15.01
GR [nmol(NADH oxidized) mg^{-1}(protein) s^{-1}]	0.076	0.054	0.063	0.049	0.060	0.006
GTase [nmol(p-nitrophenyl) mg^{-1}(protein) s^{-1}]	0.955	0.408	0.788	0.199	0.494	0.057
ACC content [pmol(C_2H_4) g^{-1}(d.m.) s^{-1}]	0.111	0.133	0.119	0.153	0.128	0.005
ACC oxidase [pmol(C_2H_4) g^{-1}(d.m.) s^{-1}]	0.208	0.286	0.269	0.347	0.286	0.006
Ethylene [pmol(C_2H_4) g^{-1}(d.m.) s^{-1}]	0.500	0.917	0.694	1.850	1.152	0.009

membranes in dehydrated tissue (Hare *et al*. 1998).

Under depleted soil moisture increase in relative stress injury (RSI) in roots was associated with the increase in lipid peroxidation and ethylene production (Table 1). The results also suggest that increased peroxidation might be mediated through H_2O_2 accumulation in roots. Sharp increase in RSI, H_2O_2 accumulation, ethylene evolution and lipid peroxidation under drought, were noticed as the characteristics of stress induced senescence in roots. The recovery in membrane integrity based on MDA content or by the check in leakage of electrolytes on re-irrigation clearly depicts that the damage to membrane integrity and permeability under drought was reversible to some extent.

Oxidative damage in pigeonpea roots under depleted soil moisture might be correlated with decreased SOD, ASC-POX, GR, GTase activities. An increase in the activity of CAT and POX could not eventually balance the diminished activities of the rest of enzymes of ASC-GSH pathway or were unable to detoxify all H_2O_2. Decline in AA content in roots under drought (Table 1) confirms the findings of Bartoli *et al*. (1999) as it is involved in removal of H_2O_2 and acts as a substrate for ASC-POX enzyme, directly reduces O_2 and regenerate reduced α-tocopherol.

Ethylene production is known to be autocatalytic, which may contribute to enhanced lipid peroxidation and reactive oxygen species (ROS) generation as also observed during this study. A positive correlation among ACC content, ACC oxidase activity and endogenous ethylene was observed with moisture stress. The convincing evidence to support these findings comes from a sharp decline in drought induced ethylene evolution upon re-irrigation of droughted plants, with a simultaneous decrease in ACC content and ACC oxidase activity. The results show that in drought affected roots, the formation of ethylene not only depended on the enhanced synthesis of ACC and ACC oxidase activity but was also on a degree of membrane injury and have direct or indirect involvement with the reactions of antioxidant defense system.

It is concluded that drought adversely affected the functioning of roots due to overproduction of ethylene, accumulation of ACC and H_2O_2 contents and increase in ACC oxidase activity, lipid peroxidation and electrolyte leakage, weakening of defense system and decrease in Ψ_w of leaf and Ψ_s and RWC of leaf and roots. However, these changes were reversible to certain extent upon re irrigation.

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