

Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties

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Received: 27 October 2008 / Revised: 24 February 2009 / Accepted: 7 March 2009 / Published online: 28 March 2009
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Abstract Drought stress hampers rice performance principally by disrupting the plant–water relations and structure of biological membranes. This study appraised the role of polyamines (PAs) in improving drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). Three PAs [putrescine (Put), spermidine (Spd) and spermine (Spm)] were used each at 10 μ M as seed priming (by soaking seeds in solution) and foliar spray. Primed and non-primed seeds were sown in plastic pots with normal irrigation in a phytotron. At four-leaf stage, plants were subjected to drought stress by bringing the soil moisture down to 50% of field capacity by halting water supply. For foliar application, 10 μ M solutions each of Put, Spd and Spm were sprayed at five-leaf stage. Results revealed that drought stress severely reduced the rice fresh and dry weights, while PAs application improved net photosynthesis, water use efficiency, leaf water status, production of free proline, anthocyanins and soluble phenolics and improved membrane properties. PAs improved drought tolerance in terms of dry matter yield and net

photosynthesis was associated with the maintenance of leaf water status and improved water use efficiency. Among the antioxidants, catalase activity was negatively related to H_2O_2 and membrane permeability, which indicated alleviation of oxidative damage on cellular membranes by PAs application. Foliar application was more effective than the seed priming, and among the PAs, Spm was the most effective in improving drought tolerance.

Keywords Oxidative damage · Polyamines · Photosynthesis · Drought stress · Leaf water status

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DHA	Dehydroascorbate
Pro	Free proline
ψ_w	Leaf water potential
LSD	Least significant difference
MDA	Malondialdehyde
A	Net rate of photosynthesis
ψ_s	Osmotic potential
PPFD	Photon flux density
PAs	Polyamines
ψ_p	Pressure potential
Put	Putrescine
ROS	Reactive oxygen species
RWC	Relative water contents
Spd	Spermidine
Spm	Spermine
g_s	Stomatal conductance
SOD	Superoxide dismutase
E	Transpiration rate
WUE	Water use efficiency

Communicated by Z. Gombos.

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Introduction

Drought stress is a great challenge to agricultural production worldwide. It results in the loss of balance between the production and dousing of reactive oxygen species (ROS) (Smirnoff 1998), thereby reducing true production potential of crop species. The ROS are highly reactive and can seriously damage plants by protein degradation, DNA fragmentation, lipid peroxidation, and ultimately cell death (Beligni and Lamattina 1999). Organelles such as chloroplasts and mitochondria are the main sites of ROS production in the plant cells (Breusegem et al. 2001). These effects lead to reduced growth and yield in a number of plant species (Abdul Jaleel et al. 2009; Farooq et al. 2008, 2009a; Hussain et al. 2008a, b; Razmjoo et al. 2008).

Of the effects of stress damage to the cellular membranes and as protective mechanism, production of stress related primary and secondary metabolites are pronounced ones (Zhu 2002; Wahid et al. 2007). Synthesis of free proline in plants is well known under a variety of stresses (Zhu 2002; Wahid et al. 2007). The phenolics (flavonoids, lignins and tannins) act as powerful antioxidants in plant cells under stress conditions (Wahid 2007). Drought stress damages the photosynthetic apparatus through its interaction with UV or/and visible radiation (Garcia-Plazaola and Becerril 2000), while phenolics, having very reactive benzene ring, can counteract these damaging effects (Bilger et al. 2001). Anthocyanins are highly water soluble and are produced to act as UV screens and osmolytes to alleviate the adverse effects of stresses (Wahid et al. 2007). However, the synthesis of both soluble phenolics and anthocyanins under the exogenous supply of polyamines (PAs) has not been investigated previously.

Polyamines including spermidine (Spd), spermine (Spm), and putrescine (Put) are small ubiquitous nitrogenous compounds. They are a recent addition to the class of plant growth regulators and also considered as a secondary messenger in signaling pathways (Davies 2004; Liu et al. 2007; Kusano et al. 2008). Richards and Coleman (1952) for the first time reported the involvement of PAs in abiotic stress tolerance. Since then, many studies reported the involvement of PAs in abiotic stress tolerance (Nayyar et al. 2005; Alcázar et al. 2006). Polyamines' interactions with membrane phospholipids implicate membrane stability under stress conditions (Roberts et al. 1986). Since PAs can act as free radical scavengers, they also protect the membranes from oxidative damages (Besford et al. 1993). Stress-tolerant plants are reported to accumulate more PAs than the sensitive ones (Lee 1997). Moreover, transgenic plants over-producing PAs displayed better stress tolerance than their wild-type counterparts (Galston et al. 1997; Kusano et al. 2008). Exogenous PAs application also improves tolerance against several abiotic stresses (Basra

et al. 1997; Nayyar and Chander 2004; Kusano et al. 2008). Nayyar et al. (2005) found that exogenous application of Put and Spd substantially improved the drought tolerance in soybean. Recently, it has been reported that endogenous production of PAs including free Spd, free Spm, and insoluble-conjugated Put is associated with improved water stress tolerance in rice (Yang et al. 2007; Farooq et al. 2009b). Seed priming with polyamines was effective in improving the emergence and seedling growth in hybrid sunflower (Farooq et al. 2007). In addition to the above, more roles for polyamines are emerging, such as signaling via inducing nitric oxide (Tun et al. 2006).

Earlier reports show that polyamines can improve stress tolerance in moderately drought tolerant plants, and these roles are mainly related to alleviation of oxidative damage (Davies 2004). Nevertheless, novel roles of PAs are to be explored in stress tolerance in various species. To our knowledge, no study has thus far explored the potential role and basis of exogenous PAs application to improve drought tolerance in a submerged plant like rice. We surmise that exogenously applied PAs may act at physiological and molecular levels to improved drought tolerance in rice. To test this hypothesis, we explored the changes in growth, photosynthetic attributes, cell water status, metabolite levels, and membrane characteristics and their possible interrelationships in order to establish involvement of PAs in drought tolerance in rice.

Materials and methods

Plant material, drought and polyamine treatments

Fine grain rice (*Oryza sativa* L. cv Super-Basmati) was used as an experimental material. The seeds were surface sterilized with 0.2% HgCl₂ solution for 5 min and thoroughly rinsed with tap water. For seed priming treatments, rice seeds were soaked in 10 μM aerated solution each of putrescine (Put), spermidine (Spd) and spermine (Spm) for 48 h at 27 ± 2°C. The ratio of seed weight to solution volume was 1:5 (w/v). After each treatment, seeds were given three surface washings with distilled water and dried back closer to original moisture level under forced air at 27°C ± 3, sealed in polythene bags and stored in a refrigerator at 5°C until use.

Treated and untreated seeds were grown in plastic pots (20 cm in diameter and 18 cm in height) in a phytotron with a photosynthetically active photon flux density (PPFD) of 300 mmol m⁻² s⁻¹, 27°C, 70–80% relative humidity, and a photoperiod of 14/10 h light/dark. Experimental design was completely randomized with five replications. Up to four-leaf stage plants from all treatments were well watered (maintained at 100% field

capacity). Afterwards, all plants were subjected to drought stress, except the control plants, which were kept well watered. Drought was maintained as 50% of field capacity by curtailing the water supply on alternate days or whenever needed. Hoagland nutrient solution (300 mL) was applied with irrigation water once in a week. For foliar spray, 10 μM solution each of Put, Spd and Spm was sprayed on leaves at five-leaf stage. Two controls were maintained, both receiving no PAs treatment as foliar application or seed treatment, one under drought conditions and the other under well-watered conditions.

All the observations, except seedling fresh and dry weight, were taken 1 week after foliar application of PAs. The experiment was terminated (3 weeks after the induction of drought stress) when 50% of stressed plants were wilted. At harvest, the seedlings were tested for vigor after carefully removing from the soil. Seedling fresh weight was determined immediately after harvest, while dry weight was taken after drying at 70°C for 7 days.

Leaf gas-exchange measurements

Net rate of photosynthesis (A), transpiration rate (E) and stomatal conductance (g_s) were measured of the penultimate fully expanded leaf using a portable infrared gas analyzer based photosynthesis system (LI-6400, LiCor, Inc., USA). Data were recorded at 09:00–10:00 a.m. 1 week after foliar PAs application. At data recording time, air relative humidity was about 75% and the ambient CO_2 concentration was 450 $\mu\text{mol CO}_2 \text{ mol}^{-1}$. Water use efficiency (WUE) was calculated as ratio between net photosynthesis and transpiration rate.

Plant water relations

Leaf water potential (ψ_w) was determined with pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA) from penultimate leaf. Frozen leaf tissues were thawed, sap expressed, centrifuged at 5,000g and osmotic potential (ψ_s) determined with an osmometer (Digital Osmometer, Wescor, Logan, UT). Leaf pressure potential (ψ_p) was computed as a difference of ψ_w and ψ_s . To determine relative water contents (RWC), fresh leaves (0.5 g) (W_f) were weighed to get fresh weight. Later these leaves were floated on water for 4 h and saturated weight (W_s) was determined. These leaves were dried for 24 h at 85°C to determine dry weight (W_d). RWC (%) were calculated as:

$$\text{RWC} = (W_f - W_d) / (W_s - W_d) \times 100\%.$$

Membrane permeability

To determine membrane permeability, leaf electrolyte leakage was measured following the protocol of Blum and

Ebercon (1981). Six leaf segments of similar size were briefly washed with distilled water and immersed in a test tube having 6 ml distilled water for 12 h at room temperature. Then electrical conductivity (EC_1) of solution was measured with a conductivity meter (Model DDS-11A, Shanghai Leici Instrument Inc., Shanghai, China). Samples were then heated in boiling water for 20 min and cooled to room temperature. The conductivity of killed tissues (EC_2) was again measured. Electrolyte leakage was measured as the ratio between EC_1 and EC_2 and expressed in percentage.

Metabolites determination

For free proline (Pro) estimation following the method of Bates et al. (1973), 0.5 g of fresh leaf material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman's No. 2 filter paper. Two milliliter of filtrate was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and absorbance was measured at 520 nm with a Shimadzu UV Spectrophotometer (Tokyo, Japan).

For determination of soluble phenolics by Julkunen-Tiitto (1985) method, fresh leaf tissue (0.1 g) was extracted in 1 ml of 80% acetone at 50°C for 1 h. An aliquot (100 μl) of the extract was reacted with 0.5 ml of Folin–Ciocalteu's phenol reagent and 2.5 ml of 20% sodium carbonate, and kept at room temperature for 20 min. The absorbance of the colored complex was taken at 750 nm using spectrophotometer (CE 2041). Tannic acid was used for the construction of a standard curve. Anthocyanins were determined after extraction of frozen fresh leaves (0.1 g) in 1 ml of acidified methanol (1% HCl v/v), vacuum filtered and quantified using spectrophotometer at 535 nm according to the method described by Stark and Wray (1989). Before anthocyanin measurement, background of the spectrophotometer (CE 2041) was set using acidified methanol.

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation following the method of Heath and Packer (1968). Leaf samples (1 g) were homogenized in 10 ml 0.1% trichloroacetic acid. The homogenate was centrifuged at 15,000g for 5 min, and 4 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added to a 1-ml aliquot of the supernatant. The mixture was heated at 95°C for 30 min and cooled rapidly in an ice bath. After centrifugation at 10,000g for 10 min, the absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The malondialdehyde content was calculated using its absorption coefficient of 155 $\text{mmol}^{-1} \text{ cm}^{-1}$.

For determination of leaf hydrogen peroxide, frozen fresh material was extracted in 0.1% (w/v) trichloroacetic acid using a pre-chilled pestle and mortar, centrifuged at 12,000g for 15 min, and supernatant used for the H₂O₂ quantification with the method of Teranishi et al. (1974).

Enzyme assays

Total extractable superoxide dismutase (SOD, EC 1.15.1.1) activity was determined following the method of McCord and Fridovitch (1969). Inhibition of color formation (measured at 560 nm) was determined by addition of 0–50 µl of the extract to a reaction mixture containing 50 mM HEPES/KOH buffer (pH 7.8), 0.05 units xanthine oxidase, 0.5 mM nitroblue tetrazolium, and 4 mM xanthine. One unit of SOD activity equaled the volume of extract needed to cause 50% inhibition of the color reaction. Catalase (CAT, EC 1.11.1.6) activity was measured following the modified method of Luck (1974). Enzyme extract (50 µl) was added to 3 ml of H₂O₂-phosphate buffer (pH 7.0). The time required for decrease in the absorbance from 0.45 to 0.40 was noted. Enzyme solution containing H₂O₂-free phosphate buffer was used as control. Enzyme activity was expressed in mmol of H₂O₂ consumed min⁻¹ mg⁻¹ chl. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was estimated according to the method of Nakano and Asada (1987) with slight modification. Ascorbate oxidation to dehydroascorbate (DHA) was followed at 265 nm in 1 ml reaction mixture containing 50 mM HEPES/KOH (pH 7.6), 0.1 mM EDTA, 0.05 mM ascorbate, 10 µl extract, and 0.1 mM H₂O₂.

Statistical analysis

The data were subjected to statistical analysis by analysis of variance using COSTAT computer software. Least

significant difference (LSD) test was applied to compare the treatment means. Correlations of growth and photosynthetic attributes were established with water relations, metabolites, membrane characteristics and antioxidants levels in the rice leaves.

Results

Upon exposure to drought stress rice growth was severely hampered, but exogenous application of polyamines assuaged the damaging effects of drought. Maximum plant height and seedling fresh and dry weight were observed from rice plants raised under well-watered conditions (Table 1). Drought stress remarkably reduced seedling growth, albeit polyamines application improved the plant growth under drought stress. Amongst PAs treatments, the maximum plant height and seedling fresh and dry weight were noted from the plants foliar treated with Spm (Spm-FA). However, Spm-FA performed similar to Spd foliar application (Spd-FA) in case of seedling dry weight and plant height (Table 1).

Maximum leaf CO₂ net assimilation rate (*A*), stomatal conductance (*g_s*) and transpiration rate (*E*) were noted in well-watered plants (CK1), while drought stress (CK2) significantly reduced these attributes. Application of PAs significantly improved the *A* but decreased *g_s* and *E* under drought stress (Table 2). Under stress conditions, maximum *A* (15.67 µmol m⁻² s⁻¹) was measured from Spm-FA being similar (15.37 µmol m⁻² s⁻¹) to that of seed priming spermine (Spm-SP). Minimum *g_s* (0.325 mol m⁻² s⁻¹) was recorded from Spm-FA, while *E* (10.01 µmol m⁻² s⁻¹) was the least in Put-FA treatment (Table 2). Maximum WUE was recorded from Spm-FA, which was at par with well-watered control. Seed priming with spermine (Spm-SP) and spermidine (Spd-SP) and Spd-FA. Minimum WUE

Table 1 Effects of polyamines application on the seedling vigor under drought stress in rice

Treatment	Seedling fresh weight (g)	Seedling dry weight (g)	Plant height (cm)
CK1	57.66 ± 1.21*	19.21 ± 0.47a	57.23 ± 1.73a
CK2	34.77 ± 1.01e	11.33 ± 0.67e	39.62 ± 2.82f
Put-SP	38.33 ± 1.33d	13.54 ± 0.61d	44.71 ± 3.01de
Spm-SP	42.21 ± 1.77c	14.69 ± 0.44c	47.25 ± 1.57c
Spd-SP	40.13 ± 2.08 cd	13.10 ± 0.34d	46.45 ± 1.35 cd
Put-FA	39.67 ± 1.63 cd	13.77 ± 0.72c	45.33 ± 1.83d
Spm-FA	46.45 ± 1.92b	16.55 ± 0.78b	49.55 ± 2.07b
Spd-FA	41.14 ± 2.07c	15.63 ± 0.66bc	47.67 ± 1.17bc

Each value indicates treatment mean ± standard error

CK1: well watered, no polyamine treatment; CK2: drought stress, no polyamine treatment; SP: seed priming; FA: foliar application; Put: putrescine; Spm: spermine; Spd: spermidine

* Means sharing the same letters in a column do not differ significantly at $P \leq 0.05$ according to LSD test

Table 2 Effects of polyamines application on the leaf CO₂ net assimilation rate (*A*) stomatal conductance (*g_s*), transpiration rate (*E*) and water use efficiency (WUE) under drought stress in rice

Treatment	Leaf CO ₂ net assimilation rate (<i>A</i>) ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Stomatal conductance (<i>g_s</i>) ($\text{mol m}^{-2} \text{s}^{-1}$)	Transpiration rate (<i>E</i>) ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Water use efficiency (WUE) (<i>A/E</i>)
CK1	18.63 ± 1.23a*	0.503 ± 0.011a	13.01 ± 1.35a	1.43 ± 0.62a
CK2	12.11 ± 1.45f	0.422 ± 0.018b	11.67 ± 1.21b	1.04 ± 0.54d
Put-SP	14.08 ± 1.33 cd	0.361 ± 0.027c	10.22 ± 1.29c	1.38 ± 0.61b
Spm-SP	15.37 ± 1.27bc	0.344 ± 0.037d	10.33 ± 1.39c	1.49 ± 0.41a
Spd-SP	14.43 ± 1.67c	0.353 ± 0.036d	10.15 ± 1.51c	1.42 ± 0.23ab
Put-FA	13.33 ± 1.88e	0.366 ± 0.027c	10.01 ± 1.26c	1.33 ± 0.55bc
Spm-FA	15.67 ± 1.39b	0.325 ± 0.036e	10.41 ± 1.41c	1.51 ± 0.43a
Spd-FA	14.55 ± 1.11c	0.343 ± 0.027d	10.43 ± 1.32c	1.40 ± 0.71ab

Each value indicates treatment mean ± standard error

CK1: well watered, no polyamine treatment; CK2: drought stress, no polyamine treatment; SP: seed priming; FA: foliar application; Put: putrescine; Spm: spermine; Spd: spermidine

* Means sharing the same letters in a column don't differ significantly at $P \leq 0.05$ according to LSD test

was noted from drought stresses plants without PA treatment (Table 2). Maximum leaf water potential (ψ_w), osmotic potential (ψ_s), pressure potential (ψ_p) and RWC were observed in well-watered plants, although drought stress drastically reduced these characteristics. Exogenous PAs application appreciably improved the water relation of drought-stressed plants. Under stress conditions, maximum ψ_w , ψ_s , ψ_p and RWC were recorded from Spm-FA (Table 3).

Among the metabolites, minimum concentrations of phenolics, anthocyanins and free proline were measured from rice plants raised under well-watered conditions. However, upon exposure to drought stress the levels of these metabolites were increased. PAs application further increased these metabolites being the maximum with Spm-FA. For phenolics, these increases were in the order: Spm-FA > Spd-FA = Put-FA = Spd-SP = Spm-SP > Put-SP > CK2 > CK1, while anthocyanins followed the order:

Spm-FA > Put-FA = Spd-FA > Spm-SP = Put-SP = Spd-SP > CK2 > CK1, and the order for Proline Spm-FA > Spd-FA = Spm-SP = Spd-SP = Put-FA > CK2 > CK1 (Table 4). There was minimum leaf H₂O₂, MDA and membrane permeability under well-watered conditions, which were significantly increased under drought stress (Table 4), although PAs application significantly lessened the leaf H₂O₂, MDA and membrane permeability under drought stress. Minimum leaf H₂O₂, MDA and membrane permeability under drought stress was observed from Spm-FA, which was similar to Spd-FA and Spm-SP in case of membrane permeability (Table 4).

Although SOD contents were decreased by drought stress, PAs application significantly improved this attribute (Table 5). Maximum CAT (10.61 $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein) contents were noted from well-watered rice plants, which were decreased significantly upon exposure to drought stress. PAs application improved the CAT contents

Table 3 Effects of polyamines application on the plant water relations under drought stress in rice

Treatment	Water potential (ψ_w) (MPa)	Osmotic potential (ψ_s) (MPa)	Pressure potential (ψ_p) (MPa)	Relative water contents (RWC) (%)
CK1	0.49 ± 0.052f*	0.96 ± 0.061f	0.47 ± 0.047a	88.33 ± 5.53a
CK2	1.18 ± 0.043a	1.31 ± 0.080a	0.13 ± 0.066f	46.41 ± 6.33e
Put-SP	1.07 ± 0.071b	1.25 ± 0.062b	0.18 ± 0.047e	50.87 ± 3.45d
Spm-SP	0.92 ± 0.062c	1.20 ± 0.057c	0.28 ± 0.061c	56.67 ± 2.66c
Spd-SP	0.95 ± 0.078c	1.22 ± 0.063c	0.27 ± 0.059c	54.53 ± 1.23c
Put-FA	0.94 ± 0.073c	1.19 ± 0.072 cd	0.25 ± 0.053d	50.44 ± 4.61d
Spm-FA	0.78 ± 0.071e	1.09 ± 0.062e	0.31 ± 0.047b	59.74 ± 2.34b
Spd-FA	0.89 ± 0.033 cd	1.17 ± 0.051d	0.28 ± 0.051c	55.51 ± 1.88c

Each value indicates treatment mean ± standard error

CK1: well watered, no polyamine treatment; CK2: drought stress, no polyamine treatment; SP: seed priming; FA: foliar application; Put: putrescine; Spm: spermine; Spd: spermidine

* Means sharing the same letters in a column don't differ significantly at $P \leq 0.05$ according to LSD test

Table 4 Effects of polyamines application on the metabolites in rice under drought stress

Treatment	Soluble phenolics ($\mu\text{g g}^{-1}$) (FW)	Anthocyanins ($\mu\text{g g}^{-1}$) (FW)	Leaf free proline content ($\mu\text{mol g}^{-1}$) (DW)	Leaf H_2O_2 ($\mu\text{mol g}^{-1}$) (FW)	MDA ($\mu\text{mol g}^{-1}$) (FW)	Membrane permeability (%)
CK1	43.25 \pm 1.26f*	25.66 \pm 2.10e	7.87 \pm 0.65e	8.47 \pm 0.88f	14.56 \pm 1.21f	12.23 \pm 1.33d
CK2	46.38 \pm 1.54e	28.47 \pm 1.78d	9.69 \pm 0.71d	20.37 \pm 1.01a	27.67 \pm 2.14a	25.43 \pm 1.67a
Put-SP	49.47 \pm 0.98d	31.71 \pm 1.32c	11.83 \pm 0.92bc	17.36 \pm 1.46b	23.32 \pm 1.33b	16.39 \pm 2.01b
Spm-SP	53.79 \pm 1.11c	32.68 \pm 1.67c	12.37 \pm 1.01b	15.77 \pm 1.56c	21.29 \pm 1.74c	15.22 \pm 1.51bc
Spd-SP	55.50 \pm 1.08bc	31.44 \pm 1.54c	11.91 \pm 0.54bc	15.69 \pm 0.84c	22.47 \pm 1.37bc	16.44 \pm 1.03b
Put-FA	57.57 \pm 1.31b	34.67 \pm 1.38b	11.77 \pm 0.53bc	14.37 \pm 1.21d	21.66 \pm 1.18c	15.56 \pm 1.41b
Spm-FA	61.61 \pm 1.06a	39.87 \pm 1.71a	14.39 \pm 1.23a	13.44 \pm 1.13e	20.43 \pm 1.21e	14.33 \pm 1.31c
Spd-FA	58.37 \pm 1.21b	36.55 \pm 1.27b	12.49 \pm 1.03b	13.52 \pm 1.09d	21.63 \pm 1.05 cd	14.49 \pm 0.51c

Each value indicates treatment mean \pm standard error

CK1: well watered, no polyamine treatment; CK2: drought stress, no polyamine treatment; SP: seed priming; FA: foliar application; Put: putrescine; Spm: spermine; Spd: spermidine

* Means sharing the same letters in a column don't differ significantly at $P \leq 0.05$ according to LSD test

Table 5 Effects of polyamines application on the antioxidants under drought stress in rice

Treatment	SOD (Unit g^{-1} protein) ^a	CAT ($\mu\text{mol min}^{-1} \text{g}^{-1}$ protein)	APX ($\mu\text{mol min}^{-1} \text{g}^{-1}$ protein)
CK1	11.47 \pm 0.37e	10.61 \pm 1.32a	9.53 \pm 0.37b
CK2	9.87 \pm 0.53f	7.47 \pm 0.64f	8.35 \pm 0.81c
Put-SP	11.49 \pm 0.83d	8.59 \pm 0.61e	8.54 \pm 0.88c
Spm-SP	13.59 \pm 0.69b	9.53 \pm 0.44d	9.69 \pm 0.67b
Spd-SP	12.39 \pm 0.64c	9.44 \pm 0.52d	8.67 \pm 0.71c
Put-FA	11.45 \pm 0.63d	9.52 \pm 0.38d	8.71 \pm 0.64c
Spm-FA	15.31 \pm 0.77a	10.57 \pm 0.83b	10.35 \pm 0.44a
Spd-FA	13.47 \pm 0.56b	9.63 \pm 0.67c	9.61 \pm 0.36b

Each value indicates treatment mean \pm standard error

* Means sharing the same letters in a column don't differ significantly at $P \leq 0.05$ according to LSD test

^a One unit of SOD activity is equivalent to the volume of extract needed to cause 50% inhibition of the color reaction

CK1: well watered, no polyamine treatment; CK2: drought stress, no polyamine treatment; SP: seed priming; FA: foliar application; Put: putrescine; Spm: spermine; Spd: spermidine; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase

under stressful conditions, and Spm-FA (10.57 $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein) was the most promising one. Likewise, APX contents were decreased by drought stress but PA application improved the APX under stress conditions. Maximum SOD (15.31 Unit g^{-1} protein) and APX (10.35 $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein) were recorded from Spm-FA treatment followed by Spm-SP and Spd-FA in both cases (Table 5).

To substantiate the validity of above findings in a submerged plant like rice, fresh and dry weight, net photosynthesis and water use efficiency were correlated with water relations, metabolites levels, membrane characteristics and antioxidant activities (Table 6). Fresh and dry weight and net photosynthesis were correlated negatively with water and osmotic potentials, but positively with leaf turgor and relative water content. Leaf H_2O_2 , MDA and membrane permeability were negatively related

to fresh, dry weight and net photosynthesis, while among the antioxidants only the activity of catalase was positively related to these attributes. None of the metabolites was correlated with growth or photosynthetic parameters. However, photosynthetic water use efficiency was negatively related to membrane permeability and positively to activities of all the antioxidants (Table 6).

Discussion

Polyamines are now being regarded as plant growth regulators and secondary messenger in signaling pathways (Davies 2004; Liu et al. 2007; Kusano et al. 2008), and play an array of physiological roles in plant growth and development (Ali 2000). Although, they induce tolerance against several abiotic stresses in plants (Nayyar et al.

2005; Yang et al. 2007; Farooq et al. 2007), mechanisms of their action during exogenous application in modulating physiological phenomena and improving drought stress tolerance are not fully understood. In this study, seed and foliar treatment with PAs appreciably improved the drought tolerance, as evident from seedling height, their fresh and dry weight (Table 1). Of the two methods of PAs application, foliar spray was more effective in displaying substantially better growth of rice, while amongst the PAs, Spm was the most effective.

Exogenous PAs application improved the photosynthetic attributes as well as water use efficiency, particularly net photosynthesis, which were severely hampered under drought (Table 2). When the available water is limiting, plants tend to close their stomata (Cornic and Massacci 1996), resulting in reduced inflow of CO₂ into the leaves for fixation. This directs more electrons for the formation of active oxygen species. As the rate of transpiration decreases, the amount of dissipated heat increases, resulting in a decline in net photosynthesis (Yokota et al. 2002). In this study, there was no relationship of water relations with stomatal conductance and transpiration rate, although it was strongly correlated with net photosynthesis (Table 6). These findings suggested that drought tolerance in rice was not due to stomatal component of photosynthesis rather improved CO₂ assimilation by Rubisco in producing photosynthate and their partitioning in dry matter yield.

Maintenance of requisite leaf water status is of paramount importance in rice (Cornic and Massacci 1996). In this study, leaf water, osmotic and turgor potentials, and

RWC were highly reduced under drought stress, whilst the exogenously applied PAs substantially improved these attributes (Table 3). It was noteworthy that foliar spray was more effective than seed priming, which is related to the fact that PAs while in direct contact with leaf surface improved the water status of epidermal cells and underlying mesophyll cells, and this effect appeared to be persistent throughout the experimental period. Seed priming with PAs was effective in producing the vigorous seedlings (Farooq et al. 2007), which were obviously better able to withstand adversaries of drought. As evident from close correlations of growth and net photosynthesis with water relations attributes (Table 6), it is believed that PAs have the ability to improve the cell water status thereby allowing rice to grow better under limited moisture supply conditions.

Foliar applied PAs due to their ability to act as growth regulator is able to modulate the plant metabolism and the production of metabolites involved in stress tolerance (Takahama and Oniki 1997). In this study, PAs application led to accumulation of high levels of free proline, soluble phenolics and anthocyanins, whilst curtailing the production of H₂O₂ and MDA and reducing the relative membrane permeability (Table 4). Accumulation pattern of these metabolites when paralleled with growth and photosynthetic characters revealed no relationships of free proline, soluble phenolics and anthocyanins, while strong negative relationships with those of H₂O₂, MDA and membrane permeability (Table 6). PAs have been reported to facilitate the accumulation of phenolic compounds and free proline to protect against oxidative damage (Olga et al.

Table 6 Correlation coefficients of some growth and gas exchange attributes of rice with water relations, metabolites and activity of antioxidant enzymes

Characteristics	Fresh weight	Dry weight	Net photosynthesis	Stomatal conductance	Transpiration rate	Water use efficiency
Water potential	-0.87**	-0.964**	-0.947**	-0.448 ns	-0.526 ns	-0.612 ns
Osmotic potential	-0.984**	-0.973**	-0.929**	-0.451 ns	-0.540 ns	-0.581 ns
Turgor potential	0.971**	0.936**	0.948**	0.437 ns	0.502 ns	0.633 ns
Relative water content	0.979**	0.899**	0.949**	0.681 ns	0.743*	0.427 ns
Free proline	-0.360 ns	-0.161 ns	-0.267 ns	-0.962**	-0.855**	0.461 ns
Hydrogen peroxide	-0.951**	-0.946**	-0.886**	-0.372 ns	-0.412 ns	-0.634 ns
MDA	-0.953**	-0.946**	-0.948**	-0.402 ns	-0.430 ns	-0.690 ns
Membrane permeability	-0.708*	-0.812*	-0.774*	0.063 ns	0.046 ns	-0.906**
Soluble phenolics	-0.241 ns	-0.046 ns	-0.211 ns	-0.864**	-0.785*	0.453 ns
Anthocyanins	-0.227 ns	0.009 ns	-0.200 ns	-0.852**	-0.727*	0.422 ns
Superoxide dismutase	0.263 ns	0.443 ns	0.362 ns	-0.627 ns	-0.413 ns	0.799*
Catalase	0.815*	0.876**	0.820*	0.008 ns	0.100 ns	0.841**
Ascorbate peroxidase	0.604 ns	0.746*	0.641 ns	-0.200 ns	0.053 ns	0.712*

ns: non-significant

Significant at: * $P < 0.05$, ** $P < 0.01$

2003; Kumar et al. 2003). Although these metabolites were accumulated, no such role of these metabolites emerged in this study for rice since none of them was related to plant fresh and dry weight or leaf photosynthesis (Table 4). This is assignable to the fact that rice, being submerged, behaves differently than non-submerged plants.

Loss of integrity of biological membranes, principally due to the oxidative damage, is another intricate effect of drought (Liu et al. 2000; Feng et al. 2003). Production of ROS, especially H_2O_2 , which is relatively long-lived ROS, is a major cause of such effects (Besford et al. 1993; Munne-Bosch and Penuelas 2003). The ROS react with proteins, lipids and DNA and impairing the normal cellular functions (Apel and Hirt 2004). However, having potential to act as free radical scavengers, PAs can protect the membranes and other macro molecules from oxidative damages (Besford et al. 1993) and thus can stabilize biological membranes under stressful conditions (Roberts et al. 1986). In this study, the membrane permeability, H_2O_2 and MDA contents of leaves under drought were significantly increased. However, PAs application alleviated drought effects as evident from substantially reduced membrane permeability, H_2O_2 and MDA contents (Table 4). ROS in plants are scavenged by a variety of antioxidant enzymes and/or lipid- and water-soluble molecules. Of these, antioxidant enzymes are the most effective against oxidative damage (Foyer and Fletcher 2001). These findings revealed that enzymatic antioxidant activities of rice seedlings were substantially induced by PAs application (Table 5). Among the antioxidant enzymes, CAT eliminates H_2O_2 in cucumber by exogenously applied salicylic acid (Shi et al. 2006). In this study, a strong negative relationship of CAT with H_2O_2 and membrane permeability (Table 6) revealed that PAs specifically enhanced CAT activity resulted in the elimination of H_2O_2 and improved membrane properties. This further confirmed the action of PAs act as ROS scavengers (Drolet et al. 1986).

To conclude, exogenously applied PAs have multiple roles in improving drought tolerance of rice. These functions are improved cell water status and alleviation of oxidative damage on the biological membranes. This suggests that maintenance of water economy through stabilized cellular structure is an important mechanism of drought tolerance in rice. Establishment of similar roles of PAs are likely to be a great step in improving drought tolerance in high water requiring plant species.

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