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PRE-CULTURING OF NODAL EXPLANTS IN THIDIAZURON SUPPLEMENTED LIQUID MEDIUM IMPROVES *IN VITRO* SHOOT MULTIPLICATION OF *CASSIA ANGUSTIFOLIA*

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An *in vitro* propagation system for *Cassia angustifolia* Vahl. has been developed. Due to the presence of sennosides, the demand of this plant has increased manyfold in global market. Multiple shoots were induced by culturing nodal explants excised from mature plants on a liquid Murashige and Skoog [8] medium supplemented with 5–100 µM of thidiazuron (TDZ) for different treatment duration (4, 8, 12 and 16 d). The optimal level of TDZ supplemented to the culture medium was 75 µM for 12 d induction period followed by subculturing in MS medium devoid of TDZ as it produced maximum regeneration frequency (87%), mean number of shoots (9.6 ± 0.33) and shoot length (4.4 ± 0.46 cm) per explant. A culture period longer than 12 d with TDZ resulted in the formation of fasciated or distorted shoots. *Ex vitro* rooting was achieved when the basal cut end of regenerated shoots was dipped in 200 µM indole-3-butyric acid (IBA) for half an hour followed by their transplantation in plastic pots filled with sterile soilrite where 85% plantlets grew well and all exhibited normal development. The present findings describe an efficient and rapid plant regeneration protocol that can further be used for genetic transformation studies.

Keywords: Ex vitro – Fabaceae – growth characteristics – tissue culture – soilrite

INTRODUCTION

Cassia angustifolia commonly known as senna is a medicinally valuable drought resistant shrub of the family Fabaceae. It is a native of Saudi Arabia and has been naturalized in India. It also flourishes in the tropical climate and also very common in Egypt, Nubia and Sudan. Leaves and pods of this plant are of great medicinal value. *C. angustifolia* contains a range of chemical compounds like Anthraquinones, resins, tannins, flavonoids, mucins, malic acid, mucilage (galactose, arabinose, rhamnose, and galacturonic acid) and tartaric acid which are biologically very active and enhances the medicinal value of this plant. This plant possesses purgative, laxative, antipyretic, cathartic, vermifuge, diuretic, cleansing, body detoxifying properties,

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which is useful in the treatment of several common and chronic diseases. The infusion of the plant is used for the treatment of diseases like anaemia, bronchitis, dysentery, fevers and hemorrhoids. It also works as an excellent blood purifier and in losing excessive weight [1].

Due to the presence of Sennosides especially Sennosides A and B, this plant has been put forward in the priority list in National as well as State Medicinal Plant Board for development. It is one of the principal herbal drugs having export potential for developed countries. India is the major supplier of the leaves and pods as well as its glycosides to the world market. Approximately 75% of the *Cassia* produced in India is exported. To fulfill the demand and to maintain the quality of the raw material used, there is a need to have an alternative system for the production of desired material and to conserve quality germplasm. Advances in biotechnology, especially in the area of *in vitro* culture techniques provide valuable tools for conservation and management of plant genetic resources. Tissue culture techniques have had a major impact on the *ex situ* conservation of plant genetic resources and importantly, disease indexed *in vitro* maintained germplasm provides an excellent means of mediating international germplasm exchange. Micropropagation through *in vitro* cell and tissue culture techniques are being used widely for the commercial propagation and revegetation of a large number of species, including many medicinal plants [6].

Cassia is exploited heavily from wild conditions by pharmaceutical companies and local tribes throughout India for medicinal purposes. Conventionally it is propagated by seeds. However, low germination percentage and poor viability restricts its propagation on large scale.

Direct regeneration through cotyledonary node and nodal segments [2, 17, 18] has already been reported earlier. Few reports on plant regeneration via cotyledon and leaflet derived calli [3, 20] and somatic embryogenesis from cotyledon [4] are available, but no one assessed the potential of nodal explants by pre-treatment with TDZ. Therefore, the objective of the present study was to optimize the concentration of TDZ for pre-treatment for a defined period of time followed by their transfer to MS medium devoid of TDZ for further development and regeneration.

MATERIALS AND METHODS

Plant material and explants source

The nodal explants (2–3 cm) were excised from a year-old plant of *C. angustifolia* maintained at the botanical garden of the university. The explants were washed thoroughly under running tap water for at least 30 min, followed by soaking in 5% (v/v) Teepol for 7 min. and finally washed thoroughly in sterile double distilled water. The explants were surface sterilized in 0.1% (w/v) $HgCl_2$ solution for 4 min followed by repeated washing with sterile distilled water.

Culture media and conditions

The culture medium used for the present work was Murashige and Skoog [13] medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Liquid MS medium was supplemented with various concentration of TDZ (5, 25, 50, 75 and 100 µM). The pH of the medium was adjusted to 5.8 with 1 N NaOH prior to autoclaving at 121 °C at 1.06 kg cm^{-2} for 20 min. All cultures were maintained in a culture room at temperature of 24 ± 2 °C, 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, Mumbai, India) and relative humidity of 60–65%.

Multiple shoot induction and proliferation

The nodal segment explants were cultured in 50 cm^3 liquid MS media supplemented with TDZ (5–100 µM) on a rotatory shaker at 100 rpm. MS medium lacking growth regulator served as control. For determining the optimal duration of exposure of the explants to the medium supplemented with TDZ, the explants from each treatment were subcultured onto MS basal medium after 4, 8, 12 and 16 d of culture. All cultures were transferred to fresh medium after every 2 weeks. The percentage of explants forming shoots and shoot length per explant were recorded after 8 weeks of culture.

Ex vitro *root formation and acclimatization*

For *ex vitro* root induction, excised shoots (3–5 cm) with 4–5 leaves were harvested and their basal portion were dipped in different concentrations of IBA (50, 100, 150, 200, 250 and 300 µM) for half an hour and subsequently planted in plastic pots containing sterile soilrite (Keltech Pvt. Ltd., Bangalore, India) under diffuse light (16/8 h photoperiod) conditions. Potted plantlets were covered with transparent polythene bags to ensure high humidity and watered every 3 days with half strength MS salt solution for 2 weeks. Polythene bags were opened after 2 weeks in order to acclimatize plants to field conditions. Data were recorded on percentage of rooting, mean number and length of roots after 4 weeks of *ex vitro* transplantation.

Statistical analysis

All the experiments were repeated three times and twenty replicates were employed for each treatment. The effects of different treatments was quantified and data was analyzed using one way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at 5% level of significance.

RESULTS

The nodal explants were cultured in liquid MS medium supplemented with TDZ at different concentrations (5.0–100 µM) on a rotary shaker at 100 rpm for 4, 8, 12 and 16 days (Table 1). After a defined duration, the explants were cultured onto MS basal medium for further growth and proliferation. The nodal segment failed to produce shoots on MS medium without TDZ. Nodal explants incubated at different concentrations of TDZ for different time duration showed initial bud break after one week. The range of percentage of shoot regenerating explants was 50–87% and the number of shoots formed per explant varied considerably and showed significant difference at

Table 1

Effect of different concentrations and durations of culture on TDZ supplemented liquid MS medium followed by their transfer to MS basal semisolid medium on shoot formation from nodal explants of *Cassia angustifolia* after 8 weeks of culture

TDZ (µM)	Culture duration (d)	Percent of regeneration	Mean number of shoots/explant	Mean shoot length (cm)
0.0	0	0.0	0.0^{k}	0.0^{h}
5.0	4	50	2.5 ± 0.40^{fgh}	1.7 ± 0.21^{ef}
	8	58	3.9 ± 0.25^{defg}	2.1 ± 0.30^{de}
	12	62	4.3 ± 0.40^{ghi}	2.5 ± 0.24^{fg}
	16	56	3.0 ± 0.22^{hij}	1.2 ± 0.10^{g}
25	4	55	3.4 ± 0.75^{cde}	2.0 ± 0.20^{bcd}
	8	63	4.6 ± 0.89^{bcd}	2.4 ± 0.29^{abc}
	12	69	5.8 ± 0.69^{defg}	3.0 ± 0.33^{cde}
	16	60	4.0 ± 0.40^{efg}	1.9 ± 0.26^{de}
50	4	71	4.4 ± 0.12^{ab}	2.9 ± 0.36^{abc}
	8	74	6.0 ± 0.22^{a}	3.6 ± 0.33^{a}
	12	80	7.6 ± 0.36^{abc}	3.9 ± 0.26^{bcd}
	16	63	5.4 ± 0.49^{bcd}	3.0 ± 0.27^{cde}
75	4	74	5.8 ± 0.61^{bcd}	3.2 ± 0.32^{bcd}
	8	79	7.0 ± 0.48^{abc}	3.8 ± 0.53^{ab}
	12	87	9.6 ± 0.33^{bcd}	4.4 ± 0.46^{bcd}
	16	66	6.5 ± 0.30^{cde}	3.1 ± 0.23^{de}
100	4	64	5.0 ± 0.60^{hij}	3.0 ± 0.14^{cde}
	8	69	5.6 ± 0.40^{fgh}	3.4 ± 0.37^{bcd}
	12	76	7.8 ± 0.21^{ij}	3.9 ± 0.43^{de}
	16	59	4.1 ± 0.13^{j}	2.7 ± 0.20^{ef}

Values represent means ± SE. Means followed by the same letter within columns are not significantly different (P = 0.05) using Duncan's multiple range test.

different concentrations and duration of exposure to liquid MS medium followed by their transfer to MS semisolid medium. Nodal explants inoculated on MS medium supplemented with 50 µM TDZ for 12 d induced 7.6 ± 0.36 shoots with 3.9 ± 0.26 cm shoot length in 80% of cultures. The highest shoot regeneration frequency (87%),

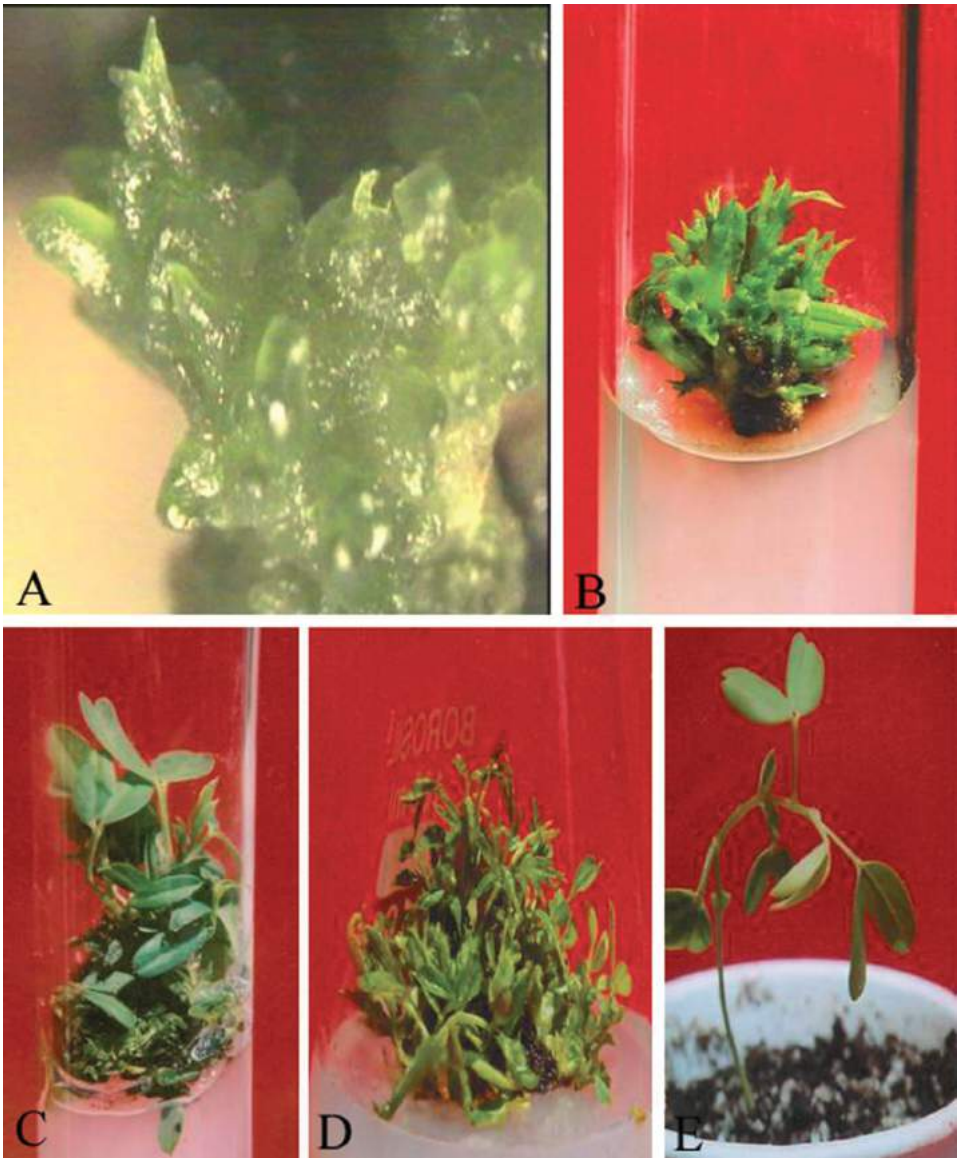

Fig. 1. (A–E) Effect of pretreatment of TDZ on shoot bud induction and plant regeneration from nodal segments of *Cassia angustifolia.* (A&B) Induction of shoot buds in MS medium after 12 d pretreatment in MS+TDZ (75µM). (C&D) Multiplication of shoots after 8 weeks in TDZ free MS medium. (E) *Ex vitro* rooted plantlet in MS + IBA (200 µM)

mean number of shoots (9.6 ± 0.33) and maximum shoot length (4.4 ± 0.46 cm) were recorded after 8 weeks at 75 µM TDZ for 12 d culture duration (Fig. 1A, B, C, D). The explants cultured on TDZ supplemented liquid MS medium for 16 d showed reduction in all the parameters evaluated and the shoots appeared stunted and deformed. Higher concentration of TDZ (100 µM) suppressed the regeneration frequency, mean number of shoots and shoot length as it induced callusing. The shoots induced from different treatments of TDZ were subcultured 3 times to hormone free MS medium for shoot multiplication at interval of 2 weeks (Fig. 1C, D).

Rooting was carried out by *ex vitro* method. The basal portion of regenerated shoots were dipped in different concentrations of IBA (50–300 µM) for half an hour and subsequently planted in plastic pots containing sterile soilrite (Table 2). The best result for rooting was recorded when shoots were dipped in 200 µM IBA as it gave the maximum frequency of rooting (80%), number of roots (5.3 ± 0.42) and root length (3.6 ± 0.30 cm) (Table 2, Fig. 1E). The rooted plantlets were successfully hardened off inside the growth room in soilrite for 4 weeks and eventually established in natural soil. The survival rate after transfer was 85%. The regenerated plant exhibited morphological characteristics similar to those of the source plant.

Table 2

The efficiency of *ex vitro* root induction from regenerated shoots of *C. angustifolia* dipped in IBA solution for half an hour. Data were taken after 4 weeks of transplantation

IBA (µM)	Percent rooting	Mean number of roots/shoot	Mean root length (cm)
50	NA	NA	NA
100	60	2.2 ± 0.13^{cd}	2.0 ± 0.24^{cd}
150	75	4.5 ± 0.30^{ab}	3.1 ± 0.20^{ab}
200	80	5.3 ± 0.42^{a}	3.6 ± 0.30^{a}
250	70	4.0 ± 0.21^{bc}	3.0 ± 0.33^{abc}
300	50	2.1 ± 0.10^{de}	2.2 ± 0.25^{bc}

Values represent means ± SE. Means followed by the same letter within columns are not significantly different (P = 0.05) using Duncan's multiple range test.

DISCUSSION

In the present study, the two-step culture used as a TDZ pretreatment that significantly enhanced the efficiency of shoot regeneration from nodal explants in Table 1. In pretreatment vs. no pretreatment experiments, pretreatment method was found to have a significant effect. Although a low range of concentrations of TDZ (1 nm–10 µM) is recommended for shoot proliferation, the result of the present study showed that a short term exposure to high concentrations of TDZ prior to culture in TDZ free MS medium enhanced shoot multiplication in *Cassia angustifolia*. On the basis of literature, it is known that TDZ acts as a potent regulator for *in vitro* propagation

system and as an effective means of induction of adventitious shoots in a number of plant species [5, 10]. It has been known to promote cytokinins-like activity similar to that of N^6 substituted adenine derivatives and has been used in tissue culture studies since 1982 [11, 17]. TDZ stimulates cytokinin activity and it is a better substitute of other cytokinins like BA and KN, which are generally used in tissue culture work [8, 22]. The effectiveness of short term exposure and stimulating effect of TDZ on bud break and multiple shoot formation has been reported earlier for several medicinal and aromatic plant species including *Hypericum perforatum* [14], *Arachis correntina* [12], *Curcuma longa* [15], *Ocimum basilicum* [19], *Curculigo orchioides* [21], *Cassia anugustifolia* [18] and *Nyctanthes arbor-tristis* [7]. It is suggested that TDZ may be needed as a trigger for initiating the proliferation of shoot meristems and further incubation on TDZ free MS medium led the explants to further development [9]. Explant which remained on the TDZ supplemented medium beyond 12 d of incubation showed reduced number of shoots which appeared stunted and deformed. Similar deformities have also been reported [15, 19, 21].

The success and cost effectiveness of Micropropagation relies on the rooting percentage and survival of the plantlets in field conditions. *Ex vitro* rooting was attempted as a means to decrease the micropropagation cost and also the time from laboratory to field. Shoots induced by TDZ and subsequently rooted *ex vitro* with IBA has also been reported in *Nyctanthes arbor tristis* [16], *Cassia angustifolia* [18].

CONCLUSIONS

The present study describes an effective, efficient and rapid multiplication protocol for large scale multiplication as well as *ex situ* conservation of this important medicinal plant. The protocol developed here could also be used for the isolation of sennoside-content by pharmaceutical companies and genetic transformation studies.

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