academicJournals

Vol. 13(9), pp. 1027-1036, 26 February, 2014
DOI: 10.5897/AJB2013.12456
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Thidiazuron-induced shoot organogenesis of *Cleome viscosa* (L) through cotyledonary explants culture

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Accepted 17 February, 2014

A reproducible protocol for direct shoot organogenesis of *Cleome viscosa,* an important medicinal weed herb was developed. The seed explants were collected primarily from field grown mature plants for *in vitro* germination on different strength of MS basal medium with or without selection of GA_3 at various concentration (0.1 to 1.0 mg/L). The highest rate of seed germination (55.3%) was noticed on full strength MS basal medium fortified with 0.5 mg/L GA_3 after 30 days of culture. The excised 7 to 10 days old cotyledonary leaf, cotyledonary node and hypocotyls explants cultured on MS medium fortified with different concentration of individual cytokinin (BA/KIN/TDZ) alone or BA+KIN or TDZ+KIN or TDZ in combinations with different auxins (IBA/NAA/IAA) influenced the frequency of adventitious microshoot formation. The rate of shoot multiplication was greatest (100%) in cotyledonary leaf explants cultured on 3.0 mg/L TDZ and 0.3 mg/L IBA tested medium after 45 days of culture. The individual microshoots were elongated well in 0.3 mg/L TDZ and 0.1 mg/L GA_3 treated medium, but more number of adventitious micro roots were developed on half strength MS medium fortified with 0.1 mg/L NAA. The regenerated healthy plants were hardened in pots containing soil mix and well established into complete state similar to that of field grown plants under greenhouse condition.

Key words: *Cleome viscosa* L, medicinal weed plant, shoot multiplication, direct organogenesis.

INTRODUCTION

Cleome viscosa L. (Capparidaceae) is commonly known as “wild or dog mustard,” found as a medicinal weed all over the plains of tropical and subtropical regions of southern, western and central parts of the India (Mali, 2010; Wake et al., 2011). The plants have devised a useful strategy of producing flowers simultaneously with maturation of fruits and seed dispersal also overlap. The events of floral biology ensure auto pollen deposition of plants being self- pollinated (Saroop and Kaul, 2011). The various constituents of these plants have shown profound therapeutic and prophylactic activities. The seeds, leaves and roots of the plant are widely used in traditional folk medicine as an antiscorbutic, analgesic, anthelmintic, antiseptic, cardiac stimulant, carminative, febrifuge, sudorific, anti-inflammatory, antimicrobial, antipyretic, hepatoprotective and immunomodulatory activities (Mali, 2010), anticonvulsant (Shah et al., 1983), antidiarrheal (Malhotra and Moorthy, 1973; Sharma et al.,

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Abbreviations: Kin, Kinetin; **TDZ,** thidiazuron; **BA,** 6-benzylaminopurine; **NAA,** naphthaleneacetic acid; **IBA,** indole-3-butyric acid; **GA_3,** gibberellic acid; **MS,** Murashige and Skoog basal medium.

1979), skin diseases (Purohit et al., 1985), itching, ulcers, leprosy, and malarial fevers (Chatterjee and Prakashi, 1991; Kirtikar and Basu, 1935; Nadkarni, 1982), antiemetic activity (Ahmed et al., 2011) and wound healing property (Panduraju et al., 2011). The seeds contain 18.3% oil, especially a mixture of amino acids, fatty acids and sucrose (Rukmini and Doesthale, 1979; Rukmini, 1978; Afaq et al., 1984; Deora et al., 2003) and minerals of potassium, phosphorus, iron, calcium, magnesium, copper in high concentration as a good source of essential nutrients required for the well being of human body for therapeutic purposes (Lavate et al., 2011).

Recently, the demands for the elite plant material in various countries are continuously increasing for medicinal purpose, because the exploitation coupled with increasing urbanization lead to a steady erosion and loss of diversity from the natural habitats of plants. *In vitro* clonal propagation is only the alternative way for the regeneration and expedites release of large number of *C. viscosa* plants under aseptic culture conditions. Earlier, there have been few reports on establishment of *in vitro* propagation through callus culture of *C. viscosa* (Anburaj et al., 2011a,b), micropropagation and shoot organogenesis from different explants of *Cleome spinosa* (Simões et al., 2004; Albarello et al., 2006; Qin et al., 2012; Albarello et al., 2013) and *Cleome gynanadra* (Rathore et al., 2013), somatic embryogenesis (Simões et al., 2010) cell suspension (Simões et al., 2012) and root culture with cryopreservation of *C. rosea* (Cordeiro et al., 2012). To develop improved methods of plant regeneration of *C. viscose*, the effect of urea-type cytokinin, and thidiazuron (TDZ) was investigated to overcome the unsatisfactory earlier findings. Some studies showed that TDZ have higher cytokinin activity to induce organogenesis in several plant species (Zhang et al., 2001; Oluk and Orhan, 2010). TDZ-induced organogenesis comprises a metabolic cataract including primary signaling event, storage, passage of endogenous plant signals and iron in plant cell, a system of secondary messengers and a simultaneous stress response (Guo et al., 2011). Therefore, TDZ emerged as an effective bioregulant in cell and tissue cultures in wide array of plant species (Li et al., 2000; Matand and Prakash, 2007). The present study was carried out to standardize a new protocol for *in vitro* seed germination combined with an efficient regeneration of *C. viscosa* from cotyledonary explants culture in optimal concentration of TDZ to formulate the strategies for the conservation of these invaluable plants in natural habitat.

MATERIALS AND METHODS

Plant material

The mature fruits of *C. viscosa* were collected from College Campus, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu, India. The fruits were dried under laboratory conditions for few days. The seeds were carefully removed mechanically from the fruits and washed under running tap water for 2 to 3 min, dried and stored at 4°C for four to five months.

Disinfection and *in vitro* seed germination

Seeds were initially sterilized with 10% *Teepol* (v/v) solution (commercial bleach solution) for 10 to 15 s and kept under running tap water for 20 min to remove dirty particles and detergents. Further processes were carried out under aseptic conditions by treating with 70% ethanol (v/v) for 1 min and rinsed with sterile distilled water for 2 min followed by 0.2% $HgCl_2$ (mercuric chloride) for 2.5 min. The surface sterilized seeds were finally washed thoroughly with sterile distilled water twice. Then, the seeds were implanted on 25×150 mm culture tube containing quarter, half and full strength MS basal salts (Murashige and Skoog, 1962), 100 mg/L myoinositol, 150 mg/L L-glutamine, 75 mg/L thiamine HCl, 100 mg/L pyridoxine, 3.0% sucrose (w/v) and 0.8% agar (w/v) (Hi-media laboratories limited, Mumbai, India) medium supplemented with or without addition of GA_3 (0.1 to 1.0 mg/L). All the cultures were initially incubated in darkness for 10 days at 25±2ºC and later transferred to 16/8 h light/dark conditions at a light intensity of 15 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Philips, India). Data on percent of seed germination was noticed after 5 to 30 days of culture.

Culture condition and direct organogenesis

Cotyledonary node, cotyledonary leaf and hypocotyl explants were isolated from 7 to 10 days old *in vitro* young seedlings of *C. viscosa* and cut into 0.5 to 1.0 cm length. The excised cotyledonary leaf and hypocotyl explants were wounded with sterile surgical blade and placed horizontally, cotyledonary node inserted vertically on MS basal salts, 100 mg/L myoinositol, 150 mg/L L-glutamine, 75 mg/L thiamine HCl, 100 mg/L pyridoxine, 3.0% sucrose (w/v) and 0.8% agar (w/v) medium fortified with different concentration of BA (3.0 to 7.0 mg/L) or KIN (0.5 to 1.5 mg/L) or TDZ (1.0 to 5.0 mg/L) or optimum concentration of BA (5.0 mg/L) and TDZ (3.0 mg/L) individually selected with various level of KIN (0.1 to 0.5 mg/L) in the first set of experiment. Further, TDZ (3.0 mg/L) alone was tested with different auxins (NAA/IAA/IBA) at various concentrations (0.1 to 0.9 mg/L). The pH of the medium was adjusted to 5.7 prior to autoclaving at 15 psi for 20 min. All cultures were incubated under 15 µmol $m^{-2}s^{-1}$ in 16 h photoperiod provided by cool white fluorescent tubes at 25±2ºC. Subcultures were done at two weeks of interval for adventitious microshoot induction. Data on percentage of response with total number of shoots per explants was recorded after 45 days of culture.

Shoot elongation, rooting and acclimatization

Individual microshoots (0.2 to 0.5 cm in length) were carefully excised from shoot clumps and transferred to MS basal medium supplemented with various concentrations of TDZ (0.0 to 0.5 mg/L) in combinations with GA_3 (0.1 mg/L) for frequency of shoot elongation after 2 weeks of culture. After shoot elongation, the individual green healthy shoots (<1.0 cm long) were carefully removed from the culture vessel and transferred to half strength MS basal salts, 1.5% sucrose and 0.8% agar medium supplemented with various concentrations of NAA or IBA or IAA (0.1 to 0.3 mg/L) for frequency of rooting after two to three weeks of culture. Data on shoot elongation, root induction frequency with total number of roots per plantlet was recorded after two to three weeks of culture. The rooted plantlets were removed carefully from the culture vessels and washed under running tap water to remove agar gels

Table 1. *In vitro* seed germination of *cleome viscosa* L.

Medium composition	*In vitro* seed germination frequency (%)			
	After 5 days	After 10 days	After 15 days	After 30 days
¼ strength MS basal salts	-	10^{d}	20^{d}	33.2^{c}
½ strength MS basal salts	-	19.1^{c}	26^{c}	37^{bc}
Full strength MS basal salts	10^{ab}	25^{b}	31.5^{b}	40^{b}
MS basal salts + 0.5 mg/L GA_3	12.2^{a}	28^{a}	47^{a}	55.3^{a}

25 seeds were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$)

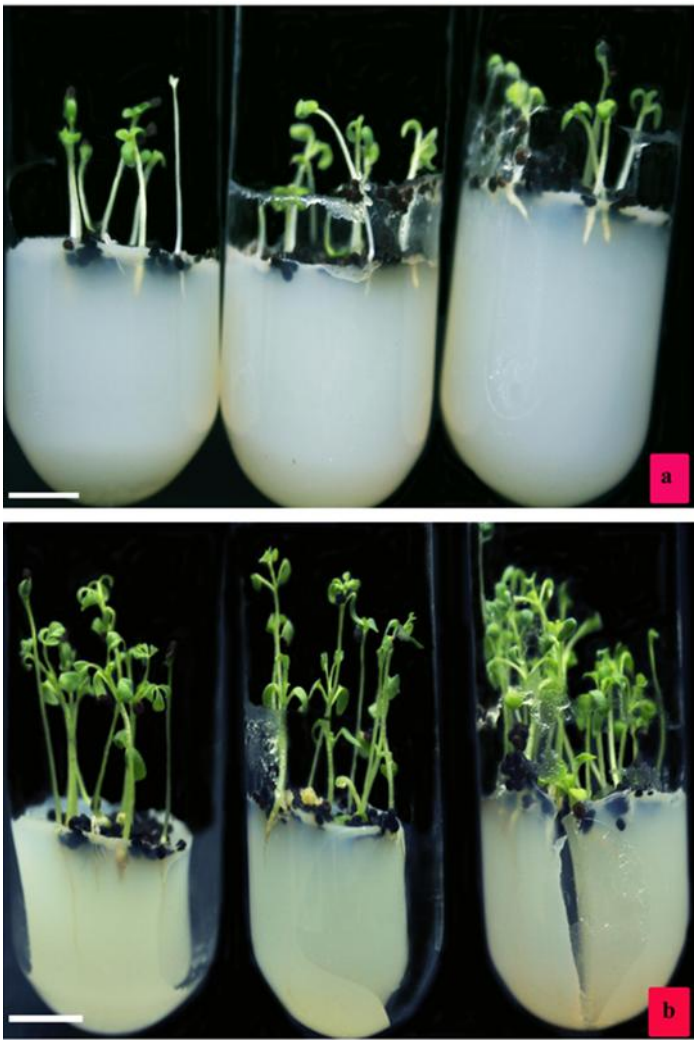

Figure 1. Effect of MS basal medium strength (a) and and GA, (b) on seed germination of *C. viscosa* L.

from the root base. The rooted plants were successfully transplanted into 6.0 cm diameter plastic pots mixed with sterile red soil, garden soil and sand in a ratio of 1:2:1. Each pot was enclosed in a clean polythene bag and maintained under a 16 h photoperiod at a light intensity of 15 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tube (Philips, India) to control high humidity. The plants were initially irrigated with autoclaved half strength MS liquid medium without sucrose at two days interval. Then, the plantlets were exposed 2 to 4 h daily to the conditions for natural humidity after five days of transfer. The well established plants were finally transferred to earthen pots containing soil mix and maintained under greenhouse condition. The rate of plant survival was observed after 2 to 4 weeks.

Statistical analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. All experiments were conducted with complete randomized block design (CRD) and factorial with different growth regulators as independent variables. Each experiment was repeated thrice. Percent of seed germination, plant regeneration and the number of shoots obtained during initial culture and subsequent transfers were tabulated. The data pertaining to frequencies of seed germination, shoot induction, shoot elongation, root induction and numbers of shoots per cultures were subjected to ANOVA test. Mean separation was carried out using Duncan's Multiple Range Test (DMRT) by SPSS (version 12.0) software package for expressing statistical significance in all culture practice.

RESULTS

In vitro seed germination response

In vitro surface sterilized seeds cultured on quarter, half and full strength of MS basal salts, 0.3% sucrose and 0.8% agar with or without addition of GA_3 at different concentrations induced germination. The seed germination was initiated under dark field for first 10 d. The onset of mean germination percentage was increased frequently in GA_3 tested medium. Although, the maximum value of seed germination (55.3%) was observed on full strength MS basal medium fortified with 0.5 mg/L GA_3 after 30 days of sowing under 16/8 h light/dark conditions about 33.2 and 37% seed germination was noticed from quarter and half strength MS basal medium, respectively (Table 1 and Figure 1a and b). The combined effect of GA_3 and dark field for the first 10 days overcame the dormancy of seeds and influenced germination significantly ($P<0.05$). The

germinated cotyledonary plants were converted into complete plants under light field.

Shoot induction and multiplication response

In the current study, cotyledonary leaf, cotyledonary node and hypocotyl explants were swelled after two to three days of culture in response to various form of plant growth regulators. Shoot bud initiation was started at axillary region of cotyledonary node and wounding sites of cotyledonary leaf and hypocotyl explants. The microshoot buds grew into tiny visible shoots in culture medium containing growth regulators. Thus, genotype dependant response with genetic variability of microshoot development was minimized based on the hormonal signal in explants. The maintenance of culture regime in subculture medium ensured continuous shoot multiplication. The present study shows that the individual or combinational usage of cytokinins, BA/KIN/TDZ stimulated microshoot induction and proliferation from three different explants of *C. viscosa*. Here, the healthy hypocotyl explants incubated on MS salts with 3.0% sucrose (w/v) and 0.8% agar (w/v) medium supplemented with BA (5.0 mg/L) influenced 57% shooting response with an increase in microshoot bud formation (10.5±0.3) and decrease in shoot height. The shoot length was decreased as the level of cytokinins increased in all culture condition. BA at 5.0 mg/L resulted to 50% shooting response with only 5.7±2.0 number of microshoots from cotyledonary node explants while cotyledonary leaf explants showed 61% shoot induction response with more number of microshoots (15.2±1.0).

Addition of KIN did not show better results for frequency of shooting in all three explants culture. Although, TDZ at 3.0 mg/L stimulated maximum of 99.1% shooting response with an average of 21.2±0.5 number of microshoots in cotyledonary leaf than cotyledonary node and hypocotyls explants culture. BA at 5.0 mg/L in combinations with 0.3 mg/L KIN influenced 73 and 53% shooting response with total of 13.5±1.9 and 7.2±1.0 microshoots from hypocotyls and cotyledonary node explants, respectively. Although, cotyledonary leaf explants showed maximum of 79.5% shooting response with an average of 17±3.7 number of microshoots in similar concentration of BA and KIN tested medium.

In other hand, cotyledonary leaf explants exhibited better shooting response (100%) with an average of 24.5±1.1 number of microshoots on 3.0 mg/L TDZ and 0.5 mg/L KIN tested medium. Hypocotyls tested medium showed moderate shooting response (96%) with total of 18±0.3 number of microshoots whereas shoot induction percentage from cotyledonary node explants was quite low (72%) with total of 11.1±1.0 number of microshoots on the above tested level of TDZ and KIN (Table 2). The shoot induction response could be dependent on their developmental state and gene expression pattern of the explants. Further, cotyledonary leaf, cotyledonary node and hypocotyl explants were subsequently tested with optimum level of TDZ along with various levels of auxins, IAA/NAA/IBA for frequency of shoot multiplication. In this case, the addition of TDZ (3.0 mg/L) and IAA (0.7 mg/L) to the medium also induced 100% shooting response with maximum of 24.9±1.0 numbers of microshoots with small amount of light green mucilaginous type of basal calli observed from cotyledonary leaf explants culture. NAA (0.5 mg/L) supported with optimum level of TDZ (3.0 mg/L) influenced minimum of 22.2±1.2 numbers of microshoots with low amount of light yellow brown calli formation in cotyledonary leaf. Hypocotyls incubated on 3.0 mg/L TDZ along with 0.5 mg/L NAA or IAA or IBA supplemented medium showed 82.2 (20±0.2), 99 (22±0.5) and 100% (24±2.0) shooting response, respectively. Cotyledonary node showed slow response to influence microshoots connection with small or less amount of basal callus formation on TDZ in combinations with IBA or NAA or IBA tested medium.

Nevertheless, cotyledonary leaf explants cultured on 3.0 mg/L TDZ in combinations with 0.3 mg/L IBA attained significant results (P <0.05) in shoot multiplication (100%) with maximum of 29.1±3.5 numbers of microshoots after 45 d of culture. Little amount of white mucilaginous basal callus was developed on medium touched portion of microshoots due to long time exposure of culture to same medium (Table 3 and Figure 2a and b). The addition of individual auxins (IBA/NAA/IAA) to the cytokinins (TDZ/BA/Zeatin/2-ip/CPPU) supplemented medium might be influenced by microshoot proliferation from basal callus of different explants culture (unpublished data). One way ANOVA analysis at the 95% confidence interval showed significant difference ($P<0.05$) in microshoot organogenesis in all cultures. Adventitious root formation was identified occasionally with few numbers of plantlets in the shoot clumps.

Elongation, rooting and acclimatization response

The combinations of hormones have some effects on shoot elongation, but they were different based on the morphological nature of microshoots. The isolated microshoots (0.2 to 0.5 cm length) placed directly on shoot elongation medium fortified with 0.3 mg/L TDZ and 0.1 mg/L GA_3 influenced maximum of 40% elongation after two weeks of culture (Figures 2c and 3). The abnormal microshoots with swollen leaf did not elongate into normal shoots. The elongated shoots were subjected to root induction medium for frequency of rooting. The reducing MS salt strength to one half normally enhanced rooting frequency but also reduced basal callus formation. Here, the individual excised plantlets subcultured on half strength MS medium supplemented with 1.5% sucrose along with different concentrations of IAA or NAA or IBA influenced adventitious root formation on stem

Table 2. Effects of cytokinins on microshoot regeneration from different explants of *Cleome viscosa* L.

MS medium composition (mg/L)	Cotyledonary leaf			Cotyledonary node			Hypocotyl		
	% Response	Mean no. of microshoots / explants	Mean shoot length (cm)	% Response	Mean no. of microshoots / explants	Mean shoot length (cm)	% Response	Mean no. of microshoots/ explants	Mean shoot length (cm)
BA									
3.0	42±2.2^{g}	7.3±1.4^{f}	0.5±2.4^{a}	21±3.5^{f}	1.0±3.3^{de}	0.5±4.0^{a}	38.2±2.5^{g}	3.0±2.7^{f}	0.6±4.0^{a}
5.0	61±1.5^{bc}	15.2±1.0^{cd}	0.4±0.7^{ab}	50±2.0^{cd}	5.7±2.0^{d}	0.4±1.0^{ab}	57±1.0^{e}	10.5±0.3^{e}	0.5±2.0^{ab}
7.0	50±0.4^{d}	15±0.7^{cd}	0.2±2.0^{bc}	40±1.0^{de}	5.0±2.6^{d}	0.2±1.6^{bc}	45±1.4^{f}	9.0±0.5^{e}	0.5±0.4^{ab}
KIN									
0.5	-	-	-	-	-	-	-	-	-
1.0	23±3.1^{h}	1.3±1.9^{g}	0.5±1.8^{a}	10±1.5^{g}	0.2±1.0e	0.1±0.5^{c}	15±1.6^{h}	0.4±0.4^{g}	0.4±2.2^{b}
1.5	14±1.0^{i}	0.5±2.0^{h}	0.5±1.3^{a}	-	-	-	9.0±2.8^{i}	0.3±1.5^{gh}	0.3±0.3^{bc}
TDZ									
1.0	80±0.5^{c}	15±1.4^{cd}	0.4±1.7^{ab}	37±0.7^{e}	7.5±1.8^{c}	0.3±1.5^{b}	62±4.0^{d}	13±1.0^{cd}	0.3±2.9^{bc}
3.0	99.1±0.4^{ab}	21.2±0.5^{b}	0.3±0.5^{b}	56.2±1.2^{c}	9±0.5^{b}	0.3±2.0^{b}	77±2.1^{c}	15±3.0^{bc}	0.2±4.2^{c}
5.0	98±1.3^{b}	20±0.7^{bc}	0.3±1.0^{b}	50±1.3^{cd}	8.8±0.1^{bc}	0.2±2.2^{bc}	76±0.2^{c}	13.8±1.5^{c}	0.2±2.0^{c}
BA + KIN									
5.0 + 0.1	63±.2.0^{f}	11.7±2.2^{e}	0.4±1.4^{ab}	25±2.5^{ef}	6±0.4^{cd}	0.4±4.4^{ab}	59.9±0.7^{de}	11±2.6^{d}	0.5±2.0^{ab}
5.0 + 0.3	79.5±0.6^{cd}	17±3.7^{c}	0.3±2.0^{b}	53±1.0^{c}	7.2±1.0^{c}	0.5±1.2^{a}	73±1.0^{cd}	13.5±1.9^{c}	0.4±0.5^{b}
5.0 + 0.5	70±1.5^{e}	15±1.5^{cd}	0.2±1.8^{bc}	48.7±1.8^{d}	7±0.7^{c}	0.3±0.7^{b}	70±1.6^{d}	9±1.7^{e}	0.4±0.4^{b}
TDZ + KIN									
3.0 + 0.1	99.5±3.6^{ab}	22±3.0^{b}	0.4±3.0^{ab}	60±1.0^{bc}	9.4±1.0^{b}	0.3±2.2^{b}	80±1.1^{bc}	15.8±2.2^{bc}	0.3±1.7^{bc}
3.0 + 0.3	100±2.0^{a}	24±2.8^{ab}	0.4±1.6^{ab}	66±1.6^{b}	10±0.5^{ab}	0.3±1.0^{b}	84±0.5^{b}	16.5±2.5^{b}	0.3±2.9^{bc}
3.0 + 0.5	100±0.9^{a}	24.5±1.1^{a}	0.3±2.5^{b}	72±0.9^{a}	11.1±1.0^{a}	0.2±1.1^{bc}	96±1.4^{a}	18±0.3^{a}	0.3±3.1^{bc}

-, No response. 45 explants were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

base of the plantlets. In this case, about 63.3 and 90% rooting response with small amount of callus interspersion was observed in half strength MS medium comprising of 0.1 mg/L IAA and IBA, respectively.

However, the plantlets on NAA (0.1 mg/L) supplemented medium exhibited significant factor ($P<0.05$) in determining 97% rooting with decreasing the level of callus interspersion after 3 weeks of culture (Table 4 and Figure 2d). For acclimatization, the rooted plants as well as the germinated plants from *in vitro* culture were transplanted into potting mix (Figure 2e) and covered with clean polythene bags to reduce 80% humidity. There was no detectable variation

Table 3. Optimum level of TDZ along with different auxins on microshoot induction in various explants of *Cleome viscosa* L.

Explants	Medium composition	% of response	Mean no. of microshoots/ explants	Shoot length (cm)	Nature of basal callus formation
Cotyledonary leaf	3.0 mg/L TDZ + 0.3 mg/L IBA	100±2.0^{a}	29.1±3.5^{a}	0.5±1.6^{a}	White mucilaginous calli
Cotyledonary node	3.0 mg/L TDZ + 0.5 mg/L IBA	98.3±1.2^{ab}	14.3±1.9^{cd}	0.4±0.7^{ab}	White mucilaginous calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L IBA	100±1.0^{a}	24±2.0^{b}	0.4±0.5^{ab}	White mucilaginous calli
Cotyledonary leaf	3.0 mg/L TDZ + 0.5 mg/L NAA	95±0.3^{b}	22.2±1.2^{bc}	0.4±1.0^{ab}	Light yellow brown calli
Cotyledonary node	3.0 mg/L TDZ + 0.1 mg/L NAA	67.5±1.7^{d}	11.4±0.2^{e}	0.3±1.4^{b}	Light yellow brown calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L NAA	82.2±1.5^{c}	20±0.2^{c}	0.3±3.0^{b}	Light yellow brown calli
Cotyledonary leaf	3.0 mg/L TDZ + 0.7 mg/L IAA	100±4.0^{a}	24.9±1.0^{b}	0.5±1.1^{a}	Light green mucilaginous calli
Cotyledonary node	3.0 mg/L TDZ + 0.5 mg/L IAA	90±2.7^{bc}	13±1.4^{d}	0.5±0.5^{a}	Light green mucilaginous calli
Hypocotyl	3.0 mg/L TDZ + 0.5 mg/L IAA	99±0.9ab	22±0.5^{bc}	0.4±2.0^{ab}	Light green mucilaginous calli

45 explants were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

among the acclimatized plants with respect to morphological and growth characteristics (Figure 2f). The plants were then transferred to earthen pots containing sterile soil mix in green house and maintained under natural conditions of day length photoperiod, temperature and humidity. The survival of *in vitro* raised plants from both seed and other explants culture were differed based on the composition and strength of medium with or without addition of growth regulators.

Although, the rate of plants survival was decreased from 99 to 95% via seed culture, but cotyledonary node, cotyledonary leaf and hypocotyl explants derived plants exhibited minimum survival rate (97 to 92.7%) for 2 to 4 weeks after transfer to green house condition (Table 5).

DISCUSSION

Effect of medium strength and GA_3 on seed germination

In general, the seed and vegetable parts of plants may pose challenges at all stages of culture. The seed coat covered with chemical inhibitors and mechanical resistance proved to be growth limiting factors to achieve better germination (Thokozani et al., 2011). The dormant seeds of various species subjected to different environmental conditions delayed germination with increasing mortality, growth reduction and low or unsuccessful reproduction (Kevin and Andrew, 2001). Thus, *in vitro* seed germination can be employed as potential method for propagation of medicinally valuable plants under aseptic condition. The seed germination rate is the "speed or velocity" of germination and can be expressed as the suitable exposure time for a defined percentage of seed to germinate (Niedz, 2008).

The present study was carried out to assess the rate of *in vitro* seed germination and conversion of normal plants of *C. viscosa* in different strength of MS basal medium with or without addition of GA_3. Seed germination frequency decreased in quarter and half strength MS medium. However, the surface sterilized seeds cultured on full strength MS basal medium fortified with 0.5 mg/L GA_3 found to be greatest for influencing 55.3% germination when compare to quarter and half strength of media tested after 30 d of culture. Similarly, Padilla and Encina (2003) reported that the various concentration of GA_3 promotes seed germination of *Annona cherimola* plants.

Influence of growth regulators on microshoot induction and proliferation

During the onset of experiment, the juvenile explants such as cotyledonary leaf, cotyledonary node and hypocotyl explants of *C. viscosa* were excised from *in vitro* young seedling and cultured on plant growth regulators supplemented medium for propagation. The regeneration via organogenesis is controlled primarily by the interaction of plant hormones, specifically cytokinins and auxins in the culture medium (Evans et al., 1981). In many cases, the hormone concentrations can-

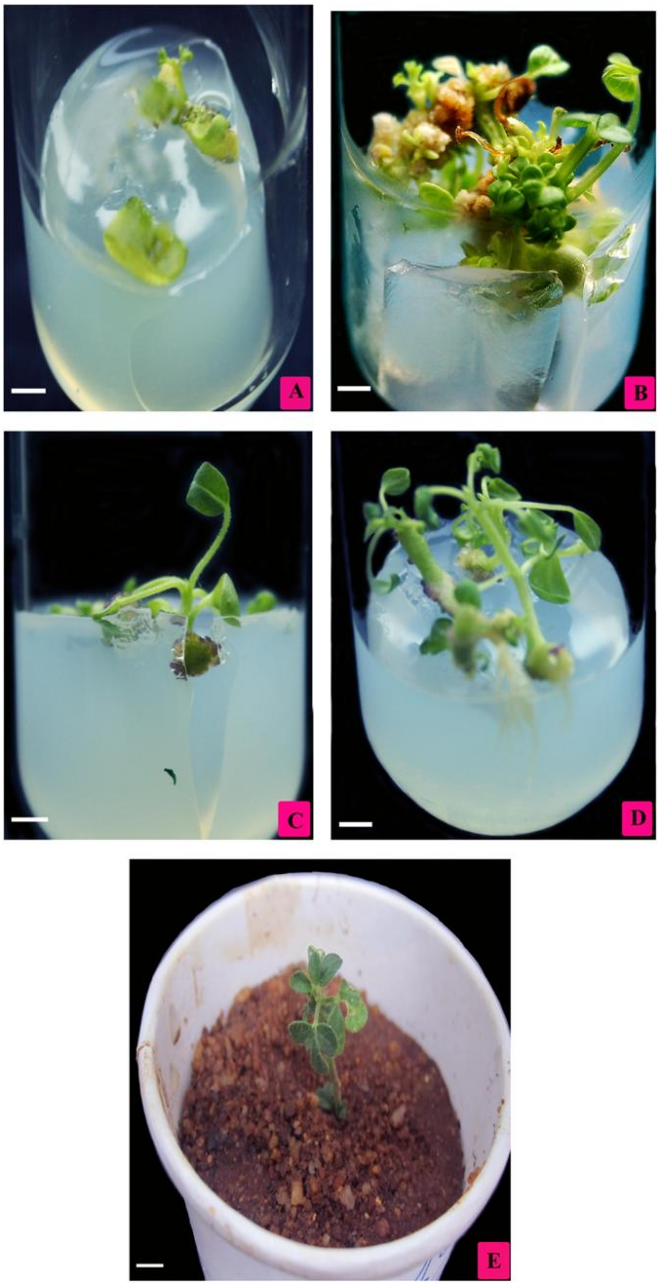

Figure 2. Effect of TDZ on efficient regeneration from cotyledonary leaf explants of *C. viscosa* L.

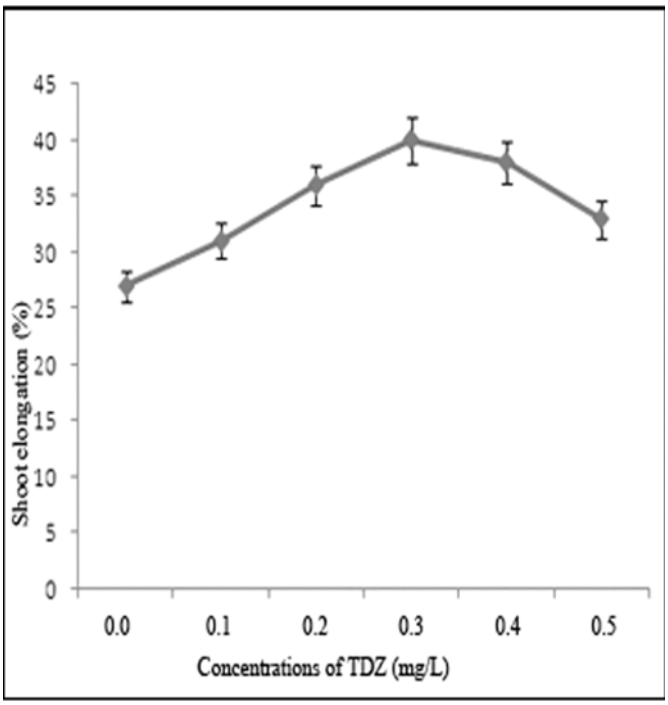

Figure 3. Effect of TDZ along with 0.1 mg/GA, on elongation.

not be the sole mechanism controlling *in vitro* developmental processes. It may be related to differences in tissue and cell differentiation and organization in all explant culture.

In the present study, cotyledonary leaf, cotyledonary node and hypocotyl explants were subjected to individual cytokinins; BA/KIN/TDZ treatment on medium showed varied response for microshoot regeneration although, cotyledonary leaf explants incubated on MS basal medium supplemented with 3.0 mg/L TDZ attained maximum of 99.1% microshoot induction response than cotyledonary node and hypocotyls explants after 45 days of culture. BA or KIN did not prove critical for high frequency microshoot induction in all three explants culture. Further, we found that no single plant growth regulator alone stimulated high frequency shoot regeneration. The enhancement of shoot induction process with subsequent development was significantly affected by combinational usage of growth regulators (BA+KIN/TDZ+KIN/TDZ+IBA/TDZ+NAA/TDZ+IAA).

Among them, TDZ at 3.0 mg/L and 0.5 mg/L KIN supple-mented medium showed 100% shooting response from cotyledonary leaf than hypocotyls and cotyledonary node explants culture. Percentage of microshoot induction per cotyledonary leaf, hypocotyls and cotyledonary node explants was decreased at optimum levels of BA and KIN treatment. In addition, optimum level of TDZ with various form of auxins (IBA or IAA or NAA) were also tested to enhance the rate of shoot multiplication from three different explants culture. In this case, the exposure of cotyledonary leaf explants on TDZ (3.0 mg/L) along with IBA (0.3 mg/L) or IAA (0.7 mg/L) and hypocotyl explants on 3.0 mg/L TDZ and 0.5 mg/L IBA treatments during induction phase have led to influence 100% microshoot growth than cotyledonary node explants. TDZ with NAA treatment did not exhibit more response in shoot multiplication from all these three explants culture.

However, cotyledonary leaf incubated on TDZ and IBA was found to be superior to influence 100% microshoots and produced maximum of 29.1±3.5 number of micro-

Table 4. Effects of auxins on adventitious rooting of *Cleome viscosa* L.

MS medium composition (mg/L)	% of response	Mean no. of roots/ plantlets	Shoot elongation (cm)	Basal callus formation
IBA				
0.1	90±1.0^{b}	2.7±0.3^{b}	1.2±0.5^{ab}	-
0.2	75±1.5^{c}	2.0±1.0^{bc}	0.9±0.1^{b}	+
0.3	60.2±0.6^{d}	1.4±1.2^{c}	0.7±1.0^{bc}	+
NAA				
0.1	97±0.7^{a}	3.8±2.0^{a}	1.8±1.5^{a}	-
0.2	95±1.0^{ab}	3.2±1.2^{ab}	1.2±2.0^{ab}	-
0.3	88±2.0^{bc}	2.6±1.0^{b}	1.0±0.3^{ab}	+
IAA				
0.1	63.3±0.9^{d}	1.4±0.5^{c}	0.7±1.0^{bc}	+
0.2	13.5±1.0^{f}	1±0.7^{cd}	0.5±0.6^{c}	+
0.3	25±3.0^{e}	0.5±1.0^{d}	0.5±1.2^{c}	++

-, Less amount basal callus; +, low amount of basal callus; ++, more amount of basal callus; 30 plantlets were taken for each experiment. Values are expressed as mean ± SE of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

shoots significantly after 45 days of culture. The long time exposure of culture (more than 2 weeks) to optimum level of TDZ in combinations with individual auxins (IBA/NAA/IAA) supplemented medium without subculturing converted the medium touched portions of explants along with microshoots to basal callus. The frequent subculture might control basal callus formation around shoot clumps in explants. The development of little amount of basal callus in regular subculture was not affected on the overall development of intact microshoots. The irregular shape of the leaves was not developed from the intact shoots or callus tissues in all cultures.

Thus, the small amount of basal callus formed around cotyledonary leaf, cotyledonary node and hypocotyl explants cultured on fresh medium influenced monopolar structure of microshoots formation (unpublished data). Earlier, Anburaj et al. (2011b) reported plant regeneration from leaf derived callus of *C. viscosa* in BAP and KIN tested medium. However, the shoot regenerative processes in explant cultures were provoked by TDZ alone and in collaboration with other plant growth regulators (Guo et al., 2011). Our results are in accordance with similar findings on efficient shoot regeneration of apple in TDZ with IBA or IAA supplemented medium (Yancheva et al., 2003).

Plant regeneration, hardening and acclimatization

The individual microshoots were excised from cotyledonary leaf, cotyledonary node and hypocotyls explants and placed on MS basal medium fortified with various concentration of TDZ and GA_3 influenced high rate of elongation of shoots. The inclusion of GA_3 alone at different concentrations had no effect on elongation of microshoots, but the addition of low level of TDZ along with GA_3 gave high response in shoot elongation after 2 weeks of culture. In similar fashion, Bhatt and Dhar (2000) reported that the supplementation of TDZ, KIN and GA_3 to the medium was generally beneficial in promoting shoot elongation of *Bauhinia vahlii* plants. Further, the elongated shoots were cultured on half strength MS basal medium comprised of 1.5% sucrose and 0.8% agar with different concentrations of individual auxins (IAA/NAA/IBA) for frequency of adventitious root formation. The rhizogenic basal calli were also observed from stem base of shoots connection with full strength MS basal medium supplemented with auxins.

Although, we have found that the half strength MS basal medium supplemented with NAA showed highest rooting response (75%) with an average of 3.8±2.0 number of adventitious microroot formation after 3 weeks of culture. Basal callus interspersion was almost controlled on half strength rooting medium with NAA. Previous study also revealed that the low level of NAA tested medium exhibited better rooting in shoot system of *C. viscosa* (Anburaj et al., 2011b). The medium devoid of growth regulators failed to develop frequency of rooting in this species. In contrast, Rathore et al. (2013) reported efficient rooting of in vitro propagated *C. gynandra* plants on MS medium with IBA, NOA (2-naphthoxyacetic acid) and activated charcoal while Albarello et al. (2006) achieved adventitious rooting of shoots from *C. spinosa* plants on MS0 medium without addition of plant growth regulators.

Table 5. The survival rate of *in vitro* raised plants in pots containing soil mixture.

Propagation	Mean no. of plants	Survival weeks after transfer to soil (%)		
		2nd week	3rd week	4th week
Seed culture	38.7±1.0[a]	99±0.9	9 7.1±0.4	95±2.0
CL/CN/HC explants culture	30±0.5[b]	97±2.0	94±1.7	92.7±1.6

CL, Cotyledonary leaf; CN, cotyledonary node; HC, hypocotyls. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

After successful root establishment, *in vitro* rooted shoots were transplanted in plastic cups containing soil mix. *In vitro* raised plants survived well and substantially grow into normal plants under greenhouse condition. They did not show any morphological abnormalities compared to donor plant during the maturation period. Therefore, the protocol developed is superior to earlier findings for high rate of multiplication of *C. viscosa* plants. This protocol could be used for the mass multiplication of this very important medicinal weed plants in a short period of time. The conservation of this plant species will cater for the growing need of pharmaceutical and biotechnological industries.

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