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SALT STRESS ENHANCES DAIDZEIN PRODUCTION IN HAIRY ROOT CULTURES OF *PSORALEA CORYLIFOLIA* L.(FABACEAE)

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Abstract

The present study was conducted to evaluate the influence of salt or water stress conditions on the production of daidzein (4.7-dihydroxy isoflavone) or its glycoside (daidzin) and to obtain correlations with enzymes of Ca^{2+} signaling phosphoprotein cascade in hairy root cultures of *Psoralea corylifolia* L (fabaceae). Culturing of hairy roots for five weeks in presence of salt (300 mM NaCl) decreased their growth, but nearly doubled daidzein production during the first two weeks of culture in comparison to untreated controls ($p < 0.01$). However, such changes could not be noted upon water stress caused by 5% polyethylene glycol. Salt stress-induced increase in daidzein production was associated with oxidative stress as evidenced by accumulation of reactive oxygen species, end products of lipid peroxidation, decreased ratio of reduced and oxidized glutathione and up-regulation of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Simultaneous increases in intracellular Ca^{2+} levels were concomitant with increased activities of Ca^{2+} dependent protein kinases and calcineurin, but decreased activity of Ca^{2+} - calmodulin dependent protein kinase. The results indicate correlations between salt stress-induced increase in daidzein production with oxidative stress and Ca^{2+}- signaling events.

Key words: Antioxidants; Ca^{2+} signaling; Daidzein; Oxidative stress; Salt stress; *Psoralea corylifolia* (Fabaceae).

Introduction

Isoflavonoids are naturally produced in legumes as a consequence of plant-pathogen interactions for protecting the host from microbial or insect invasions (Dixon *et al*., 2002). Among various such phytoalexins, much interest has recently been evinced in optimizing the production of daidzein (4.7-dihydroxy isoflavone), because of its potential health benefits to humans (Jia *et al*., 2003; Setchell *et al*., 1981). This phytoestrogen is found in whole plant and produced in high levels by callus cultures of *Psoralea* species (Bouque et al., 1998), as well in hairy roots of *Psoralea* (Bourgaud *et al*., 1999). It is also known that isoflavonoid synthesis, involving the phenylpropanoid pathway, can generally be induced by various biotic and abiotic stress factors (Dixon and Paiva, 1995; Tsukamoto *et al*., 1995). The genes coding for key enzymes in isoflavonoid synthesis, such as isoflavone synthase and isoflavone reductase, are reportedly induced by environmental stress conditions (Dixon *et al*., 2002). In order to enhance isoflavone synthesis in plants, different approaches including genomic profiling studies and metabolic engineering have also been vigorously initiated in recent years (Liu *et al*., 2002; Yu *et al*., 2003).

Recent studies conducted by our group have resulted in establishing genomic and metabolomic profiles of hairy root clones of *Psoralea corylifolia* L. that were transformed with *Agrobacterium rhizogenes* (Gouri *et al*., 2005). Clone-specific compound profiles have resulted in the identification of a Ps I clone with enhanced production of total isoflavonoids. Daidzein production by this clone was also much greater (> 1% of dry weight) compared to the earlier report by Bourgaud *et al* (1999). Previous studies have also indicated close interrelations between daidzein production and stress as well as its recognition as an antioxidant (Pietta *et al*., 2000). These observations prompted us to examine whether abiotic stress conditions can further enhance daidzein production in the hairy root clone. The present study was thus carried out to evaluate the influence of salt and water stress conditions on daidzein production. The obtained results indicate that increased daidzein production in salt stress was concomitant with oxidative stress and increased intracellular Ca^{2+} contents. Since basic events of Ca^{2+}- signaling are well recognized to be involved in the initiation of secondary metabolite production, preliminary correlations were also obtained in the present study between daidzein production and enzymes involved in Ca^{2+} signaling events.

Materials and Methods

Hairy root cultures and stress conditions

Hairy root cultures of *Psoralea corylifolia* were established using *Agrobacterium rhizogenes* strain ATCC 15834 (Gauri *et al*., 2005). The hairy roots originating from different pieces of single seedling hypocotyls were considered as separate clones. The hairy root clones were separated based on their origin and growth pattern. Out of five clones, Ps 1 is selected for this study. Hairy root cultures of Ps 1 clone were maintained on Murashige and Skoog (1962) basal (MSB) medium containing cefotaxime (250 mg L^{-1}) for 2 months followed by atleast nine months on MSB medium without any antibiotics in dark with routine sub culturing for every four- weeks. The stabilized cultures were employed for further analysis. Weighed inoculum (250 mg roots) of hairy roots was sub-cultured on MSB for 4 weeks and then transferred onto MSB medium with 8 g L^{-1} agar containing either NaCl (3 mM, 30 mM and 300 mM) or polyethylene glycol (3%, 4% and 5 %) of different concentrations. The roots were harvested at weekly intervals for upto 5 weeks and analyzed parameters related to oxidative stress, Ca^{2+}-dependent protein kinases and phosphatases apart from determination of daidzein and daidzin production.

Growth analysis and estimation of daidzein and daidzin

Hairy roots cultures grown on MSB media containing either NaCl (3 mM, 3 mM and 300 mM) or polyethylene glycol (3%, 4% and 5%) of different concentrations upto 5 weeks and growth pattern was analyzed by recording fresh weights at an interval of one week. Four replicates were used for analysis. 100 mg hairy roots powder was extracted with 10 ml methanol at room temperature for 2 days, filtered through Whatman filter paper no.1 and concentrated to 1ml. 5 µl of root extract in 495 µl of 100 ppm Fluorescein (MW=332; as internal standard) was filtered through cellulose nitrate filter (Sartorius AG, Germany, 0.45 mm) and chromatographed on reversed phase C18 column (3.9 ´ 150 mm; Waters corporation, MA, USA) using Gilson HPLC system (GILSON, France S.A., Villiers le Bel, France) by employing gradient of acetonitrile 20-40% as

mobile phase (Gauri *et al*., 2005). Merck (Mumbai, India) solvents were used. Daidzein (MW=254) and its glycoside, daidzein (MW=416) were quantitated by employing standards obtained from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of oxidative stress indicating parameters

Reactive oxygen species (ROS) were measured by employing a cell permeable non-fluorescent probe, 10 µM 2', 7'-dichlorodihydrofluorescein diacetate (2', 7' DCFDA) in 0.01% DMSO incubated with hairy roots (20 mg) immersed in 0.1 M potassium phosphate buffer pH 7.0 (PBS) for 30 minutes at 37^0C ± 1 in dark and washed with PBS three times as described by Liu and Schnable (2002). The roots were homogenized in PBS and supernatant were collected by centrifuging at 10,000 × g for 10 min at 4^0C and fluorescence intensity formed upon reactions with ROS was measured (λ_{Ex}= 490 nm, λ_{Em} = 520 nm) as described by Davidson *et al*., (1996).

For measuring the oxidative stress indicator parameters, hairy roots were homogenized at 4^0C and cytosolic extracts were isolated from homogenates by centrifugation at 15000 × g for 30 min at 4^0C in appropriate buffers as recommended by the concerned procedures (Jayashree and Subramanyam, 1999). Protein content in the extracts was determined by Bradford's method (1976).

Products of lipid peroxidation were measured as thiobarbituric acid reactive substances (TBARS) in cytoplasmic extracts according to the method described by Ernster and Nordenbrand (1967) employing malondialdehyde (MDA) as the reference standard.

Reduced (GSH) and oxidized (GSSG) forms of glutathione concentrations in hairy roots protein free filtrates were measured as per Hissin and Hilf (1976). GSH and GSSG react with a fluorescent reagent *o*-phthalaldehyde to yield a fluorescent complex and the intensity was measured at λ_{Ex}= 350 nm, λ_{Em} = 420 nm respectively.

Superoxide dismutase (SOD) activity was assayed in hairy roots extracts by measuring the ability of the enzyme to inhibit super oxide anion-dependent auto oxidation of pyrogallol as described by Marklund and Marklund (1974). One unit of SOD represents the amount of enzyme that inhibits 50% of the rate of auto-oxidation of pyrogallol min^{-1} mg $protein^{-1}$ under the defined assay conditions.

Glutathione peroxidase (GPx) was assayed in hairy roots extracts as per the method of Martinez *et al* (1979) involving degradation of cumene hydroperoxide by the enzyme accompanied by oxidation of GSH to GSSG. The fluorescence of the complex formed between GSSG and *o*-phthalaldehyde was measured at λ_{Ex}= 350 nm, λ_{Em} = 420 nm. One unit of the enzyme activity represents µg of oxidized glutathione (GSSG) formed min^{-1} mg $protein^{-1}$.

Measurement of Intracellular Ca^{2+} levels and assay of Ca^{2+}- signaling enzymes

For determining intracellular Ca^{2+} contents, hairy roots (20 mg) were immersed in phosphate buffered saline (PBS) pH 7.0 and incubated with 10 µM crimson red for 30 minutes at 28 ± 1^0C in the dark. The tissues were washed thrice with PBS and cytoplasmic extracts were isolated by

centrifuging the homogenates at 10,000 × g for 10 min at 4^0C. Fluorescence intensity of the dye bound with free Ca^{2+} was measured in 70 µL cytoplasmic extracts (λ_{Ex}= 590 nm, λ_{Em} = 615 nm) in a spectrofluorimeter (Perkin Elmer, LS-3B) as described by Haugland (1997). Ca^{2+} contents, expressed in nanomolar concentrations, were derived from dissociation constants of crimson red (K_d = 187 nM).

Ca^{2+}- dependent protein kinase (CDPKs: EC 2.7.1.37) activity was determined in hairy root cultures essentially as per Anil *et al*. (2000). Roots were homogenized in presence of ice-cold Tris buffer (40 mM, pH 7.2) containing 1 mMβ- mercaptoethanol, 5 mM EGTA, 1 mM PMSF and leupeptin (4 µg/ ml). The homogenate was clarified by centrifugation at 4°C (25, 000x g) and protein contents were determined in supernatants as per Bradford (1976). In a total volume of 150 µL, the reaction mixture contained 1 mg mL^{-1} histone III-S, Ca^{2+}/ EGTA buffer, (50 mM HEPES pH 7.2, 10 mM $MgCl_2$, 0.25mM EGTA) with or without 0.2 mM $CaCl_2$ and 50 mg of the protein sample. The reaction was initiated by 10 µM [ã-^{32}P]-ATP (5000 µCi/pmol). After incubation at 37^0C for 10 min, keeping on ice rapidly terminated reactions and the reaction mixture was spotted on phosphocellulose (P81) filter papers. After washing the strips twice with 75 mM phosphoric acid, followed by distilled water, incorporation of [ã-^{32}P]-ATP into the substrate was determined by liquid scintillation counting of dried paper strips in 3 mL of Bray's solution (toluene cocktail made of 0.3% 2, 5-diphenyloxazole (PPO) and 0.01% dimethyl POPOP) using a Beckman model LS 1807 liquid scintillation counter (Fullerton, CA, USA) after appropriate quench corrections. One unit of the enzyme is defined in terms of pmoles of [ã-^{32}P]-ATP incorporated into the substrate min^{-1} mg $protein^{-1}$.

Ca $^{2+}$- calmodulin-dependent protein kinase II activity was assayed as described by Hanson *et al*. (1989). Homogenates of hairy roots in a buffer containing 25 mM Tris (pH 7.4) 1mM EDTA, 2 mM NaF, 1 mM β-mercaptoethanol and 0.02% Triton X 100 were clarified by centrifugation at 4°C (25,000x g, 30 min). Reactions were conducted in a total volume of 50µL containing 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.4 mM $CaCl_2$, 5 mg/mL calmodulin, 1 mg/mL bovine serum albumin, 10 mM syntide 2 and 20 µM [ã-^{32}P]-ATP (2 mCi/ mmol) and initiated by addition of hairy root extracts (50 µg protein). After incubation of the contents for 5 minutes, the reactions were terminated by addition of ice-cold 30% TCA (10 µL) and centrifuged (10,000 x g, 10 min at 4^0 C). The incorporation of [ã-^{32}P]-ATP into syntide 2 was determined by applying the reaction mixture on P81 phosphocellulose paper strips in Bray's solution as described earlier. Radioactivity incorporated in presence of 5µM calmidazolium (inhibitor of the enzyme) was considered as blank. One unit of enzyme denotes the incorporation of 1×10^{6} dpm of [ã-^{32}P]-ATP into the synthetic substrate min^{-1} mg $protein^{-1}$.

For assaying the activities of total phosphatase as well as of calcineurin (CaN; EC 3.1.3.16), homogenates of hairy roots were made at 4^0C in a buffer containing Tris (50 mM pH 7.8), 3 mM $MgSO_4 7H_2 0$, 1 mM EGTA, 0.5 mM DTT, 0.02% sodium azide, 0.1 mM PMSF. The contents were clarified by centrifugation at 4°C (20,000xg, 30 min) and used for phosphatase assays. Total phosphatase and calcineurin activities were assayed according to Wang and Pallen (1983) employing p-nitrophenyl phosphate as the substrate. One unit of the enzyme denotes µmole of p-

nitrophenol released from the substrate min^{-1} mg $protein^{-1}$. Calcineurin activity was assayed by measuring phosphate released from the synthetic RII phosphopeptide using malachite green and employing a commercial kit (Calbiochem). One unit of calcineurin specific activity denotes μmoles of free phosphate released from the substrate min^{-1} mg $protein^{-1}$. Simultaneously, calmodulin and calcineurin contents in the extracts were determined by competitive ELISA methods employing monoclonal antibodies specific to bovine brain calmodulin or calcineurin (Padma and Subramanyam 1999).

Data were expressed as Mean ± SD obtained from atleast three independent experiments. Statistical significance of the obtained results was verified by Student's t-test using a commercial package (SigmaPlot 5.05). $p < 0.05$ was considered as significant.

Results and Discussion

The primary objective of this investigation was to obtain improved production of daidzein by hairy root cultures of *Psoralea corylifolia,* and to examine possible correlations with enzymes of Ca^{2+} signaling phosphoprotein cascade. Even though other plants like *Pueraria* contain more of this phytoestrogen, *Psoralea* is enriched in daidzein (Bouque *et al*., 1998). In comparison to the whole plant, hairy roots induced by *Agrobacterium rhizogenes* represent an attractive alternative to callus and cell cultures for producing secondary metabolites in plants (Bhagyalakshmi *et al*., 2004, Shi and Kintizois, 2003). Hairy root cultures of *Psoralea* species transformed by *A. rhizogenes* produce higher levels of daidzein compared to whole plants and callus cultures and the production might be increased upto 0.6% of dry weight (Bouque *et al*., 1998). Recent studies in our laboratory have also shown enhanced daidzein production to as much as 1 g 100 g^{-1} dry weight by Ps I clone of hairy root cultures (Gouri *et al*., 2005). In view of the above, we conducted the present investigation to examine and validate the influence of stress conditions on daidzein production by Ps I clone of *P. corylifolia* hairy root cultures.

One of the important observations made during this study was related to the variable influences of stress conditions induced either by salt or water stress. Hairy root cultures subjected to 300 mM NaCl for 5 weeks showed decreased growth as compared to unstressed cultures. Hairy roots (~ 10 g fresh wt) cultured under salt stress conditions exhibited a dose-dependent decrease over 5 weeks of growth, resulting in 32% ($p < 0.01$) decline in growth at the end of the experimental period. Salt stress also resulted in cumulative increase in the production of daidzein and its glycoside with an initial increase of > 60% in the first two weeks, which was not sustained over subsequent periods; only 21% increase was recorded at the end of the fifth week of salt stress in comparison to untreated cultures of same growth periods (Table 1). The production of aglycone (daidzein) was significantly increased during the second and third weeks accounting to nearly 35% of total amount of aglycone and glycone (daidzin) production. The relative ratio between aglycone/glycoside was also enhanced from 0.23 (observed in untreated controls) to 0.54 during these periods indicating that salt stress caused specific increase in aglycone production but not of the glycone (daidzin). Apparently glycation of daidzein towards formation of daidzin is limited under these conditions. This may perhaps, be attributed to enhanced metabolic channeling of daidzein for the formation of pterocarpans whose synthesis is known to be induced by biotic and abiotic stress factors (Dixon and Paiva, 1995).

In comparison to such results obtained upon salt stress, water stress induced by 5% polyethylene glycol (PEG) did not result in any change either in growth or the production of daidzein or daidzin (Table 1) by hairy root cultures. But for certain observations made with water-stressed rosemary plants that showed enhanced diterpene production (Munne-Bosch *et al*., 1999), such a stress is not known to significantly influence plant secondary metabolite production. Thus, experimental focus in the present study was mainly on salt stress caused by 300 mM NaCl, which resulted in substantial increase in daidzein production to an extent of 18mg g^{-1} dry weight of hairy roots concomitant with their decreased growth.

Table: 1. Influence of salt or water stress on daidzein production by hairy root cultures of *Psoralea corylifolia*. Daidzein and its glycoside were quantitated from a calibration graph obtained with three different concentrations of each compound. All the values are represented as Mean ± SD of triplicates. *p <0.01 as compared to controls.

Growth period (Weeks)	Growth condition	Total Daidzein (mg g^{-1} dry wt)	Daidzein (aglycone) (mg g^{-1} dry wt)	Aglycone/ Total daidzein (%)	Aglycone/Glycone Daidzein
0 (Before exposure	Control	10.47	1.97 ± 0.01	18	0.21
1	Control 300 mM NaCl 5% PEG	12.62 20.62 11.94	2.38 ± 0.02 3.91 ± 0.04 2.08 ± 0.04	18 19 17.4	0.23 0.23 0.21
2	Control 300 mM NaCl 5% PEG	24.58 40.9* 27.36	4.28 ± 0.04 14.6 ± 0.7* 4.66 ± 0.03	17.4 35.7* 17	0.21 0.55* 0.2
3	Control 300 mM NaCl 5% PEG	35.09 50.26* 36.06	6.19 ± 0.05 17.56 ± 0.8* 6.26 ± 0.2	17.6 34.9* 17.3	0.21 0.53* 0.21
4	Control 300 mM NaCl 5% PEG	45.03 60.48* 45.96	7.73 ± 0.9 18.08 ± 1* 7.96 ± 0.1	17.1 29.9* 17.3	0.2 0.42* 0.21
5	Control 300 mM NaCl 5% PEG	50.41 62.1* 51.08	9.11 ± 0.5 16.70 ± 0.9* 8.78 ± 0.3	18 26.9* 17.1	0.22 0.37* 0.21

It is known that the isoflavone production in plants is enhanced by different abiotic factors such as light (Lal *et al*., 2003), temperature (Zobayed *et al*., 2005) and UV-B treatment (Deavours and Dixon, 2005), apart from biotic factors including microbial and fungal invasions (Dixon *et al*., 2002). Involvement of various factors including reactive oxygen species as well as changes in ionic flux and phosphorylation status have all been invoked in this regard (Dixon *et al*., 2002). We are yet to completely comprehend the metabolic basis of stress induced isoflavone production and parameters indicative of oxidative stress conditions that were measured in hairy root cultures subjected to salt stress.

Salt-induced enhancement of daidzein production observed in the present study provided us an appropriate model system to obtain further insights in this regard. As depicted in Table 2, salt stress caused by 300 mM NaCl resulted 122% and 43 % increase in accumulation of reactive oxygen species (ROS) and thiobarbituric acid-reactive substances (TBARS), respectively, at the end of 5 weeks, as compared to controls ($p< 0.01$). In consonance with these observations, thiol status of hairy root cultures was altered in response to salt stress, as evidenced by 57% decrease in the ratio of reduced and oxidized glutathione (GSH/GSSG) contents at the end of 5 weeks. Further, salt stress also resulted in up regulation of antioxidant enzymes in hairy root cultures. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were enhanced to the extent of 43% and 40%, respectively, in comparison to untreated cultures ($p< 0.01$; Table 2).

A closer examination of the above indicators of oxidative stress over the 5-week period revealed that salt stress influences these parameters more profoundly in the very first two weeks of exposure to 300 mM NaCl ($p< 0.01$). Salt stress induced increase of isoflavone production was found concomitant with oxidative stress as evidenced by increase in the accumulation of ROS (~170%), end products of lipid peroxidation (~115%), and decreased the ratio of GSH/GSSG (~82%). The activities of antioxidant enzymes were also up regulated under these conditions as evidenced by increase in activities of SOD (~91%) and GPx (~59%) under these conditions. It is pertinent to note that such indicators of oxidative stress became more apparent at times corresponding to maximal production of daidzein. This is in conformity with earlier reports indicating accumulation of ROS and increased antioxidants in plants under salt stress conditions (Hasegawa *et al*., 2000).

Various abiotic stress conditions, including exposure to cold, drought and salt, are well-recognized to cause transient increase in cytosolic Ca^{2+} that may be derived in plants either from influx from the apoplastic space or release from internal stores (Knight, 2000). In view of the oxidative stress conditions observed in the present study, it was of interest to ascertain whether salt stress alters intracellular Ca^{2+} levels. In comparison to untreated controls experiencing rapid increase in intracellular Ca^{2+} contents under salt stress conditions (300 mM NaCl) and reaching maximal levels (from ~ 40 to 105 nM) at the end of second week ($p < 0.01$), the period that coincides with oxidative stress and daidzein production by hairy roots. Continued exposure of hairy roots to salt stress resulted in sustained increase of Ca^{2+} levels that were ~ 90 nM during subsequent time periods. In comparison to these results, PEG-induced water stress in hairy roots resulted only in moderate increase of intracellular Ca^{2+} contents (from ~ 40 nM to 55 nM) at the end of first week followed by further increase to ~ 75 nM at the end of 5 weeks (Fig. 1).

Upon ascertaining salt stress-induced increases in Ca^{2+} contents, we investigated possible downstream events of Ca^{2+}- signaling since earlier reports also indicated such a possibility (Xiong and Schumaker, 2002). It is known that increased intracellular Ca^{2+} levels can be perceived directly by key signaling enzymes involved in protein phosphorylation cascades. Importantly, activation of SOS3 (Salt Overlay Sensitive) family of Ca^{2+} sensors (Ishitani *et al*., 2000; Liu and Zhu, 1998) as well as expression of stress-regulated genes (Yu *et al*., 2003) was closely associated with Ca^{2+}-coupled phosphoprotein cascade. For example, the Ca^{2+}- dependent protein kinases (CDPKs) were induced by abiotic stress suggesting that they might be involved in stress signaling

Table: 2. Influence of salt or water stress on oxidative stress indicators in hairy root cultures of *Psoralea corylifolia*. RFI: Relative Fluorescence Intensity. All values are represented as Mean ± SD of triplicates. *p <0.01 as compared to controls.

Growth period (weeks)	Growth condition	ROS content (RFI 20 mg hairy root^{-1})	TBARS contents (μmoles mg protein^{-1})	GSH/GSSG contents (Ratio)	SOD activity (Units mg protein^{-1})	GPx activity (Units mg protein^{-1})
0 Week (Before exposure)	Control	375 ± 5	6.0 ± 0.36	24 ± 1.8	1.72 ± 0.8	6.0 ± 0.5
1	Control	383 ± 4	5.9 ± 0.46	26 ± 2.1	1.87 ± 0.7	5.7 ± 0.5
	300 mM NaCl	959 ± 7*	10.04 ± 0.3*	5.2 ± 0.13*	3.28 ± 0.23*	8.83 ± 0.3*
	5% PEG	378 ± 4	6.12 ± 0.18	23.8 ± 0.9	1.59 ± 0.07	6.06 ± 0.3
2	Control	411 ± 6.3	6.14 ± 0.28	15.3 ± 0.4	2.08 ± 0.15	7.8 ± 0.07
	300 mM NaCl	1110 ± 34*	13.2 ± 0.16*	2.8 ± 0.12*	4.18 ± 0.1*	12.4 ± 0.3*
	5% PEG	365 ± 22	6.33 ± 0.28	10.4 ± 0.07	2.14 ± 0.09	7.74 ± 0.16
3	Control	422 ± 8	6.18 ± 0.56	9.87 ± 0.12	2.59 ± 0.08	8.52 ± 0.16
	300 mM NaCl	1104 ± 17*	12.7 ± 0.14*	2.94 ± 0.1*	4.2 ± 0.1*	12.4 ± 2*
	5% PEG	422 ± 6	5.87 ± 0.07	7.32 ± 0.05	2.62 ± 0.2	8.31 ± 0.04
4	Control	506 ± 14	8.04 ± 0.17	7.11 ± 0.05	2.8 ± 0.01	8.96 ± 0.03
	300 mM NaCl	1200 ± 18*	12.6 ± 0.11*	2.65 ± 0.1*	4.28 ± 0.1*	13 ± 0.5*
	5% PEG	518 ± 12	7.75 ± 0.07	5.4 ± 0.28	2.75 ± 0.15	9.81 ± 0.04
5	Control	541 ± 6	8.56 ± 0.07	5.45 ± 0.07	3.13 ± 0.15	9.2 ± 0.08
	300 mM NaCl	1202 ± 15*	12.2 ± 0.16*	2.31 ± 0.02*	4.49 ± 0.09*	12.9 ± 0.7*
	5% PEG	538 ± 9	8.78 ± 0.03	5.11 ±0.06	2.94 ± 0.1	10.2 ± 0.15

(Sheen, 1996). In addition, earlier studies also suggested that calmodulin might play an important role in mediating cell signaling events induced by stress. Sequence similarities between SOS3 and the regulatory subunit of the Ca^{2+}-calmodulin dependent protein phosphatase, calcineurin (Liu and Zhu, 1998) lend credence to this. Thus, we measured the intracellular contents of calcineurin and calmodulin under salt-stress conditions (Table 3.) and assayed the activities of CDPKs, Ca^{2+}- calmodulin dependent protein kinase II and calcineurin in hairy roots with enhanced daidzein production under conditions of salt stress.

Table: 3. Influence of salt stress on intracellular calmodulin and calcineurin contents in hairy root cultures of *Psoralea corylifolia*. All values are represented as Mean ± SD of triplicates.

Growth period (weeks)	Growth condition	Calmodulin (µg mg protein^{-1})	Calcineurin (µg mg protein^{-1})
0 Week (Before exposure)	Control	1.02 ± 0.01	2.01 ± 0.01
1	Control	1.05 ± 0.01	2.09 ± 0.03
	300 mM NaCl	1.07 ± 0.01	2.13 ± 0.03
2	Control	1.0 ± 0.01	2.01 ± 0.1
	300 mM NaCl	1.04 ± 0.01	2.06 ± 0.08
3	Control	1.15 ± 0.06	1.94 ± 0.07
	300 mM NaCl	1.12 ± 0.1	1.94 ± 0.05
4	Control	1.09 ± 0.01	2.03 ± 0.02
	300 mM NaCl	1.07 ± 0.01	2.12 ± 0.04
5	Control	1.12 ± 0.1	2.01 ± 0.05
	300 mM NaCl	1.06 ± 0.02	2.05 ± 0.09

Activity of CDPKs was enhanced during first two weeks (from ~13 to ~22 units) of salt stress as shown in Fig. 2 coinciding with maximal daidzein production ($p < 0.01$). Similarly, the activity of calcineurin (apart from that of total phosphatases) was also enhanced in response to salt stress at periods corresponding to maximal production of daidzein. Total protein phosphatase activity was increased (55-83% increase) over five weeks of salt stress and maximal at the end of second week (83% increase) in response to salt stress (Fig. 4.) as compared to respective controls ($p < 0.01$). Notably, the activity of calcineurin was increased (75-100% increase) upon salt stress ($p < 0.01$). Once again, such an increase in calcineurin activity was maximal at the end of second week (100%) in response to salt stress as compared to respective controls (Fig. 5.).

By contrast, activity of Ca^{2+}- calmodulin dependent protein kinase II was decreased in response to salt stress (Fig. 3). Initial activity of CaMK II (~0.105 units) decreased by about 92% at the end of second week of salt stress (300 mM NaCl) without any further decrease in its activity upto the end of experimental period ($p < 0.01$). These observations are in consonance with similar reports made earlier, wherein intracellular events of salt stress are mediated by the enzymes involved in Ca^{2+}- dependent phosphoprotein cascade (Marquez and Serrano, 1996; Pardo *et al*., 1998). However, observed alterations in the relative contents of calmodulin and calcineurin remained at steady state levels during salt stress. It was noted that salt stress, but not water stress, could alter the activities of these enzymes.

The results in present study concluded that culturing *Agrobacterium*- induced hairy root cultures of *Psoralea corylifolia* in the presence of 300 mM NaCl resulted in significant enhancement of daidzein production, accounting to 18 mg g^{-1}, much higher than previous observations made by other investigators. Oxidative stress conditions as well as increase in Ca^{2+} contents and in continuation with altered activities of concerned Ca^{2+}- mediated protein kinases and phosphatases were evident at times corresponding to salt stress-induced increase in daidzein production. The results suggest possible interrelations between oxidative stress and Ca^{2+}- signaling under salt-stress, which are relevant to enhanced daidzein production by hairy root cultures.

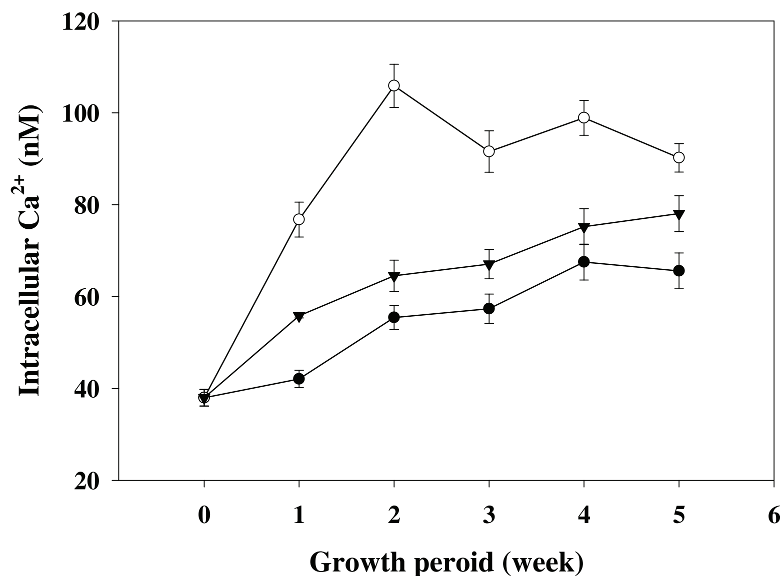

Fig. 1. Influence of salt or water stress on intracellular Ca^{2+}: control (-●-), salt stress (-○-) and water stress (-▼-) conditions are shown.

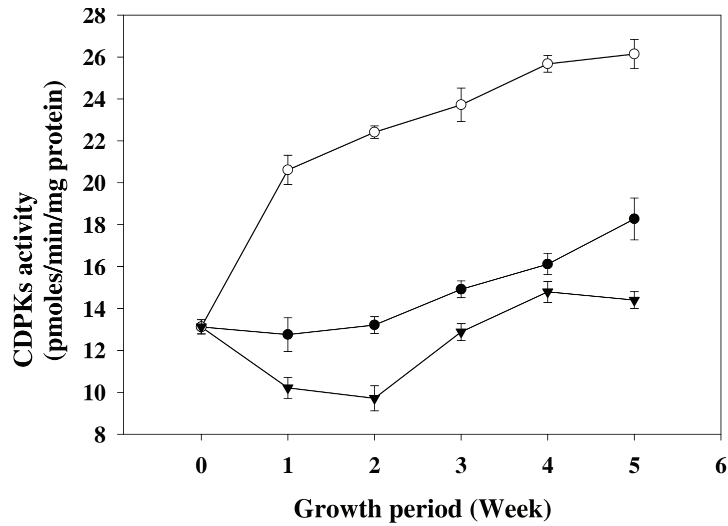

Fig. 2. Influence of salt or water stress on Ca^{2+}- dependent protein kinase activity: control (-●-), salt stress (-○-) and water stress (-▼-) conditions are shown.

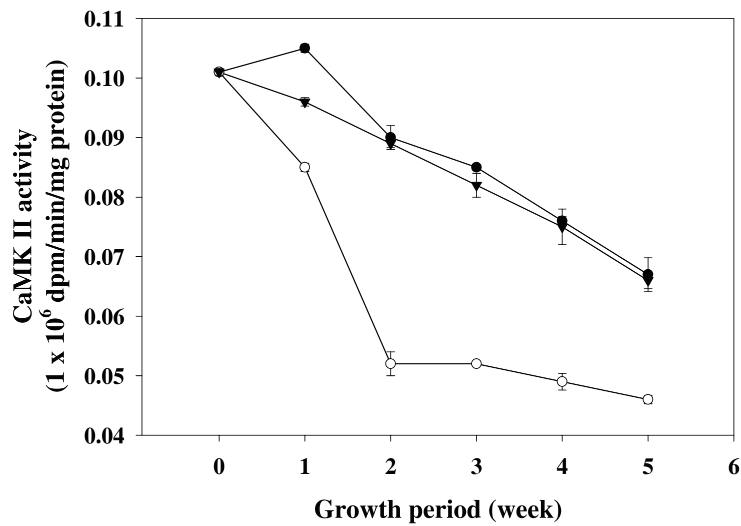

Fig. 3. Influence of salt or water stress on Ca^{2+}- calmodulin dependent protein kinase II activity: control (-●-), salt stress (-○-) and water stress (-▼-) conditions are shown.

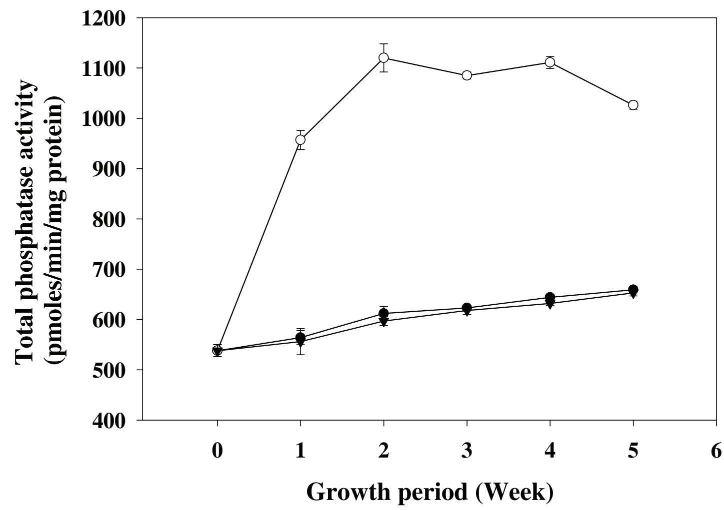

Fig. 4. Influence of salt or water stress on total phosphatase activity: control (-●-), salt stress (-○-) and water stress (▼-) conditions are shown.

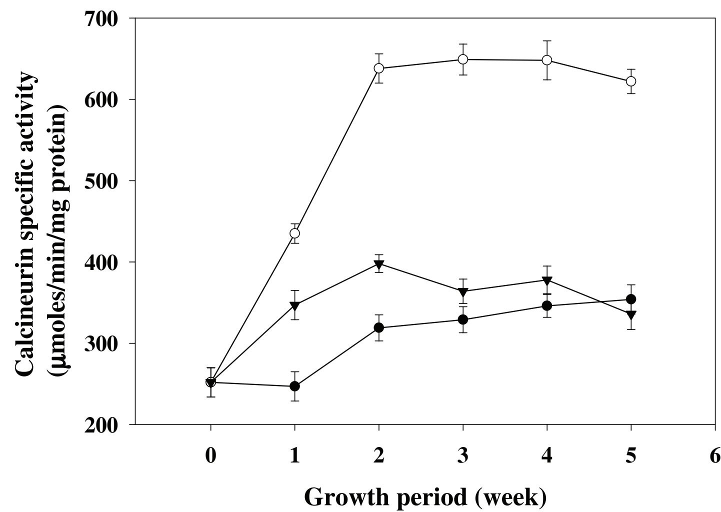

Fig. 5. Influence of salt or water stress on calcineurin specific activity: control (-●-), salt stress (-○-) and water stress (▼-) conditions are shown.

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References

Anil, V.S., Harmon, A.C. and Rao, K.S. (2000). Spatio-accumulation and activity of calcium dependent protein kinases during embryogenesis, seed development and germination in sandalwood, *Plant Physiol.*, 122: 1035-1044.

Bhagyalakshmi, N., Thimmaraju, R., and Narayan, M.S. (2004). Various hexoses and di-hexoses differently influence growth, morphology and pigment synthesis in transformed root cultures of red beet (*Beta vulgaris*), *Plant Tiss. Organ. Cult*., 78: 183-195.

Bradford, M.M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, *Anal. Biochem*., 72: 248-254.

Bourgaud, F., Bouque, V. and Guckert, A. (1999). Production of flavanoids by *Psoralea* hairy root cultures, *Plant Cell Tiss. Organ Cult.*, 56: 97–104.

Bouque, V., Bourgaud, F., Nguyen, C. and Guckert A. (1998). Production of daidzein by callus cultures of *Psoralea* species and comparison with plants, *Plant Cell Tiss. Organ Cult.*, 53: 35–40.

Davidson, J.F., Whyte, B., Bissinger, P.H. and Schiestl R.H. (1996). Oxidative stress is involved in heat induced cell death in *Saccharomyces cerevisiae, Proc. Natl. Acad. Sci.*, 93: 5116-5121.

Deavours, B.E. and Dixon, R.A. (2005). Metabolic engineering of isoflavonoid biosynthesis in Alfalfa, *Plant Physiol.*, 138: 2245-2259.

Dixon, R.A. and Paiva, N.L. (1995). Stress induced phenylpropanoid metabolism, *The Plant Cell,* 7: 1085-1097.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S.S. and Wang, L.J. (2002). The phenylpropanoid pathway and plant defense - a genomics perspective, *Mol. Plant Pathol.*, 3: 371-390.

Ernster, L. and Nordenbrand, K. (1967). Microsomal lipid peroxidation, *Met. Enzymol.,* 10: 574-580.

Gauri, A., Reddy, V.D., Giri, C.C., Rao, K.V., Lakshmi, V.V.S., Prabhakar, S., Vairamani, M., Thippeswamy, B.S. and Bhattacharya, P.S. (2005). Amplified fragment length polymorphism and metabolomics profiles of hairy roots of *Psoralea corylifolia* L, *Phytochemistry,* 66: 2441–2457.

Ha, H., Lee, Y.S., Lee, J.H., Choi, H. and Kim, C. (2006). High performance liquid chromatographic analysis of isoflavones in medicinal herbs, *Arch. Pharm. Res.*, 29: 96-101.

Hanson, P.I., Kapiloff., Lou, L.L., Rosenfield, M.G. and Schulman, H. (1989). Expression of a multifunctional Ca^{2+}/ calmodulin dependent protein kinase and mutational analysis of its autoregulation, *Neuron*, 3: 59-70.

Hasegawa,P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000). Plant cellular and molecular responses to high salinity, *Annu. Rev. Plant Mol. Plant Physiol*., 51: 463-499.

Haugland, R.P. (1997). *Handbook of fluorescent probes and research chemicals*. Molecular Probes, Eugene, Oregon.

Hissin, P. and Hilf, R.A. (1976). Flourimetric method for determination of oxidized and reduced glutathione in tissues, *Anal. Biochem*., 74: 214-226.

Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Wei, M. and Zhu, J.K. (2000). SOS3 function in plant salt tolerance requires myristoylation and calcium binding, *Plant Cell*, 12: 1667-1677.

Jayashree, T. and Subramanyam, C. (1999). Antiaflatoxigenic activity of eugenol is due to inaibition of lipid peroxidation, *Lett. Appl. Microbiol*., 28: 179-183.

Jia, T.L., Wang, H.Z., Xie, L.P., Wang, X.Y. and Zhang, R.Q. (2003). Daidzein enhances osteoblaanst growth that may be mediated by increased bone morphogenetic protein (BMP) production, *Biochem. Pharmacol*., 65: 709–715.

Knight, H. (2000). Calcium signaling during abiotic stress in plants, *Int. Rev. Cytol*., 195: 269-325.

Lal, A., Warber, S., Kirakosyan ,A., Kaufman, P.B. and Duke, J.A. (2003). Upregulation of isoflavonoids and soluble proteins in edible legumes by light and fungal elicitor treatments, *J. Altern Complement. Med*., 9: 371-378.

Liu, C-J., Blount, J.W., Steele, C.L. and Dixon, R.A. (2002). Bottlenecks for metabolic engineering of isoflavone glycoconjugates in *Arabidopsis, Proc. Natl. Acad. Sci.,* 99: 14578-14583.

Liu, J. and Zhu, J.K. (1998). A calcium sensor homolog required for plant salt tolerance, *Science*, 280: 1943-1945.

Marklund, S. and Marklund, G. (1974). Involvement of superoxide anion radical in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.*, 47: 469-474.

Marquez, J.A. and Serrano, R. (1996). Multiple transduction pathways regulate the sodium extrusion gene PMR2/ENA1 during salt stress in yeast, *FEBS Letters* 382: 89-92.

Martinez, J.I.R., Launay, J.M. and Dreux, C. (1979). A sensitive fluorimetric assay for glutathione activity: Application to human blood platelets, *Anal. Biochem*., 98: 154-159.

Munne-Bosch, S., Schwarz, K. and Alegre, L. (1999). Enhanced formation of a tocopherol and highly oxidized abietance diterpenes in water stressed rosemaary plants, *Plant Physiol*., 121: 1047-1052.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant* 15: 473–497.

Padma, S. and Subramanyam, C. (1999). Extracellular calcineurin: Identification and quantification in serum and amniotic fluid, *Clin. Biochem*., 32: 491-494.

Pardo, J.M., Reddy, M.P., Yang, S., Maggio, A., Huh, .H., Matsumoto, T., Coca, M.A., D'Urzo, M.P., Koiwa, H., Yun, D.J., Watad, A.A., Bressan, R.A. and Hasegawa, P.M. (1998). Stress

signaling through Ca^{2+}/calmodulin dependent protein phosphatase calcineurin mediates salt adaptation in plants, *Proc. Natl. Acad. Sci*., 95: 9681-9686.

Pietta, P. (2000). Flavonoids as antioxidants, *J. Nat. Prod*., 63: 1035-1042.

Setchell, K.D.R., Lawson, A.M., Borriello, S.P., Harkness, R., Gordon,M. and Morgan, D. (1981). Lignan formation in man microbial involvement and possible roles in relation to cancer, *Lancet*, 2: 4–7.

Sheen, J. (1996). Ca^{2+}-dependent protein kinases and stress signal transduction in plants, *Science*, 274: 1900-1902.

Shi, H.P. and Kintizois, S. (2003). Genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots, *Plant Cell Rep*., 21: 1103-1107.

Tsukamoto, C., Shimada, S., Igita, K., Kudou, S., Kokubun, M., Okubo, K. and Kitamura, K. (1995). Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development, *J. Agric. Food Chem*., 43: 1184-1192.

Wang, J.H. and Pallen, C.J. (1983). Calmodulin stimulated dephosphorylation of p-nitrophenyl phosphate and free phosphotyrosine by calcineurin, *J. Biol. Chem*., 258: 8550-8553.

Xiong, L., Schumaker, K.S. and Zhu, J.K., (2002). Cell signaling during cold, drought and salt stress, *The Plant Cell*, 14: S165-S183.

Yu, O., Shi, J., Hession, A.O., Maxwell, C.A., McGonigle, B. and Odell, J.T. (2003). Metabolic engineering to increase isoflavone biosynthesis in soyabean seed, *Phytochemistry*, 63: 753-763.

Zobayed, SM., Afreen, F and Kozai, T. (2005). Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort, *Plant Physiol. Biochem*., 43: 977-84.

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