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Reserpine Accumulation in NaCl Treated Calli of *Rauvolfia tetraphylla* L.

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ABSTRACT: *Rauvolfia tetraphylla* L. is an endangered plant, known for its medicinal properties. It contains various indole group alkaloids with reserpine most prominent among them. Reserpine is a hypotensive agent that is in great demand for the modern pharmaceutical industries. In the present work, leaf explants were induced to produce calli by using the phytohormones 2,4-D, NAA, IBA and IAA. Among these, 9 µM 2,4-D was found suitable for maximum callus induction (95%). The calli produced in this hormone concentration were subjected to NaCl salt treatment (0, 25, 50, 75 and 100 mM) and the effect of salinity on callus growth and reserpine accumulation was observed. The callus growth was normal up to 50 mM concentration of NaCl and there was a reduction in growth of the calli at 75 mM salt treatment whereas at 100 mM concentration complete cessation of callus growth was noticed. An increase in reserpine accumulation was noticed with increases measured up to 75 mM NaCl concentration.

KEYWORDS: Callus, NaCl, Reserpine, 2,4-D, *Rauvolfia tetraphylla* L.

The genus *Rauvolfia* belongs to the family Apocynaceae that consists of around one thousand species, five of which are native to India[1]. *R. tetraphylla* is economically important because of the presence of alkaloids, which are localized in the roots[2]. The roots are useful in the treatment of hypertension, cardiovascular diseases and as a tranquilizing agent. The extract of the root is valuable for intestinal problems. Roots are believed to stimulate uterine contraction in case of difficult delivery[3]. About 30 indole alkaloids are reported in *Rauvolfia* and reserpine holds the first place among them. Other frequently reported alkaloids are ajmalicine, reserpitine, deserpitine, sarpagine, rescinnamine and yohimbine[4]. Tryptophan is the starting material in the biosynthetic pathway of reserpine that is converted to tryptamine by tryptophan decarboxylase enzyme. Tryptamine is combined with secologanin in the presence of strictosidine synthetase enzyme and yields strictosidine. Various enzymatic conversion reactions lead to the synthesis of reserpine from strictosidine[5]. *R. tetraphylla* roots are often used as a substitute to the *R. serpentina* roots which gained export value in recent years. *R. tetraphylla* L. is a woody shrub that grows up to 1½ m in height. Tender parts of this plant are puberulous. Leaves are four at each node, elliptic and ovate. Inflorescence develops in axillary or terminal, 5-7 flowered corymb. Flowers are white or yellowish white. Fruit is a drupe and seeds are ovoid[6]. The indiscriminate collection and limited cultivation of both *R. tetraphylla* and *R. serpentina* made these plants unavailable normally and they are listed as endangered[7, 8]. When plant material is rare and difficult to acquire, or when chemical synthesis is not possible and cost and demands are high, plant tissue culture technology provides a valuable alternative to obtain the desired product[9]. Plant tissue culture and hairy root cultures are promising potential alternative sources for the production of high value secondary metabolites of industrial importance[10, 11]. By controlling the composition of the culture medium and the environment, secondary metabolite synthesis may be enhanced *in vitro*. Cheng and Cheng[12] successfully produced more reserpine in calli of some Chinese herbs, in a quantities approaching *in situ* level, by modifying the medium components. Certain secondary compounds produced by plants under the influence of various biotic and abiotic factors that are called as 'elicitors'. Ag^+ for example, has been employed as an abiotic elicitor to stimulate the secondary metabolite diterpenoid tanshinones in hairy root cultures of *Salvia miltiorrhiza*[13]. Although there are reports available on *in vitro* regeneration of *R. tetraphylla* L.[14], no attempts have been made to enhance the alkaloid production in this plant. Hence the present study was undertaken with an objective of enhancing alkaloid production in *R. tetraphylla* L. under tissue culture condition by using NaCl salt as an elicitor or stress component.

Leaf explants of field grown plants were collected and surface sterilized with 0.1% mercuric chloride (W/V) for four minutes. The sterile explants were grown on

callus induction medium consisting MS salts, B_5 vitamins, 30 g/l sucrose and 8 g/l agar with various auxins such as IAA, IBA, NAA and 2,4-D ranging from 1-10 µM concentration and the pH of the medium was adjusted to 5.7. The medium was sterilized at 15 lb for 15 minutes. The cultures were exposed to 16/8 hr light/ dark condition by using cool white fluorescent tubes (40 µM $m^{-2}s^{-1}$).

NaCl at increasing concentrations (25, 50, 75 and 100 mM) was added into the callus induction medium consisting of 9 µM 2,4-D (from the previous experiments this was found to be the best callus inducing hormone at the mentioned concentration) to test the effect of salinity on callus induction from the leaf explants. Calli grown in the medium fortified with 9 µM 2,4-D were maintained as controls and NaCl was added in the same medium at 25 mM, 50 mM, 75 mM and 100 mM concentrations in which the calli were inoculated to test the effect of NaCl salinity on callus growth. For each treatment 12 explants were used and all the experiments were repeated thrice. The data were recorded periodically and the results were expressed as mean values in the tables.

Biomass parameters such as growth value and moisture content were analyzed after five subcultures of the calli in the respective treatment medium. The callus was cut into approximately 50 mg pieces and inoculated on callus induction medium in control and treatment conditions. After 30 days of culture, the initial and final weight of the calli grown in control and various treatments was recorded and the growth value was calculated. The percentage of moisture content of the callus was calculated by using the fresh weight and dry weight of the callus. The calli after collection from the medium, were weighed and the values were recorded as fresh weight. The calli were dried at 40°C for 24 hours and the dry weight was recorded.

The dried callus was extracted with methanol, acidified with 0.1 N HCl and then neutralized with NH_4OH[15]. The supernatant was evaporated under vacuum and the weight was recorded as crude alkaloid content. Crude alkaloid was dissolved in a few drops of ethanol and subjected to TLC and reverse phase HPLC analysis by using reserpine (SRL products, India) as standard. Reserpine content in the crude extract was calculated and tabulated.

Callus initiation from edges and wounded regions of the leaf explants was observed after three weeks of inoculation. Only 2,4-D was found to induce callus from the leaf explants at the concentration of 9 µM. The superiority of 2,4-D on callus induction has been documented in several studies[16, 17, 18]. Moreover, combinations of BAP or KN with 2, 4-D were proved to be ineffective for callus induction. This result is contrary to the observations in another medicinal plant, Olivera *et al*.[19] reported an optimal callusing response in auxin + cytokinin combinations in *Aspidosperma ramifloram*.

NaCl greatly affected callus induction whereas callus growth was not significantly affected at the corresponding salt concentration (Table 1). Salinity did not affect callus induction at 25 mM NaCl whereas at concentrations of 50 mM and 75 mM a reduction in callus induction was observed. 100 mM salt treatment completely stopped the callusing response from the leaf explants. Salinity enhanced callus growth at 25 mM concentration, but at 50 mM concentration, there was no difference observed when compared to control conditions. At 75 mM concentration, the callusing response was reduced and at 100 mM concentration callus growth had stopped.

Table 1. Effect of NaCl salinity on callus induction and callus growth in *R. tetraphylla* L.

Treatment	Percentage of callus induction	Percentage of callus growth
2,4-D 9 µM (Control)	90	90
2,4-D+ NaCl		
9 µM +25 mM	90	92
9 µiM +50 mM	78	90
9 µM +75 mM	43	78
9 µM +100 mM	-	-

The growth value and percentage of moisture content were calculated and tabulated (Table 2) for calli obtained in various concentrations of NaCl. The moisture content of the callus decreased with increasing concentration of NaCl. The growth value decreased in saline treatments compared to controls.

Table 2. Effect of NaCl salinity on biomass and growth value in the callus of *R. tetraphylla* L.

Treatment	F. Wt. of callus (g)	D. Wt. of callus (g)	Moisture content (%)	Growth value (%)
Control (9 µM 2,4-D)	0.3354	0.0826	75.37	46
9 µM 2,4-D +25 mM NaCl	0.2738	0.0850	68.96	48
9 µM 2,4-D +50 mM NaCl	0.2448	0.0815	66.71	25
9 µM 2,4-D +75 mM NaCl	0.1554	0.0581	62.61	12

The crude alkaloid and the reserpine yield were tabulated (Table 3). The reserpine content was detected in the calli (control and treated) whereas there was no reserpine production detected in leaves of *in vivo* plants. This is contrary to the report by Oliveira *et al*.[19] who had

Table 3. Effect of NaCl on crude alkaloid and reserpine content in calli of *R. tetraphylla* L.

Treatment	Crude alkaloid content (mg/g D.Wt.)	Reserpine content (mg/g D.Wt.)
Reserpine (standard)	-	0.1 mg –1.0 mg/ml
Control (9 μM 2,4-D)	5.0	0.9
9 μM 2,4-D+25 mM NaCl	5.5	1.2
9 μM 2,4-D+50 mM NaCl	4.6	1.35
9 μM 2,4-D+75 mM NaCl	3.7	0.5

reported that the alkaloid content was reduced in the calli of *Aspidosperma ramiflorum* in comparison to the parent plant. In the control callus (9 μM 2,4-D) the reserpine content was calculated as 0.9 mg / g of callus dry weight. Consistent with our work, Baskar Rajan[20] reported that 2,4-D was found the best regulator for the callus induction and production of the alkaloid solasodine in *Solanum eleagnifolium.*

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