Salinity Stress Enhances Production of Solasodine in *Solanum nigrum* L.

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Various *in vitro* grown tissues (non-regenerative callus, regenerative callus and microshoot derived leaves) of *Solanum nigrum* L. were cultured under salinity stress (0—150 mM NaCl) for enhanced production of solasodine, a steroidal alkaloid and an alternative to diosgenin, which is used as a precursor for the commercial production of steroidal drugs. The role of plant growth regulators and various concentrations of NaCl during *in vitro* production of solasodine was studied. The *in vitro* yield was compared with the yield from leaves of field grown plant. Solasodine content was maximum (2.39 mg/g dry wt.) in regenerative callus when grown on medium added with 150 mM NaCl; followed by *in vitro* raised leaf of microshoot. Quantitative estimation of solasodine was carried out using a new HPTLC method, which is validated for its recovery and precession. The proposed HPTLC method showed a good linear relationship (r^2=0.994) in 50—2000 ng/spot concentration ranges. The data demonstrate that the solasodine production in cultures was growth dependent.

Key words *Solanum nigrum*; solasodine; non-regenerative callus; regenerative callus; Murashige and Skoog (MS) medium; salinity

The genus Solanum includes about 1400 species.[1] *Solanum nigrum* is an important medicinal plant and contains solasodine, a steroidal glycoalkaloid, which is considered as a potential alternative to diosgenin for commercial synthesis of various steroidal drugs.[2] Solasodine can be converted to dehydropregnenolone and shows a close similarity to diosgenine (Fig. 1). It is found with a series of sugar residues attached to the oxygen atom at C-3; most common forms are the triglycosides and solamargin.

Solasodine is widely regarded as defensive allelochemical against a number of pathogens and predators.[1] It may cause disruption of cell membranes and the inhibition of acetylcholine esterase, a key enzyme in nerve impulse transmission.[3] In recent times, the mixtures of solasodine glycosides have been used successfully for the treatment of certain human skin carcinomas.[4]

The plant cell and tissue culture techniques have been used to produce secondary metabolites over the last 50 years.[5] Various strategies involving callus/suspension[6] and hairy root cultures[6—8] have been employed for the production of solasodine. However, production of solasodine from other cultures using differentiated callus is little explored. The metabolite content is regulated by various factors including basal medium components, carbon source and photoperiod *etc*.[9,10] Plant growth regulators have been tried to stimulate alkaloid synthesis in different cell cultures including *S. nigrum*,[11,12] but the influence of salinity as elicitor, has not been conducted in a big way for secondary metabolite synthesis. The present report describes the production of solasodine from three cultures (undifferentiated callus, differentiated callus and microshoot leaf) in response to various growth regulators and NaCl. A new HPTLC method has been used and described for quantitative estimation of solasodine.

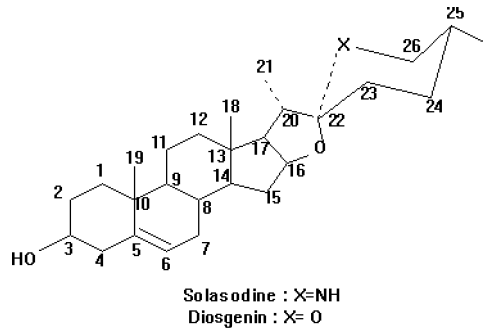

Fig. 1. Solasodine and Closely Related Diosgenin (Luckner, 1984)

Experimental

***In Vitro* Study** Leaf, apical bud and node/internode were the major explant sources. Collected from Herbal Garden of Hamdard University, New Delhi, the explants were surface sterilized in 0.1% $HgCl_2$ for 4 min, and rinsed three times in sterile distilled water. All the cultures were grown on MS[13] (Murashige & Skoog's) basal medium. The medium contained 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Company). For callus initiation, MS medium was supplemented with different plant growth regulators like 2,4-dichloro-phenoxy acitic acid (2,4-D), napthaline acitic acid (NAA), and indole-3-acitic acid (IAA). Regeneration study was carried out to achieve emergence of plantlets from two-weeks-old normal callus treated with various levels of 6-benzylamino purine (BAP), NAA and IAA either individually or in combination on MS medium. *In vitro* shoot formation (axillary shoot induction) was achieved using nodal explants on MS added with various levels of BAP and kinetin (KIN). The pH of the media was adjusted to 5.8 before sterilization (at 121 °C for 20 min). The cultures were kept in a growth room at a temperature of 25±2 °C light/20 °C dark and provided with cool, white fluorescent light with a 16 h photoperiod (Fluorescent F40 T12/CW/EG) lamp at a photon flux density of 100 μmol m^{-2} S^{-1}. The data obtained from the experiments conducted twice with five replicates per treatment, were analyzed statistically by application of *t*-test.

NaCl Treatment The best responsive cultures (regenerating cultures maintained on 0.75 mg/l BAP+2 mg/l NAA, non-regenerating callus maintained on 0.25 mg/l 2,4-D, and microshoots maintained on 0.5 mg/l BAP) were subjected to NaCl treatments in order to study the response of salinity stress on cultures. MS medium supplemented with optimized plant growth regulator (PGR) levels was separately treated with 25, 50, 100 and 150 mM NaCl. The pH of the medium was adjusted to 5.6—5.8 before autoclaving and cultures were inoculated. These cultures were regularly sub-cultured at the interval of four weeks on the same PGR and NaCl treatment. The quantification of solasodine of each culture was made after 12 weeks of incubation.

Quantification of Solasodine by HPTLC The content of solasodine in field grown plants and in *in vitro* cultures was estimated using solasodine (MP Biomedicals, LLC Germany) as standard.

Sample Preparation A modified Bradley's method[14] was used for extraction in which dried and powdered field grown plant (leaf) and lyophilized *in vitro* cultures (500 mg each) were refluxed separately in 15 ml

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of 1 M HCl on water bath for 2 h. It was filtered and washed with the same solvent. The pH of filtrate was adjusted to 10 using a dilute ammonia solution (10% v/v); basic solution was extracted with $CHCl_3$ (20 ml each) thrice to ensure complete extraction. The $CHCl_3$ extracts were pooled and evaporated on water bath (below 40 °C). Dried extracts were further dissolved in methanol for application to TLC plate for quantification.

Standard Solasodine One mg/ml solution of standard solasodine was prepared in methanol and 1 ml of this solution was diluted to 10 ml with methanol to get 100 μg/ml solution, both of these solutions were used for application on TLC plate for preparation of standard plot. The UV spectrum of standard solasodine solution in methanol (50 μg/ml) was recorded using a UV spectrophotometer (UV-1601, Shimadzu Japan) for authentication. The λ_{max} obtained (205, 224, 280 nm) was matched with that as reported for standard solasodine.[15)]

HPTLC Instrumentation and Conditions The samples were spotted in the form of bands (width 3 mm) with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm×10 cm with 0.2 mm thickness, E. Merck, Germany) using a Camag Linomat V (Switzerland). A constant application rate of 80 nl/s was employed and space between two bands was 5.2 mm. The slit dimension was kept at 4 mm×0.1 mm, and a 20 mm/s scanning speed was employed. The mobile phase consisted of chloroform : methanol (5 : 1, v/v). Linear ascending development was carried out in twin trough glass chamber, saturated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 65 mm. Subsequent to the development; TLC plates were dried with air current provided by an air-dryer. Densitometric scanning was performed on Camag TLC scanner IV (absorbance mode 510 nm) after spraying with anisaldehyde sulphuric acid and dried at 100 °C. Deuterium and tungsten lamps were used as radiation sources.

Calibration Curves of Solasodine Different volumes of standard solutions of solasodine 0.5, 1, 2, 3, 4, 5 and 6 μl of 100 μg/ml and 0.7, 1 and 2 μl of 1000 μg/ml were spotted in triplicate on TLC plate to obtain 50, 100, 200, 300, 400, 500, 600, 700, 1000 and 2000 ng per spot of solasodine, respectively. The data of peak area *vs.* solasodine concentration were treated by linear least-square regression and the regression equation thus obtained from standard curve, was used to estimate solasodine in different samples.

Recovery Studies The accuracy of the present method was checked in samples by recovery studies in which, pre-analyzed samples were spiked with extra 50, 100 and 150% of the standard solasodine; the mixtures were reanalyzed by adopting the proposed method. The experiment was repeated six times, average recovered solasodine content was quantified using regression equation, and the % recovery was calculated accordingly.

Precision The precision of the proposed method was obtained by determining intra- and inter-day variation in which six replicates of solasodine at two concentration levels (200 and 400 ng per spot) were carried out and the Percent Relative Standard Deviation (RSD) was calculated.

Quantification of Solasodine in Different Samples Samples of *in vitro* culture and field-grown plant (leaf), 5 μl each, were applied in triplicate on TLC plate for quantification of solasodine. The alkaloid yield was quantified by using regression equation of calibration curve.

Results and Discussion

Solasodine production has been monitored and assessed from various *in vitro* cultures, therefore we cultured and cultivated callus (regenerative and non regenerative) and microshoots in different PGR added media. Solasodine production analysis was made when the cultures were in maximum growth stage; the responses are summarized hereunder:

Callus Induction Leaf and nodal explants from field-grown *S. nigrum* responded differently when grown on media added with different auxins [2,4-D, NAA and IAA (0.25, 0.5, 1.0, 2.0 mg/l)]; 2,4-D was observed to be more effective for callus induction (Fig. 2), while NAA and IAA were effective only at high concentrations (Figs. 3, 4). Among various PGR concentrations, 0.25 mg/l 2,4-D was most responsive and induced profuse callus; the nodal callus was friable, white and fast-growing, while the leaf callus was compact and light yellow, and grew relativity slow. The callus formation started from two nodal ends and later on the whole explant was covered with callus; in case of leaves, it started from margins. High concentration (2.0 mg/l) of 2,4-D resulted in decreased callus formation. The calluses were periodically transferred to the optimized medium (0.25 mg/l 2,4-D) for continuous growth and subjected to 0—150 mM NaCl treatment for solasodine production. Callus was induced from all the explants but differed in biomass and growth rate. Efficient callus induction and maintenance were noted only on MS with 2,4-D (0.25 mg/l). This may be because 2,4-D is much more stable and less inactivated during culture processes compared with other auxins.[16)] Similar results have been reported earlier, establishing clearly the superiority of 2,4-D over other auxins in inducing callus and antagonizing any organized development.[17—19)] In *Solanum nigrum* nodal explant was more responsive and induced profuse callus, compared with leaf. The use of leaf as explant was less responsive giving little production of callus, observed in some previous studies.[19—21)]

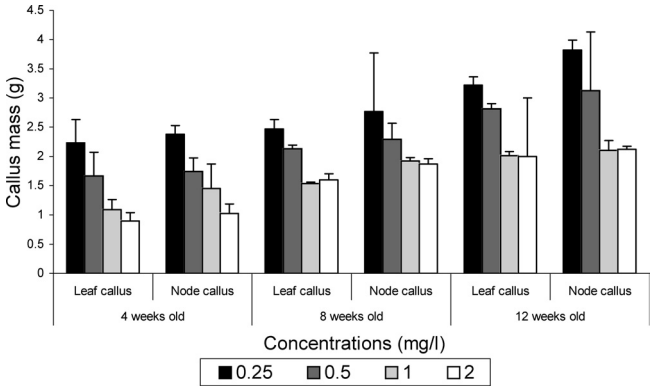

Fig. 2. Leaf and Nodal Callus Growth (g) on Various Concentrations of 2,4-D (mg/l) on MS Medium (Initial Weight of Callus: 1 g)

Table 1. Leaf and Node Derived Callus Cultured on MS Medium Supplemented with BAP and IAA or NAA

MS+PGR (mg/l)	Percentage of explants producing shoots		Mean number of shoots/explant	
	Leaf	Node	Leaf	Node
BAP+IAA				
0.25+1.0	—	—	—	—
0.50+0.25	—	—	—	—
0.50+0.50	—	—	—	—
0.75+0.25	8.72±2.00	6.22±2.33	3.45±2.01	2.98±2.00
0.75+0.50	10.77±2.55	20.22±3.00	6.11±2.45	6.12±1.85
0.75+0.75	6.15±1.33	8.12±2.33	5.11±2.11	6.00±2.01
1.0+0.50	2.10±1.66	3.06±1.55	3.10±2.04	3.20±1.11
BAP+NAA				
0.75+0.25	8.22±1.66	10.27±2.10	6.17±1.95	8.22±1.33
0.75+0.50	48.72±2.66	32.11±1.63	7.12±2.14	6.03±1.23
0.75+0.75	52.11±1.12	61.15±2.55	7.19±2.04	6.18±2.01
0.75+2.0	81.12±3.02	78.21±2.01	14.15±2.00	18.71±1.22
1.0+0.50	69.18±0.12	70.22±1.22	10.31±3.00	9.43±1.00
2.0+0.25	45.15±1.22	41.92±1.22	6.11±2.01	5.93±1.22
2.0+0.50	47.01±0.11	39.41±2.00	6.03±2.00	6.05±1.00

The data on shoot number and percentage of response were recorded after 15 d of culture. The values (mean±S.D.) are based on five individual readings.

Shoot Regeneration Induced calluses (3—4 weeks old) placed on regeneration medium added with several combinations and concentrations of PGRs did not exhibit a high regeneration frequency. A significant variation in shoot regen-

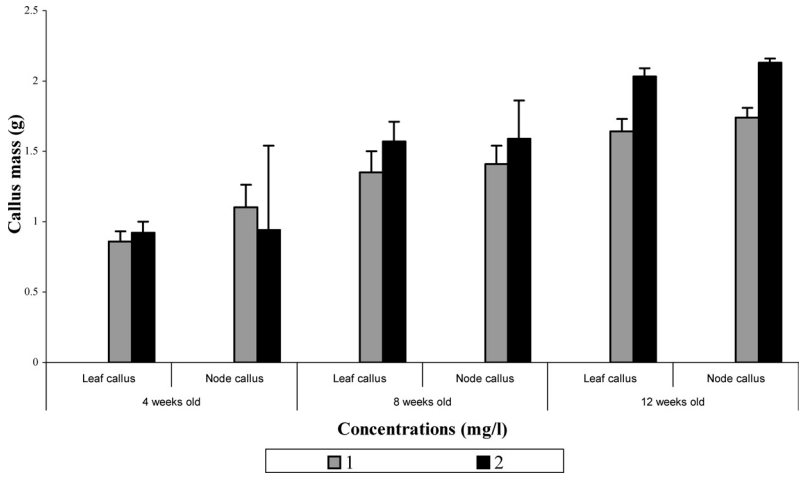

Fig. 3. Leaf and Nodal Callus Growth (g) on Various Concentrations of NAA (mg/l) on MS Medium

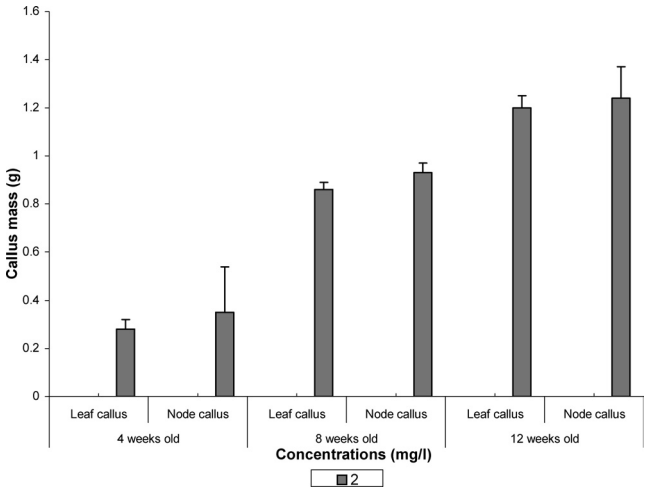

Fig. 4. Leaf and Nodal Callus Growth (g) on Various Concentrations of IAA (mg/l) on MS Medium

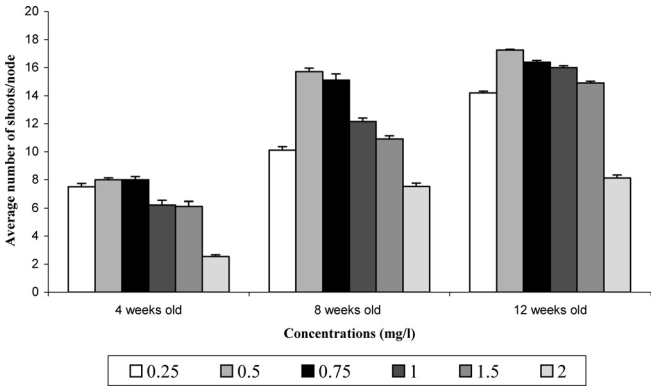

Fig. 5. Nodal Shoot Multiplication on MS Medium Added with Concentrations of BAP

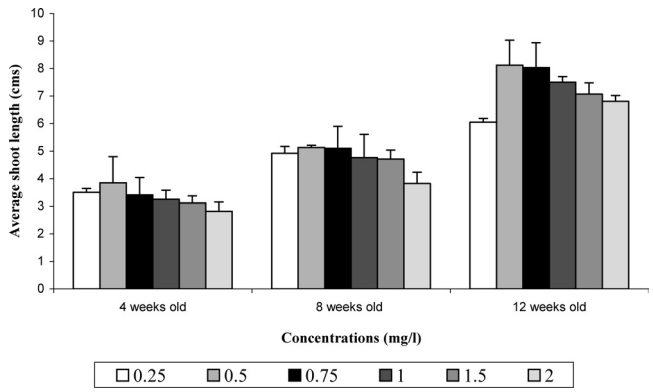

Fig. 6. Nodal Shoot Length (cm) on MS Medium Added with Concentrations of BAP

eration rate was observed when leaf- and node-derived calluses were cultivated on MS, either with BAP+IAA or with BAP+NAA, (Table 1). Among the concentrations and combinations tested, maximum regeneration was observed in MS+BAP (0.75 mg/l)+NAA (2 mg/l), in which 81% leaf calluses and 78.21% node calluses produced shoots; MS+BAP (1 mg/l)+NAA (0.5 mg/l) was also effective. Regeneration was very poor with other combinations. On media amended with BAP+IAA (0.25+1.0, 0.50+0.25, 0.50+0.50), however, no regeneration was noted. In the present study, high frequency of shoot regeneration was noted in MS when supplemented with BAP and NAA. Similar morphogenetic responses have been observed and described for several other medicinally important plants.[18,22,23]

Shoot Multiplication and Shoot Growth Axillary shoot induction and multiplication were also achieved when nodal explants were cultured on MS, amended with BAP or KIN. Shoot induction and growth rate were highly influenced by the nature and concentrations of cytokinins applied. After one week, the explants showed apparent differentiation of buds. The maximum number of shoots per explant (17.25 shoots, after 12 weeks), with a mean length of 8.12 cm, was observed on MS supplemented with 0.50 mg/l BAP, followed by MS+0.75 mg/l BAP (Figs. 5, 6). The response was poor with higher concentration (2.0 mg/l) of KIN, which produced a few shoots (5.44) with average height of 6.75 cm (Figs. 7, 8). The optimum concentration of PGR showing best response in terms of growth and production of biomass (MS supplemented with 0.50 mg/l BAP) was continued for solasodine analysis.

Quantification of Solasodine in Various Tissues The solvent system developed produced a sharp and compact peak of solasodine (*Rf* 0.22±0.05, Fig. 9). Densitometric

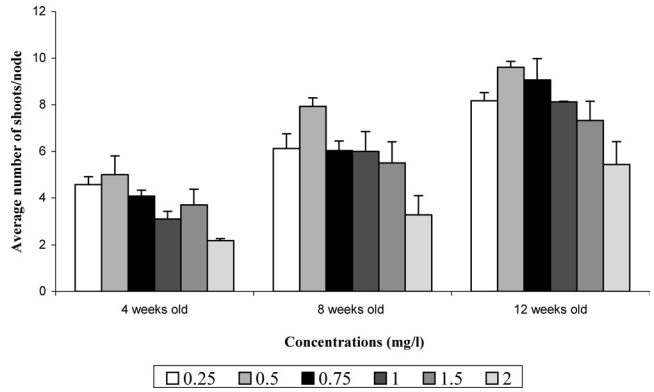

Fig. 7. Nodal Shoot Multiplication on MS Medium Added with Concentrations of Kinetin

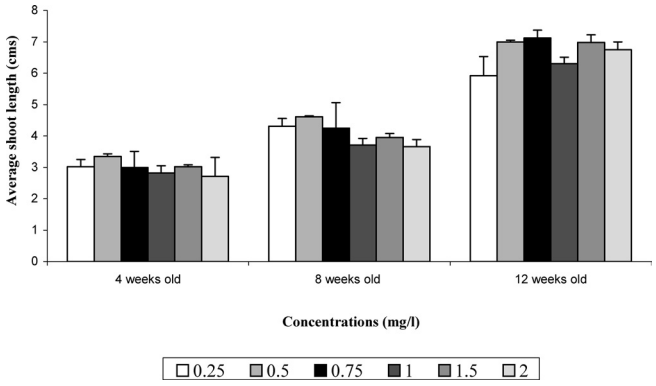

Fig. 8. Nodal Shoot Length (cm) on MS Medium Added with Concentrations of Kinetin

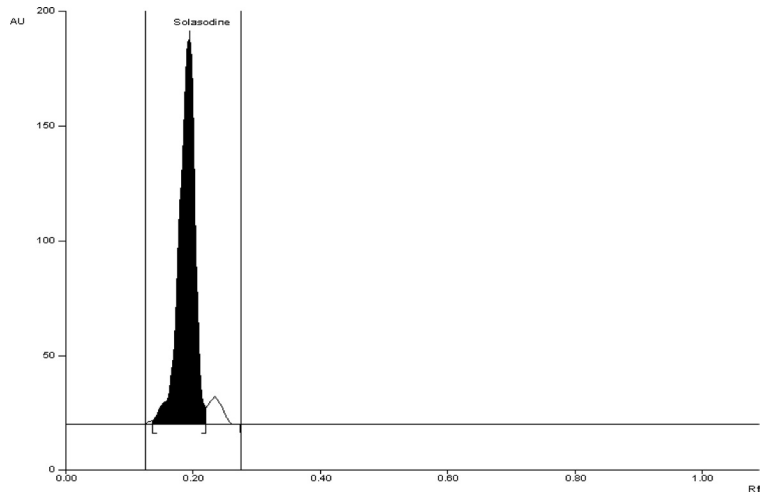

Fig. 9. HPTLC Chromatogram of Standard Solasodine (700 ng/Spot)

analysis of solasodine was carried out at 510 nm absorbance mode. The regression analysis data showed a good linear relationship, with $r=0.997$ ($r^2=0.994$), with respect to peak area and concentration in the range of 50—2000 ng per spot. The regression equation obtained was $Y=2.0233X+923.04$ with respect to peak area. The proposed method when used for solasodine extraction and subsequent estimation (after spiking with 50, 100 and 150% of additional drug) afforded recovery of 99.18—100.88% (Table 2). The intra- and inter-day variation of alkaloid at two different concentration levels showed a low % Relative Standard Deviation (0.95—1.57%, Table 3). The proposed HPTLC method is simple, can be used over a wide range of concentrations with good recovery and does not require any tedious pre chromatographic derivatization and multiple extraction procedures.[24] The content of solasodine in different samples was analyzed from the regression equations using values of area obtained from wincats software. The mean values of samples are presented in Fig. 10.

Salt treatments have had a great impact on solasodine content at all the three morphogenetic stages (each culture 12 weeks old, Fig. 10). The minimum content of solasodine (0.512 mg/g dry wt.) occurred in field-grown plant leaf, whereas the maximum content (2.020 mg/g dry wt.) was found in *in vitro* leaf. In the non-regenerative callus, maximum content of solasodine (1.298 mg/g dry wt.) was obtained with 100 mM NaCl. In case of regenerative callus (showing maximum shoot emergence on MS+BAP (0.75 mg/l)+NAA (2 mg/l)), maximum content of solasodine (2.398 mg/g dry wt.) was noted at 150 mM NaCl-supple-

Table 2. Recovery Studies[a)]

Excess drug added to the analyte (%)	Theoretical content (ng)	Recovery (%)	% RSD
0	200	99.18	1.13
50	300	99.23	0.95
100	400	99.99	0.97
150	500	100.88	0.33

a) $n=6$.

Table 3. Intra- and Inter-day Precision of HPTLC Method[a)]

Amount (ng per spot)	Mean (area)	S.D.	% RSD	S.E.M.
a. Intra-day				
200	1325.035	15.47	1.17	6.32
400	1733.115	16.46	0.95	6.72
b. Inter-day				
200	1324.70	20.83	1.57	8.50
400	1732.28	18.92	1.09	7.72

a) $n=6$.

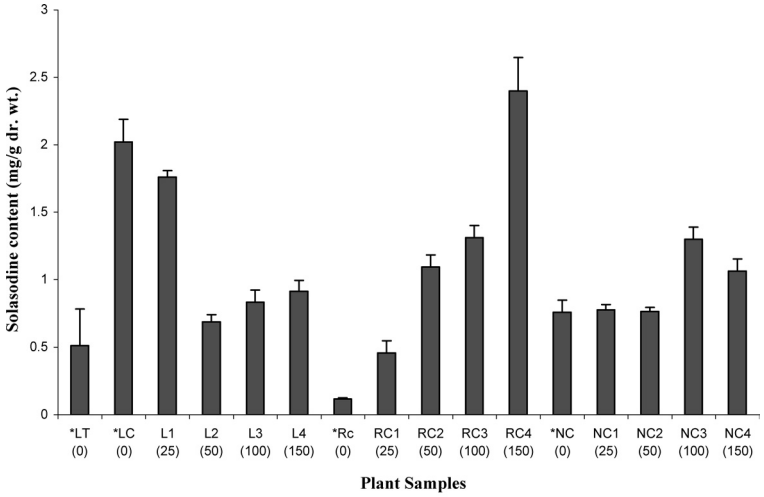

Fig. 10. Effect of NaCl on Solasodine (mg/g Dry Wt.) in Various Samples as Analysed by HPTLC (Mean±S.D.)

mented medium. We noted enhanced solasodine production in some cultures compared to field-grown plants. Among various cultures, regenerating callus treated with 150 mM NaCl contained the highest solasodine, followed by *in vitro* raised leaf (without salt treatment). Our results corroborate the earlier reports,[25,26] where enhanced alkaloid was noted with increased NaCl level. Various biotic and abiotic factors, used as elicitors, have been reported to increase secondary metabolite yield; application of NaCl enhances alkaloid production.[27] In tomato hairy root cultures increased jasmonic acid was noted on 100 mM NaCl amended media.[28]

The biosynthesis of solasodine starts from acetyl coenzyme (A), later converts to mevalonic acid, *via* mevalonic-acid pathway, in which cholesterol, a key intermediate of solasodine, is synthesized. High salinity seems to enhance *in vitro* cholesterol production, which in turn increases solasodine in tissues, or the enhanced yield may be due to over expression of genes. As *Solanum nigrum* is an important and proven medicinal plant, the present protocol offers possibility of enhanced production of solasodine, using NaCl as an efficient and economical elicitor source.

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