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Effect of kinetin on starch and sucrose metabolising enzymes in salt stressed chickpea seedlings

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Abstract

Higher amylase activity in cotyledons of kinetin treated salt stressed (75 mM NaCl) chickpea (*Cicer arietinum* L. cv. PBG-1) seedlings, as compared to salt stressed seedlings was observed during a growth period of 7 d. The activities of acid and alkaline invertases were maximum in shoots and minimum in cotyledons under all conditions. The reduced shoot invertase activities under salt stress were enhanced by kinetin with a simultaneous increase in reducing sugar content. Kinetin increased the activities of sucrose synthase (SS) and sucrose phosphate synthase (SPS) in both the cotyledons and shoots of stressed seedlings. Kinetin appears to increase the turnover of sucrose in the shoots of stressed seedlings.

Additional key words: amylase, *Cicer arietinum*, invertase, sucrose synthase, sucrose phosphate synthase.

Introduction

Salinity induced growth reduction could be due to osmotic stress and toxic level of ions within plants (Munns *et al.* 1995, Kinraide 1999). In chickpea salt stress has been reported to reduce growth, photosynthesis, nodulation and activities of the enzymes of sucrose breakdown (Soussi *et al.* 1998, 1999). The accumulation of sucrose under salt stress as a result of decreased sucrolytic activity might be important in growth of tomato fruits (Balibrea *et al.* 1999). Plant growth regulators have been found to play an important role in plant responses to stress (Amzallag *et al.* 1990, 1992). Growth reduction induced by salt stress could be due to the altered endogenous hormonal contents as decreased cytokinin and gibberellic acid and increased abscisic acid contents have been reported in plants growing under salt stress (Kuiper *et al.* 1988, Roy *et al.* 1995). Therefore, a study on the effect of plant growth regulators on the enzymes of sugar metabolism under salt stress can provide a relevant information about the biochemical basis of responses of plants to salt stress. In this paper, the effect of kinetin on enzymes of starch mobilization and sucrose metabolism in chickpea seedlings growing under salt stress is discussed.

Materials and methods

Germination of seeds: Chickpea (*Cicer arietinum* L. cv. PBG-1) seeds were washed with tap water, sterilized with 0.1 % $HgCl_2$ for 5 min, again washed thoroughly with sterilized water under aseptic conditions and then cultured aseptically, in 250 cm^3 conical flasks, on Murashige and Skoog (1962) medium, without sucrose. In the second and third sets of treatments, 75 mM NaCl or 75 mM NaCl + 6 μM kinetin were added. The flasks were kept in an incubator at 25 ± 1 °C under dark. The chickpea seedling consists of cotyledon, shoot and root. The activities of enzymes and contents of reducing sugar and sucrose were determined in cotyledons, shoots and roots separately at 3, 5 and 7 d after sowing (DAS).

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Abbreviations: DAS - days after sowing; SS - sucrose synthase; SPS - sucrose phosphate synthase.
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Extraction and determination of enzyme activities: Amylases were extracted by crushing the tissues (100 - 500 mg fresh mass) with 20 mM sodium acetate buffer (pH 5.0) containing 1 mM $CaCl_2$. Amylase activity was determined according to Miyagi *et al.* (1990) and reducing sugars formed were estimated as described previously (Kaur *et al.* 1998).

Sucrose synthase (SS) and sucrose phosphate synthase (SPS) were extracted by grinding the tissues (100 - 500 mg) in a chilled mortar with a pestle with 3 - 4 cm^3 of 100 mM HEPES (pH 8.2), containing 10 mM EDTA, 15 mM KCl, 5 mM $MgCl_2$, 2 mM sodium diethyl dithiocarbamate and 5 mM β-mercaptoethanol (Kerr *et al.* 1987). Insoluble polyvinylpolypyrrolidone (100 mg per g tissue) was also added while extracting the enzymes. The extract was centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatant was made free from reducing sugars by chromatographing it on *Sephadex G-25* column using 10 mM HEPES buffer (pH 7.0). The assay mixture of sucrose synthase consisted of 7.5 mM UDP-glucose, 30 mM fructose, 27.5 mM $MgCl_2$ and 0.5 M HEPES buffer (pH 8.2) and enzyme in total volume of 0.14 cm^3. After incubation at 37 °C for 30 min, the reaction was stopped by adding 0.02 cm^3 of 30 % NaOH and the contents were kept in boiling water for 10 min to destroy free fructose. Sucrose formed was determined by the anthrone reagent. For estimating SPS activity, the assay procedure was the same as described for sucrose synthase except that fructose was replaced by fructose-6-phosphate and reaction mixture also contained 15 mM NaF.

Invertases were isolated by crushing the tissues with chilled 0.1 M NaCl (Dey 1986). Acid and alkaline invertase activities were determined (Kaur *et al.* 2000). The protein content in the enzyme extract was determined by method of Lowry *et al.* (1951).

Extraction and estimation of soluble sugars: The tissue were extracted twice with hot 80 % ethanol and then twice with hot 70 % ethanol. The extracts containing soluble sugars were concentrated by evaporating ethanol at 50 °C under vacuum. Total free and reducing sugars were determined colorimetrically using the reactions with phenol (Dubois *et al.* 1956) and arsenomolybdate (Nelson 1944), respectively. Sucrose in the sugar extract was completely hydrolyzed with excess of acid invertase (*Sigma*, St. Louis, USA). After invertase hydrolysis, glucose (free + bound) was determined by glucose oxidase and peroxidase reaction (Gascon and Lampen 1968). Glucose released from sucrose hydrolysis was calculated by subtracting the free glucose content from total glucose and sucrose content was determined.

Extraction and estimation of starch: Starch was estimated from the sugar free residue left after the extraction of soluble sugars (Davis 1984). The residue containing starch was dried at 50 °C and then after an addition of 5 cm^3 of distilled water, kept in a boiling water bath for 1 h. Starch was hydrolysed completely with excess of amyloglucosidase and the glucose thus formed was estimated (Nelson 1944).

Results

The specific activity of amylase in cotyledons and roots of stressed seedlings was reduced in comparison to control, however, in cotyledons, the reduction was less in the presence of kinetin (Table 1). The depletion of starch was observed in cotyledons with the progress of seedling growth but the starch content was higher in cotyledons of stressed seedlings as compared to control and kinetin treated seedlings (Table 2). The specific activity of amylase in shoots of stressed seedlings at 3 and 5 DAS was higher and at 7 DAS was comparable with that of

Table 1. Effect of kinetin (6 μM) on the specific activity of amylase [nmol(reducing sugars formed) mg^{-1}(protein) s^{-1}] in chickpea seedlings under salt stress (75 mM NaCl) at different days after sowing (DAS). Means ± SD of three replicates. Differences significant in comparison with NaCl at * - $P < 0.01$ or ** - $P < 0.05$; in comparison with control at *** - $P < 0.01$ (Student's *t*-test).

Tissue	Treatment	3 DAS	5 DAS	7 DAS
Cotyledon	control	1.99 ± 0.15**	3.65 ± 0.49*	12.61 ± 0.99**
	NaCl	1.49 ± 0.13	2.32 ± 0.41	4.98 ± 0.33
	NaCl + kinetin	1.99 ± 0.14**	4.64 ± 0.66**	8.10 ± 0.76**
Shoot	control	2.82 ± 0.19	5.97 ± 0.66	23.90 ± 2.00
	NaCl	3.65 ± 0.16***	30.87 ± 5.64***	24.07 ± 2.98
	NaCl + kinetin	1.82 ± 0.15*	24.56 ± 1.00	11.95 ± 1.49
Root	control	6.30 ± 0.50**	5.47 ± 0.49*	3.82 ± 0.83
	NaCl	2.49 ± 0.30	3.98 ± 0.17	4.15 ± 0.60
	NaCl + kinetin	1.82 ± 0.16*	2.16 ± 0.50**	2.16 ± 0.49**

Table 2. Effect of kinetin (6 μM) on sugar composition of cotyledons, shoots and roots [mg g^{-1}(f.m.] of chickpea seedlings under salt stress (75 mM NaCl). Means ± SD of three replicates. Differences significant in comparison with NaCl at ** - $P < 0.01$; in comparison with control at * - $P < 0.01$ (Student's t-test).

Tissue	DAS	Treatment	Reducing sugars	Sucrose	Starch
Cotyledons	3	control	0.80 ± 0.03	2.3 ± 0.03	139 ± 8.2
		NaCl	0.74 ± 0.02	4.1 ± 0.02*	167 ± 7.5*
		NaCl + kinetin	0.95 ± 0.05	4.2 ± 0.19	150 ± 1.3
	5	control	1.29 ± 0.16	3.0 ± 0.19	110 ± 2.8
		NaCl	0.88 ± 0.02	6.3 ± 0.02*	142 ± 3.3*
		NaCl + kinetin	1.12 ± 0.26	6.0 ± 0.04	119 ± 2.0
	7	control	1.04 ± 0.05	4.0 ± 0.06	90 ± 4.8
		NaCl	0.36 ± 0.02	4.9 ± 0.12	120 ± 2.7*
		NaCl + kinetin	0.88 ± 0.11	5.5 ± 0.42	110 ± 9.4
Shoots	3	control	6.60 ± 0.04**	1.6 ± 0.04	4.0 ± 0.22
		NaCl	4.00 ± 0.29	5.2 ± 0.14*	3.0 ± 0.40
		NaCl + kinetin	6.90 ± 0.37**	7.5 ± 0.49	3.9 ± 0.07
	5	control	9.00 ± 0.35**	1.0 ± 0.04	4.2 ± 0.06
		NaCl	6.90 ± 0.33	5.2 ± 0.02*	2.6 ± 0.12
		NaCl + kinetin	11.50 ± 0.13**	4.0 ± 0.05	3.4 ± 0.16
	7	control	6.00 ± 0.71	0.9 ± 0.02	2.0 ± 0.06
		NaCl	6.60 ± 0.47	1.5 ± 0.13*	1.9 ± 0.11
		NaCl + kinetin	9.10 ± 1.90**	3.2 ± 0.45	3.3 ± 0.46
Roots	3	control	2.00 ± 0.04	1.9 ± 0.04	1.6 ± 0.05
		NaCl	3.10 ± 0.03	3.8 ± 0.02*	1.4 ± 0.13
		NaCl + kinetin	3.60 ± 0.12	2.3 ± 0.14	2.5 ± 0.05
	5	control	2.20 ± 0.01	1.1 ± 0.01	2.0 ± 0.16
		NaCl	2.30 ± 0.16	2.6 ± 0.01*	2.2 ± 0.05
		NaCl + kinetin	3.20 ± 0.45	2.4 ± 0.31	2.7 ± 0.18
	7	control	1.60 ± 0.13	1.2 ± 0.09	2.2 ± 0.10
		NaCl	1.20 ± 0.12	2.3 ± 0.02*	1.7 ± 0.01
		NaCl + kinetin	1.50 ± 0.19	2.2 ± 0.25	2.3 ± 0.15

Table 3. Effect of kinetin (6 μM) on the specific activity of acid invertase [nmol(sucrose hydrolysed) mg^{-1}(protein) s^{-1}] in chickpea seedlings under salt stress (75 mM NaCl) at different days after sowing (DAS). Means ± SD of three replicates. Differences significant in comparison with NaCl at * - $P < 0.01$ or ** - $P < 0.05$ (Student's t-test).

Tissue	Treatment	3 DAS	5 DAS	7 DAS
Cotyledon	control	0.14 ± 0.01	0.21 ± 0.03	0.10 ± 0.01
	NaCl	0.11 ± 0.01	0.17 ± 0.01	0.06 ± 0.01
	NaCl + kinetin	0.15 ± 0.02	0.18 ± 0.02	0.09 ± 0.01
Shoot	control	2.16 ± 0.25*	3.16 ± 0.12**	1.48 ± 0.13
	NaCl	1.41 ± 0.20	2.41 ± 0.18	1.76 ± 0.16
	NaCl + kinetin	2.75 ± 0.28*	4.55 ± 0.22**	2.63 ± 0.13*
Root	control	1.43 ± 0.06	1.28 ± 0.06	0.68 ± 0.05
	NaCl	1.60 ± 0.13	1.46 ± 0.13	0.56 ± 0.10
	NaCl + kinetin	1.80 ± 0.16	1.66 ± 0.06	0.45 ± 0.05

normal seedlings. On addition of kinetin, the activity was reduced in shoots and roots of stressed seedlings (Table 1).

Acid and alkaline invertase activities were maximum in shoots and minimum in cotyledons (Tables 3, 4). The reducing sugar content was found to be very low in cotyledons (Table 2). The reduced specific activities of acid and alkaline invertases in shoots of stressed seedlings were increased by kinetin. The shoots of stressed seedlings had a lower reducing sugar content

than control seedlings at 3 and 5 DAS and it was increased with kinetin (Table 2). With the progress of seedling growth, acid and alkaline invertase activities in roots decreased (Tables 3, 4). It was observed that acid invertase activity was negatively correlated with amylase in cotyledons of control, salt stressed and kinetin treated salt stressed seedlings (correlation coefficient, r = -0.66 to -0.71). Acid and alkaline invertases were positively correlated with each other in cotyledons (r = 0.95 to 0.98) and roots (r = 0.92 to 0.98) but the correlation was weak

Table 4. Effect of kinetin (6 μM) on the specific activity of alkaline invertase [nmol(sucrose hydrolysed) mg^{-1}(protein) s^{-1}] in chickpea seedlings under salt stress (75 mM NaCl) at different days after sowing (DAS). Means ± SD of three replicates. Differences significant in comparison with NaCl at * - $P < 0.01$ or ** - $P < 0.05$ (Student's t-test).

Tissue	Treatment	3 DAS	5 DAS	7 DAS
Cotyledon	control	0.10 ± 0.01	0.12 ± 0.01	0.08 ± 0.01
	NaCl	0.09 ± 0.01	0.10 ± 0.01	0.07 ± 0.00
	NaCl + kinetin	0.11 ± 0.02	0.11 ± 0.01	0.08 ± 0.01
Shoot	control	1.55 ± 0.18**	1.30 ± 0.13*	0.73 ± 0.05**
	NaCl	1.13 ± 0.08	1.08 ± 0.11	0.35 ± 0.04
	NaCl + kinetin	1.45 ± 0.20**	1.43 ± 0.14*	0.88 ± 0.14**
Root	control	0.75 ± 0.06	0.63 ± 0.08	0.33 ± 0.06
	NaCl	0.86 ± 0.05	0.88 ± 0.04	0.38 ± 0.04
	NaCl + kinetin	0.95 ± 0.05	0.76 ± 0.08	0.58 ± 0.05

Table 5. Effect of kinetin (6 μM) on the specific activity of sucrose synthase [nmol(sucrose synthesized) mg^{-1}(protein) s^{-1}] in chickpea seedlings under salt stress (75 mM NaCl) at different days after sowing (DAS). Means ± SD of three replicates. Differences significant in comparison with NaCl at * - $P < 0.01$ or ** - $P < 0.05$ (Student's t-test).

Tissue	Treatment	3 DAS	5 DAS	7 DAS
Cotyledon	control	0.06 ± 0.01**	0.09 ± 0.01*	0.13 ± 0.01**
	NaCl	0.10 ± 0.01	0.12 ± 0.01	0.18 ± 0.01
	NaCl + kinetin	0.11 ± 0.01	0.20 ± 0.04**	0.28 ± 0.02**
Shoot	control	0.23 ± 0.01**	0.36 ± 0.03**	0.13 ± 0.01**
	NaCl	0.36 ± 0.04	0.48 ± 0.02	0.20 ± 0.02
	NaCl + kinetin	0.47 ± 0.01**	0.59 ± 0.03**	0.33 ± 0.06**
Root	control	0.28 ± 0.01**	0.38 ± 0.06	0.18 ± 0.01**
	NaCl	0.40 ± 0.04	0.40 ± 0.04	0.35 ± 0.06
	NaCl + kinetin	0.29 ± 0.02	0.51 ± 0.06*	0.58 ± 0.05**

Table 6. Effect of kinetin (6 μM) on the specific activity of sucrose phosphate synthase [nmol(sucrose synthesized) mg^{-1}(protein) s^{-1}] in chickpea seedlings under salt stress (75 mM NaCl) at different days after sowing (DAS). Means ± SD of three replicates. Differences significant in comparison with NaCl at * - $P < 0.01$ or ** - $P < 0.05$ (Student's t-test).

Tissue	Treatment	3 DAS	5 DAS	7 DAS
Cotyledon	control	0.02 ± 0.00*	0.05 ± 0.01*	0.08 ± 0.01**
	NaCl	0.05 ± 0.01	0.08 ± 0.01	0.13 ± 0.02
	NaCl + kinetin	0.06 ± 0.01	0.11 ± 0.01*	0.20 ± 0.03*
Shoot	control	0.11 ± 0.01	0.05 ± 0.01*	0.04 ± 0.01
	NaCl	0.10 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
	NaCl + kinetin	0.17 ± 0.02**	0.11 ± 0.02*	0.07 ± 0.01**
Root	control	0.10 ± 0.01	0.20 ± 0.01	0.16 ± 0.01
	NaCl	0.17 ± 0.02	0.24 ± 0.05	0.31 ± 0.02
	NaCl + kinetin	0.17 ± 0.02	0.27 ± 0.04	0.35 ± 0.03

in shoots of control, salt stressed and kinetin treated salt stressed seedlings.

The specific activities of SS and SPS in cotyledons at 5 and 7 DAS and in shoots of stressed seedlings at 3, 5 and 7 DAS were increased with kinetin (Tables 5, 6). Acid invertase activity is negatively correlated with SPS in cotyledons (r = -0.37 to -0.78) whereas amylase is strongly, positively correlated with SS (r = 0.96 to 0.99) and SPS (r = 0.93 to 0.98) in cotyledons of control, NaCl and NaCl + kinetin treated seedlings. In general, the sucrose content was found to be more in cotyledons, shoots and roots of salt stressed and kinetin treated salt stressed seedlings in comparison with control (Table 2).

Discussion

It was observed in previous experiments that 75 mM NaCl caused a decrease of about 50 % in germination and 60 - 70 % in lengths and mass of roots and shoots in chickpea seedlings, as compared with control. Kinetin at 6 μM concentration, when added to a medium containing 75 mM NaCl, was found to be effective in promoting germination and growth of stressed chickpea seedlings (Kaur *et al.*.1998).

The growth of seedlings is dependent upon starch mobilization from the cotyledons and availability of sucrose as a carbon source for meeting the energy demands of the growing tissues. Sucrose is invariably the sugar which is transported from cotyledons to embryonic axis during germination. Acid invertase is involved in phloem unloading in shoots thereby converting the sucrose unloaded to apoplast into hexoses and resulting in continued export of sucrose from the phloem (Patric 1990). Amylase has been found to be strongly positively correlated with SS and SPS in cotyledons of control, salt stressed and kinetin treated, salt stressed seedlings. It appears that in cotyledons amylase and sucrose synthesizing enzymes are acting synergistically to maximize their effect. However, the decreased amylase activity in cotyledons of stressed seedlings could result in reduced formation of glucose from starch, thereby leading to a decreased synthesis of sucrose and its reduced supply to embryonic axis. This process seems to be partially reversed by exogenous application of kinetin (Table 1). The increased amylase activity in cotyledons of stressed seedlings with kinetin resulted in an enhanced mobilization of cotyledonary starch and better growth as indicated by their lower starch content as compared to untreated salt stressed seedlings (Tables 1, 2). The amylase activity in cotyledons of water stressed seedlings has also been reported to be enhanced by kinetin (Kaur *et al.* 2000).

Kinetin also increased the activities of SS and SPS in cotyledons of stressed seedlings (Tables 5, 6) thereby indicating that kinetin enhanced the conversion of starch to sucrose in cotyledons of stressed seedlings (Tables 1, 5, 6). Kinetin reduced the activity of amylase in shoots of salt stressed seedlings while stimulated the activities of SS and SPS (Tables 1, 5, 6). However, in shoots amylase activity was negatively correlated with SPS. It appears that in shoots amylolytic products of starch are possibly utilized mainly for energy needs instead of being converted to sucrose. The decreased growth of stressed seedlings could be due to the lower invertase activity in shoots which would result in decreased availability of reducing sugars needed for growth (Tables 2, 3, 4). Furthermore under stressed conditions supply of sucrose from cotyledons to shoots has been reported to be restricted (Gupta *et al.* 1993). Under such conditions a low invertase activity will further enhance the effect of salt stress in reducing growth. The increased growth of kinetin treated stressed seedlings could be due to increased invertase activity in shoots thus providing more reducing sugars for growth and more availability of sucrose as a result of increased SS and SPS in cotyledons and shoots (Table 2). Increased activities of both SS and SPS and invertases in shoots of kinetin supplied stressed seedlings could result in higher turnover of sucrose in shoots In certain aspects, the effect of kinetin was tissue specific. For example, addition of exogenous kinetin increased the cotyledonary amylase activity of stressed seedlings whereas it decreased the amylase activity in shoots and roots (Table 1).

On the basis of the present studies, it can be concluded that kinetin helps to counteract the effects of salt stress by altering the activities of both starch degrading and sucrose metabolising enzymes.

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