# *In vitro* development of Salt Tolerant Plants in *Scoparia dulcis* L.

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## Abstract

An effective tissue culture system to regenerate *Scoparia dulcis* (Scrophulariaceae), an important multipurpose – medicinal herb was established. The optimized protocol resulted in plant formation from nodal explants via organogenesis. Callus induction was obtained on MS basal medium supplemented with 2.0 µM TDZ and 3% sucrose. Proliferation and elongation of shoots – derived from callus – was achieved on medium supplemented with 4.0µM TDZ and 1.0 µM IBA. Rooted shoots were successfully acclimatized and transferred to soil. Callus cultures were also screened for salt tolerance using direct selection methods and their growth responses were examined. Despite the presence of salt in culture medium resulting in the loss of regeneration potential in callus, plantlets were obtained from culture media with 17.2 mM, 34.4 mM and 51.6 mM NaCl and subsequently regenerated in salinity soil. The salt tolerant callus was showed comparatively lesser fresh weight, dry weight and protein content. The development of salinity tolerant plants was achieved from 17.2 mM NaCl containing medium with 64% survival rate.

Key words: *Scoparia dulcis,* salt tolerant plant, NaCl, thidiazuron, callus

## Introduction

Callus and cell culture possess an enormous potential for the plant improvement and can also be used as a tool for various *in vitro* analysis (Silva *et al.,* 2005). *Scoparia dulcis* has been used as a remedy for treating diabetes mellitus in India and hypertension in Taiwan. It has been revealed that numerous chemical studies on isolated chemicals from *S. dulcis* that includes coumarins, phenols, saponins, tannins, amino acids, flavonoids, terpenoids and catecholamines (Hayashi *et al.,* 1996, Hayashi *et al.,* 1997, Hayashi *et al.,* 1999, Ratnasooriya *et al.,* 2005). Most plant species are particularly sensitive to a low concentration of sodium. The potential of physiological responses to differentiate between salt sensitive and salt tolerant species or individuals is now being examined. Of considerable use for the development of salt tolerance in S. dulcis depends on the availability of a technique that could identify salt tolerance within a species. Looking at such a response in vitro may assist in providing information about the salt tolerance mechanisms, leading to further capacity to increase salt tolerance (Sharry and Abedini, 2001). Although callus induction of *S. dulcis* has been already investigated (Nkembo *et al.,* 2005; Saitoh *et al.,* 2007), there are no reports on the effects of salt on plant development. The aim of the first part of this work was to develop an efficient and reproducible protocol for *in vitro* propagation via organogenesis of *S. dulcis* in order to obtain vigorous plants that might be used for commercial purpose. Second was to examine the possibility of obtaining NaCl tolerance in *S. dulcis* through the *in vitro* selection of callus, and their subsequent regeneration. Comparative growth characteristics of the callus culture and to follow the dynamics of the proteins synthesis during growth were determined.

## Materials and Methods

### Collection of explants and Surface sterilization

*S. dulcis* were collected freshly from Madras Christian College campus, Chennai, India. Nodal segments were used as the explant source. Explants were surface-sterilized in 0.1 % mercuric chloride, and rinsed three times in sterile distilled water.

### Culture media and growth conditions

Murashige and Skoog (1962) vitamins, macro and micronutrient at full concentration with 3% (w/v) sucrose gelled with 8 g/L agar was used throughout the experiment. The pH of

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all media was adjusted to 5.8-6.0 and sterilized by autoclaving for 17 min at 121ºC at 15 lbs. The cultures were maintained at 25±2°C under a 16/8 h (day/night) photoperiod with light supplied at an intensity of 3000 lux. Plantlets were grown under continuous cool, white light fluorescent tubes (General Electric) at 25ºC in a plant tissue culture chamber before their transplantation into the greenhouse.

### Callus induction and shoots proliferation

The freshly harvested leaf and nodal explants was cultured on MS medium supplemented with 2.0 µM thidiazuron (TDZ). The freshly formed callus was subcultured after 21 days for shoot proliferation on basal MS medium supplemented with 4.0 µM TDZ and 1.0 µM Indole butyric acid (IBA) (Karthikeyan and Prasad, 2009).

### Rooting and gardening

Elongated shoots – 4-5 cm in length with 4 or 5 compound leaves were transferred MS media supplemented with 2.5 µM IBA (Karthikeyan *et al.,* 2009). The formation of healthy shoots and roots make sure that this is ready to harden. The rooted plants were removed from the culture tubes, washed with sterile distilled water, and transferred to protrays with sterile cow dung: coco peat: sand (1:1:1 v/v/v). The plantlets were placed at 70% to 80 % humidity, 25 ± 2 °C under a 12-hours photoperiod for acclimatization. After the plants get acclimatized, the plants were transferred to pot with farmyard mixture: sand (1: 1 v/v) and placed in the green house (Karthikeyan *et al.,* 2009). After three weeks of development, these hardened plants were transferred to the field and the survival rate was recorded. Twenty cultures were used per treatment and each experiment was repeated at least three times. Percentage of success was scored four weeks after culture.

### *In vitro* selection of salt tolerance

*In vitro* selection of salt tolerant plants of *S. dulcis* has been accomplished by screening highly morphogenic explants cultured on high NaCl media. Media were prepared with different concentrations of NaCl (17.2, 34.4, 51.6, 68.8 mM). The treatments in which callus survived were subcultured for shoot elongation. The shoots were put on rooting medium with the addition of NaCl. Data on survival, organogenesis and fresh weight were recorded after each week of culture. Regenerated plants were transferred to saline soil for acclimatization and hardening.

### Determination of callus growth and protein determination

The development of callus was monitored at 4 days intervals up to 32 days and a growth curve was determined based in fresh and dry weigh of the callus. The callus obtained after 28 days were lyophilized, macerated and submitted to extraction with 0.1 M glycine-HCl pH 2.6, 0.1 M sodium acetate (NaOAc) pH 4.0, 0.1 M NaOAc pH 6.0, 0.1 M Tris-HCl pH 8.0 and 0.1 M Na-borate pH 10.0 buffers, while all the buffers contains 0.15 M NaCl. The samples were sonicated for 1 hour and clarified by centrifugation at 12,000 x g for 20 min at 4°C. The clear supernatants were used for determination of soluble protein content. The protein content in the different fractions were performed according to Bradford (1976), using bovine serum albumine (BSA) as standard.

## Results and Discusion

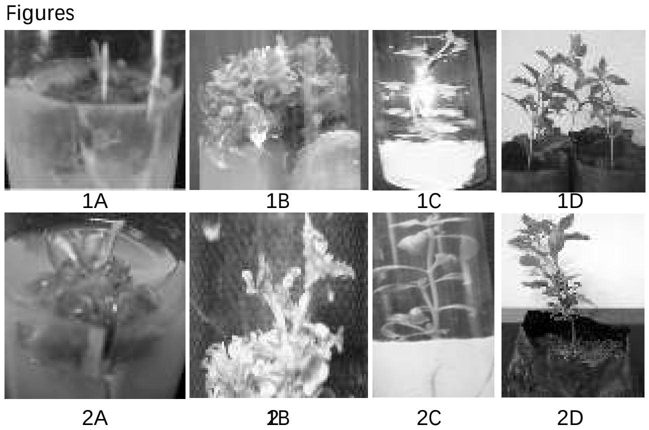
### Callus induction and shoot proliferation

The induction of callus from nodal explants were effective on MS medium supplemented with 2.0 µM TDZ (Fig. 1A). When portions of these callus were subcultured for shoot elongation. The elongation of shoots were effective on medium supplemented with 0.8 mg/L TDZ and 0.2 mg/L IBA (Fig. 1B). As reported in *Glycine max* (L.) Merr. (Kaneda *et al.,* 1997), *Rosa damascene* (Kumar *et al.,* 2001) and *Artemisia judaica* L. (Liu *et al.,* 2003), the presence of TDZ in the culture medium proved vastly superior to the treatment with BAP in case of *S. dulcis* (Karthikeyan *et al.,* 2009), and the rate of proliferation was significantly higher in TDZ-containing media. Maximum of 24 shoots/g of fresh weight of callus was obtained.

### *In vitro* selection of salt tolerance

Explant necrosis was observed at 51.6 and 68.8 mM of NaCl. At 17.2 mM NaCl the response was slightly similar to the control. The gradual decline in the growth was recorded at 34.4 and 51.6 mM NaCl. *In vitro* selection of salt tolerant plants of *Brassica juncea* L. (Indian mustard) cv. Prakash has been accomplished by screening highly morphogenic cotyledon explants cultures on high NaCl media (Jain *et al.* 1991). In our work, high NaCl media also resulted in explant necrosis. The

Figures




Control i.e. without Salt stress
1A – Nodular callus
1B – Multiple shoots
1C – Regenerated sapling
1D – Hardening in Green house

17.2 mM NaCl (salt) stress
2A – Callus
2B – Multiple shoots
2C – Regenerated sapling
2D – Hardening in Green house

callus on 17.2 mM NaCl gave a higher growth mass and was significantly higher than other treatments (Fig. 2A). Work with callus adapted to salinity is characterized by their poor regeneration (Binh *et al.* 1992). The presence of high concentration of salt in the culture medium produced significant loss in regeneration potential at 68.8 mM. The loss of growth potential may be due to increased osmotic potential of the saline medium; increased osmotic potential affects water and nutrient uptake, which may in turn inhibit the metabolic activities necessary for shoot initiation and growth (Vijayan *et al.* 2003). Callus did not lose its regeneration capacity when a portion was subcultured onto medium with NaCl for shoot proliferation.

### Comparison of Callus growth and protein content

Growth curves of callus were plot based on fresh and dry weights. Callus presented sigmoid growth curves with lag, exponential, linear and stationary phases. The adaptive period (lag phase), in which the fresh and dry weight of calli increases slowly, occurred up to 3rd day in control and 5th day in 17.2 mM NaCl. According to Scragg and Allan (1993), the lag phase is considered an energy producing period. The fast growth phases (exponential and linear phases) occurred between 3rd and 15th day in control while 5th to 19th day of treatment with 17.2 mM NaCl. The exponential or biosynthetic phase is the period of maximum cellular division and greatest growth rate of the callus, while the linear phase is the period in which the cells grow but the cellular division decreases (Scragg and Allan, 1993). In stationary phase, the rate of cellular division is gradually reduced and then remains constant. According to Smith (2000), in the stationary phase occurs deprivement of nutrients in culture medium and a reduction of the $O_2$ amounts inside the cells.

The protein content was analyzed during the callus growth. The results showed that between 0 to 10th day and 0 to 15th day the protein content was decreased in control and in treatment with 17.2 mM NaCl respectively. This reduction possibly occurred due to an adaptation period of callus in the culture medium. The increase in the protein levels between 10th and 16th day in control and 15 to 20th day in 17.2 mM NaCl of culture could be related to mitotic activity during the exponential and linear growth phases. But Gutmann *et al.* (1996) observed an increment in the protein levels in the cells of *Larix leptoeuropaea* during the first 2 weeks of culture, followed by a decrease of this content during the following weeks. The decrease of the protein levels during to stationary phase in control and NaCl treatments correlates with the physiological stress related to diminishing nutrition in the medium, ageing and cell degeneration.

### Rooting and acclimatization

Rooting of *S. dulcis* and its subsequent establishment in outdoor was better achieved with a low concentration of auxins. Of the various auxins tested for root induction, IBA proved to be the most effective (Thakur *et al.* 1998; Karthikeyan *et al.,* 2009) (Fig. 1C). Whole plants were grown at 27ºC and 50 ± 5% relative humidity in the culture chamber. In agreement with Thakur *et al.* (1998), we observed that high relative humidity is necessary for plantlet survival. As opposed to Thakur *et al*. 1998, in the current experiment, a maintenance period of high humidity was not necessary for some time after transplantation to the greenhouse. Plants were subsequently transferred to larger pots and gradually acclimated under greenhouse conditions with variable relative humidity (35-60%). Plants were phenotypically normal (Fig. 1D) and had a good growth rate. All plants transferred to soil conditions survived.

| NaCl mM | Callu | | | In vitro plant regeneration (days) | Hardening (days) |
|---|---|---|---|---|---|
| | Fresh weight g | Dry weight g | Protein mg/g | | |
| 0 | 1.9±0.73 | 0.52±0.94 | 18.2±0.88 | 63 | 42 |
| 17.2 | 1.6±0.45 | 0.45±0.64 | 16.8±0.62 | 89 | 54 |
| 34.4 | 0.9±0.86 | 0.27±0.58 | 15.6±0.48 | - | - |
| 51.6 | 0.7±0.56 | 0.15±0.66 | 15.1±0.76 | - | - |

Table: Effect of NaCl on callus growth, protein content and plant development

Whole plants from NaCl medium were grown under greenhouse conditions in salinity soil (Fig 2D). Their performance on saline soil was carried out to ascertain if their salinity tolerance that was expressed under *in vitro* conditions was also observed in saline soil.

## Conclusions

This protocol established the potential to produce plantlets from leaf and nodal explants through indirect organogenesis. The saplings under salt stress were showed comparatively lesser survival and larger duration of development than the saplings under control. The callus under salt stress was showed comparatively lesser fresh weight also protein content than the control. Plant regeneration with a high percentage conversion of explants to plants will allow adjustment of the methodology to use in vitro selection for abiotic stress resistance. In addition, successful differentiation and multiplication of shoots and subsequent rooting of *S. dulcis* shows the feasibility of this technique for large-scale production of planting stock. It is very important to note that high shoot proliferation rate, conversion to plants and survival under field conditions was observed in this present study. There has been much interest in the development of medicinal plants tolerant to a biotic stress, primarily salinity. This study provides an understanding of the response of callus to salinity, which is important for future studies aimed at developing strategies for selecting and characterizing somaclonal variants tolerant to salt stress. The overall conclusion that can be drawn from this work is that *in vitro* screening is clearly effective for developing salinity-adapted regenerated plants of *S. dulcis.*

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