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Response of alfalfa to putrescine treatment under drought stress

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Abstract

Alfalfa (*Medicago sativa* L. cv. Siwa 1) seeds were germinated in polyethylene glycol (PEG 4000) of different concentrations and with or without putrescine. The decrease in water potential of the PEG solution reduced germination rate, germination percentage, and growth criteria (*e.g.*, hypocotyl length, fresh and dry masses of shoot and root), while the root length was increased. The decrease in water potential also reduced the contents of total soluble and reducing sugars, and proteins, and activities of α- and β-amylases and invertase, while increased protease activity. Putrescine treatment improved germination and all growth criteria and increased the activity of the hydrolytic enzymes except protease. In a pot experiment, drought stress was imposed by decreasing the soil moisture. Growth criteria, contents of proteins, chlorophyll *a*, *b* and carotenoids, as well as Hill reaction activity decreased while the hydrolytic enzyme activity and total soluble and reducing sugar contents increased under drought stress. Putrescine treatment decreased the activity of the hydrolytic enzymes and increased the polysaccharide, protein and photosynthetic pigment contents, and Hill reaction activity.

Additional key words: carotenoids, chlorophyll, enzyme activity, germination, growth, Hill reaction, polyethylene glycol.

Introduction

Gill *et al.* (2002) reported that under stress conditions induced by mannitol, germination of sorghum decreased markedly. However, high percentage of ungerminated seeds germinated after washing with distilled water. Reduced germination under water stress may be attributed to the development of an osmotically enforced "dormancy", which may be adaptive strategy of seeds to prevent germination under stressful environment, thus ensuring proper establishment of seedlings (Singh *et al.* 1996, Prado *et al.* 2000). Chazen *et al.* (1995) attributed the inhibitory effect of polyethylene glycol (PEG) on plant growth to the inhibition on root-water transport which in turn affects leaf development, or to reduced oxygen availability in PEG solution due to high viscosity of PEG.

Photosynthesis is reduced under drought stress; non-stomatal inhibition of photosynthesis normally accounts for a larger proportion of photosynthetic inhibition as water potential becomes lower (Ogren and Öquist 1985). This inhibition includes reduction in electron transport, photophosphorylation or activity of Rubisco (Björkman *et al.* 1981, Mayoral *et al.* 1981). Several *in vivo* studies demonstrated that water stress resulted in damage of oxygen evolving complex of PS 2 (Toivenen and Videvar 1988, Masojidek *et al.* 1991). Photosynthetic pigments were also affected by drought stress; Björkman *et al.* (1981) observed degradation of chlorophyll and chlorophyll-protein complexes.

The activity of some enzymes are reduced by water stress, while the activity of others, such as those involved in hydrolysis and degradation reactions, may be increased by stress (Hsiao 1973). Analysis of α-amylase activity in 8 rice cultivars indicated that drought resistance was correlated with high amylase activity (Wang *et al.* 1991). Jacobsen *et al.* (1986) added that water stress enhances the expression of α-amylase genes in barley. The activity of proteinase in litchi leaves increases under drought stress (Chen and Liu 1999). Perez and Lalher (1995) and Wang *et al.* (1991) reported that salt or drought stress induced conversion of hexoses, sucrose and starch into sugar alcohols (polyols) and proline. Bolarin *et al.* (1995) indicated that soluble sugars play an important role in osmotic regulation of cells during germination.

Polyamines accumulate in relatively high quantities in resting seeds (Felix and Harr 1987). The polyamines accumulated in embryos of *Avena fatua* and *Oryza sativa*, but their content in endosperms was low. Zeid (2004) reported that exogenous Put treatment (0.01 mM)

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Abbreviations: Car - carotenoids; Chl - chlorophyll; FC - field capacity; PEG - polyethylene glycol; Put - putrescine; Spd - spermidine; Spm - spermine.

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increased germination and growth of bean under normaland NaCl-induced stress conditions, may be due to the activation of amylase and protease during germination. Sung and Jae (1995) observed retardation of chlorophyll loss in excited leaves Chinese cabbage under the dark by polyamines. Compared with putrescine and spermidine, spermine retarded the loss of chlorophyll and electron transport activities to a greater extent in wheat leaves during dark incubation. (Subhan and Murthy 2001). Concerning with enzyme activity, it was found that spermine, spermidine or putrescine significantly increased α-amylase activity under salt stress in barley (Tipirdamaz *et al*. 1995). Exogenous polyamines (spermidine and spermine) strongly reduced protease activity in tobacco cultured *in vitro* (Scaramagli *et al*. 1999).

The present work aims to indicate the effect of the putrescine on germination and growth of alfalfa under drought stress. Its effect on the activity of some hydrolases, as well as photosynthetic pigments and Hill reaction activity were also studied during the first four days of growth and after 21 d.

Materials and methods

Seeds of alfalfa (*Medicago sativa* L. Siwa 1) were sterilized with sodium hypochlorite solution (2.5 %) for 3 min and washed thoroughly with distilled water. Then, the seeds were soaked in distilled water or putrescine (0.01 mM) for 8 h. Putrescine (Put) concentration was chosen after carrying out a preliminary experiment, in which the seeds were soaked in a series of different concentrations of Put. Put concentration at 0.01 mM was the most effective concentration on germination and early growth of alfalfa under PEG-induced water stress. The seeds were then transferred to sterile Petri dishes containing two sheets of *Whatman No. 1* filter paper moistened with 10 cm^3 of distilled water or PEG solution (-0.2, -0.4, -0.6 or -0.8 MPa). Each Petri dish contained 20 seeds and each treatment was replicated five times. The seeds were allowed to germinate at 25 °C in the dark. In a pot experiment, water stress was induced by decreasing the water supply to the soil, to be subjected to 60, 45, or 30 % of the field capacity (FC) after one week from sowing. Plants grown at 60 % of the field capacity were considered as control plants. Put (0.01 mM) was added once to the Hoagland’s nutrient solution.

Shoot and root lengths, fresh masses, and dry masses were measured at germination and seedling stages. For enzyme assay, plant material was prepared by macerating the tissues with a chilled pestle and mortar at 0 - 4 °C. The tissue homogenate was centrifuged at 10 000 *g* for 20 min and the supernatant obtained was used directly for determining enzyme activity. For assaying the activity of α- and β-amylases, 3,5-dinitrosalicylic acid reagent was used according to Bergmeyer (1974). Acid-invertase enzyme was assayed by the method of Malik and Singh (1984). Protease activity was assayed according to the method described by Bergmeyer (1974). Fresh leaves were extracted in 70 % ethanol and completed to a known volume with distilled water and used for estimation of total sugars using anthrone reagent (Umbreit *et al*. 1959) and soluble proteins (Lowry *et al*. 1951), while the reducing sugars were determined by using Nelson reagent according to Naguib (1964). Photosynthetic pigments were estimated in 85 % acetone extracted leaves according to Metzner *et al*. (1965). For isolation of chloroplasts, according to the method of Aronoff (1946) and Osman *et al.* (1982), fresh leaves were blended in cold buffer with 0.4 M sucrose, (pH 7.8), 3 mM $MgCl_2$, 4 mM sodium ascorbate and 0.1 % bovine serum albumin. The suspension was centrifuged at 4 ºC (1 min at 800 *g*). The pellet was resuspended in the isolation buffer and centrifuged for 5 min at 300 *g* and the supernatant was then centrifuged for 10 min at 1000 *g*. Chloroplasts (residue) were resuspended in the buffer solution. Hill reaction of the isolated chloroplasts was measured by using potassium ferricyanide as electron acceptor.

Statistical analysis was carried out according to Snedecor and Cochran (1980) using analysis of variance and the significance was determined using LSD values at $P = 0.05$ and 0.01.

Results and discussion

Low water potential delayed initiation of seed germination, slowed the rate of germination and decreased final germination percentage. The germination percentage of unstressed seeds was 73 % at the first day of germination and increased to 88 % at the fourth day. Seeds under water stress showed a progressive reduction in germination percentage with decreasing the external water potential, from 0 to -0.8 MPa. It was 47 % at the first day and 73 % at the fourth day, at the water potential -0.8 MPa (Table 1). Activity of α- and β-amylase in the unstressed seeds was higher at the first day and gradually decreased with the lapse of time. Decreasing the water potential of the external medium resulted in a marked reduction in the activity of α- and β-amylase (Table 1). Hsiao (1973) reported that the activity of α-amylase increases in the vegetative tissues exposed to drought stress, while its activity decreases when the germinating seeds were stressed. The protease activity increased from the first to the fourth day, and with decreasing the water potential. The increase in protease activity may compensate the reduced content of soluble sugars under water stress, which is required for germination and the subsequent growth, through gluconeogenesis (Zeid 2004). The contents of soluble sugars and reducing sugars in the growing seeds were higher at the first day of germination and decreased gradually with the lapse of time. Decreasing water potential induced a great reduction in the content of soluble sugars partially due to the inhibition of amylases activity (Table 1). Put treatment may activate amylases and invertase and consequently, increase the content of soluble sugars.

Since cell expansion is dependent on cell water

Table 1. Effect of PEG-induced water stress and putrescine (0.01 mM) treatment on germination percentage [%], activities of α- and β-amylases, invertase and protease [µg g^{-1}(f.m.) s^{-1}], and contents of sugars and soluble proteins [mg g^{-1} (d.m.)] in 4-d-old alfalfa seedlings.

Put [mM]	Ψ_s [MPa]	Germination	α-Amylase	β-Amylase	Invertase	Protease	Reducing sugars	Soluble sugars	Soluble proteins
0	control	88	12.8	22.1	45.4	13.8	0.77	10.3	20.2
	-0.2	82	12.0	23.1	40.9	14.4	0.44	9.2	19.8
	-0.4	82	11.1	19.3	38.7	15.6	0.39	7.5	18.0
	-0.6	79	10.3	8.1	37.5	17.2	0.28	6.5	10.4
	-0.8	73	3.8	5.07	35.2	18.0	0.23	4.2	9.0
0.01	0	97	24.7	33.3	47.2	13.0	1.49	11.0	21.8
	-0.2	89	17.3	24.3	43.2	13.7	0.84	10.5	20.9
	-0.4	87	13.9	21.6	41.4	15.3	0.64	9.0	19.2
	-0.6	82	11.3	16.4	39.5	16.4	0.44	7.3	18.9
	-0.8	80	4.2	8.8	37.0	17.5	0.40	6.3	15.4
LSD at 0.05		1.1	1.2	2.2	0.6	0.3	0.08	0.4	1.0
LSD at 0.01		1.5	1.6	2.9	0.8	0.4	0.10	0.6	1.4

Table 2. Effect of PEG-induced water stress and putrescine treatment on hypocotyl and root length [cm] and shoot and root fresh and dry masses [mg] of alfalfa seedlings (4-d-old).

Put [mM]	Ψ_s [MPa]	Hypocotyl length	Shoot fresh mass	Shoot dry mass	Root length	Root fresh mass	Root dry mass	Hypocotyl/ root ratio
0	control	3.7	232.4	12.4	3.5	64.4	3.5	1.05
	-0.2	3.3	199.8	12.5	4.4	61.0	3.4	0.75
	-0.4	3.0	181.0	10.2	4.4	58.2	3.4	0.68
	-0.6	2.7	173.8	9.4	4.9	45.0	3.1	0.55
	-0.8	1.8	146.5	9.2	5.9	41.8	2.9	0.30
0.01	0	4.1	265.1	13.1	4.4	104.5	3.9	0.93
	-0.2	4.1	244.1	12.5	4.9	93.3	4.0	0.83
	-0.4	4.4	202.0	10.8	5.4	79.1	3.9	0.81
	-0.6	3.7	197.2	11.0	6.1	72.0	3.7	0.60
	-0.8	2.3	183.0	9.9	7.1	70.0	3.5	0.32
LSD at 0.05		0.4	15.0	0.2	0.5	6.0	0.6	
LSD at 0.01		0.6	19.0	0.3	0.7	8.0	0.8	

Table 3. Effect of putrescine treatment on hypocotyl and root length [cm] and shoot and root fresh and dry masses [mg] of alfalfa seedlings (21-d-old) grown under drought stress (FC - field capacity).

Put [mM]	FC [%]	Shoot length	Shoot fresh mass	Shoot dry mass	Root length	Root fresh mass	Root dry mass	Shoot/ root ratio
0	60 (control)	7.9	273.0	15.2	10.8	85.4	7.40	0.72
	45	6.4	200.9	11.3	12.6	111.9	8.80	0.50
	30	4.5	152.4	9.3	12.9	91.8	8.00	0.35
0.01	60	10.3	314.0	17.6	12.0	101.1	10.0	0.85
	45	9.1	253.1	13.1	15.2	310.0	22.0	0.59
	30	7.7	180.5	10.2	14.2	110.2	11.0	0.54
LSD at 0.05		1.3	7.0	0.5	1.0	6.0	0.6	
LSD at 0.01		1.7	9.5	0.7	1.3	7.0	0.8	

potential, developing cells expand less and cell size is smaller under stress conditions (Barlow 1986). The response of hypocotyl and root length to water stress was different (Table 2). Hypocotyl length was reduced with decreasing the available water content, while the root length increased and the hypocotyl/root ratio decreased with decreasing the external water potential. The development of roots under drought stress is of particular significance to plants, since an extensive and efficient root system is an important drought-avoidance mechanism. However, the fresh and dry masses of shoots and roots decreased with reducing the external water potential (Table 3).

Put treatment resulted in a beneficial effects on germination of alfalfa seeds under normal and stress conditions. Its application increased the rate of germination and the final germination percentage at all concentrations applied. The final germination percentage of unstressed seeds increased from 88 to 97 %, and from 73 to 80 % in water stressed seeds at -0.8 MPa. This may be attributed to the stimulation of the activity of α- and β-amylases during seed germination. The activation of these hydrolytic enzymes was accompanied by increased content of soluble sugars which transfer to the embryo axes to serve as precursors for early growth and the different metabolic processes. Therefore, it has been observed that growth criteria, *e.g.*, length of hypocotyls and roots, fresh and dry masses of shoots and roots, as well as the ratio of hypocotyl/root length increased with the application of Put. Treatment of Put showed an inhibitory effect on protease activity under both stressed and unstressed conditions. This finding supports the results obtained by Scaramagli *et al.* (1999).

Growth criteria of alfalfa seedlings (21-d-old), *e.g.*, shoot and root lengths, fresh and dry masses of shoots and roots were markedly decreased as a result of drought stress (Table 3). Plant organs differed in their sensitivity to water stress, shoot seems to be more sensitive than roots to water stress. Therefore, the ratio of shoot/root length decreased with increasing drought stress.

The growth inhibiting effects of drought stress may be correlated with the reduction in photosynthesis, which could be partially attributed to the decreased content of the photosynthetic pigments (Chl *a*, Chl *b*, and carotenoids), and the partial inhibition of the Hill reaction (Table 4). Several *in vivo* studies demonstrated that water stress resulted in damage of oxygen evolving system of PS 2 (Masojidek *et al*. 1991, Rensburg and Kruger 1993).

Table 4. Effect of putrescine treatment on contents of photosynthetic pigments [mg g^{-1}(d.m.)] and activity of Hill reaction [μmol(ferricyanide) g^{-1}(Chl) s^{-1}] in leaves of alfalfa seedlings (21-d-old) under drought stress.

Put [mM]	FC [%]	Chl *a*	Chl *b*	Car	Chl *a*/*b*	Chl *a*+*b*/Car	Hill reaction
0	60 (control)	0.70	0.30	0.22	2.33	4.54	32.7
	45	0.62	0.21	0.15	2.95	5.18	26.7
	30	0.49	0.16	0.07	3.06	9.28	18.1
0.01	60	0.83	0.41	0.31	2.02	4.00	43.5
	45	0.75	0.33	0.22	2.27	4.90	36.2
	30	0.60	0.29	0.13	2.06	6.84	19.7
LSD at 0.05		0.01	0.01	0.002			1.5
LSD at 0.01		0.01	0.01	0.003			1.8

Table 5. Effect of putrescine treatment on the activities of α and β-amylases, invertase and protease [μg g^{-1}(f.m.) s^{-1}], and the contents of sugars and soluble proteins [mg g^{-1} (d.m.)] in leaves of alfalfa seedlings (21-d-old) under drought stress.

Put [mM]	FC [%]	α-Amylase	β-Amylase	Invertase	Protease	Reducing sugars	Soluble sugars	Polysaccharides	Soluble proteins
0	60 (control)	50.3	62.4	23.2	0.23	23.3	42.5	45.4	18.0
	45	44.2	59.3	25.3	0.52	44.6	60.3	13.3	15.7
	30	55.9	66.5	29.9	0.73	53.9	63.1	7.3	10.2
0.01	60	43.2	58.6	20.1	0.23	20.6	30.9	60.5	18.2
	45	40.1	58.6	21.7	0.52	36.4	54.3	28.5	15.8
	30	50.7	59.0	27.3	0.59	42.4	60.4	11.3	11.3
LSD at 0.05		1.1	3.5	0.3	0.03	2.2	2.0	2.0	0.3
LSD at 0.01		1.4	4.8	0.5	0.04	2.4	2.7	2.7	0.5

In leaves of alfalfa seedlings (21-d-old), activity of α- and β-amylases, decreased at the moderate drought stress (45 % F.C.), but increased at severe stress (30 % F.C.) while invertase and protease activities increased with increasing drought stress (Table 5). Increased amylase activities were correlated with the increased content of total sugars, while content of polysaccharides decreased. The increased protease activity was accompanied with reduced content of proteins. The reducing sugars content increased in parallel with the invertase activity. These results are in accordance with the findings of Pelleshi *et al*. (1997) and Chen and Liu (1999). One of the plant adaptations to drought stress is the increased content of organic solutes such as sugars and amino acids for osmoregulation (Zeid and El-Semary 2001).

The activities of α- and β-amylases, invertase and protease decreased in response to Put treatment. This finding may indicate that Put treatment partially alleviated the adverse effects of drought stress. Scaramagli *et al*. (1999) found that polyamines such as spermidine and spermine inhibit the activity of the acid protease *in vitro* by 70 %. The Put treatment increased contents of polysacchrides, proteins, Chl *a*, Chl *b* and carotenoids and photosynthetic activity (Hill reaction) under all levels of soil moisture. Zheleva *et al*. (1994) attributed the retention of photosynthetic pigments caused by PAs, to their interaction with thylakoid membrane. Subhan and Murthy (2001) reported that the activities of the whole chain of electron transport, photosystem 1 and 2, and absorbed excitation energy distribution in the favour of system 1, were protected by PAs. All these events suggest that Put application reduces the sensitivity of alfalfa to drought stress.

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