Short Communication

TDZ–induced high frequency shoot regeneration in *Cassia sophera* Linn. via cotyledonary node explants

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ABSTRACT

Cassia sophera Linn. is an important medicinal plant belonging to family Fabaceae. It is extensively used in Homeopathy and is well known for its medicinal properties. The present study describes a simple, efficient and reproducible regeneration system for *in vitro* propagation of *C. sophera* through cotyledonary node (CN) explant excised from 21 d old axenic seedlings. Explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of thidiazuron (TDZ). Multiple shoots were produced on all the concentrations of TDZ; however 2.5 µM concentration proved to be optimal for the production of maximum number of shoots. To avoid adverse effects of prolonged exposure to TDZ in long term establishment, the cultures were transferred to TDZ free MS medium fortified with various concentrations of 6- benzyl aminopurine (BA) for multiplication, proliferation and elongation of induced shoots. Emergence of new shoot buds and multiplication continued up to second subculture passage and maximum number (14.9 ± 1.4) of shoots obtained on MS + BA (1.0 µM). Best rooting response was observed on half strength MS containing Indole-3-butyric acid (IBA) (1.0 µM). Regenerated plantlets were successfully acclimatized and hardened off inside the culture room and then transferred to green house with 100 % survival rate. **[Physiol. Mol. Biol. Plants 2010; 16(2) : 201-206]** *E-mail : ashahzad.bt@amu.ac.in, shahzadanwar@rediffmail.com*

Keywords : *Cassia sophera, Cotyledonary node, TDZ, In vitro regeneration, Acclimatization*

Abbreviations : *BA – 6-benzylaminopurine, IBA – Indole-3-butyric acid, MS – Murashige and Skoog, NAA – α-Naphthalene acetic acid, TDZ – Thidiazuron*

INTRODUCTION

Plants have been an important source of medicine for thousands of years. Even today majority of world's population still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the source of many modern medicines. Medicinal plant species are, still to a large extent, gathered and collected from the wild and relatively few genera are cultivated on a commercial scale. This exploitation coupled with increasing urbanization, has lead to a steady erosion and loss of diversity from the natural habitats of these plants. *In vitro* regeneration or micropropagation is the best alternative to overcome these hurdles and it holds tremendous potential for rapid multiplication and production of high quality medicines from them (Murch *et al*., 2000). *In vitro* protocols producing quality planting stock on a large scale have been developed for several medicinal plants such as *Rauvolfia tetraphylla* (Faisal and Anis 2002); *Pterocarpus marsupium* (Husain *et al*., 2006); *Acacia sinuata* (Shahzad *et al*., 2006); *Cannabis sativa* (Lata *et al*., 2009) and *Veronica anagallis-aquatica* (Shahzad *et al*., 2010) through various strategies of micropropagation.

Cassia sophera Linn. is a very important medicinal plant belonging to family Fabaceae. Various parts of the plants are used in Homeopathy. The plant also exhibits anticancer, antidiabetic, antidiuretic and anti-inflammatory properties. The leaves of the plant contain "sennosides" and their juice is known to cure a large number of diseases such as asthma, bronchitis, cough, jaundice, skin diseases, rheumatic and inflammatory fever (Anonymous, 1992). The bark in the form of infusion and the powdered seeds, mixed with honey are given in diabetes and the root is administered internally with black pepper for snake bite (Kirtikar *et al*., 1980).

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Infusion of leaves is also useful in gonorrhea and syphilitic sores (Ghani 1998). The seed extract exhibited important pharmacological effects like analgesic, hypnotic, sedative and antiepileptic effects (Bilal *et al*., 2005). Conventionally the plant can be propagated through seeds. The seeds however remain viable for a short period and germinate poorly. Because of its wide spectrum of medicinal properties and potential there is a need to develop an efficient regeneration system for mass propagation. Thus, the present study describes a micropropagation protocol for rapid multiplication of *C. sophera* through cotyledonary node (CN) explant.

MATERIALS AND METHODS

Plant material and explant collection

The mature and dried pods of *C. sophera* were collected from the plants growing in the botanical garden of the Department. Seeds were isolated from the pods, washed thoroughly under running tap water for 30 min and kept in 1 % (w/v) Bavastin (Carbendenzim Powder, BASF, India Ltd) for 20 min and then treated with 5 % (w/v) Teepol (a liquid detergent) for 15 min. Subsequent sterilization was done under laminar air flow hood, seeds were surface disinfected with 70 % (v/v) ethanol for 40 s followed by 0.1 % mercuric chloride ($HgCl_2$) for 5 min and then finally rinsed 5 times with sterile double distilled water (DDW) to remove surface sterilants. Surface sterilized seeds were incubated aseptically under controlled conditions on hormone free half strength MS (Murashige and Skoog, 1962) medium with 3 % (w/v) sucrose and 0.8 % agar. Cotyledonary node (CN) explants excised from 7, 14, 21 and 28 days old axenic seedlings were used to induce multiple shoots.

Culture media and culture conditions

Murashige and Skoog (MS) medium supplemented with 3 % (w/v) sucrose and 0.8 % (w/v) agar was used throughout the experiments. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. All the cultures were maintained at 24 ± 2 °C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40W, Philips, India) and with 50 - 60 % relative humidity.

Shoot induction and multiplication

The CN explants of different ages (7, 14, 21 and 28 d) excised from axenic seedlings were cultured on MS medium supplemented with different concentrations (0.1, 0.25, 5.0, 1.0, 2.5, 5.0, 7.5 and 10.0 µM) of thidiazuron (TDZ) or without TDZ. Data for percentage shoot regeneration, shoot number per explant and shoot length was recorded after 6 wk of culture. To facilitate proper growth and development of shoots, the TDZ induced cultures of optimum concentration were transferred to MS basal medium as well as MS medium supplemented with different concentrations (0.5, 1.0 and 2.5 µM) of 6-benzylaminopurine (BA). All the cultures were transferred to fresh medium at six week intervals and data for multiplication and growth of shoots was recorded up to three subculture passages.

In vitro rooting

For complete plant development regenerated microshoots (4-5 cm) were excised and transferred to rooting medium comprising of MS basal and half strength MS without any growth regulator or with auxins – indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) at various concentrations (0.5, 1.0 and 2.5 µM). The rooting percentage, number of roots per shoot and root length was recorded after 6 weeks of culturing.

Hardening and acclimatization

Plantlets with well developed root system were removed from the rooting medium, washed properly under running tap water to remove any adherent gel and transferred to thermocol cups containing sterilized soilrite. Thermocol cups were covered with transparent polythene bags to ensure high humidity and irrigated with ¼ strength MS salt solution (without vitamins) for initial 2 weeks followed by tap water. Hardening and acclimatization was done under diffuse light conditions (16:8 h photoperiod). Polythene bags were removed gradually in order to acclimatize plantlets, after 4 weeks they were successfully transferred to earthen pots containing garden soil and maintained in green house under normal day length conditions.

Data collection and statistical analysis

All the experiments were conducted with 10 replicates per treatment and repeated three times. Data for shoot induction, shoot multiplication and rooting experiments were recorded after 6 weeks of culture. The data were analyzed statistically using SPSS ver. 10 (SPSS INC. Chicago, USA). The significance of difference among means was carried out using Duncan's multiple range test at 5 % probability level and the results were expressed as a mean ± SE of three repeated experiments.

RESULTS AND DISCUSSION

Induction of multiple shoots

The sterilized seeds of *C. sophera* germinated under aseptic conditions on hormone free half strength MS medium within 1 wk with 90 % germination rate. CN explants excised from axenic seedlings of different age (7, 14, 21 and 28 d) were used to induce multiple shoots on MS medium supplemented with different concentrations (0.1–10 µM) of TDZ or without TDZ. Among CN explants of different ages, 21 days old explants showed best results in terms of early response, regeneration potential and number of shoots per explant (data not shown). Age of explant plays a major role in multiple shoot regeneration in a number of plant species including *Vicia faba* (Khalafalla and Hattori, 1999), *Morus alba* (Thomus, 2003) and *Pterocarpus marsupium* (Husain *et al*., 2007).

TDZ, a substituted phenylurea, is known to be more effective than all adenine type cytokinins in inducing high frequency shoot organogenesis as the most prevalent type of regeneration in legumes. (Amutha *et al*., 2006). TDZ has an immense cytokinin potential in shoot organogenesis in a number of plant species including legumes (Murthy *et al*., 1998; Mroginski *et al*., 2004 and Husain *et al*., 2007). It is well established that the concentration of TDZ is highly sensitive for the induction of multiple shoots and their proper growth and development as compared to other cytokinins. Thus, in the second experiment, a wide range of different concentrations of TDZ was evaluated for the selection of optimal concentration, to induce multiple shoots in *C. sophera* using CN explants of 21 d old aseptic seedlings. Explants cultured on TDZ free MS basal (control) did not show any morphogenic change and died within 1 wk without showing any response. Initial response was noticed by swelling and enlargement of the explant tissue within 1 wk of inoculation. Differentiation of multiple shoot buds started after 3 wk of culture, these shoots buds were induced directly from the axil of each explant and later on grow in size to develop into healthy shoots (Fig. 1A). Multiple shoots were induced in all the concentrations of TDZ and the number increases with an increase in the concentration from 0.1-2.5 µM. However, beyond the 2.5 µM of TDZ a sudden decrease in explant response was noticed which ultimately affect the number of shoots per explant as well as shoot length. The MS medium containing TDZ (2.5 µM) induced a maximum of 6.7 ± 0.2 shoots per explant and 93 % regeneration frequency with an average shoot length 2.3 ± 0.1 cm. Similarly in large number of studies it was well advocated that comparatively lower concentration of TDZ is needed for high frequency shoot regeneration (Murthy *et al*., 1998; Raghu *et al.*, 2006; Chhabra *et al*., 2008). It was further observed that the higher concentrations of TDZ resulted in reduction of shoot length and heavy callusing, similar inhibitory effects have also been reported in other plant species such as *Murraya koinegii* (Bhuyan *et al*., 1997) and *Oroxylum indicum* (Dalal and Rai, 2004). TDZ (N- phenyl-1, 2, 3-thidiazol-5-ylurea) has a unique mode of action with intrinsic cytokinin like activity (Mok *et al*., 1982). Application of TDZ may increase the levels of endogenous cytokinins by inhibiting the action of cytokinin oxidase and this response is concentration dependent (Hare and Van Staden, 1994).

Proliferation and elongation of shoots

Multiple shoots induced by TDZ exhibited stunted growth and also the prolonged exposure to TDZ supplemented medium resulted in distortion and fasciation in the regenerated shoots. Similar results have also been reported by Murch *et al*., 2000 and Khurana *et al*., 2005. Cytokinins generally stimulate the proliferation of multiple shoots but inhibit their further

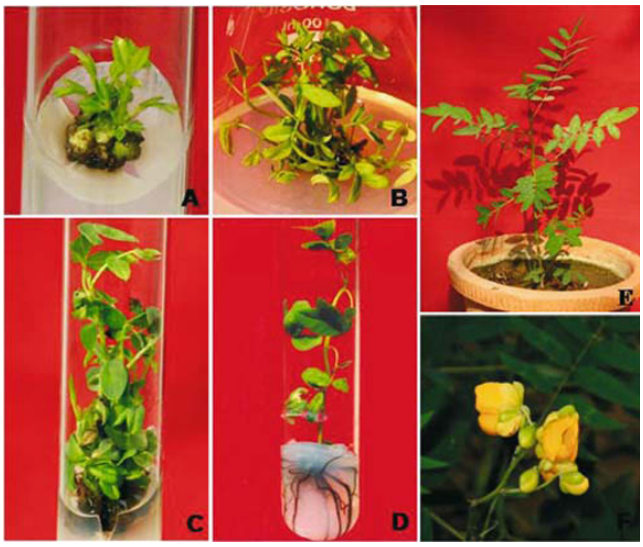

Fig. 1. *In vitro* shoot regeneration and plant development in *C. sophera* through CN explant. **A.** Induction of multiple shoots on TDZ supplemented medium. **B & C.** Proliferation and elongation of TDZ induced shoots on MS + BA (1.0 µM). **D.** *In vitro* rooting on ½ MS + IBA (1.0 µM). **E.** A well developed acclimatized plant in soil. **F.** Flowering twig of an acclimatized plant.

Table 1. Effect of different concentrations of TDZ on multiple shoot induction from CN explant of *C. sophera*

TDZ (µM)	% Response	Mean no. of shoots/explant	Mean shoot length (cm)
0.1	40.0 ± 1.2^{g}	3.2 ± 0.1^{d}	1.3 ± 0.1^{cde}
0.25	49.0 ± 2.1^{ef}	4.0 ± 0.3^{c}	1.5 ± 0.2^{cd}
0.5	61.0 ± 2.1^{d}	4.6 ± 0.3^{c}	1.6 ± 0.2^{bc}
1.0	79.7 ± 2.6^{b}	5.3 ± 0.2^{b}	2.1 ± 0.2^{ab}
2.5	93.0 ± 1.5^{a}	6.7 ± 0.2^{a}	2.3 ± 0.1^{a}
5.0	69.7 ± 3.2^{c}	2.8 ± 0.2^{de}	1.3 ± 0.1^{cde}
7.5	55.7 ± 2.3^{de}	2.5 ± 0.2^{e}	1.0 ± 0.2^{e}
10.0	45.7 ± 3.5^{fg}	2.2 ± 0.2^{e}	0.8 ± 0.2^{e}

-Data recorded after 6 weeks of culture.
-Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are significantly not different (P = 0.05) using Duncan's test.

growth and elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity (Huettman and Preece, 1993). In order to avoid deleterious effects of TDZ, the clumps of induced shoot buds were seperated from the parent tissue and transferred to growth regulator free MS basal medium as well as MS medium containing different concentrations (0.5, 1.0 and 2.5 µM) of BA for further growth and elongation of shoots (Fig. 1B). When TDZ induced cultures were transferred to MS basal medium no further growth and development of shoots occurred, shoots failed to elongate and ultimately shed all their leaves after 3 weeks. Among different concentrations of BA investigated, 1.0 µM showed better response and the number of shoots per explant as well as shoot length increased up to second subculture passage at an interval of 6 wk each (Fig. 1C). In the second subculture passage an average of 14.9 ± 1.4 shoots with an average 5.8 ± 0.2 cm shoot length was recorded (Table 2). During third subculture passage a reduction in number of shoots was observed. Similar strategy of using TDZ for multiple shoot induction and another cytokinin for elongation of induced shoots has been adopted by several workers in other plant species such as *Morus alba* (Thomus, 2003); *Acacia sinuata* (Vengadesan *et al*., 2002); *Cajanus cajan*

Table 2. Effect of different concentrations of BA on TDZ (2.5 µM) induced cultures of *C. sophera* for further multiplication and elongation.

BA (µM)	Mean no. of shoots/explant	Mean shoot length (cm)
0.5	10.2 ± 1.6^{ab}	5.0 ± 0.1^{b}
1.0	14.9 ± 1.4^{a}	5.8 ± 0.2^{a}
2.5	9.5 ± 1.3^{b}	4.2 ± 0.2^{c}

-Data recorded after 6 weeks of culture.
-Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are significantly not different (P = 0.05) using Duncan's test.

Table 3. Effect of different concentrations of auxins on *in vitro* rooting in microshoots of *C. sophera*.

Treatment	% Rooting	Mean no. of roots/shoot	Mean root length (cm)
MS basal	42.3 ± 1.5^{e}	1.8 ± 0.1^{c}	2.4 ± 0.2^{e}
½ MS	65.0 ± 1.7^{d}	2.4 ± 0.2^{c}	2.7 ± 0.2^{de}
½ MS + IBA (0.5 µM)	83.7 ± 2.0^{b}	3.8 ± 0.2^{b}	3.4 ± 0.2^{cde}
½ MS + IBA (1.0 µM)	93.7 ± 2.4^{a}	5.7 ± 0.5^{a}	5.6 ± 0.5^{a}
½ MS + IBA (2.5 µM)	80.3 ± 2.6^{b}	4.0 ± 0.2^{b}	5.2 ± 0.3^{ab}
½ MS + NAA (0.5 µM)	72.3 ± 1.5^{c}	4.0 ± 0.2^{b}	5.0 ± 0.4^{ab}
½ MS + NAA (1.0 µM)	78.3 ± 1.2^{b}	4.2 ± 0.1^{b}	4.2 ± 0.4^{bc}
½ MS + NAA (2.5 µM)	67.0 ± 1.7^{cd}	3.6 ± 0.2^{b}	3.6 ± 0.4^{cd}

-Data recorded after 6 weeks of culture.
-Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are significantly not different (P = 0.05) using Duncan's test.

(Eapen *et al*., 1998) and *Pterocarpus marsupium* (Husain *et al*., 2007)

In vitro rooting and plant acclimatization

For the development of complete plantlets, *in vitro* raised microshoots of appropriate length (4-5 cm) were excised from the parent tissue and transferred to rooting medium comprised of half or full strength MS medium with or without auxins (IBA and NAA) at different concentrations (Table 3). All the treatments applied in the present study induced rooting, with root initiation occurring approx. 2 wk after the transfer of shoots to the rooting medium. Medium free of growth regulator induced relatively few and smaller roots in 65.0 % and 42.3 % of the shoots in half and full strength media respectively. The induction of roots in auxin free medium may be due the endogenous level of hormones in the regenerated microshoots (Minocha, 1987). Addition of IBA and NAA to half strength MS medium facilitates better rhizogenesis. Best rooting response (93.6 %) were obtained on half strength MS medium containing IBA (1.0 μM) where maximum number of roots per shoot (5.7 ± 0.5) with maximum shoot length (5.6 ± 0.5) were obtained after 6 wk of cultures (Fig. 1D). Roots were healthy, thick and having well developed secondary branches. Superiority of IBA over other auxins in root formation has also been reported in other plant species such as *Cunila galoides* (Fracro and Echeverrigaray, 2001); *Clitoria ternatea* (Shahzad *et al*., 2007) and *Cassia siamea* (Parveen *et al*., 2010).

Plantlets with well developed root system were isolated from the rooting medium and hardened off inside the growth room as described in materials and methods. Regenerated plantlets were successfully transferred to greenhouse conditions with 90 % survival rate and no morphological variation was recorded in leaf morphology, inflorescence pattern and fruit development when compared to *in vivo* plants (Fig. 1E & F).

CONCLUSIONS

In conclusion, the protocol described in this study can be used for the efficient production of *C. sophera*. Such plants could be used as a source of tissues for the biochemical characterization of medicinally active compounds and will increase the opportunities for the use of this medicinal plant in both the traditional and modern medical health care.

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