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Alterations in Nitrogen Metabolites after Putrescine Treatment in Alfalfa under Drought Stress

I.M. Zeid and Z.A. Shedeed
Department of Botany, Faculty of Science, Helwan University, Cairo 11792, Egypt

Abstract: Alfalfa (*Medicago sativa*, Siwa 1) seeds were subjected to drought stress during germination by using polyethylene glycol (PEG 4000) for studying the changes in some enzyme activities involved in nitrogen metabolism and the content of nitrogenous compounds during the first four days of growth after putrescine (Put) treatment. Decreasing the external water potential reduced activities of glutamate-pyruvate transferase (GPT), glutamate-oxaloacetate transferase (GOT) and RNase. Some free amino acids such as proline and glycine increased, while alanine and aspartic acid decreased. Nucleic acids content also decreased. Polyamines e.g., spermidine (Spd) and spermine (Spm) increased at the water potential -0.4 MPa. Put treatment increased activities of GOT, GPT and RNase. Furthermore, Put treatment increased nucleic acids content and the endogenous polyamines under drought stress. Drought stress was imposed during seedling stage by decreasing soil moisture content. GOT, GPT and RNase activities increased in leaves of alfalfa seedlings under drought stress. Soluble nitrogenous compounds accumulated under drought stress, while nucleic acids content decreased. Except glutamic acid, all free amino acids detected increased under drought stress. Put treatment decreased activities of GOT, GPT and RNase, as well as reduced the accumulation of the total soluble nitrogenous compounds, but increased DNA, RNA and protein contents.

Key words: Amino acids, enzyme activity, germination, nucleic acids, polyamines

INTRODUCTION

Polyamines e.g., putrescine, spermidine and spermine are a group of nitrogen containing compounds accumulate in plants in response to environmental stress. Polyamines are low molecular weight polycations and are present in all living organisms. Putrescine is the obligate precursor for spermidine and spermine. Polyamines are found in all organisms, in a wide variety of capacities. They bind to nucleic acids (Flink and Pettijohn, 1975), for example, play an essential role in growth of thermophilic bacteria (Muhtich *et al.*, 1983) and they are essential for cell viability and are correlated with, or required for a variety of physiological events. There are many reviews for polyamine involvement in various growth and developmental phases: cell division, embryogensis, rooting and flowering.

Many workers cleared the changes in nitrogen metabolism under water stress conditions. Rabe (1990) indicated that, any stress condition causing glucose depletion and/or reduced growth or impaired plant health will result in NH_3-NH_4 accumulation early in the stress period. The detoxification process in which excess free ammonia in the cell is removed results in the accumulation of nitrogen containing compounds. Cao *et al.* (2002) found that DNA and RNA content decreased in leaves of *Malus hupensis* (drought resistant) and *M. sieversii* (drought tolerant) under water stress, while DNase and RNase activities increased. The reduction in DNA and RNA content and increased activities of DNase and RNase were lower in the drought tolerant variety. The inhibition of DNase by polyamines *in vitro* is probably dependent on the interaction of the PAs with the substrate and not with the enzyme (Shoemaker *et al.*, 1983), though this may indicate a mechanism by which nucleic acid degradation can be prevented by polyamines *in vivo* (Kaur-Sawhney *et al.*, 1978). Ribonuclease activity in water stressed wheat was inhibited by soaking seeds in 10 mM of spermidine but stimulated by root treatment with this solution. In another experiment, potato tuber discs show a considerable rise in RNase activity on incubation for 24 h, but this can be completely blocked by spermidine or spermine (Altman, 1982). In addition, 5 mM spermidine applied to roots inhibited protease activity in both stressed and unstressed plants (Kubis *et al.*, 1991).

Zeid and Shedeed (2006) reported that Put treatment (0.01 mM) increased germination percentage of alfalfa seeds under drought stress, through increasing activity of the hydrolytic enzymes α- and β-amylases and

Corresponding Author: I.M. Zeid, Department of Botany, Faculty of Science, Helwan University, Cairo 11792, Egypt

invertase during germination. Moreover, Put treatment increased growth criteria of alfalfa seedlings and alleviated the adverse effects of drought stress through increasing polysaccharides, protein and chlorophyll content and Hill reaction activity, while the hydrolytic activity decreased.

The present study aims to elucidate the variations in the nitrogenous compounds as affected by Put treatment in alfalfa grown under drought stress. Activity of GOT, GPT and RNase, as well as the changes in the free amino acid pool during germination and seedling stages were investigated. Endogenous polyamines e.g., putrescine, cadaverine, spermidine and spermine were also investigated during germination stage.

MATERIALS AND METHODS

Seeds of alfalfa (*Medicago sativa* L. siwa 1) were sterilized with sodium hypochlroite 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. They were then transferred to sterile Petri-dishes containing two sheets of Whatman No.1 filter paper moistened with 10 mL distilled water (control), or subjected to water stress by using 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, drought stress was induced by decreasing the water supply to the soil to be subjected to 30, 45, or 60% of the field capacity after one week from sowing. Plants grown at 60% of the field capacity were considered as control plants. Hoagland's nutrient solution with half-strength was used for irrigation. Putrescine (0.01 mM) was added once to the nutrient solution.

Chemical analysis: Borate buffer (pH 8.0) extract of dry leaves was used according to Naguib (1969) for determination of soluble nitrogenous compounds e.g., free ammonia-N, nitrate-N, amino acid-N, peptide-N and total soluble-N. Total nitrogen was measured by digesting the dry leaves in 50% sulphoric acid and 35% perchloric acid and its ammonia content was estimated using Borthelot reaction which carried out according to Chaney and Marbach (1962). DNA was measured according to Dische and Schwartz (1973) by using diphenylamine reagent. RNA was determined using the method adopted by Ashwell (1957) using orcinol reagent. Ribonuclease enzyme was assayed by the method described by Malik and Singh (1984). The transferring enzymes, glutamate-pyuvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) were assayed according to the method described by Horsman and Wellburn (1975). Extraction and separation of free amino acids was carried out by thin-layer chromatography technique according to Toro *et al.* (2003). Polyamines determination was carried out by HPLC (Redmond and Tseng 1979) in the National Research Center, Giza, Egypt.

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

PEG in the external medium reduces the available water required for absorption and germination of seeds, which affect germination rate and the final germination percentage (Zeid and Shedeed, 2006). The present results showed a gradual reduction in DNA and RNA contents with decreasing the external water potential, indicating a great reduction in growth rate. This was associated with a decline in some enzyme activities e.g., RNase and the aminotransferases GOT and GPT (Table 1) due to the low water content required for enzyme activity and germination events. However, Zeid and Shedeed (2006) observed a significant increase in protease activity during germination of alfalfa seeds, which may result in more production of amino acids and consequently sugars, through the process of gluconeogenesis, which transfer to the growing embryo axes to compensate the reduced sugar content under drought stress. Put treatment (0.01 mM) significantly increased DNA and RNA contents and stimulated the activity of RNase, GOT and GPT during germination under all levels used of water potentials (from 0 to -0.8 MPa) may be due to improving the water use efficiency of the germinating seeds.

Table 1: Effect of putrescine treatment on the activities of RNase, GOT and GPT ($\mu g\ g^{-1}$ f.m. s^{-1}) and nucleic acids content ($\mu g\ g^{-1}$ f.m.) during germination of alfalfa seeds under PEG-induced water stress (4-day-old)

Put (mM)	Ψs (MPa)	RNase	GOT	GPT	DNA	RNA
0	Control	24.2	0.035	0.043	10.0	2.0
	-0.2	21.5	0.032	0.041	9.2	1.1
	-0.4	19.1	0.029	0.037	8.7	0.6
	-0.6	15.5	0.025	0.034	7.5	0.2
	-0.8	12.7	0.021	0.031	5.9	0.1
0.01	0	25.9	0.042	0.049	10.6	4.1
	-0.2	22.8	0.036	0.044	9.9	3.1
	-0.4	21.6	0.032	0.040	9.5	1.1
	-0.6	20.0	0.026	0.037	8.0	0.7
	-0.8	14.6	0.024	0.033	6.6	0.3
LSD at 0.05		0.42	0.002	0.001	0.3	0.2
LSD at 0.01		0.60	0.003	0.002	0.4	0.3

Table 2: Effect of putrescine treatment on the endogenous polyamines ($\mu g\ g^{-1}$ f.m.) content in alfalfa under PEG-induced water stress (4-day-old)

Put (mM)	Ψs (MPa)	Putrescine	Cadaverine	Spermidine	Spermine	Total polyamines
0	Control	46.39	3.36	5.26	nd	55.01
	-0.4	45.45	3.18	31.92	2.41	82.96
	-0.8	1.93	0.18	2.8	nd	4.91
0.01	0	53.18	4.20	5.08	nd	62.46
	-0.4	61.19	5.93	68.57	3.44	139.13
	-0.8	35.2	2.21	23.15	2.14	62.7

ND: Not Detected

Decreasing the water potential to -0.8 MPa considerably decreased polyamines content. However, in response to water stress at the moderate level (-0.4 Mpa), Spd and Spm contents increased and the increment of Spd was more obvious (Table 2). This response may be involved in mechanisms of adaptation to water deficit stress in alfalfa. Put treatment increased the polyamines content under drought stress and the highest level was observed at -0.4 MPa. Many workers e.g., Serafini *et al.* (1980) and Kadioglu *et al.* (2002) indicated a rise in polyamines content and synthesis before and during nuclear DNA synthesis in plants. Stimulation of rapid PA synthesis probably one of the first events occurring after dormancy break (Komada *et al*., 1994). Bachrach (1973) suggested that at cellular pHs, PAs are polycations and thus bind readily to such important cellular polyanions as DNA, RNA, phospholipids and acidic protein residues. PAs also facilitate conformational changes, which do have serious consequences for DNAs protein interaction in the cell (Heby and Persson, 1990). PAs stabilize other double helical structures such as stems and loops in mRNA, rRNA and tRNA conformation through binding to specific sites, which may be the basis of polyamines stimulatory effects on DNA, RNA and protein.

Variations in the balance of the free amino acid pool after four days from sowing under different levels of external water potentials were illustrated in comparison with control unstressed seeds (Fig. 1a). The rearrangement of the amino acid pool involved a strong increase in the amount of glycine, glutamate and isoleucine, followed by valine and proline and then serine and arginine, while the aspartic acid and alanine decreased. The decreased content of aspartate and alanine under water stress may be due to the reduced activity of GOT and GPT, which resulted also in the accumulation of glutamate. Put treatment, on the other hand, increased GOT and GPT activities during germination to reduce the decreased values of aspartate and alanine. Glycine, glutamate, valine and proline content also increased in Put treated seeds (Fig. 1b).

Table 3: Effect of putrsecine treatment on the activities of RNase, GOT and GPT ($\mu g\ g^{-1}$ f.m. s^{-1}) and nucleic acids content ($\mu g\ g^{-1}$ f.m.) in leaves of alfalfa seedlings under drought stress (21-day-old)

Put (mM)	FC (%)	RNase	GOT	GPT	DNA	RNA
0	60 (Control)	2.21	0.24	0.19	1.10	2.59
	45	4.01	0.33	0.26	0.90	2.03
	30	5.50	0.37	0.34	0.79	1.85
0.01	60	2.00	0.18	0.17	2.20	3.20
	45	3.30	0.28	0.23	1.20	2.75
	30	4.90	0.35	0.31	0.95	2.13
LSD at 0.05		0.21	0.01	0.01	0.22	0.30
LSD at 0.01		0.30	0.02	0.02	0.35	0.45

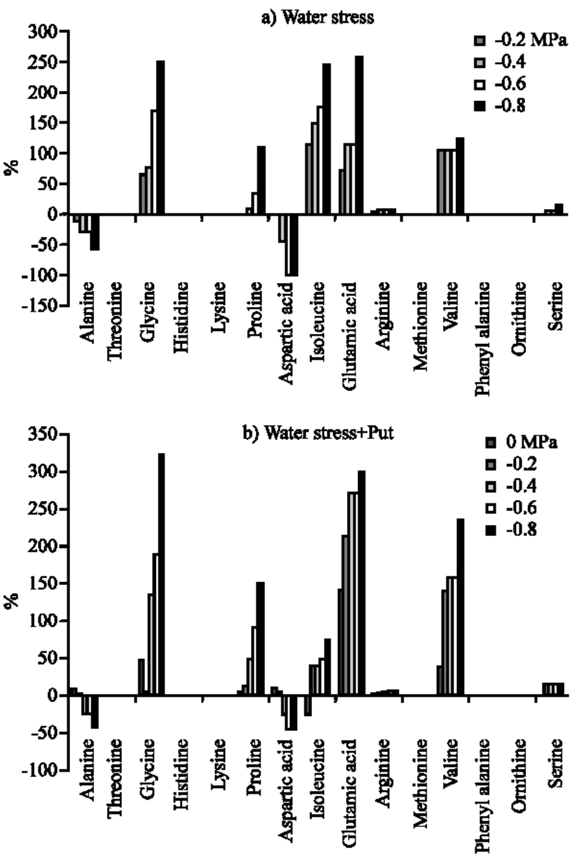

Fig. 1: Variations in the composition of the free amino acid pool in alfalfa seedlings (4-day-old) as affected by Put treatment and PEG-induced water stress, as compared with the control untreated seedlings. Data show how each single amino acid increase or decrease its percentage proportion in the free amino acid pool following the treatment

Activities of RNase, GOT and GPT were reversed in the seedling leaves (21-day-old) compared with the germinating seeds (4-day-old). Their activities increased with decreasing the soil moisture content (Table 3). The increased activity of RNase indicates a reduction in growth rate, while the increased activity of GOT and GPT

Table 4: Effect of putrescine treatment on nitrogenous compounds (mg g^{-1} d.m.) content in shoots of alfalfa seedlings under drought stress (21-day-old)

Put (mM)	FC (%)	Amm-N	Nitrate-N	Amino acid-N	Peptide-N	Total soluble-N	Total insoluble-N	Total-N
0	60 (Control)	0.370	1.73	5.30	11.3	25.3	45.0	70.3
	45	0.553	1.95	6.22	11.5	27.9	42.4	70.3
	30	0.690	2.10	8.50	14.6	30.0	37.3	67.3
0.01	60	0.162	1.43	3.93	11.2	23.0	48.9	71.9
	45	0.376	1.67	5.10	11.5	25.6	44.9	70.5
	30	0.470	1.80	6.70	12.6	28.8	40.9	69.7
LSD at 0.05		0.01	0.06	0.5	0.3	0.4	0.4	0.4
LSD at 0.01		0.02	0.08	0.7	0.5	0.6	0.6	0.6

explain the accumulation of amino acids under drought stress. Soluble nitrogenous compounds e.g., ammonia, nitrate, amino acids, peptides, as well as the total soluble nitrogen increased under drought stress, while the total insoluble nitrogen and the total nitrogen content decreased (Table 4). Accumulation of soluble nitrogenous compounds may play an important role in cellular osmotic regulation under drought stress conditions (Zayed and Zeid, 1998; Zeid and El-Semary, 2001). The reduction in protein-N (or insoluble-N) content in plants grown under water deficit conditions might be attributed to decreased synthesis and increased protease activity as suggested by Rose (1988). The accumulated ammonia under drought stress conditions supports the postulation of Miflin and Lea (1980), that the drought-induced ammonia accumulation may be due to the diminution of anabolic processes (e.g., protein synthesis and growth), mean while, there is no feedback inhibition for N uptake and nitrate reduction, leading to subsequent detoxification by sequestering ammonia into nitrogen-containing compounds (e.g., proline). Reddy *et al.* (1990) suggested that the higher activities of NAD and NADP-dependent glutamate dehydrogenase enzymes may form a mechanism to assimilate excess ammonia under drought stress. The observed accumulation of nitrate-N with increasing water deficit may be explained by the observation of Reddy *et al.* (1990) and Zeid (2004) who recorded a decrease in nitrate reductase activity under drought stress conditions and Foyer *et al.* (1998) who found that this reduction was accompanied with a decrease in nitrate reductase transcript. Put treatment reduced the accumulation of the soluble nitrogenous compounds and increased the insoluble and the total nitrogen content in drought stressed and unstressed plants, due to enhancing the incorporation of peptide-N into protein synthesis.

Free amino acids e.g., aspartate, alanine and proline strongly increased in the seedling leaves (21-day-old), followed by serine, valine and arginine and then threonine, glycine and histidine, while glutamate markedly decreased with the progressive reduction in soil moisture content (Fig. 2a). Put treatment increased the accumulation of some amino acids e.g., proline, valine, aspartic acid, glycine and threonine, as well as increased

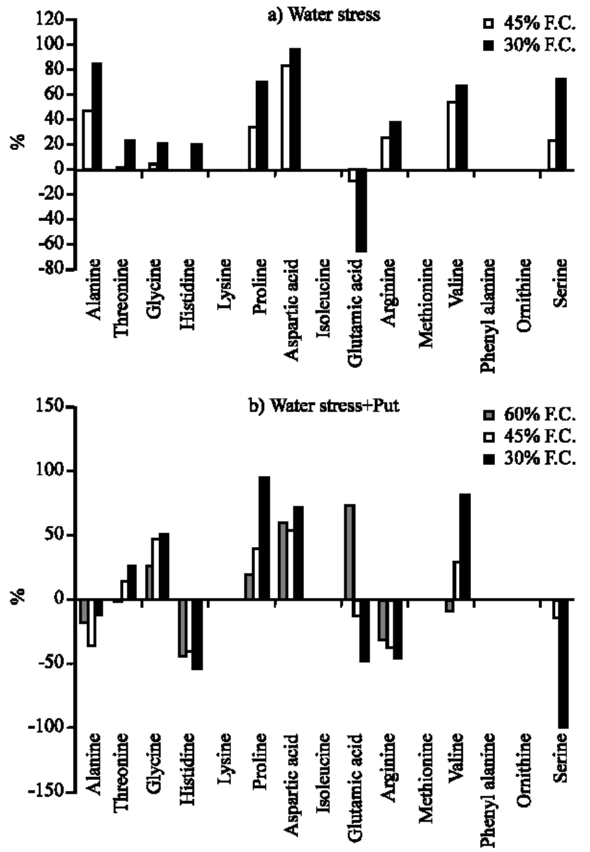

Fig. 2: Variations in the composition of the free amino acid pool in leaves of alfalfa seedlings (21-day-old) as affected by Put treatment under drought stress, as compared with the control untreated seedlings. Data show how each single amino acid increase or decrease its percentage proportion in the free amino acid pool following the treatment

the glutamate content as compared with the Put-untreated seedlings. Other amino acids e.g., serine, arginine, histidine and alanine decreased by Put treatment (Fig. 2b). The accumulation of proline may be attributed to its stimulated synthesis via glutamate pathway (Buhl and Stewart, 1983), or due to the inhibition of proline dehydrogenase, or proline oxidase under stress conditions (Kumari and Veeranjaeyulu, 1996). The increased level of arginine may be attributed to its synthesis from glutamate (as amine donor) through ornithine (Ranieri *et al*., 1989). Photorespiration may rise briefly as stress develops and stomata close (Clarke and Dureley, 1984). This may explain the increase in glycine and serine content. The observed increase in aspartate and alanine agree with the observations of Reddy *et al*. (1990) who pointed out that the enhanced activities of GOT and GPT may suggest the conversion of keto acids into amino acids in the presence of accumulated ammonia. The increased accumulation of alanine and aspartic acid was correlated with the increased activity of GPT and GOT enzymes, respectively under drought stress, which was accompanied with a reduction in glutamate content. Put treatment resulted in reducing the accumulation of alanine and aspartate due to the decreased activities of GPT and GOT, which were associated with increased accumulation of glutamate in comparison with the untreated seedlings.

Drought stress was also accompanied with a reduction in nucleic acids content in the seedling leaves. This may be correlated with the increased activity of RNase under drought stress (Table 3). Similar results have been obtained by Chen and Liu (1999) and Barathi *et al*. (2001). Put treatment decreased activity of RNase at all levels used of soil moistures. These results support the findings of Altman (1982) who found that RNase activity could be completely blocked by spermidine or spermine. This may indicate a mechanism by which nucleic acid degradation can be prevented by polyamines which stabilize the double helical structures of DNA and RNA as suggested by Shoemaker *et al*. (1983) and Heby and Persson (1990).

In conclusion, Put treatment increased enzyme activities e.g., the aminotransferases GOT and GPT during germination stage, which provide the embryo axes with the required amino acids for growth, but reduced their activities in the leaves during the seedling stage in comparison with the untreated seedlings, indicating an improve in water use efficiency under drought stress. Put treatment reduced RNase activity and increased nucleic acids content in the drought stressed leaves. It also reduced the total soluble nitrogen content, while the total insoluble nitrogen content increased i.e., increased the anabolic processes during seedling stage. These positive effects of Put treatment may be attributed to the increased polyamines content, which in turn stimulate DNA, RNA and protein synthesis.

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