CHAPTER 13

NITROGEN ASSIMILATING ENZYMES IN NaCl-SENSITIVE AND RESISTANT CELL LINES

13.1 INTRODUCTION

Nitrate is the predominant form of nitrogen available to cells growing on culture medium and the growth of cells is largely influenced by the acquisition and reduction of nitrate nitrogen and its incorporation into amino-acids and proteins. In view of the decreased growth of glycophytic cells on salt supplemented medium, uptake and reduction of nitrate assume central importance in cells exposed to saline stress. Nitrate reductase (NR, E.C. 1.6.6.1) localized in cytoplasm is the first enzyme in the pathway of nitrate assimilation and catalyses the reduction of nitrate to nitrite, which is further reduced to ammonia by nitrite reductase in chloroplast. 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). Many studies have reflected the interdependence of GS and GOGAT enzymes since they are known to work in conjunctions (Lea and Miflin, 1974). However, reductive amination of 2-oxoglutarate is also catalyzed by glutamate dehydrogenase (GDH, E.C. 1.4.1.2) under certain nutritional and environmental conditions (Srivastava and Singh, 1987). The GS/GOGAT pathway (Tempest *et al.,* 1970; Lea and Miflin, 1974; Dougall and Bloch, 1976) involves incorporation of ammonia into glutamate to form glutamine by the enzyme glutamine synthetase. GOGAT, in turn removes the amide group from glutamine in the presence of α-ketoglutarate to form two moles of glutamate. The GS/GOGAT pathway is operative not only under conditions of ammonia limitations, but also under a variety of physiological and environmental conditions, including stress (Miflin and Lea, 1976; Miranda-Ham and Loyole-Vargas, 1988). GDH pathway has been suggested to be involved in ammonia assimilation in roots of non-stressed plants (Loyola-Vargas *et al.,* 1988; Miranda-Ham and Loyola-Vargas, 1988). But, Miranda-Ham and Loyola-Vargas (1988) have shown that 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. Thus, the effect of NaCl stress on nitrogen assimilation are controversial and have been studied in only a few plants (Klyshev *et al.,* 1979; Kasymbekov *et al.,* 1980; Miranda-Ham and Loyola-Vargas, 1988; Rao and Gnanam, 1990). Such studies on nitrogen assimilating enzymes during callus growth under salt stress are largely missing.

In the present study, effect of salt-stress on the activity of NR (*in-vivo* and *in-vitro*), GDH (aminating and deaminating) and GOGAT in callus lines of *Vigna radiata* which differ in salt-resistant was undertaken with the view to understand the mechanism of nitrogen assimilation under salt stress and to achieve possible correlation between the behaviour of these enzymes and the degree of salt tolerance in *Vigna radiata*.

13.2 MATERIALS AND METHODS

Salt-resistant (isolated at 300 mol m^{-3} NaCl) and salt-sensitive callus lines were grown in petri dishes (100 mm x 17 mm) containing 20 ml of modified PC-L2 medium supplemented with 0,100,200,250 and 300 mol m^{-3} NaCl. The petridishes were sealed with parafilm and incubated under 16-h photoperiod (80 μ mol m^{-2} s^{-1}) at 25 $\pm$ 2°C. For each treatment, 10 calli pieces (each 25 $\pm$ 2 mg) per dish and twelve replicate dishes were used. At intervals of 7 days, callus from 3 petridishes of each treatment was removed and analyzed for enzymatic studies.

Nitrate reductase : The activity of nitrate reductase was assayed either by *in-vivo* (Srivastava, 1975) or by *in-vitro* (Stevens and Oaks, 1973) methods with slight modifications.

In-vivo assay : 250 mg callus tissue was taken in black vials in 20 ml capacity containing 8 ml of 0.1 M sodium phosphate buffer (pH 7.4), 1 ml of 0.2 M KNO_3 and 1 ml of 25% n-propanol. These vials were sealed and incubated in dark for 30 minutes at 30°C. At the end of incubation period, the vials were placed in a boiling water bath for complete extraction of NO_2^-. The vials were centrifuged and NO_2^- was determined in the supernatant in 1 ml of aliquot by adding 1 ml of 1% sulphanilamide in 1 N HCl and 1 ml of 0.01% N (1-naphthylethylene diamine dihydroxy chloride). The color was allowed to develop for 20 minutes and recorded at 540 nm. The amount of nitrite was calculated as μ moles NO_2^- h^{-1} g^{-1} FW with the help of standard curve prepared from sodium nitrite.

In-vitro assay

Enzyme extraction : 250 mg of callus tissue was homogenized with 2 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.1% casein, 0.01 M cystein and 0.03 M EDTA - sodium salt at 0-4°C. The homogenate was centrifuged at 20,000 x g at the same temperature for 20 minutes.

Enzyme assay : The assay mixture contained 2 ml of 0.1 M sodium phosphate buffer (pH 7.4), 0.5 ml of 0.2 M KNO_3, 0.3 ml of NADH (1 mg/ml) and 0.2 ml enzyme preparation. The reaction was started by the addition of enzyme and this assay mixture was incubated at 30°C for 30 minutes. The reaction was stopped by adding 1 ml of 1% sulphanilamide in 1N HCl and color development was achieved by adding 1 ml of 0.01% N (1-naphthylethylene diamine dihydroxychloride). After 20 minutes absorbance at 540 nm was measured using Bausch and Lomb spectronic-20.

The enzyme activity was expressed in terms of production of μ moles of NO_2^- h^{-1} g^{-1} FW. The amount of NO_2^- was estimated from a standard curve prepared from sodium nitrite as *in-vivo* assay.

GDH

Enzyme extraction : A crude enzyme extract was prepared by homogenizing 250 mg of callus tissue in 2 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM cysteine, 0.5% Triton-X, 0.5 mM EDTA-sodium salt and 2 mM $CaCl_2$ at 4°C. The homogenate was centrifuged at 6000 x g at the same temperature for 45 minutes. The clear supernatant was used as enzyme preparation.

Enzyme assay : Both aminating and deaminating GDH activity was determined spectrophotometrically following the oxidation and reduction of NAD(H) (Bulen, 1956).

The assay mixture of NADH-GDH consisted of 1.5 ml of 100 mM Tris-HCl (pH 7.5), 0.3 ml of 0.2 M 2-oxoglutarate, 0.3 ml of 1.5 M ammonium sulfate, 0.2 ml of 0.1 mM calcium chloride, 0.6 ml of 1 mM NADH and 0.1 ml of enzyme preparation giving a final volume of 3.0 ml. Each component of the assay mixture was dissolved in the buffer. The reaction was started by adding enzyme preparation and the decrease in absorbancy was recorded continuously for 5 minutes at 340 nm in a double bean spectrophotometer (model CE 594).

The assay mixture of NAD^+ - GDH consisted of 1.8 ml of 100 mM Tris-HCl buffer (pH 7.5), 0.5 ml of 0.2 M glutamic acid, 0.6 ml of 1.0 mM NAD^+ (each dissolved in buffer) and 0.1 ml of enzyme preparation giving a find volume of 3 ml. The increase in absorbance at 340 nm was recorded for 5 minutes. The amount of NAD(H) oxidised or reduced was calculated from a standard curve of NADH.

GOGAT

Enzyme extraction : 250 mg callus tissue was ground and extracted in a mortor with 2 ml of a medium containing 200 mM Tris-HCl (pH 7.5), 2 mM EDTA-sodium salt, 50 mM KCl, 1% (v/v) β-mercapoethanol and 5% (w/v) Triton-X-100 at 0-4°C. Thee homogenate was centrifuged at 20,000 xg for 20 minutes at 0°C.

Enzyme assay : The enzyme was assayed spectrophotometrically following the oxidation of NADH (Srivastava and Ormrod, 1984). The assay mixture contained 0.4 ml of 20 mM L - glutamine, 0.4 ml of 5 mM 2-oxoglutarate, 0.1 ml 100 mM KCl, 0.6 ml 1 mM NADH and 0.5 ml of enzyme preparation in a final volume of 3 ml completed with 100 mM Tris-HCl (pH 7.5). The reaction was started by adding L-glutamine immediately following the enzyme preparation. The decrease in absorbance was recorded for 5 minutes at 340 nm in a double beam spectrophotometer. The amounts of NADH oxidised was calculated from a standard curve of NADH.

Estimation of protein : Protein in each enzyme extraction was estimated by the method of Lowery *et al.*, (1951) after precipitation with equal volume of 20% TCA. Bovine serum albumin was used as standard.

The data presented are average value ± S.E. of atleast two independent series of experiments each with three replicate determinations. Paired t-test was applied whenever required to test for significance of difference.

13.3 RESULTS

In-vivo NR activity :

The effect of increasing NaCl levels on NR activity in NaCl sensitive and resistant callus lines with time is shown in Table 1. NR activity in non-stressed NaCl sensitive callus increased during early days of growth upto 14 days and decreased thereafter. Salt treatment caused an increase in enzyme activity thoroughout the growth period, the increase being greater later in growth at low (100-200 mol m^{-3}) NaCl concentration. At 28-d of growth, 100 mol m^{-3} NaCl caused a 4-fold increase in enzyme activity in NaCl sensitive callus, while at 300 mol m^{-3} NaCl. NR was almost the same as in the control calli of sensitive line.

NR-activity in non-stressed NaCl-resistant callus decreased with the growth of callus but increased during early days of growth under low levels (100-200 mol m^{-3}) of salt upto 14 days as well as at high salt levels (250-300 mol m^{-3}) on 28 day of growth. On 28 day NaCl at 300 mol m^{-3} caused a 2.5-fold increase in enzyme activity in resistant calli.

The NaCl-resistant callus in the absence or presence of salt maintained low levels of NR activity compared with NaCl sensitive callus and increased enzyme activity due to NaCl stress except at 250-300 mM on 28 day of growth was also less in resistant calli compared with sensitive ones. In other words, salt-sensitive line is characterized by high levels of NR activity and a greater increase under low concentration of salt compared with resistant line which maintained higher NR activity at 250 and 300 mM NaCl on 28 day of growth.

In-vitro NR activity

In-vitro NR activity of NR sensitive calli in the absence of NaCl increased upto 14 day of growth and, thereafter, decreased considerably (Table 2). However, in the presence of NaCl, the enzyme activity increased at almost all the salt levels throughout the growth period.

NR activity of NaCl tolerant calli under unstressed conditions increased over the entire growth period. Salt treatments caused an increase in NR activity in NaCl-resistant calli only during early days of growth upto 14 days and decreased thereafter.

NaCl-sensitive calli under NaCl stress maintained high *in-vitro* activity of NR than NaCl-resistant calli throughout the growth period. However, in the absence of NaCl, *in-vitro* NR activity former was higher only upto 14 days of growth than the latter.

NADH-GDH activity

NADH-GDH activity of NaCl-sensitive 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 thereafter decreased, while at 28-d the GDH activity remain unaffected at lower concentrations (100-200 mol m^{-3}) compared to those grown in the absence of salt (control). GDH activity in tolerant calli at 300 mM NaCl, declined from 7 day onwards though it was significantly higher than control upto 21 day of culture (Table 3).

The NaCl-sensitive calli showed decrease in GDH activity at all the salt concentrations throughout the growth period except on 28-d at low NaCl concentrations (100 to 200 mol m^{-3}) where significant ($P > 0.05$) increase was observed over the control.

A comparison of both the lines showed that NADH-GDH activity in the presence of NaCl was higher in NaCl-resistant line than the sensitive line almost throughout the growth period.

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 day, NaCl-sensitive calli in the absence of NaCl showed higher NAD-GDH activity than tolerant callus line whereas in the presence of NaCl, enzyme activity was much lower in the former than the latter at all the salt concentrations compared to their respective control (Table 4).

GOGAT

NADH-GOGAT activity in both NaCl-sensitive and resistant lines growing in the absence of salt 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 control (Table 5).

13.4 DISCUSSION

The NR activity in NaCl sensitive calli under salt stress increased throughout the growth period, while in resistant calli increased at low NaCl concentrations during early growth and at higher concentrations during 4th week of growth. Enhancement of NR activity under salinization has also been reported in *Zea* mays seedlings (Aliva and Klyshev, 1975), *Suaeda maritima* (Billard and Boucaud, 1982) and in *Phaseolus aureus* (Misra and Dwivedi, 1990). However, NR is also reported to be inhibited by saline stress (Lal and Bhardwaj, 1987; Rao and Gnanam, 1990). 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 control cultures. The induction of enzyme synthesis by salt stress, therefore, may play a role in the salt tolerance

expressed by *Vigna* culturees. In the present study, the resistant calli selected at 300 mol m^{-3} NaCl exhibit an efficient nitrate reduction at NaCl concentration on which they were selected on 28-d of growth. This seems to be an adaptation to saline stress. These results indicate that maintenance of high levels of enzymes can be a method for coping with salt stress as proposed by Billard and Boucaud (1982).

The NADH-GDH activity in the presence of NaCl was higher in tolerant calli than NaCl sensitive calli thoroughout the growth period. Such increase in GDH activity by saline treatment has also been reported from a number of sources (Rao *et al.,* 1981; Subhashini and Reddy, 1990). However, GDH is also reported to be inhibited by saline stress (Kasymbekov *et al.,* 1980; Misra and Dwivedi, 1990). The increase in NADH-GDH activity under saline conditions may be the consequence of increased ammonia which accumulate under these conditions. The accumulation of ammonia is very harmful to metabolism (Givan, 1979) and it may be detoxified by increased levels of GDH. Thus, increased activity of NADH-GDH in tolerant cells under stress may play a vital role in protecting the cells from the stress effect. It is speculated that salt stress may increase NADH-GDH activity in salt tolerant line by increasing the amount of enzymatic protein or/and by modulating the activity of the existing enzyme molecules. On the other hand, salt stress has completely inhibited NAD-GDH activity in both the cell lines after 7 days of growth. Similar inhibition in deaminating activity of GDH has been reported in the presence of ammonia (Singh and Srivastava, 1982). These results indicates that salt stress affect both the aminating and deaminating activity of GDH.

GOGAT activity was found to decreased under saline treatment in both the NaCl - resistant and sensitive calli throughout the growth period, except in the former where activity increased during second week of growth. Decreased in GOGAT activity under salt stress has also been reported earlier (Billard and Boucaud, 1982). However, increase in GOGAT activity under saline stress has also been reported by *Miranda*-Ham and Loyala-Vargas (1988) and Misra and Dwivedi (1990).

Thus, the nitrogen assimilation in salt tolerant calli under salt stress was found to be characterized by high level of NR and GDH activity. Concomitantly with low GOGAT activity.

An over view of the data presented suggests that during NaCl treatment, when NO_3^- reduction is occuring very efficiently and GS and GOGAT is inefficient, NH_4^+ is probably assimilated by GDH pathway. However, whether the shift in enzyme level would also imply a shift in NH_4^+ assimilation from GS-GOGAT to GDH pathway during salt stress cannot be predicted with certainity at this stage of study since high levels of enzymes activity as such are not sufficient to permit NH_4^+ assimilation, other factors such as concentration of ATP,

reductants, α - ketoglutarate and glutamine pool etc. may also be critical in controlling the actual rate of NH_4^+ assimilation (Givan, 1979). On the other hand, the sensitive calli under salt stress assimilate NH_4^+ by coaction of NADH-GDH and GS-GOGAT pathway.

Table 1. In vivo nitrate reductase activity in control and salt calli of NaCl-sensitive and resistant callus lines of Vigna radiata cv.K 851 at different days of growth

Callus line	NaCl concentration (mol m^{-3})	Nitrate reductase activity, μ mole NO_2^- h^{-1} g^{-1} FW			
		Days after growth			
		7	14	21	28
Resistant	0	2.43 ± 0.11 (100)	2.17 ± 0.98 (100)	1.74 ± 0.08 (100)	0.41 ± 0.03 (100)
	100	6.04 ± 0.12 (247)	4.11 ± 0.10 (189)	2.37 ± 0.05 (136)	0.39 ± 0.01 (95)
	200	3.99 ± 0.10 (163)	3.72 ± 0.04 (171)	1.32 ± 0.15 (76)	0.61 ± 0.01 (148)
	250	1.17 ± 0.07 (48)	1.98 ± 0.09 (91)	1.04 ± 0.01 (59)	1.06 ± 0.01 (255)
	300	0.59 ± 0.01 (24)	1.04 ± 0.01 (48)	0.99 ± 0.12 (56)	1.04 ± 0.01 (251)
Sensitive	0	5.13 ± 0.05 (100)	7.46 ± 0.07 (100)	2.12 ± 0.04 (100)	1.04 ± 0.01 (100)
	100	11.18 ± 0.05 (217)	8.39 ± 0.07 (112)	6.99 ± 0.07 (329)	4.41 ± 0.03 (423)
	200	11.29 ± 0.18 (219)	8.90 ± 0.17 (119)	4.61 ± 0.05 (216)	3.82 ± 0.05 (366)
	250	9.65 ± 0.13 (188)	7.75 ± 0.12 (103)	3.12 ± 0.16 (146)	2.17 ± 0.01 (208)
	300	4.67 ± 0.18 (91)	7.60 ± 0.06 (101)	3.64 ± 0.08 (172)	1.16 ± 0.02 (111)

Values are mean ± SE based on three independent observations.
Values relative to control are given in parentheses as percentages

Table 2. In vitro nitrate reductase activity in control (in absence of salt) and salt stressed calli of NaCl-sensitive and resistant callus lines of Vigna radiata cv.K 851 at different days of growth

Callus line	NaCl concentration (mol m^{-3})	Nitrate reductase activity, μ mole NO_2^- h^{-1} g^{-1} FW			
		Days after growth			
		7	14	21	28
Resistant	0	1.58 ± 0.04 (100)	1.84 ± 0.03 (100)	2.35 ± 0.18 (100)	2.55 ± 0.16 (100)
	100	1.76 ± 0.14 (111)	4.35 ± 0.03 (236)	2.29 ± 0.06 (97)	0.91 ± 0.02 (35)
	200	3.40 ± 0.29 (215)	5.37 ± 0.21 (291)	1.58 ± 0.14 (67)	0.53 ± 0.01 (20)
	250	1.30 ± 0.01 (82)	5.01 ± 0.24 (272)	1.06 ± 0.06 (45)	0.76 ± 0.05 (29)
	300	0.94 ± 0.00 (59)	2.41 ± 0.12 (130)	1.06 ± 0.06 (45)	0.65 ± 0.0 (25)
Sensitive	0	3.67 ± 0.20 (100)	4.18 ± 0.13 (100)	0.62 ± 0.09 (100)	0.80 ± 0.01 (100)
	100	2.91 ± 0.11 (79)	4.28 ± 0.14 (102)	3.97 ± 0.18 (632)	1.12 ± 0.00 (138)
	200	5.60 ± 0.26 (152)	6.13 ± 0.08 (146)	0.67 ± 0.06 (107)	1.17 ± 0.03 (144)
	250	6.60 ± 0.08 (180)	5.83 ± 0.12 (139)	0.76 ± 0.07 (121)	1.43 ± 0.04 (177)
	300	2.11 ± 0.06 (57)	6.07 ± 0.22 (145)	1.30 ± 0.10 (207)	1.12 ± 0.00 (138)

Values are mean ± SE based on three independent observations.
Values relative to control are given in parentheses as percentages.

Table 3. Effect of increasing concentration of NaCl on NADH-GDH activity in NaCl-sensitive and resistant callus lines of Vigna radiata cv.K-851 at different days of growth under increasing concentrations of NaCl.

Callus line	NaCl concentration (mol m^{-3})	Enzyme activity, n mol NADH (mg protein)$^{-1}$ min^{-1}			
		Days after growth			
		7	14	21	28
Resistant	0	437.4 ± 30.5 (100)	244.2 ± 14.3 (100)	230.7 ± 7.0 (100)	192.2 ± 18.2 (100)
	100	380.1 ± 27.8 (87)	152.3 ± 6.2 (62)	177.6 ± 18.4 (77)	194.1 ± 8.1 (100)
	200	[b]732.6 ± 21.7 (167)	454.2 ± 63.4 (185)	217.1 ± 5.3 (94)	200.7 ± 9.0 (104)
	250	[a]2048.6 ± 19.2 (468)	[a]530.7 ± 4.2 (217)	196.1 ± 29.9 (85)	158.2 ± 10.1 (79)
	300	[a]2825.1 ± 12.6 (645)	[a]478.5 ± 12.2 (196)	[a]409.9 ± 7.4 (177)	120.5 ± 6.7 (62)
Sensitive	0	1316.2 ± 15.3 (100)	518.2 ± 13.8 (100)	238.2 ± 10.5 (100)	141.64 ± 9.7 (100)
	100	330.7 ± 22.0 (25)	403.4 ± 4.2 (77)	202.5 ± 11.9 (85)	183.7 ± 10.2 (129)*
	200	912.3 ± 19.7 (69)	295.2 ± 5.0 (56)	186.5 ± 14.3 (78)	183.9 ± 7.2 (129)*
	250	288.6 ± 0.0 (22)	485.7 ± 39.2 (93)	116.6 ± 0.0 (49)	128.3 ± 6.7 (90)
	300	[b]1558.1 ± 14.8 (118)	89.9 ± 9.4 (17)	138.1 ± 13.9 (58)	48.9 ± 2.7 (34)

Values are mean ± SE based on three independent observations. Values relative to control are given in parentheses as percentages.

Table 4. Effect of increasing concentration of NaCl on NAD^+-GDH activity in NaCl-sensitive and resistant callus lines of Vigna radiata cv. K-851 at 7 days of growth under increasing concentration of NaCl.

NaCl concentration mol m^{-3}	Enzyme activity, n mol NADH (mg protein)$^{-1}$ min.$^{-1}$	
	Callus lines	
	Sensitive	Resistant
0	2112.1 ± 11.7 (100)	671.3 ± 7.8 (100)
100	264.5 ± 2.5 (12)	537.1 ± 4.7 (80)
200	892.6 ± 8.9 (42)	986.5 ± 8.2 (147)
250	962.2 ± 7.9 (45)	1062.2 ± 11.2 (158)
300	81.7 ± 3.2 (4)	793.2 ± 8.9 (118)

Values are Mean ± SE based on three independent observations.
Values relative to control are given in parentheses as percentages.

Table 5. Effect of increasing concentrations of NaCl on glutamate synthase (NADH) activity in NaCl-sensitive and resistant callus lines of Vigna radiata cv. K-851 at different days of growth.

Callus line	NaCl concentration mol m^{-3}	Enzyme activity, n mol NADH oxidised (mg protein)$^{-1}$ min.$^{-1}$			
		Days after growth			
		7	14	21	28
Resistant	0	651.0 ± 5.1 (100)	217.3 ± 1.8 (100)	127.7 ± 41.2 (100)	72.1 ± 2.4 (100)
	100	337.1 ± 2.9 (51.7)	351.8 ± 4.6 (162)	94.4 ± 10.7 (74)	41.0 ± 2.7 (57)
	200	342.4 ± 2.7 (52)	458.2 ± 6.6 (211)	55.6 ± 13.2 (43)	25.1 ± 0.0 (35)
	250	388.5 ± 3.0 (59.6)	364.1 ± 3.2 (167)	23.2 ± 0.0 (18)	13.3 ± 1.5 (18)
	300	527.2 ± 5.2 (80.9)	292.7 ± 15.1 (134)	29.7 ± 0.0 (23)	14.3 ± 0.7 (20)
Sensitive	0	707.1 ± 6.8 (100)	532.6 ± 29.8 (100)	55.4 ± 4.7 (100)	68.5 ± 16.5 (100)
	100	790.8 ± 7.0 (112)	473.6 ± 4.2 (89)	33.7 ± 0.0 (61)	34.4 ± 2.3 (50)
	200	601.2 ± 5.9 (85)	453.7 ± 4.3 (85)	20.4 ± 0.8 (37)	21.6 ± 2.4 (31)
	250	640.5 ± 5.6 (90)	278.1 ± 11.7 (52)	30.2 ± 3.0 (54)	13.3 ± 2.9 (19)
	300	580.7 ± 5.4 (82)	227.5 ± 3.4 (42)	22.7 ± 1.9 (61)	15.3 ± 3.0 (22)

Values are Mean ± SE based on three independent observations.
Values relative to control as given in parentheses as percentages.