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Effect of NaCl on nitrate reductase, glutamate dehydrogenase and glutamate synthase in *Vigna radiata* calli

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Abstract

The effect of NaCl stress on the activities of nitrate reductase (NR), glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) in callus lines of *Vigna radiata* which differ in salt resistance, was studied at weekly intervals upto 28 d of growth. After 28 d, the NaCl resistant callus (selected at 300 mM NaCl) at NaCl concentrations higher than 200 mM maintained higher NR activity than non-selected line. NaCl stress also affects aminating and deaminating activities of GDH. The NADH-GDH activity in the presence of NaCl was higher in the resistant than non-selected line. On the other hand, NAD-GDH activity in both the lines was completely inhibited after 7 d of growth. The increased activity of NADH-GDH in resistant calli may play a vital role in protecting the cells from toxic effect of increased endogenous level of ammonia which probably accumulates due to efficient NO_3^- reduction. NADH-GOGAT activity was found to decrease under salt stress in both the callus lines. Nitrogen assimilation in salt-resistant calli under salt stress was found to be characterized by high NR and NADH-GDH activities, concomitantly with low GOGAT activity.

Additional key words: mung bean, NaCl-resistant and -sensitive calli, nitrogen assimilating enzymes.

Introduction

Nitrate reductase (NR, E.C. 1.6.6.1.) catalyses the reduction of nitrate to nitrite, which is further reduced to ammonia by nitrite reductase (NiR, E.C. 1.7.1.1.). Further assimilation of ammonia takes place primarily with the help of enzymes, glutamine synthetase (GS, E.C. 6.3.1.2.) and glutamate synthase (GOGAT, E.C. 1.4.7.1.). The second alternative pathway is operative not only under conditions of ammonia limitations, but also under a variety of stresses (Miflin and Lea 1976, Miranda-Ham and Loyola-Vargas 1988). GDH pathway has been suggested to be

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involved in ammonia asimilation in roots of non-stressed plants (Loyola-Vargas *et al.* 1988, Miranda-Ham and Loyola-Vargas 1988). There is a switch from one pathway of ammonia assimilation to another depending on the nature of stress and the tissue in which the process takes place (Miranda-Ham and Loyola-Vargas 1988). The effects of NaCl stress on nitrogen assimilation are controversial (Miranda-Ham and Loyola-Vargas 1988, Rao and Gnanam 1990) and studies on nitrogen assimilating enzymes during callus growth under salt stress are largely missing.

In the present study, effect of salt-stress on the activities of NR (*in vivo*), NADH-GDH (aminating) and NAD-GDH (deaminating) and GOGAT in callus lines of *Vigna radiata* which differ in salt resistance was undertaken.

Materials and methods

Establishment of callus lines: Salt-resistant callus line was isolated as described by Gulati and Jaiwal (1993) by exposing 25 ± 2 mg fresh mass callus pieces (obtained after one subculture from leaf explants) on agar solidified PC-L2 (Phillips and Collins 1979) medium supplemented with BAP (1 mg dm^{-3}), NAA (0.5 mg dm^{-3}), 2,4-D (0.5 mg dm^{-3}) and 300 mol m^{-3} NaCl, a concentration which caused 70 % inhibition of growth of wild type cells.

Effect of NaCl on calli: The salt-selected (resistant) and non-selected lines were grown in Petri dishes (100 × 17 mm) containing 20 cm^3 of PC-L2 medium supplemented with different concentrations of NaCl (0 - 300 mol m^{-3}). The Petri dishes were sealed with parafilm and incubated under 16-h photoperiod, irradiance of 80 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C. For each treatment, ten callus pieces (each 25 ± 2 mg) per dish and twelve replicate dishes were used. At intervals of 7 d, callus from three Petri dishes of each treatment was removed and used for enzymatic studies.

Enzyme extraction and protein assay: 250 mg callus tissue was ground and extracted in 2 cm^3 of buffer. The GDH extraction buffer (pH 7.5) contained 100 mol m^{-3} Tris-HCl, 1 mol m^{-3} cysteine, 0.5 % Triton-X, 0.5 mol m^{-3} EDTA-sodium salt and 2 mol m^{-3} $CaCl_2$. The GOGAT extraction buffer contained 200 mol m^{-3} Tris-HCl, 2 mol m^{-3} EDTA-sodium, 50 mol m^{-3} KCl, 1 % β-mercaptoethanol and 5 % Triton-X. After centrifugation, to remove cell debris, the supernatants were used for the enzyme assays.

Soluble protein was estimated by the method of Lowry *et al.* (1951) after precipitation with equal volumes of 2 % TCA and using bovine serum albumin as standard.

Enzyme assays: GDH activities both aminating and deaminating were determined as decrease and increase in absorbance at 340 nM due to NAD(H) oxidation and reduction according to Bulen (1956). NADH-GDH assay buffer contained 100 mol m^{-3} Tris-HCl (pH 7.5), 200 mol m^{-3} 2-oxoglutarate, 1500 mol m^{-3} ammonium sulphate, 0.1 mol m^{-3} $CaCl_2$ and 1 mol m^{-3} NADH. NAD^+-GDH assay mixture

contained 100 mol m^{-3} Tris-HCl (pH 7.5), 200 mol m^{-3} glutamic acid and 1.0 mol m^{-3} NAD^+.

GOGAT activity was determined after the method of Srivastava and Ormrod (1984) by following the decrease in absorbance at 340 nm due to NADH oxidation. For GOGAT, the assay mixture contained 100 mol m^{-3} Tris-HCl (pH 7.5), 20 mol m^{-3} L-glutamine, 5 mol m^{-3} 2-oxoglutarate, 100 mol m^{-3} KCl and 1 mol m^{-3} NADH.

In vivo NR activity in fresh callus was determined by the method of Srivastava (1975) with some modifications. The NR extraction buffer contained 100 mol m^{-3} sodium phosphate buffer (pH 7.4), 200 mol m^{-3} KNO_3 and 25 % *n*-propanol. The endogenous nitrate pool was measured indirectly by analysing the anaerobic nitrite production by the callus tissue in the dark.

NR activity was expressed in terms of production of moles of NO_2 per fresh mass unit per s, while GDH and GOGAT activities were expressed as specific activities.

The data presented are mean value ± SE of at least 2 independent series of experiments each with three replicate determinations. Paired *t*-test was applied whenever required to test significance of difference.

Results and discussion

***In vivo* NR activity:** NaCl treatment caused an increase in NR activity in NaCl-non-selected callus, the increase was greater at longer treatment and at lower NaCl concentrations (100 - 200 mol m^{-3}). After 28 d, 100 mol m^{-3} NaCl caused a 4-fold increase in NR activity, while at 300 mol m^{-3} NaCl NR was almost the same as in the non-stressed calli (Table 1).

Table 1. *In vivo* nitrate reductase (NR) activity during growth of control (without NaCl) and salt stressed (100 - 300 mol m^{-3} NaCl) calli of non-selected and NaCl-resistant callus lines of *Vigna radiata* cv. K 851. Values are means ± SE based on three independent observations. Percentages of control are given in parentheses.

Callus line	NaCl [mol m^{-3}]	NR activity [μmol(NO_2) g^{-1}(f.m.) s^{-1}] 7 d	14 d	21 d	28 d
NaCl-resistant	0	0.68±0.03 (100)	0.60±0.27 (100)	0.48±0.02 (100)	0.12±0.01 (100)
	100	1.68±0.03^b(247)	1.14±0.02^b(190)	0.66±0.01^b(137)	0.11±0.00 (92)
	200	1.11±0.02^b(163)	1.03±0.01^b(171)	0.37±0.04 (76)	0.17±0.00^b (142)
	250	0.33±0.02 (48)	0.55±0.02 (91)	0.29±0.00 (59)	0.30±0.00^{ab}(250)
	300	0.16±0.00 (25)	0.29±0.00 (48)	0.27±0.03 (56)	0.29±0.00^{ab}(242)
non-selected	0	1.43±0.01 (100)	2.07±0.02 (100)	0.59±0.01 (100)	0.29±0.00 (100)
	100	3.11±0.01^b(217)	2.33±0.02^b(112)	1.94±0.02^b(329)	1.23±0.01^{ab}(424)
	200	3.14±0.05^b(219)	2.47±0.05^b(119)	1.28±0.01^b(217)	1.06±0.01^{ab}(366)
	250	2.68±0.04^b(188)	2.15±0.03 (103)	0.86±0.04^b(146)	0.60±0.00^b (207)
	300	1.30±0.05 (91)	2.11±0.02 (102)	1.01±0.02^b(171)	0.32±0.00^b (110)

^{a} - significance of difference between resistant and non-selected callus line at $P < 0.05$,

^{b} - significance of difference between control and salt stressed one of the respective line at $P < 0.05$.

NR-activity in NaCl-resistant callus increased under lower concentrations (100 - 200 mol m^{-3}) of salt upto 14 d and at higher NaCl concentrations (250 - 300 mol m^{-3}) after 28 d of treatment; *e.g.* 300 mol m^{-3} NaCl caused a 2.4-fold increase in enzyme activity.

The NaCl-resistant callus in the absence of NaCl maintained lower NR activity compared with non-selected callus. However, the former maintained higher NR activity at lower NaCl concentrations during early growth and at higher NaCl concentrations during later stages of growth.

Enhancement of NR activity under salinity has also been reported in *Zea mays* seedlings (Aliva and Klyshev 1975), *Suaeda maritima* (Billard and Boucard 1982) and in *Phaseolus aureus* (Misra and Dwivedi 1990). However, NR is also reported to be inhibited by NaCl stress in *Pisum sativum* (Lal and Bhardwaj 1987) and *Arachis hypogaea* (Rao *et al.* 1981). The increase in enzyme activity seems to be due to the direct influence of salt stress on enzyme synthesis as salt stressed *Vigna* cultures possessed 33 - 66 % more protein than non-stressed cultures (data not shown). The induction of enzyme synthesis by salt stress, therefore, may play a role in the salt tolerance expressed by *Vigna* cultures. In the present study, the resistant calli exhibit an efficient nitrate reduction after 28 d of treatment at 300 mol m^{-3} NaCl, a concentration lethal to non-selected callus. This indicates that maintenance of higher levels of enzymes can be a mechanism for coping with salt stress.

NADH-GDH activity of non-selected and resistant calli in the absence of NaCl decreased with the age of the cultures. The GDH-activity of resistant calli in the presence of 100 to 250 mol m^{-3} NaCl increased upto 14 d and thereafter decreased, thus after 28 d at 100 - 200 mol m^{-3} NaCl, the GDH activity was similar to that in

Table 2. Effect of increasing concentrations of NaCl on NADH-GDH activity during growth of non-selected and NaCl-resistant callus lines of *Vigna radiata* cv. K-851. Values are means ± SE based on three independent observations. Percentages of control are given in parentheses.

Callus line	NaCl [mol m^{-3}]	Enzyme activity [nmol(NADH) mg^{-1}(protein) s^{-1}] 7 d	14 d	21 d	28 d
NaCl-resistant	0	7.29±0.50 (100)	4.07±0.23 (100)	3.84±0.11 (100)	3.20±0.30 (100)
	100	6.33±0.46 (87)	2.53±0.10 (62)	2.96±0.30 (77)	3.23±0.13 (100)
	200	12.21±0.36^{c}(167)	7.57±1.05 (185)	3.61±0.08 (94)	3.34±0.15 (104)
	250	34.14±0.32^{b}(468)	8.84±0.07^{b}(217)	3.26±0.49 (85)	2.63±0.16 (82)
	300	47.08±0.21^{b}(645)	7.97±0.20^{b}(196)	6.83±0.12^{b}(177)	2.00±0.11 (62)
non-selected	0	21.93±0.25 (100)	8.63±0.23 (100)	3.97±0.17 (100)	2.36±0.16 (100)
	100	5.51±0.36 (25)	6.72±0.07 (77)	3.37±0.19 (85)	3.06±0.00^{a}(129)
	200	15.20±0.32 (69)	4.92±0.08 (57)	3.10±0.23 (78)	3.06±0.12^{a}(129)
	250	4.18±0.00 (22)	8.04±0.65 (93)	1.94±0.00 (49)	2.13±0.11 (90)
	300	25.96±0.08^{c}(118)	1.49±0.15 (17)	2.30±0.23 (58)	0.81±0.04 (34)

^{a} - significance of difference between resistant and non-selected callus line at $P < 0.05$,

^{b} - significance of difference between control and salt stressed one of the respective line at $P < 0.05$,

^{c} - significance of difference between control and salt stressed one of the respective line at $P > 0.05$

calli grown in the absence of NaCl (control). GDH activity in resistant calli at 300 mol m^{-3} NaCl was higher than control upto 21 d of culture (Table 2).

The non-selected calli showed decrease in GDH activity at all the salt concentrations throughout the growth period except on 28-d at NaCl concentrations 100 to 200 mol m^{-3} where increase was observed.

A comparison of both the lines showed that NADH-GDH activity in the presence of NaCl was higher in NaCl-resistant line than the non-selected line almost throughout the growth period. Such increase in GDH activity by saline treatment has also been reported in groundnut (Rao *et al.* 1981) and rice callus (Subhashini and Reddy 1990). Contrary to this, GDH is found to be inhibited by NaCl stress in *Phaseolus aureus* (Misra and Dwivedi 1990). The increased activity of NADH-GDH in resistant cells under stress may play a vital role in protecting the cells. The increased activity of NADH-GDH may be due to acceleration in *de novo* synthesis of the enzyme protein and/or by modulating the activity of the existing enzyme under stress conditions.

NAD-GDH activity: Both the callus lines in the presence or absence of NaCl did not show any NAD-GDH activity after 7 d of growth. On 7 d non-selected calli in the absence of NaCl showed higher NAD-GDH activity than resistant callus line whereas in the presence of NaCl. Enzyme activity was much lower in the former than the latter at all the salt concentrations (Table 3).

Table 3. Effect of increasing concentrations of NaCl on NAD-GDH activity during growth of non-selected and NaCl-resistant callus lines of *Vigna radiata* cv. K-851. Values are means ± SE based on three independent observations. Percentages of control are given in parentheses.

NaCl [mol m^{-3}]	Enzyme activity [nmol(NADH) mg^{-1}(protein) s^{-1}] non-selected line	resistant line
0	35.20 ± 0.19 (100)	11.18 ± 0.13 (100)
100	4.40 ± 0.04 (12)	8.95 ± 0.07 (80)
200	14.87 ± 0.14 (42)	16.44 ± 0.13[b] (147)
250	16.03 ± 0.13 (45)	17.70 ± 0.18[b] (158)
300	1.36 ± 0.05 (4)	13.22 ± 0.14 (118)

[b] - significance of difference between control and salt stressed one of the respective line at $P < 0.05$.

The inhibition in deaminating activity of GDH has been reported in the presence of ammonia in maize seedlings (Singh and Srivastava 1982). These results indicate that salt stress affects both the aminating and deaminating activity of GDH.

NADH-GOGAT activity in both non-selected and resistant lines grown in the absence of NaCl decreased with the age of cultures, whereas in the presence of NaCl, enzyme activity in the former decreased throughout the growth period while in the latter increased upto 14 d thereafter decreased till 28 d compared to their controls (Table 4). Decrease in GOGAT activity has also been reported in *Suaeda maritima* (Billard and Boucard 1982). In contrast, GOGAT is found to be increased under

saline stress in *Canavalia ensiformis* (Miranda-Ham and Loyola-Vargas 1988), *Phaseolus aureus* (Misra and Dwivedi 1990) and *Lycopersicon esculentum* and *L. pennellii* (Rus-Alvarez and Guerrier 1994).

Table 4. Effect of increasing concentrations of NaCl on glutamate synthase (NADH) activity during growth of non-selected and NaCl-resistant callus lines of *Vigna radiata* cv. K-851. Values are means ± SE based on three independent observations. Percentages of control are given in parentheses.

Callus line	NaCl [mol m^{-3}]	Enzyme activity [nmol(NADH oxidised) mg^{-1}(protein) s^{-1}] 7 d	14 d	21 d	28 d
NaCl-resistant	0	10.85±0.08 (100)	3.62±0.03 (100)	2.12±0.68 (100)	1.20±0.04 (100)
	100	5.61±0.04 (51)	5.86±0.07 (162)	1.57±0.17 (74)	0.68±0.04 (57)
	200	5.70±0.04 (52)	7.63±0.11 (211)	0.92±0.22 (43)	0.42±0.00 (35)
	250	6.47±0.05 (59)	6.06±0.05 (167)	0.38±0.00 (18)	0.22±0.02 (18)
	300	8.78±0.08 (81)	4.87±0.25 (134)	0.49±0.00 (23)	0.24±0.01 (20)
non-selected	0	11.78±0.11 (100)	8.87±0.49 (100)	0.92±0.07 (100)	1.14±0.27 (100)
	100	13.18±0.11 (112)	7.89±0.07 (89)	0.56±0.00 (61)	0.57±0.03 (50)
	200	10.02±0.09 (85)	7.56±0.07 (85)	0.34±0.01 (37)	0.36±0.04 (31)
	250	10.67±0.09 (90)	4.63±0.19 (52)	0.50±0.05 (54)	0.22±0.04 (19)
	300	9.67±0.09 (82)	3.79±0.05 (42)	0.38±0.04 (41)	0.25±0.05 (22)

Thus, the nitrogen assimilation in salt resistant calli under salt stress was found to be characterized by high NR and GDH activities, concomitantly with low GOGAT activity.

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