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Phenotypic aberrations during micropropagation of *Soymida febrifuga* (Roxb.) Adr. Juss

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Abstract

Like most of the medicinal plants *Soymida febrifuga* (Meliaceae) possess significance for its valuable secondary metabolites. Multiplication of this endemic plant is limited by difficulty in rooting of stem cuttings, high seedling mortality rates and low seed viability period. Hence efficient protocols for *in vitro* mass propagation has been established from field grown and aseptic seedlings explants. Strikingly, we observed aberrant structures such as vitrified shoots, faciated shoots, albino shoots as well shoot necrosis during its micropropagation. These phenotypic maladies were observed during organogenesis and rooting. Compared to other abnormalities, shoot necrosis nonetheless was frequent and pronounced leading to plant death. Shoots when subjected to rooting also displayed necrosis which was controlled by transferring to MS medium containing various concentrations and combinations of calcium levels, activated charcoal, glucose, fructose and auxins. Microshoots initiated roots on half strength MS medium with IBA and IAA individually or in combination within two weeks. MS half strength solid medium supplemented with CAN (556 mg l^{-1}), CAP (1.0 mg l^{-1}), IAA (2.0 mg l^{-1}) and IBA (2.0 mg l^{-1}) in combination was found to be more efficient in showing high frequency (95%) of root regeneration. Rooted plantlets were successfully hardened and 70-85% of regenerated plants were successfully acclimatized to natural environment. *In vitro* derived plantlets were morphologically similar to *in vivo* plants.

Abbreviations: STN- shoot tip necrosis, CAP- calcium pantothenate, CAN- calcium nitrate, BA – 6, Benzyladenine, IBA – Indole 3 Butyric Acid, IAA – Indole 3 Acetic Acid, KN-kinetin, MS – Murashige and Skoog,

Introduction

Mahogany family (Meliaceae) covers more than 50 genera with more than 1,000 species distributed in tropical and subtropical regions of western peninsula and Indomalaysia. *Soymida febrifuga* (Roxb.) Adr.Juss. or (Syn. *Swietenia febrifuga* Roxb). (Meliaceae) commonly called Chandravallabha (Sanskrit), Indian red wood, Bastarol cedar (English), Somi, Somidha, Sumi (Telugu) is an indigenous lofty deciduous medicinal tree and monotypic endemic to India (Anonymous, 1952). Bark of this plant possess a resinous bitter principle used in Ayurveda (Yoganarasimhan, 1996), adapted for gargles, vaginal infections, enemata, rheumatic swellings, diarrhoea, dysentery and fevers (Chopra *et al*., 1956). Bark is antimalarial (Kirtikar and Basu, 2003), anti-inflammatory (Diwan and Singh, 1993) and antiplasmodial (Simonsen *et al*., 2001). Like most of the medicinal plants, *Soymida febrifuga* has highest significance for its valuable secondary metabolites.

Tissue culture of tree species has been progressing successfully from the past few years to overcome the problems to a great extent and assessment of the regeneration potential of tissues has been undertaken as it is a prerequisite for the forest tree improvement. Traditional multiplication of this plant is limited by difficulty in rooting of stem cuttings, high seedling mortality rates and low seed viability period. Hence protocols have been established for rapid *in vitro* propagation of *S. febrifuga* by using different explants (Chiruvella *et al*., 2011; 2013). Here we report aberrant structures such as vitrified shoots, faciated shoots, albino shoots as well shoot necrosis observed during micropropagation of this plant. These phenotypic abnormalities were noticed both during caulogenesis and rhizogensis. Such aberrant structures hampered the micropropagation of this endemic medicinal plant.

Materials and methods

Plant collection and Seedling establishment

Nodes and seeds were excised from mature trees growing

in Divyaramam nursery, Tirupathi, India during late July-August, 2007-2009. The explants were initially washed with running tap water for 15 min followed by detergent for 15 min, followed by repeated washing in running tap water until all traces of detergent were removed. Then they were rinsed 4-5 times in distilled water. Surface sterilization of these explants was then made by keeping them in 70% alcohol for 15 sec followed by rinsing for 3 times in sterile distilled water. Finally the explants were treated with 0.05-0.1% $HgCl_2$ for 5 min (Chiruvella *et al.,* 2011; 2013). The surface sterilization was followed by 5-6 rinses in sterile distilled water. The explants were blotted on sterile filter paper discs before planting them vertically on agar gelled MS medium.

Culture Initiation, Conditions and Shoot Multiplication

Initial experiments were designed for the selection of most appropriate explant and suitable medium. MS medium with different concentrations (0.5-5.0 mg l^{-1}) and combinations of cytokinins (BA, CM, 2-iP, TDZ and Kinetin) and auxins (IAA and NAA) were used to study their effects on shoot induction and multiplication. Subculturing was done by transferring the micro-shoots to fresh shoot induction medium. Subculturing was carried out at regular intervals of 30 days after inoculation. Visual observations of the cultures were made at every transfer during subculturing, and the effects of different treatments were studied based on their response. All cultures were incubated in a culture room at 25 ± 2°C with a relative humidity of 50-60% and 16 h photoperiod at a photon flux density of 15-20E m^2/s^{-1} from white cool fluorescent tubes. Each treatment had 20 replicates, and the experiments were conducted thrice. Explants were vertically placed on agar gelled MS medium taken in 25 x 150 mm culture tubes or 150 ml conical flasks sealed with aluminum foil.

Root Initiation and Field Establishment

The microshoots of 4-5 cm length were dissected from proliferated shoot cultures and inoculated on agar gelled medium containing full strength, half strength and quarter strength MS salts with/without different auxins. After 6-8 weeks of inoculation, rooting frequency and the number of roots with other characters like callusing were recorded. The rooted microshoots were planted in plastic trays containing vermiculite, covered with plastic sheets, and kept in mist chamber. The plastic trays were kept open during night and covered with plastic sheets during the day. After one week the plantlets were exposed to direct light during morning hours. Then they were transferred to root trainers containing vermicompost and sand (1:1) for 10 days, and 10 ml of autoclaved ¼ MS salts were directly supplied to substratum in root trainers. After one week the plantlets were transferred to plastic bags containing a mixture of soil organic manure and sand in 2:1:1 ratio. The plants in plastic bags were removed from the mist chamber and kept in open shade and watered twice a day. After one week they were transferred to the field.

Results and Discussion

In vitro developed plants sometimes exhibit aberrant structures during morphogenesis. Signals necessary for organ formation appear to be incomplete, interrupted or inaccurate, so that correct determination is not achieved. This leads to the development of incompletely formed or even gross abnormal structures, which are liable to arise during any sequence of direct or indirect (Bowes, 1970) morphogenesis conducted *in vitro*. Abnormalities in tissue cultures and in the plants produced from them often increase in frequency if the cultures are maintained for a longer period. During micropropagation of *S. febrifuga*, *in vitro* cultures showed some kinds of abnormalities (Fig. 1, 2). That atypical structures formed during micropropagation of *Soymida febrifuga* are discussed below.

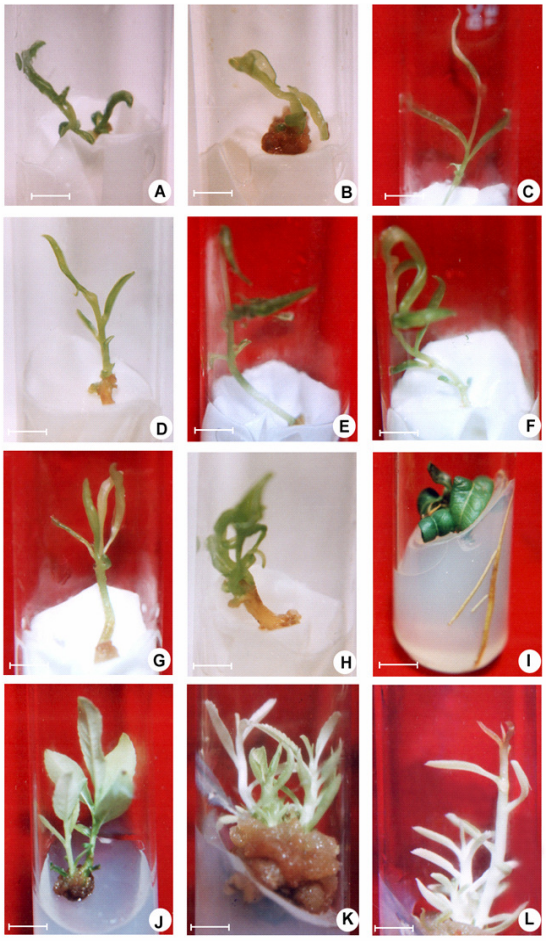

Fig. 1. Phenotypic aberrations during *In vitro* propagation of S. febrifuga. (A-F) Vitrified shoots developed on liquid medium containing high concentrations of cytokinins(5.0-7.0 mg l-l BA / Kn) (Bar = 2.0, 1.9, 3.9, 2.7, 2.8, 2.4 mm) (G-H) Fasciated shoots developed on liquid medium containing high concentrations of cytokinin (5.0 mg l^{-l} BA) (Bar = 3.7, 1.9 mm) (I) Rhizogenesis of *In vitro* leaf on medium containing Kn (Bar = 5.4 mm) (J) Partial albino shoots from direct shoot cultures on medium containing 4.0 mg l^{-l} Kn (Bar = 5.2) (K,L) Complete albino shoots from indirect shoot cultures on medium containing 7.0 mg l^{-l} BA (Bar = 5.2, 4.9 mm)

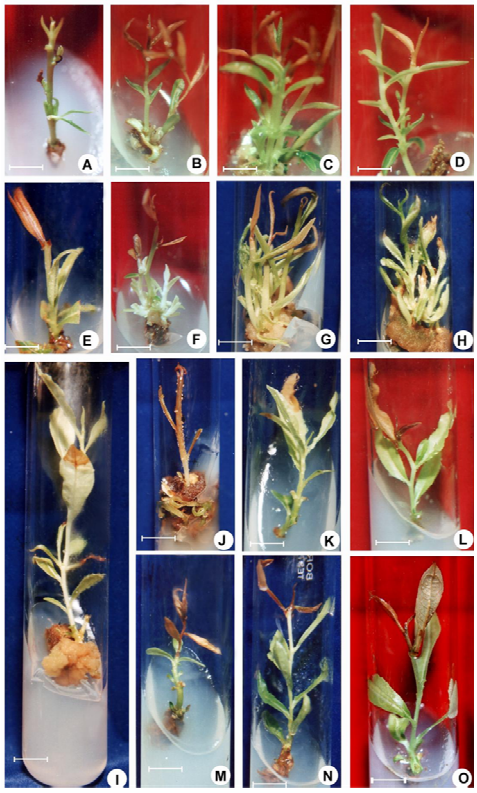

Fig. 2. Shoot tip necrosis in cultures of *S. febrifuga*. (A-F) Shoot necrosis in proliferating direct shoot cultures (Bar = 2.0, 2.6, 2.0, 2.1, 3.4, 3.3 mm) (G-J) Shoot tip necrosis in proliferating indirect shoot cultures (Bar = 3.3, 4.9, 6.0, 6.9 mm) (K-O) Shoot tip necrosis in cultures kept for rooting (Bar = 4.9, 5.2, 5.2, 6.2, 5.0 mm)

Vitrification

This is a physiological disorder frequently affecting woody plants during their *in vitro* propagation. It may be considered as morphological response to non-wounding stress conditions (Davis *et al.,* 1977) excess of some mineral ions or cytokinins and low light intensity levels. The tissue culture environment some times induces an abnormal growth, a known as vitrification or hyperhydricity (Hazarika, 2006). Glassy, hyperhydrated nature of vitrified shoots were observed in the present study during direct multiple shoot production that were induced at higher concentration of cytokinins i.e. BA at 0.1 mg l^{-1} and Kn (>8 mg l^{-1}) (Fig. 1A–F).

Recently Mahdiyeh (2011) demonstrated the occurrence of vitrification in high cytokinin containing medium. Vitrification in the present study was also observed in liquid cultures and those cultures that received less light intensity i.e., cultures that were far from light source. Some cultures recovered to normal morphology after some period on transferring to higher light intensity. Vitrified plants appear glassy, often hyperhydrated and do not survive on transferring from tissue culture to the *ex vitro* environment, that is why vitrification is problematic for the plant tissue culturist (Debergh *et al.,* 1992). Vitrification may also be induced by very high relative humidity that exists *in vitro* because of the closed nature of culture vessels (Wardle *et al.,* 1983; Short *et al.,* 1987; Capellades *et al.,* 1990; Thomas *et al.,* 2000). Phan and Hegedus (1986) have attributed the problem of vitrification to the deficiency of lignin, which is essential to the structure of cell walls and other tissues.

Lighting in plant tissue culture is usually provided by cool white, fluorescent tubes (Debergh and Read, 1991). Such light provides a photosynthetic photon flux density (PPFD) of around 30-50 µmol photons $m^{-2}s^{-1}$ only (Goncalves *et al.,* 1994), where as the PPFD measured in full sunlight is of 1500-2000 µmol $m^{-2}s^{-1}$ in the field (Chaves, 1994). Consequently *in vitro* plants are generally growing at close to the light compensation point. Such low irradiation could not elicit the transpiration pull and consequently plants become hyperhydrated. Reduced BA concentration in medium (Soni and Kaur, 2014) and usage of aerated capped culture bottles minimized vitrification (Sharma and Mohan, 2006).

Shoot fasciation

During micropropagation of *S. febrifuga*, shoot cultures showed a phenotypic aberration form with multiple shoot apexing. This abnormal character is called shoot fasciation. Shoot fasciation *in vitro* was observed generally at high cytokinin concentrations. Fasciated shoots appeared with short length having thick multiple shoots at the apex (meristematic tissue). High frequency of occurrence of shoot fasciation was found with BA (> 5.0 mg l^{-1} BA). Once after it is induced, it is difficult to remove this abnormality. Attempts to alleviate the medium to remove this abnormality failed. Shoot fasciation is a physiological problem rather than a genetical aberration (George, 1996). We observed high frequency of *in vitro* fasciation in MS medium containing cytokinins (Fig. 1 G, H). In this regard, there are numerous similar observations made by others supporting our notion (Yusnita *et al.,* 1990; Preece et al. 1991; Huetteman and Preece, 1993; Shirani *et al.,* 2009; Martin *et al.,* 2006; Fatemeh and Maheran, 2010; Najmeh *et al.,* 2011).

It has been suggested that fasciations might be the result of the growth of a single apical meristem and, alternatively, that fasciations are due to the adhesion of several sites of growth (Ivan and Kitin, 2011) or to a hormonal imbalance within plants. Recently, Peter Kitin *et al.* (2005) reported a comparative histological study between normal and fasciated shoots of *Prunus avium* generated *in vitro*. In heritable fasciation has been observed in a number of experimental systems and the *CLAVATA I* and *CLAVATA 3* (Fletcher *et al.,* 1999) genes have been shown to be associated with fasciation of stems of Arabidopsis.

Albino Shoots

It is observed that some shoots regenerated through tissue culture especially callus regenerated ones were without chlorophyll (albinos). They were either complete or partial albinos. In the present study we observed partial albinos during direct organogenesis (Fig.

1J) while complete albinos arised during indirect organogenesis (Fig. 1K,L). These albino shoots were observed randomly in all types of media in the present study but frequently at higher cytokinin concentrations of BA and KN. These albinos survived *in vitro* but grow more slowly than their green counter parts as observed by Chin (1980). But these shoots could not be rooted and transplanted as they lack plastids. Partial albinos some time can be made green by repeated sub culturing in the present study as done by Rotem-Abarbanell and Breiman (1989).

Albinos, occasionally found in broad leaved genera were reported from many *in vitro* cultured shoots (Mehra and Jaidka, 1979; Lassocinski, 1985; Noh and Minocha, 1990). The inability of plants to produce chlorophyll is due to changes that occur in both nuclear and chloroplast genes and also changes in ploidy (Park and Walton, 1989). The nature of the medium used, especially the concentration of KNO_3 has been shown to influence the frequency with which albino plants occurs (Feng and Ouyang, 1988). Albino shoots and plants can also result from the culture of explants from chimeras as said by Rotem-Abarbanell and Breiman (1989) and Cervelli (1987). Recently crucial factors were identified for albino shoot primordial to green plantlets during *in vitro* propagation (Mohiuddin *et al.,* 2011.

Shoot Necrosis

Application of tissue culture techniques for micropropagation of this tree is seriously hampered by apical necrosis of shoots and leaves in culture. Shoot tip necrosis or apical necrosis is a phenomenon in which the terminal portion of the shoot becomes dark and dies. Necrosis strictly describes the death of the parts of plant tissues in enclosed vessels can readily lead to death of the whole culture. In the current investigation, this problem is mainly associated at the stage of induction of proliferating shoots directly (Fig. 2A–F) and indirectly (Fig. 2G–J) from juvenile explants of aseptic seedlings and also in cultures kept for rooting which is termed as "shoot-tip necrosis". The most obvious symptom is an initial discoloration of the shoot tip of young shoots (1 to 3 weeks) after subculture. This discoloration usually progresses to an actual death of the shoot tip (necrosis). In some cases, the shoot tip continues to grow slowly or even outgrow the problem, leaving behind a region on the stem with distorted or poorly developed leaves. After shoot tip death, the role of apical dominance is taken over by one of the lateral buds, generally that nearest the apex and the plant survives. The shoot usually branches and in severe cases these branches show necrosis and also branch again, thus producing a "witches broom" (George, 1996). Necrosis is common in rapidly growing shoot cultures and prolonged subcultures, if shoots were allowed to grow longer than 3 cm (Amin and Jaiswal, 1988). Necrosis of shoot apex is considered to be a physiological disorder associated with *in vitro* rooting and is reported to cause severe loss of cultures more commonly trees *Camelia sinensis, Castanea dentata, Malus domestica, Pistacia vera, Populus spp., Quercus sps., Sassafras randaiense, Salix and Butea monosperma* (Srivasatva and Joshi, 2013; Bairu *et al.,* 2009; Thakur and Kanwar, 2008; Martin, 2007; Kulkarni and Souza, 2000). Shoot tip necrosis and vitrification are the two common unresolved problems in micropropagation of trees. Gribble *et al.* (2003), has reviewed the impact of environment on the growth of vitrified plants in order to pave the way for further research on this aspect. We figured out the possible ways to control *in vitro* shoot tip necrosis in *S. febrifuga* associated with actively growing cultures and cultures kept for rooting by supplementing MS medium with

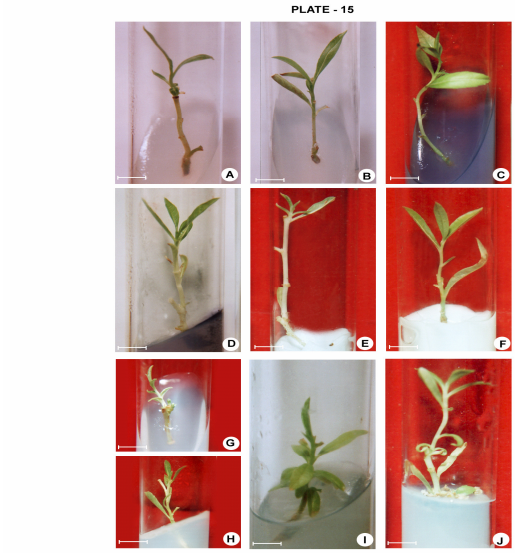

Fig. 3.Control of *In vitro* shoot tip necrosis (STN) in *S. febrifuga* (A, B) Inhibition of STN on MS full strength solid medium fortified with 556 mg l^{-1} CAN 1.0 mg l^{-1} CAP (Bar = 4.8, 4.9 mm) (C, D) Inhibition of STN on MS half strength solid medium supplemented with 556 mg l^{-1} CAN 1.0 mg l^{-1} CAP 20 mg l^{-1}AC along with 100 mg l^{-1} fructose (Bar = 4.7, 4.8 mm) (E, F) Inhibition of STN on MS full strength liquid medium fortified with 556 mg l^{-1} CAN 1.0 mg l^{-1} CAP (Bar = 5.8, 5.6 mm) (G, H) Inhibition of STN on MS quarter strength solid medium fortified with 556 mg l^{-1} CAN 1.0 mg l^{-1} CAP (Bar = 6.0, 6.0 mm) (I) Inhibition of STN on MS full strength solid medium fortified with 556 mg l^{-1} CAN (Bar = 3.8 mm) (J) Inhibition of STN on MS full strength solid medium fortified with 1.0 mg l^{-1} CAP (Bar = 4.9 mm)

Conclusion

The above described phenotypic abnormalities observed during *in vitro* culture hampered the micropropagation of *Soymida febrifuga*. We conclude high concentrations of cytokinins induced vitrification, fasciated shoots and development of albinos. Hence the usage of cytokinins such as BA, kinetin and coconut milk in establishing optimized cuture systems during *in vitro* propagation needs to be carefully monitored in order to obtain normal plantlets. Further we also conclude shoot tip necrosis is a physiological disorder caused by calcium deficiency.

calcium supply (Fig.3J) (Chiruvella *et al.,* 2011).

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Author contributions

Conceived and designed the experiments: KKC,AM,GRG. Performed the experiments: KKC. Analyzed the data: KKC, AM, GRG. Wrote the paper: KKC, AM, GRG.

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