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ORIGINAL PAPER

Effect of arginine and urea on polyamines content and growth of bean under salinity stress

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Abstract Prior to sowing, seeds of bean (*Phaseolus vulgaris* L.) were treated with 4 mM arginine or 0.1% urea, as nitrogen source. The seeds were then subjected to salinity stress. Arginine and urea treatments stimulated germination of both unstressed and salinity-stressed seeds. It was interesting to observe that the increased germination percentage in response to arginine and urea treatments was associated with increased content of polyamines, particularly putrescine (Put), spermidine (Spd) and spermine (Spm). Growth of the seedlings was also improved by application of arginine and urea, which was also associated with increased content of the polyamines Spd and Spm, while the Put content decreased. Total soluble sugars were much accumulated in response to arginine and urea treatments under salinity stress for cellular osmoregulation. The ratio of K^+/Na^+ increased in the leaves by application of arginine and urea, indicating a more alleviation to the adverse effects of salinity stress. Changes in proteinogenic amino acids were also investigated.

Keywords Amino acids · Germination · Growth · K/Na ratio · Polyamines

Abbreviations

Cad	Cadaverine
Put	Putrescine
Spd	Spermidine
Spm	Spermine

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Introduction

Robe (1990) reported that a number of N-containing compounds (NCCs) accumulate in plants subjected to environmental stress (e.g., mineral deficiencies, water and salinity stress, temperature stress, acid stress, pathological stress and exogenously supplied ammonia). The most frequently accumulating NCCs include amides (glutamine and asparagine), amino acids (arginine, proline, citruline, ornithine) and the amine, putrescine (Put). The specific NCCs that accumulate are determined by the stressed plant species and the nature of the stress. Tabatabaei (2006) reported that increasing N concentration up to 200 mg l^{-1} of NH_4NO_3 in salt-sensitive cultivars of olive is favorite in counteracting the adverse effects of salinity.

Salinity is one of the major abiotic stresses affecting plant agriculture worldwide (Jimenez et al. 2007). Gama et al. (2007) mentioned that plants growing under saline conditions are stressed basically in three ways: (1) reduced water potential in the root zone causing water deficit, (2) phytotoxicity of ions such as Na^+ and Cl^- and (3) nutrient imbalance by depression in uptake and/or shoot transport. This is attributed to the fact that Na^+ competes with K^+ for binding sites essential for cellular function (Tester and Davenport 2003). Flores et al. (1990) and Tamai et al. (1999) reported that plants subjected to salt stress suffer from growth suppression, which may be via changing their endogenous polyamine contents. The inhibitive effect of salt on seed germination and seedling growth was alleviated in varying degrees, and dramatically, by polyamine pretreatments, particularly with lower levels of salt (Kürsat et al. 2007). Santa et al. (1997) suggested that the initial polyamine accumulation in leaf discs of tomato was due to the osmotic effect induced by salinity, but the levels rapidly began to decrease as the saline ions were accumulated

from the medium. Capell et al. (2004) found that transgenic pants expressing *Datura adc* produced much higher levels of Put under stress, promoting Spd and Spm synthesis and ultimately protecting the plants from drought. Groppa and Benavides (2008) reported that the rise in Put is mainly attributed to the increase in arginine decarboxylase (ADC) activity as consequence of the activation of ADC genes and their mRNA levels. On the other hand, free radicals are now accepted as important mediators of tissue injury and cell death. The polycationic nature of polyamines, positively charged at physiological pH, has attracted the attention of researches and has led to the hypothesis that polyamines could affect physiological systems by binding to anionic sites, such as those associated with nucleic acids and membrane phospholipids. These amines, involved with the control of numerous cellular functions, including free radical scavenger and antioxidant activity, have been found to confer protection from abiotic stresses but their mode of action is not fully understood yet. However, the structure of many types of proteins and the activities of many enzymes are modulated by polyamine binding (Drolet et al. 1986; Lovass 1991). Exogenous application of Put or its precursor arginine successfully overcomes the harmful effects induced by NaCl in stressed plants, e.g., rice (Line and Kao 1995), wheat (El-Shintinawy and Hassanein 2001) and bean (Zeid 2004). Harms and Langebartels (1985) found that the increased urea concentration in the nutrient solution markedly increased Put contents of *Phaseolus vulgaris* cell suspensions.

From the above-mentioned reviews on the role of polyamines in protection of stressed plants, the present study aimed to stimulate the polyamine synthesis by the application of a nitrogen fertilizer such as urea, in comparison with arginine as the precursor for Put synthesis, to counteract the adverse effects of salinity on germination and growth of bean. Activity of the hydrolytic enzymes, amylase and protease, was assayed during germination period. Photosynthetic pigments, photosynthetic activity (Hill reaction), photosynthate products (sugars), as well as the ratio of K/Na ions were measured in response to arginine and urea pretreatments. Changes in the proteinogenic amino acids composition were also studied.

Materials and methods

Plant material

In a preliminary experiment, seeds of bean (*P. vulgaris* L.) were immersed in a series of concentrations of urea, or arginine for 12 h at 25°C. Then, the seeds were germinated in sand culture and supplied with Hoagland's nutrient solution, which was adjusted to one of the following osmotic potentials: −0.2, −0.4, −0.6 and −0.8 MPa by adding NaCl and $CaCl_2$ (Zayed and Zeid 1998). The moisture of the sand culture was maintained at 60% of the field capacity all over the experimental period. It has been observed that the plumule growth and the development of the first two foliage leaves were inhibited at −0.4 MPa. However, the presowing treatment with 0.1% urea and 4 mM arginine was the most effective concentrations for stimulation of the plumule development and seedling growth under this osmotic potential. Therefore, the seeds were grown at −0.2 MPa of salt stress in presence or absence of urea or arginine.

Enzyme activity

Activity of the hydrolytic enzymes, amylase and protease was assayed in the germinating seeds after 4 days from sowing. The cell-free extract of the plant material was prepared at 0–4°C by macerating the seeds with a chilled-pestle and mortar. 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 amylase, soluble starch was used as the enzyme substrate in phosphate buffer (pH 7.0) and the produced maltose was measured by using 3,5-dinitrosalicylic acid reagent as described by Rick and Stegbauer (1974). Protease activity was assayed according to the method described by Gallop et al. (1957) using casein as substrate in phosphate buffer (pH 7.2) and Folin phenol as the color reagent for measuring the produced peptides.

Chemical analyses

Polyamines content was measured in the growing seeds after 4 days from sowing, as well as in the fresh leaves of 21-day-old seedlings. Determination of polyamines was carried out by HPLC (Redmond and Tseng 1979) in the National Research Center, Giza, Egypt. Photosynthetic pigments [chlorophyll (Chl *a*, Chl *b*) and carotenoids (Car.)] were estimated in 85% acetone-extracted leaves according to Metzner et al. (1965). Photosynthetic activity was measured as Hill reaction activity of the isolated chloroplasts. 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°C (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 1,000*g*. Chloroplasts (residue) were resuspended in the buffer solution. Hill reaction of the isolated chloroplasts was

measured by using potassium ferricyanide as electron acceptor.

Proteinogenic amino acids were assayed in the leaves by using Eppendorff Biotronik Amino Acid Analyzer model 300 in the National Research Centre, Giza, Egypt. Fresh leaves and roots of bean seedlings were extracted in 70% ethanol and completed to a known volume with distilled water and used for estimation of total soluble sugars using anthrone reagent (Umbreit et al. 1959). Potassium and sodium ions were measured photometrically in acid-digested samples using a Corning-400 Flame Photometer.

Statistical analysis

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

Arginine treatment increased germination percentage of control seeds from 76 to 86%, while the urea treatment increased it to become 96%. Salt-induced stress (−0.2 MPa) reduced germination percentage, which may be due to the osmotic stress and the toxic effect of sodium and chloride ions, as reported by Waisel (1989). Treatments with arginine and urea also increased germination percentage of salt-stressed seeds from 72% to become 79 and 80%, respectively (Table 1). Activity of amylase enzyme significantly decreased under salinity stress, suggesting a reduction of the translocated sugars into the embryo axes during germination and early growth. On the other hand, activity of protease enzyme markedly increased under salinity stress (Table 1). These results support the view of Zeid and Shedeed (2006) who reported that the increased activity of protease enzyme under stress conditions may compensate the reduced sugar content during germination through the process of gluconeogenesis. Arginine and urea treatments significantly increased amylase and protease activity in control and salt-stressed seeds during germination. Reduction of germination percentage under salt-induced stress was associated with a reduction in polyamines content (Table 1). However, arginine and urea treatments increased the total content of polyamines, particularly Put, Spd and Spm. The increment was more obvious when using urea. These results suggest that the reduction in germination percentage under salinity stress could be partially attributed to the decline in polyamines content, which may also affect the enzyme activities. Benavides et al. (1997) concluded that polyamines might be involved in the germination process and in response to salinity-induced stress. Increasing germination percentage in response to arginine and urea treatments could also be attributed to the enhancement of polyamines synthesis, particularly Put, Spd and Spm, which may stimulate activity of the hydrolytic enzymes.

Salinity-induced stress considerably reduced plant growth criteria, e.g., root and shoot lengths, leaf fresh and dry masses, and leaf area of bean seedlings. Lovelli et al. (2000) recorded similar results and added that salinity increases leaf senescence. However, all growth criteria were significantly increased by application of arginine (4 mM) and urea (0.1%) under control and salt-induced stress conditions (Table 2). It was interesting to observe that Put and Cad content increased in leaves of salt-stressed seedlings (Table 2), while Spd decreased and Spm was not affected. However, arginine and urea treatments resulted in increasing growth criteria of the seedlings, which was associated with increased content of Spd and Spm, whereas Put and Cad levels declined. These results support the postulations of Tiburcio et al. (1986) and Capell et al. (2004) who suggested that the polyamines Spd and Spm have a protective role under adverse environmental conditions. The increment was more obvious when using urea.

Table 1 Effect of arginine and urea on germination percentage (%), amylase activity [μg maltose g^{-1} (f.m) s^{-1}], protease activity [μg peptides g^{-1} (f.m) s^{-1}] and polyamines content (μg g^{-1} f.m.) of bean seeds during germination (4-day-old)

Treatments	Germ.	Amylase	Protease	Put	Cad	Spd	Spm	Total polyamines
Control	76	2.1	0.029	2.04	0.02	2.46	1.72	6.24
Arg.	86	2.6	0.046	12.21	nd	4.03	7.24	23.48
Urea	92	2.67	0.052	5.62	0.34	30	23.1	59.06
Salts	72	1.22	0.066	0.454	0.09	1.22	nd	1.764
Salts + Arg.	79	1.76	0.042	0.625	0.18	1.23	4.13	6.165
Salts + urea	80	1.81	0.044	1.59	nd	1.9	5.86	9.37
LSD at 0.05		0.21	0.013					
LSD at 0.01		0.29	0.017					

nd not detected

Table 2 Effect of arginine and urea on growth [root and shoot lengths (cm), leaf fresh and dry masses (mg), leaf area (cm^2)] and polyamines content (μg g^{-1} f.m.) in leaves of bean seedlings

Treatments	Root length	Shoot length	Leaf f.m.	Leaf d.m.	Leaf area	Put	Cad	Spd	Spm	Total polyamines
Control	4.9	28	1.84	0.184	13.6	3.13	0.03	4.38	2.06	9.6
Arg.	5.5	35.1	2.493	0.226	18.3	4.07	nd	4.91	3.44	12.42
Urea	5.8	34.2	2.727	0.247	18.3	11.5	1.34	5.78	5.5	24.12
Salts	4.4	24.1	1.232	0.166	6.7	5.56	1.46	2.45	2.06	11.53
Salts + arg.	5.2	30.1	1.628	0.188	12.6	1.3	0.01	4.56	6.82	12.69
Salts + urea	5.1	29.9	2.248	0.242	13.9	2.78	0.07	13.68	29.65	46.18
LSD at 0.05	0.4	4	0.02	0.002	2.1					
LSD at 0.01	0.6	5.6	0.03	0.003	2.7					

nd not detected

Table 3 Effect of arginine and urea on photosynthetic pigments [mg g^{-1}(d.m.), photosynthetic activity [μ mol (ferricyanide) g^{-1} (chlorophyll) s^{-1}], total soluble sugars content [mg g^{-1} d.m.] and their percent of increase or decrease from the control, and K^+/Na^+ ratio in leaves and roots of bean seedlings

Treatment	Chl *a*	Chl *b*	Car	Total pigments	Photosynthetic activity	Sugars in leaves	Percentages of changes	Sugars in roots	Percentages of changes	K/Na in leaves	K/Na in roots
Control	11.94	7.61	2.52	22.07	9.49	105.5	0	19.26	0	414	10.06
Arg.	13.92	8.48	3.05	25.45	12.74	103	−2.3	28.24	46.6	414	13.22
Urea	14.2	8.91	3.06	26.17	13.04	101.5	−3.7	26.31	36.6	437	15.59
Salts	9.04	6.47	1.8	17.31	7.19	110.7	4.9	40.27	109.1	52.11	4.45
Salts + arg.	12.62	8.05	2.55	23.22	9.93	135.6	28.5	51.46	167.2	62.37	4.69
Salts + urea	13.67	8.48	2.68	24.83	10.7	124.4	17.9	45.52	136.3	84.26	4.81
LSD at 0.05	1.99	0.36	0.87		2.21	5.1		1.3			
LSD at 0.01	2.86	0.52	1.24		3.29	7.4		1.9			

The reduction of growth under salinity-induced stress was paralleled with a reduction in total photosynthetic pigments, e.g., Chl *a*, Chl *b* and Car. content, as well as the photosynthetic activity (Hill reaction activity). Application of arginine and urea significantly increased chlorophyll content and activity of photosynthesis (Table 3), which in turn led to increasing growth under salinity-induced stress. This effect of arginine and urea could be partially attributed to the increased content of polyamines (PAs). Besford et al. (1993) attributed the positive effects of PAs on chlorophyll and carotenoid levels to preservation of the thylakoid membranes at the site of chlorophyll–protein complex. Demetriou et al. (2007) observed that certain polyamine changes (putrescine reduction) were correlated with changes in the structure and function of the photosynthetic apparatus, such as the increase in the functional size of the antenna and the reduction in the density of active photosystem II reaction centers. They concluded that the exogenously added Put was used to compensate for this stress condition and to adjust the above-mentioned changes, so that to confer some kind of tolerance to the photosynthetic apparatus against enhanced NaCl-salinity and permit cell growth even in cell concentrations that under natural conditions would be toxic.

Variations in the composition of proteinogenic amino acids as affected by arginine and urea treatments under salinity-induced stress were indicated in Fig. 1, as percentage of increase or decrease from the control. Salinity-induced stress highly increased the leaf content of histidine, alanine, isoleucine, tyrosine and lysine; followed by leucine, serine, threonine and glycine; and then glutamate and phenylalanine. On the other hand, salinity decreased proline, aspartate, valine and to some extent, arginine content. Reduction of these proteinogenic amino acids may indicate their accumulation in the free amino acid pool, for cellular osmoregulation (Zeid 2007). Arginine and urea treatments much increased the cellular content of arginine, glutamate, phenylalanine and tyrosine, whereas they reduced the increment of serine, glycine, alanine, leucine, isoleucine and histidine, but much decreased the proteinogenic proline, aspartate and glycine under salinity stress, which may be due to their accumulation in the free amino acid pool via the proteolytic activity. This alteration in the proteinogenic amino acids under salinity stress, as well as in response to arginine and urea treatments, indicates occurrence of some changes at the gene expression level.

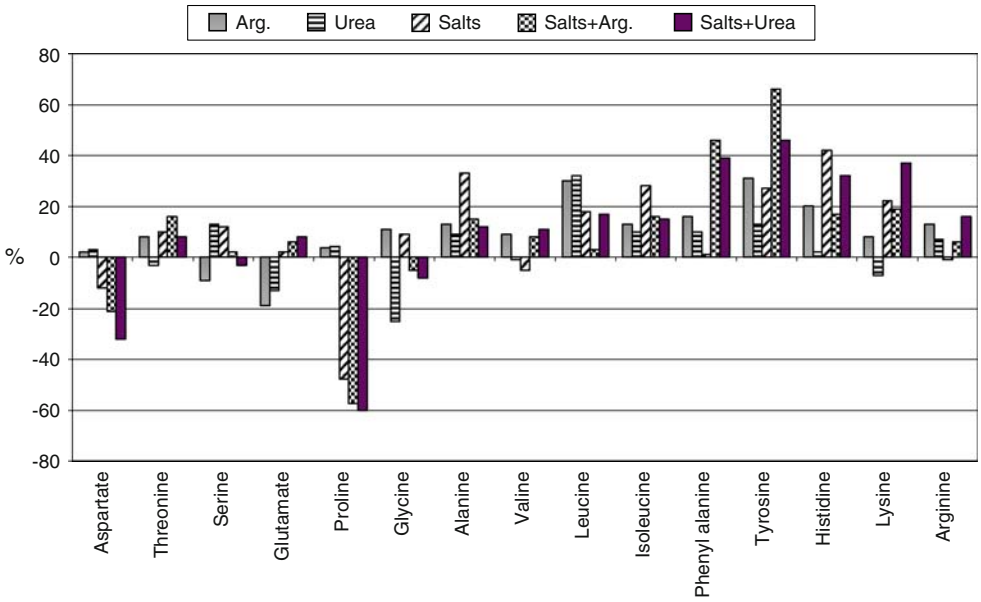

Fig. 1 Variations in the composition of the proteinogenic amino acids in leaves of bean seedlings (21-day-old), as affected by arginine and urea treatments under salinity stress, as compared with the control untreated seedlings. Data show how each single amino acid increase or decrease its percentage proportion following the treatments

Plant analysis showed a much accumulation of Na ions in roots of salt-stressed plants. Therefore, the ratio of K^+/Na^+ was very low in the roots, particularly under salt stress. On the other hand, K^+/Na^+ ratio was very high in leaves of control plants, but it was markedly reduced under salinity stress. Tester and Davenport (2003) reported that Na^+ in salt-stressed plants compete with K^+ for binding sites essential for cellular function. However, application of arginine and urea increased K^+/Na^+ ratio, particularly in the leaves. These results indicate an improvement in salinity tolerance, since the sodium ion concentration decreased in leaves of bean seedlings treated with arginine and urea. The effect of urea was more pronounced (Table 3).

Sugars, as organic solutes, were accumulated in leaves and roots of salt-stressed plants, but their accumulation was higher in response to arginine and urea application (Table 3). The increased content of organic solutes is considered as a very important phenomenon for cellular osmoregulation under drought stress (Zayed and Zeid 1998) and salt stress (Zeid 2004) to enable root cells to absorb water under low water potential. Moreover, it was interesting to observe that the leaf content of total soluble sugars was much higher than in the roots, but the percent of increasing under salinity-induced stress was much higher in the roots than in the leaves, indicating adaptation of the root cells to the osmotic stress.

Conclusion

The reduction of germination percentage of bean seeds under salinity-induced stress could be partially attributed to the decline in polyamines content. Arginine and urea treatments increased germination percentage of both control and salt-stressed seeds via the increased content of the polyamines Put, Spd and Spm, which may stimulate activity of the hydrolytic enzymes amylase and protease during germination. Arginine and urea treatments significantly increased growth, chlorophyll content and photosynthetic activity of the seedling leaves, which could be attributed to the increased content of Spd and Spm under control and salt stress conditions. These results suggest that Spd and Spm had an important role in alleviation of the adverse effects of salinity during germination and early growth of seedlings. Alterations in the proteinogenic amino acids content under salinity and in response to arginine and urea pretreatments indicate alterations at the gene expression level.

The decreased content of sodium ions in salt-stressed leaves in response to arginine and urea indicates an increase in salinity tolerance. Arginine and urea treatments also increased the accumulation of soluble sugars, particularly in the roots for cellular osmoregulation against the external osmotic stress of salts.

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