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In vitro Shoot Regeneration and Control of Shoot Tip Necrosis in Tissue Cultures of *Soymida febrifuga* (Roxb.) A. Juss.

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Abstract

Soymida febrifuga (Roxb.) A. Juss., (Meliaceae) an indigenous lofty deciduous tree, endemic to India, has extensive pharmacological activity. Continuous destruction of plants due to environmental and geopolitical instability has posed a major threat to endemic tree species. *In vitro* propagation techniques are also problematic due to high incidence of shoot tip necrosis (STN). The effect of various culture conditions on regeneration and STN of *Soymida febrifuga* were investigated. Nodal segments from field grown plants were used as explants and cultured on MS supplemented with different concentrations of BA, KN, NAA and IAA. The synergistic effect of BA (2 mg/l) and NAA (0.2 mg/l) induced a mean of 6.3 ± 0.09 shoots from the nodal explants with a frequency of 80.4%. Frequent subculturing of nodal explants from *in vitro* derived shoots increases the number of multiple shoots, but the regenerated shoots exhibited the symptoms of STN. Various factors such as strength of the media, different carbon sources activated charcoal and different calcium sources have been investigated for reducing the incidence of shoot necrosis. STN can easily be recovered by increasing the levels of calcium. Transfer of shoots showing early signs of necrosis to half strength MS supplemented with CN (556 mg/l), CP (1.0 mg/l), AC (20 mg/l) and fructose (100 mg/l) facilitated recovery of more than 98% of the shoots. Rooted plantlets produced, using the optimized protocol, were acclimatized successfully.

Introduction

Soymida febrifuga (Roxb.) A. Juss., commonly called Chandravallabha (Sanskrit), Indian red wood, Bastarol cedar (English), Somi, Somidha, Sumi (Telugu) is an indigenous lofty deciduous medicinal tree, a monotypic genus of Meliaceae

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endemic to India (Anon. 1952) distributed in the dry forests of Southern and Central India. The bark contains a resinous bitter principle, is a popular drug in indigenous medicine used in Ayurveda (Yoganarasimhan, 1996) credited with astringent and antiperiodic properties. A decoction of the bark forms a good substitute for oak-bark and can be well adapted for gargles, vaginal infections, enemata, rheumatic swellings, in the treatment of diarrhoea, dysentery and fevers as a bitter tonic in general debility (Chopra et al. 1956). Bark is antimalarial (Kirtikar and Basu 2003), anti-inflammatory (Diwan and Singh 1993) and antiplasmodial (Simonsen et al. 2001) in action. The wood makes high quality furniture with the appearance of mahogany employed for frames, stiles and also a potential source of indigenous tanning material, gum and fiber (Anon. 1952). Methyl angolensate, a secondary metabolite isolated from callus of *Soymida febrifuga*, found to possess antimicrobial and antileukaemic properties (Chiruvella et al. 2007, 2008). Conventionally this plant is propagated through seeds, have low germination capacity and seedlings are more prone to insect attack. Propagation through stem cuttings is cumbersome that restricts propagation due to difficulty in rooting.

The large scale destruction of natural habitat due to population pressure and overexploitation has become a major threat to important bioresources (Sangeeta and Buragohain 2005). Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology (Amin et al. 2003). Tissue culture propagation and its importance in conservation of genetic resources and clonal improvement have been described by many workers (Barz et al. 1977, Datta and Datta 1985, Kukreja et al. 1989 and Jusekutty et al. 1993). One objective of this study was to develop rapid and efficient propagation of *S. febrifuga* using nodal explants.

Shoot tip necrosis is a major obstacle in the successful propagation of certain species by tissue culture. The symptoms of STN are browning and die back of buds and the youngest leaves (Bairu et al. 2009). The first assumption in seeing STN is that it is caused by nutrient deficiency. The symptoms of nutrient deficiency of less mobile elements such as calcium (Ca) and boron (B) (Raven 1977) first appear in the meristematic regions and young leaves whereas symptoms of excessive amounts of these minerals are first observed on the older leaves (Barghchi and Alderson 1996). The problem of non-pathogenic dieback or shoot necrosis *in vitro* has been associated with complex set of factors such as salt formulation, growth regulators, use of additives like charcoal to medium, sugar source, frequency of subculturing affecting it, physiological disorders associated with rooting, sulphur content and NH_4/NO_3 ratio and pH fluctuations (Vieitez et al. 1989, De Block 1990, Kataeva et al. 1991, Lakshmi and Raghava 1993,

Mackay et al. 1995, Piagnani et al. 1996, Grigoriadou et al. 2000, Seling et al. 2000, Wang and Van Staden 2001 and Jain et al. 2009).

Shoot tip necrosis causes severe loss of cultures in several woody species (Kataeva et al. 1991, Hammatt and Ridout 1992, Sita and Swamy 1993, Amo-Marco and Lledo 1996, Xing et al. 1997, Kulkarni and D'Souza 2000). Different approaches have been reported to overcome the loss of cultures by different means (Alderson et al. 1987), (Martin 2002, Martin et al. 2007, Jain et al. 2009, Bairu et al. 2009), (Valles and Boxus 1987), (Kulkarni and D'Souza 2000) (McCown and Sellmer 1987). The second objective of this study highlights the occurrence of shoot tip necrosis followed by the death of plants at the shoot multiplication and rooting stages of micropropagation and its alleviation through the modification of culture media.

Materials and Methods

Tender twigs from 15 - 20 years old mature trees of *Soymida febrifuga* (Roxb.) growing in Divyaramam nursery, Tirupati, A.P, India were collected, defoliated and sectioned into 2 - 3 nodal segments. They were washed under running tap water and then treated with a solution of Labolene (5% v/v) for 10 min and finally surface sterilized with $HgCl_2$ (0.05% w/v) for 5 min. Lastly, the material was washed three times with autoclaved distilled water to remove any trace of $HgCl_2$. The nodal segments were excised from the disinfected material and divided into 1.0 - 1.5 cm pieces with at least one node in each explant. The basal medium used for all the experiments was MS formulation containing 30 g/l sucrose, 8 g/l agar (Merck India Ltd., Mumbai) and supplemented with BA and Kn at various concentrations either used alone or in combination with IAA and NAA. The media were adjusted to pH 5.7 ± 0.1 and autoclaved at 1.1 kg/cm^2 for 20 min at 121°C. Cultures were incubated at 25 ± 1°C 16 hr at 2000 - 3000 lux of cool white fluorescent light. All the chemicals used in media preparation were of analytical grade. The hormones were Sigma make. The shoots that proliferated from primary explants were isolated and subcultured on fresh medium several times for bulking up shoot culture material.

STN is a major problem when culturing *Soymida febrifuga*. The rates of necrosis and death at the shoot multiplication and rooting stages were recorded as a percentage of 100 cultures. Several methods for alleviating necrosis at the shoot multiplication stage were tried. The effects of culture media (MS, half, one-fourth strength), different calcium sources (calcium chloride; calcium ammonium nitrate and calcium pantothenate), activated charcoal (AC) and different carbon sources (glucose and fructose) were studied in the present study on the inhibition of necrosis. Browned leaves (before the stage of complete browning)

were removed from the necrotic shoots before the shoots were used for culture on the various media tested to alleviate the malady.

Well-grown shoots (3 - 4 cm) recovered from the necrotic cultures were excised from proliferating cultures and implanted on half strength MS supplemented with either of NAA, IBA or IAA (0.1 - 1.0 mg/l) for rooting. Non-necrotic culture derived plantlets with well developed roots were taken out of the test tubes, washed with sterilized double distilled water to remove all the traces of agar and transferred to small cups containing soil, sand and vermicompost (1 : 1 : 1) covered with polythene bags, well irrigated with liquid half strength MS without sucrose and hardened for four weeks in a humidity chamber. Fully acclimatized plants were shifted to bigger pots containing the same potting mixture and left to grow for one year in the greenhouse with a watering frequency of once per week.

Each culture tube with one shoot explant was considered as one replicate. Each treatment in each set of experiments consists of 15 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data were statistically analyzed using one way ANOVA and means were compared using the DMRT at the 0.05% level of significance.

Results and Discussion

Nodal explants from field grown mature plants of *S. febrifuga* were cultured on MS supplemented with BA, KN, TDZ and 2-iP at various concentrations either used alone or in combination with IAA and NAA for proliferation of axillary shoots. Direct shoot regeneration of *S. febrifuga* was achieved by proliferation of already existing axillary buds. Nodal explants as the best source of multiple shoot induction have also been suggested in case of other woody medicinal plants, such as *Rauvolfia serpentine* Benth. Ex Kurz., *Emblica officinalis* Gaertn. and *Enicostemma hyssopifolium* (Willd.) Verd (Roy et al. 1995, Rahaman et al. 1999, Seetharam et al. 2002).

Among the different cytokinins used, within ten days of culture initiation BA and Kn showed positive response for the induction of multiple shoots whereas TDZ and 2-iP failed to show organogenic response from the axillary buds (data was not shown in the table). BA alone was sufficient to trigger the growth of axillary bud of nodal explants. Irrespective of the concentrations of BA used, the axillary buds sprouted within ten days after culture initiation. Lower concentrations of BA supported activation and sprouting of dormant axillary bud also supported multiplication, which was best at 2.0 mg/l BA. When the explants were cultured on MS + BA, the frequency of regeneration and the number of shoots per explant (Table 1) was maximum at 2.0 mg/l and thereafter decreased with further increase in BA. The proliferation efficiency was

significantly higher producing shoots in 72.5% of cultured explants with 4.89 ± 0.09 shoots per explant and 3.4 ± 0.06 cm average length of shoots per culture (Fig. 1B,C). Cytokinins especially BA reported to overcome apical dominance, release lateral buds for dormancy and promote shoot formation. In the present study, it was found that BA was more effective than other cytokinins used for shoot multiplication from axillary bud and the effectiveness of BA in promoting *in vitro* axillary shoot production is well documented in different plants by Nobre et al. 2000, Mandal et al. 2001, Gisele and Thomas 2005, Avani et al. 2006.

Table 1. Effect of different PGR in MS on shoot organogenic response of mature nodal explants.

PGR (mg/l)				Mature nodal explants		
BA	KN	NAA	IAA	% of shoot regeneration	Mean number of shoots/explant	Mean length of shoots (cm)
2.0	-	-	-	72.5 ± 0.76^{ki}	4.89 ± 0.09^{l}	2.2 ± 0.07^{def}
2.0	-	0.2	-	80.4 ± 0.74^{n}	6.3 ± 0.09^{m}	3.4 ± 0.06^{ij}
2.0	-	-	0.2	44.6 ± 0.42^{f}	3.1 ± 0.08^{i}	2.1 ± 0.10^{cdef}
3.0	-	-	-	65.7 ± 0.91^{i}	4.2 ± 0.08^{jk}	1.8 ± 0.09^{cd}
3.0	-	0.2	-	77.4 ± 0.59^{mn}	4.5 ± 0.11^{k}	2.4 ± 0.12^{efg}
3.0	-	-	0.2	72.8 ± 0.59^{kl}	2.8 ± 0.06^{hi}	2.2 ± 0.10^{cde}
5.0	-	-	-	42.5 ± 0.76^{f}	2.1 ± 0.08^{defg}	1.5 ± 0.06^{ab}
5.0	-	0.2	-	66.9 ± 0.58^{ij}	2.0 ± 0.08^{bcde}	2.2 ± 0.08^{def}
5.0	-	-	0.2	56.5 ± 0.42^{g}	1.8 ± 0.06^{bcde}	1.0 ± 0.13^{a}
-	2.0	-	-	75.5 ± 0.43^{lm}	2.0 ± 0.05^{bcdef}	3.5 ± 0.09^{ij}
-	2.0	0.2	-	18.1 ± 0.39^{a}	2.3 ± 0.09^{fg}	4.3 ± 0.14^{k}
-	2.0	-	0.2	32.1 ± 0.48^{c}	1.8 ± 0.08^{bcd}	2.4 ± 0.11^{efg}
-	3.0	-	-	44.6 ± 0.49^{f}	2.0 ± 0.09^{bcde}	3.2 ± 0.09^{hi}
-	3.0	-	-	69.8 ± 0.95^{jk}	1.7 ± 0.07^{bc}	3.8 ± 0.11^{jk}
-	3.0	-	0.2	62.1 ± 0.60^{h}	1.2 ± 0.07^{a}	2.0 ± 0.09^{cde}
3.0	1.0	0.2	-	37.4 ± 0.37^{de}	2.5 ± 0.07^{gh}	2.5 ± 0.14^{fg}
3.0	1.0	-	0.2	32.0 ± 0.73^{c}	2.1 ± 0.09^{cdefg}	1.8 ± 0.07^{cd}
3.0	2.0	0.2	-	38.1 ± 0.54^{e}	2.2 ± 0.07^{efg}	2.8 ± 0.06^{gh}
3.0	2.0	-	0.2	34.7 ± 0.48^{cd}	1.7 ± 0.10^{b}	1.4 ± 0.08^{ab}
3.0	1.0	0.2	0.2	42.5 ± 0.58^{f}	2.5 ± 0.06^{gh}	2.1 ± 0.12^{def}
3.0	2.0	0.2	0.2	25.5 ± 0.76^{b}	2.0 ± 0.07^{bcde}	1.8 ± 0.06^{cd}
3.0	1.0	-	-	44.5 ± 0.72^{f}	4.6 ± 0.09^{k}	1.6 ± 0.07^{bc}
3.0	2.0	-	-	56.2 ± 0.63^{g}	4.0 ± 0.08^{j}	2.3 ± 0.09^{efg}

Values represented above are the means of 20 replicates. Mean values having the same letter in each column don't differ significantly at $p \leq 0.05$ (Tukey test)]

Increasing concentrations of BA (> 3.0 mg/) did not improve any of the parameters but reduced shoot proliferation. Eeswara et al. (1998) and Fracaro and Echeverrigaray (2001) state that high concentrations of cytokinins caused the

production of small shoots which failed to elongate, and also resulted in the formation of leaves with abnormal shape and or hyperhydric shoots in some plant species. At most of concentrations BA was more effective in inducing proliferation of axillary shoots while Kn was considerably less effective. Begum et al. (2002) also reported that BA was more effective than Kn for proliferation and the development shoots.

In this experiment different combinations of cytokinin and auxin were tested. Of these, the most effective combination for axillary shoots proliferation was 2 mg/l BA + 0.2 mg/l NAA producing highest percentage (80.4) of shoot regeneration, with 6.3 ± 0.09 shoots per culture and an average length of 3.4 ± 0.06 cm shoots per culture were observed (Fig. 1D).

Fig. 1. Regeneration of multiple shoots *in vitro* from nodal explants obtained from field grown *S. febrifuga* plants. A. MS containing 2.0 mg/l BA @ 0.2 mg/l IAA (Bar = 5.4 mm). B,C. MS containing 2.0 mg/l BA (Bar = 6.3, 6.2 mm). D. MS containing 2.0 mg/l BA @ 0.2 mg/l NAA (Bar = 6.9 mm).

Frequent subculturing the nodal explants from *in vitro* derived shoots increases the number of multiple shoots but the regenerated shoots exhibited the

symptoms of STN at the shoot multiplication and rooting stages after 15 days randomly in all cultures. The death of the apical shoot resulted in lateral shoot emergence. The lateral shoot that developed first assumed the role of the apical shoot and was the next to be affected by STN, with necrosis continuing hierarchically until all shoots were affected. The browning/blackening of the margin and tip of young leaves was the first symptom of the malady and finally resulted in the whole shoot browning/blackening and dying (Fig. 2E-J). Necrosis by the exudation of phenolics has been reported in cultured apple (Standardi and Romani 1990) and *Quercus* sp. (Bellarosa 1988). In present study on *S. febrifuga*, necrosis was not much associated with phenolic exudation.

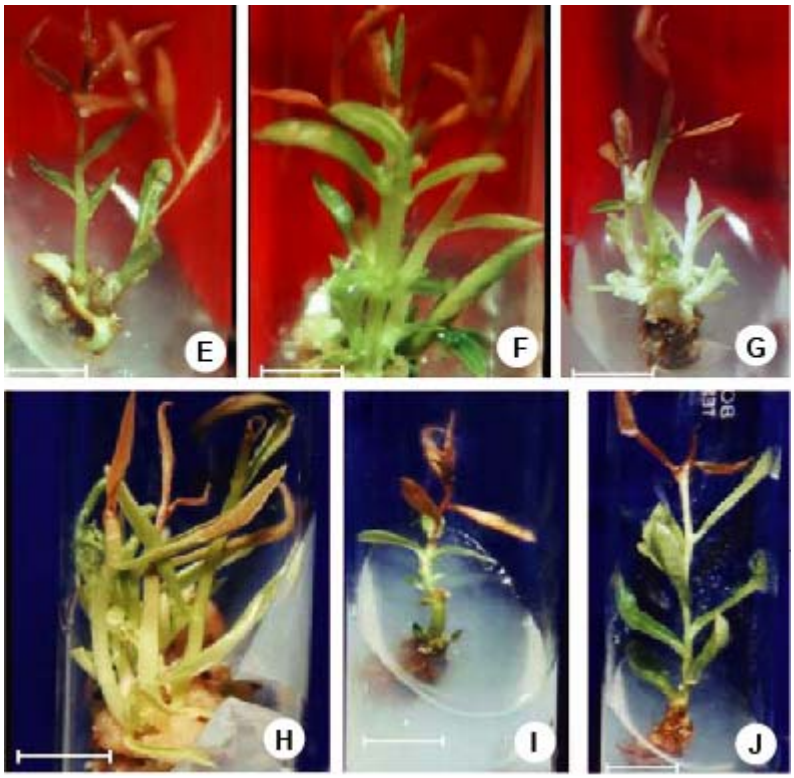

Fig. 2. *In vitro* grown shoots of *S. febrifuga* showing necrotic symptoms. E-H. Micro shoots showing the symptoms of rigorous STN on shoot multiplication medium (Bar = 2.6, 2.0, 3.3, 3.3 mm). I,J. Micro shoot tip necrosis in cultures kept for rooting (Bar = 5.2, 6.2 mm).

Dieback of the shoot tips has been reported in *Gymnema sylvestre* (Komalavalli and Rao 2000), *Holostemma adakodien* (Martin 2002) and *Rotula aquatica* (Martin 2003). STN causes severe loss of cultures in several woody species (Kataeva et al. 1991, Hammatt and Ridout 1992, Sita and Swamy 1993, Amo-Marco and Lledo 1996, Xing et al. 1997, Kulkarni and D'Souza 2000). Loss of regenerants due to such symptoms was also observed in *Eucalyptus tereticornis*

(Gill et al. 1993), *Solanum nigrum* (Ara et al. 1993), *Rauvolfia serpentine* (Ilahi 1993) and *Rosa damascena* (Kumar et al. 1995).

Subculture of *S. febrifuga* shoots at the onset of necrosis to plant growth regulator-free and fresh shoot multiplication medium (i.e. at an interval of 20 days) proved to be unsuccessful in circumventing the problem of necrosis and dieback of *in vitro* shoots. The frequency of necrosis and death of shoots at the shoot multiplication as well as rooting stages increased as the number of subculture increased. The necrotic shoots resumed fresh growth and multiplication after transfer onto shoot multiplication medium, but started to show the symptoms between 17 and 21 days. The shoots transferred on basal medium without PGRs exhibited the problem after 22 days, while the shoot multiplication rates declined significantly. The occurrence of necrosis after several subcultures (as in this study) has been reported in *Psidium guajava* (Amin and Jaiswal 1988) and *Cercis canadensis* (Yusnita et al. 1990).

In order to reduce the loss of shoots by STN, different strategies were exploited to alleviate the problem and the results are summarized as follows. The transfer of necrotic shoots to fresh shoot multiplication media containing different strengths (full, half and quarter strengths) medium, different sources and concentrations of calcium (*viz.*, calcium chloride (25 - 150 mg/l; CP (0.5 - 2.0 mg/l); CN (278 - 1112 mg/l), different carbon sources (glucose and fructose (50 - 200 mg/l) and different concentrations of activated charcoal (10 - 50 mg/l) were attempted. Recovery of the shoots was more when the transfer of shoots was done with initial signs of necrosis and did not exhibit further necrosis and dieback for over 30 days.

Subculture of necrotic shoots to shoot multiplication media containing different strengths of mineral nutrients tested for the inhibition of shoot tip necrosis, half strength MS proved to be the best when compared with full strength and quarter strength MS. In the present study, addition of different levels of fructose was effective in reducing shoot tip necrosis. Effect of two monosaccharide sugars (glucose and fructose) at equimolar concentrations tested, fructose at 200 mg/l proved to be better in the control of STN. The effectiveness of fructose as in the present study has been reported in *Butea* (Kulkarni and D'Souza 2000).

The first assumption in seeing STN is that it is caused by nutrient deficiency of less mobile elements such as calcium and boron first appears in the meristematic regions and young leaves (Bairu et al. 2009, Raven 1977). Some report that the use of higher concentrations of Ca alleviates or controls the STN (Dyson and Digby 1975, Vieitez et al. 1989, Singha et al. 1990, Barghchi and Alderson 1996, Piagnani et al. 1996, Wang and Van Staden 2001, Chang and Miller 2005, Martin et al. 2007). In contrast, other reports show that high Ca

concentrations in the culture media significantly increase the percentage of STN (Grigoriadou et al. 2000). Increasing Ca concentrations in macadamia rooting

Table 2. Effect of MS, CAN, CAP, AC, glucose and fructose in the inhibition of shoot tip necrosis in *S. febrifuga*.

Type of media	CAN	CAP	AC	Glucose	Fructose	Frequency of inhibition (%)
MS	278	-	-	-	-	21.0 ± 0.56 [a]
	556	-	-	-	-	80.2 ± 0.77 [k]
	834	-	-	-	-	64.5 ± 0.71 [h]
	1112	-	-	-	-	50.0 ± 0.44 [f]
	-	0.5	-	-	-	32.3 ± 0.25 [b]
	-	1.0	-	-	-	56.4 ± 0.48 [g]
	-	2.0	-	-	-	45.1 ± 0.95 [e]
	-	-	10	-	-	40.2 ± 0.47 [d]
	-	-	20	-	-	74.6 ± 0.43 [j]
	-	-	50	-	-	50.3 ± 0.50 [f]
	-	-	-	100	-	45.4 ± 0.74 [e]
	-	-	-	200	-	29.7 ± 0.46 [b]
	-	-	-	-	100	50.2 ± 0.71 [cf]
	-	-	-	-	200	35.9 ± 0.58 [c]
Half strength MS	556	1.0	-	-	-	92.5 ± 0.50 [m]
	834	1.0	-	-	-	70.5 ± 0.61 [i]
	556	1.0	20	100	-	86.9 ± 0.68 [l]
	834	1.0	20	100	-	63.1 ± 0.32 [h]
	556	1.0	20	-	100	94.3 ± 0.27 [mn]
	834	1.0	20	-	100	68.5 ± 0.25 [i]
	556	1.0	20	100	-	96.7 ± 0.31 [no]
	834	1.0	20	100	-	78.9 ± 0.29 [k]
	556	1.0	20	-	100	98.1 ± 0.40 [o]
	834	1.0	20	-	100	84.8 ±0.58 [i]
Quarter strength MS	556	1.0	20	100	-	51.5 ± 0.54 [f]
	834	1.0	20	100	-	39.4 ± 0.42 [d]
	556	1.0	20	-	100	58.2 ± 0.50 [g]
	834	1.0	20	-	100	40.6 ± 0.42 [d]

Values represented above are the means of 20 replicates. Mean values having the same letter in each column do not differ significantly at $p \leq 0.05$ (Tukey Test).

experiments failed to stop STN with concentrations higher than 6 mM aggravating the problem (Bhalla and Mulwa 2003). Of all the methods used by Martin et al. 2007, for alleviating shoot necrosis, including shortening the culture

period, altering the media salt strength, use of various plant growth regulators, different levels of sucrose, fructose, silver nitrate only the addition of calcium chloride proved to be effective.

In the current study application of increased levels of $CaCl_2$ did not ameliorate the disorder. Addition of higher levels of calcium chloride in shoot multiplication as well as rooting medium did not result in complete alleviation of necrosis and this may be due to chloride toxicity caused by the supra-optimal level of calcium chloride in the medium. Excess calcium may also produce deficiencies in magnesium and potassium. Although calcium can be present in millimolar concentrations within the plant as a whole, calcium ions are pumped out of the cytoplasm of cells to maintain the concentration at around only 0.1 lM. This active removal of Ca^{2+} from the protoplasm is necessary to prevent the precipitation of phosphate and interference with the function of Mg^{2+} (George 1993).

To overcome this difficulty, MS was modified, by adding the different concentrations of calcium nitrate (CN) individually or in combination with calcium pantothenate (CP) to correct the calcium deficiency. High concentrations of CN (> 556 mg/l) and CP (>1.0 mg/l) individually were not proficient in inhibiting STN, but the synergistic effect of both CN and CP in the same medium facilitated 92.5% recovery of the shoots. The addition of AC at 20 mg/l to media even advantageously regulated the plant growth *in vitro*, due to higher affinity for calcium in terms both of uptake and ion translocation. Transfer of shoots showing early signs of necrosis to half strength MS supplemented with CN (556 mg/l), CP (1.0 mg/l), AC (20 mg/l) and fructose (100 mg/l) facilitated recovery of more than 98.5% of the shoots (Table 2) (Fig. 3K-O).

An *in vitro* rooting experiment reiterates the importance of auxins on root induction. Among the three auxins used, IBA alone was most effective when compared with IAA and NAA for root induction. The IBA improved rooting efficiency and the superiority of IBA in rhizogenesis was also envisaged by many workers (Palacios et al. 2001, Liu et al. 2002, Soniya and Das 2002, Das and Rout 2002, Tiwari et al. 2002, Martin et al. 2003 and Prasad 2004). Occurrence of necrosis in some plants species is regarded as a physiological malady associated with rooting. Shoot tip necrosis in European chestnuts and oaks during the rooting stage was shown to be due to calcium deficiency, lack of cytokinins and presence of auxins in the culture medium (Vieitez et al. 1989). Xing et al. (1997) demonstrated alleviation of necrosis in American chestnut by the use of an auxin-free calcium-rich rooting medium with a reduced level of cytokinin. In the present study, though the rooting medium contains only IBA, additional level of calcium to rooting medium effectively curtailed the malady. Efficient rooting of proliferated shoots without STN was induced on half strength MS solid medium

supplemented with CN (556 mg/l), CP (1.0 mg/l) and IBA (2.0 mg/l) in combination was found to be more efficient in showing high frequency (95.0%) of root regeneration (5.1) with mean root length of 5.3 cm (Fig. 3P,Q) within four weeks of culture. These *in vitro* derived plants were better acclimatized under *ex vitro* conditions and finally established in soil under green house conditions with 75 - 80% survival rate (Fig. 3R).

Fig. 3. Recovery of STN, rooting and acclimatization of *S. febrifuga.* K-O. Recovery of normal shoots from necrotic shoots on half strength MS shoot multiplication medium supplemented with CN (556 mg/l), CP (1.0 mg/l), AC (20 mg/l) and fructose (100 mg/l) (Bar = 6.9; 4.1; 5.5, 6.4; 5.0 mm). P. Recovery of *in vitro* derived micro shoots from STN on half strength MS rooting medium supplemented with CN (556 mg/l), CP (1.0 mg/l) and IBA (2.0 mg/l) medium (Bar = 4.9 mm). Q. Rooted plants ready for acclimatization (Bar = 10.0 mm). R. Fully acclimatized and greenhouse grown plant (Bar = 11.9 mm).

Through this study a protocol for *in vitro* multiplication, control of shoot tip necrosis and regeneration of complete plantlets has been established. This is perhaps the first report on *in vitro* plant regeneration of *Soymida febrifuga* (Roxb.) A. Juss. These results may be of some importance as a pioneering study on tissue culture of this medicinal plant.

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