OPTIMIZED PLANT TISSUE CULTURE PROTOCOL FOR *IN VITRO* MORPHOGENESIS OF AN ENDANGERED MEDICINAL HERB *Ceropegia ensifolia* Bedd.

[PROTOCOLO ÓPTIMO DE CULTIVO DE TEJIDOS *IN VITRO* PARA MORFOGÉNESIS DE LA HERBACEA MEDICINAL AMENAZADA *Ceropegia ensifolia* Bedd.]

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SUMMARY

In the present study effect of different concentrations of sucrose and Thidiazuron (TDZ) on *in vitro* morphogenesis of *C. ensifolia* was investigated and rapid micropropagation protocol was developed from *in vitro* derived nodal explants. Among the sucrose concentrations tested, medium concentration (3%) of sucrose induced maximum number of healthy shoots (6.5±0.68). The shoots formed in this concentration are normal and elongated rapidly. Whereas at higher concentrations delayed shoot induction with stout shoots stunted in their growth was observed. Among different concentrations of TDZ tested healthy shoots with well developed leaves were formed in lower concentration i.e. 0.2 mg/l. the number of shoots formed in this concentration was limited to 3.6±0.18 shoots/explant. The shoots raised *in vitro* were best rooted on MS medium supplemented with 0.1 mg/l α-Naphthaleneacetic acid (NAA) in combination with 0.05 mg/l 6-Benzyladenine (BAP). The maximum root induction (84%) with mean number of roots of 6.7±0.26 and with mean root length 3.5±0.28 cm was observed after six weeks of inoculation. Complete plantlets developed *in vitro* were acclimatized successfully with 85% survival in field conditions.

Keywords: *Ceropegia ensifolia;* Thidizuron (TDZ); sucrose; multiple shoots.

RESUMEN

Se estudió diferentes concentraciones de sucrose y thidiazuron (TDZ) en la morfogenésis *in vitro* de *C. ensifolia* y se desarrolló un protocolo rápido de micropropagación *in vitro* a partir de explantes nodales. Entre las concentraciones de sucrosa evaluadas, la concentración media (3%) indujo el mayor número de rebrotes saludables (6.5±0.68). Los rebrotes obtenidos a esta concentración fueron normales y elongaron rápidamente. A concentraciones mayores se retrasó la inducción del rebrote y se observó brotes robustos atrofiados en su crecimiento. Entre las concentraciones de TDZ se observaron rebrotes saludables con hojas bien desarrolladas a concentraciones bajas (0.2 mg/l) donde el número de rebrotes fue limitado a 3.6±0.18 rebrotes por explante. Los rebrotes cultivados *in vitro* enraizaron mejor en medio suplementado con 0.1 mg/l de ácido α-Naphthaleneacetico (NAA) en combinación con 0.05 mg/l 6-Benzyladenine (BAP). La máxima inducción de rebrotes (84%) con un promedio de 6.7±0.26 longitud media de raíz de 3.5±0.28 cm fue observado 6 semanas posteriores a la inoculación. Plantuelas completamente desarrolladas *in vitro* fueron exitosamente aclimatizadas con un 85% de sobrevivencia en condiciones de campo.

Palabras clave: *Ceropegia ensifolia*; thidizuron (TDZ); sucrosa; rebrotes múltiples.

INTRODUCTION

It is a well-known fact that worldwide thousands of plant species are endangered and facing extinction with the current trend of their exploitation and destruction (Poi *et al*., 2010; Wyse *et al*., 2009). According to the International Union of Conservation of Nature (IUCN), it is anticipated that the current species extinction rate is between 1000 and 10,000 times higher than it would naturally be. Biodiversity conservation is of global concern which requires a holistic approach. Recognizing the need, the

biodiversity convention was adopted by United Nations in 1992 (Sharma and Sharma, 2013). Convention on Biodiversity seeks to conserve the endangered plants at their genetic, species and ecosystem levels. Nonetheless it is now recognized that *ex situ* techniques can be efficiently used to complement *in situ* methods, and they represent conservation of highly endangered and rare species (Ramsay *et al*., 2000). Recently, a combination of *ex situ* and *in situ* conservation methods of a critically endangered plant (*Ceropegia fantastica* Sedgw.) has been demonstrated (Chandore *et al*., 2010). Nowadays *in vitro* multiplication has become an advanced tool for protecting medicinal plants, particularly rare and endangered species (Khan *et al*., 2012).

The species of *Ceropegia* as a whole are under threat, owing to either destructive collection or habitat degradation. They are not only genetically depleted but also are scarcely available. These species are of economic importance (Jagtap and Singh, 1999) due to their starchy edible tubers with medicinal value (Murthy and Kondamudi, 2011). The tubers of this genus contain sugars, starch, fats, albuminoids, gums and crude fibers which are useful as nutritive tonic (Kirtikar and Basu, 1935). The active compound of tuberous roots is the alkaloid cerpegin which is active against diarrhea and dysentery inflammation of gums and delirious fevers of parturition (Nadkarni, 1976). Recently this genus has attracted the attention of several biologists for conservation, since many of the *Ceropegia* species are listed in RED data book of Indian medicinal plants (Srinivasarao *et al*., 2010). Furthermore, the application of advanced tissue culture protocols is of economic use for the industrial production and consequently, conservation of *Ceropegia* species. However, for effective reintroduction of these plants, *in vitro* morphogenesis protocols must be established along with the habitat conservation and other appropriate *ex vitro* strategies. *Ceropegia ensifolia* Bedd. (Asclepiadaceae) is an endemic, perennial, tuberous twining herb distributed originally isolated from Peermadu Ghat of Kerala. This species is now extended its distribution in Tamil Nadu, i.e. at high wavy mountains in Theni district of Western Ghats, India. In recent days the population of this species is very rare due to habitat degradation. Conventional propagation of this plant species is apparently difficult, owing to low fruit and seed setting, less viability of seeds, poor seed germination and delayed rooting of seedlings. Therefore we propose an *in vitro* propagation method as an alternative technique for propagation of this endangered species and reestablishment of this plant population in natural habitat.

In vitro plant cells, tissues and organ cultures are not fully autotrophic, establishing a need for carbohydrates in culture media to maintain the osmotic potential and serve as energy and carbon sources for developmental processes (Chae, 2013). A variety of carbon sources viz. glucose; maltose, sucrose, dextrose etc are used in culture media depending upon genotypes, species and specific stages of growth (Bozena and Szczerba, 1991). However, sucrose is extensively used as a major transport-sugar in the phloem sap of many plants (Lemoine, 2000). In micropropagation systems, morphogenetic potential of plant tissues can greatly be manipulated by varying concentration of sucrose (Yaseen *et al*., 2013). In addition to that TDZ (*N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea) is a substituted phenyl urea compound which was developed for mechanized harvesting of cotton bolls and emerged as a highly effective bioregulant of morphogenesis in plan tissue culture (Guo *et al*., 2011). Unlike conventional phytohormones, it was proved that TDZ individually contented the requirements of various regenerative responses of many different plant species (Sanikhani *et al*., 2006). However, in previous studies it has shown that TDZ exhibited both auxin and cytokinin like effects, although, it is chemically, different from auxins and cytokinins (Murthy *et al*., 1998). It exhibits strong cytokinin like activity and promotes the proliferation of axillary shoots (Faisal *et al*., 2005). In addition to that TDZ also releases the lateral bud dormancy and stimulates adventitious organ regeneration (Mroginski *et al*., 2004). These interesting findings on TDZ raised many tantalizing questions for researching in plant morphogenesis. The purpose of the present work was to investigate the effects of sucrose and TDZ on morphogenesis of *C. ensifolia* using nodes with axillary buds as explants. So for the reports are not available on *in vitro* micropropagation for the regeneration of *C.ensifolia*.

MATERIALS AND METHODS

In the present study nodal segments derived from the *in vitro* grown plantlets were used as explants to study the effect of different concentrations of sucrose and TDZ on *in vitro* morphogenesis of *C. ensifolia*. In order to study the effect of sucrose on morphogenesis of *C. ensifolia,* single nodal segments were separated (1 cm long) and cultured on Murashige and Skoog (MS) media containing varying concentrations of sucrose (1 to 5%) along with BAP (3.0 mg/l) respectively. The pH of the media was adjusted to 5.6 to 5.8. Then the agar (0.8%) was added to the media and autoclaved for 20 min at 121 ^{o}C and15 lbs pressure. The cultivation was performed at 25±2°C with optimized room conditions for 6 weeks under fluorescent lightening (Philips) of 3000 lux (16/8, D/N cycle) at the top of the culture vessels. The plantlets grown *in vitro* were harvested from all replicate vessels of a treatment and evaluated their morphogenesis status. The parameters considered

were a) percentage of shooting response, b) number of shoots per explants, c) shoot height (cm).

In order to study the influence of TDZ on shoot proliferation of *C. ensifolia*, nodal segments was inoculated on MS basal media augmented with TDZ at different concentrations (0.1 to 3.0 mg/l). The explants derived from sprouted shoots were transferred periodically to the fresh medium and in each subculture the transfer of explants were carried out for every six weeks. The frequency of explants sprouting, number of shoots formed per explants and shoot length after six weeks of culture were recorded as observations to know the optimum concentration of TDZ for effective shoot proliferation in *C. ensifolia*.

Attempts were made to root the cloned shoots under *in vitro* conditions by using various combinations of NAA, BAP and Kinetin (KN). The healthy shoots were harvested and cultured on 0.6% agar gelled MS medium supplemented with 0.1% NAA in combination with different concentrations of BAP and KN. Plantlets with well developed shoots and roots were taken out from the culture vials and were cleaned with distilled water to remove traces of agar. Roots of each plantlet were treated with 0.5% fungicide (carbendazim 50% WP) solution before planting into plastic cups contained sterile soil, sand and vermicompost in different ratios. The pots were covered with polythene covers; these hardened plantlets were irrigated with sterilized 1/4 MS salt solution devoid of sucrose for initial 2 to 3 days and were maintained in culture room at 25 ± 1°C with photoperiod of 16 h light. Subsequently after 4 to 6 weeks the covers were pored to allow free gaseous exchange with the outer environment and then removed to expose the plantlets to *ex vitro* conditions. Primary hardened plants after two weeks were transferred to earthen pots and irrigated with tap water and observed for their further growth.

Each experiment was repeated three times and twenty replicates were maintained. The data regarding number of shoots formed, shoot length, number of roots formed and root length was recorded periodically (six weeks) for shoot multiplication and rooting respectively. Raw data were subjected to analysis of variance (ANOVA) carried out by the SPSS 20 (SPSS Inc. Chicago, IL, USA). The values with $P \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The carbon sources (sucrose) serves as energy and osmotic agents to support the growth of plant tissues. In the present study different concentrations of sucrose were added in the media to know their effect on shoot initiation and proliferation. Different concentrations of sucrose showed varied responses during plant morphogenesis of *C. ensifolia*. The optimum response that is healthy shoot formation was observed when 3.0 mg/l sucrose was added to the medium (Table – 1). The explants inoculated in this medium showed fast sign of shoot sprouting when compared with the explants inoculated on other media. The shoots formed in this medium are good to observe with maximum number of shoots (6.5±0.68) and with maximum shoot length (8.4±0.25 cm) (Fig – 1B). These cultures survived for a longer duration in culture conditions. The shoots which are formed in the lower concentrations of sucrose are with linear stems with pale yellowish green leaves (Fig – 1A). Initially in the lower concentration (1.0 mg/l) the fast sign of shoot sprouting was observed, which developed into thin linear shoots very rapidly and latter these shoots under gone weathering and failed to survive for longer durations. The explants taken from these shoots were failed to sprout in further subcultures. Whereas at higher concentrations of sucrose the initial shoot sprouting was delayed for longer durations (nearly 2 weeks) and the shoots formed in these concentrations are stout and stunted in their growth (Fig – 1C). Browning of the medium was also observed due to leaching of phenolics from the base in to the medium. Swelling of the base of explants and tuber formation was also noticed when higher concentrations of sucrose present in the medium (Fig – 1F). The level of sucrose in tissue culture can affect the growth and development of the plants. To address this problem, several researchers showed that sucrose at 3% level was optimum for healthy shoot regeneration (Vinterhalter and Vinterhalter, 1999: Adjei, 2001, Gauchan, 2012).

In *C. ensifolia* lower concentrations of TDZ played a prominent role in shoot induction. The shoots that are formed at lower concentration of TDZ showed excellent results in their morphogenesis especially with healthy stem and broad dark green elongated leaves which resembled the plants of the natural environment. The maximum shoot sprouting (90) percentage with mean number of shoots (3.6±0.18) and mean shoot length (10.1±0.81 cm) was observed on MS medium supplemented with 0.2 mg/l TDZ (Table–2, Fig–1D). However, with the increase in concentration of TDZ, decrease in shoot sprouting percentage and shoot number and length was observed. The shoots that are formed at higher concentrations of TDZ showed reduction in leaf size (Fig–1E). Even though the shoots contain reduced leaf size, the nodal explants derived from these shoots are good in their shoot sprouting and produced healthy shoots in further subcultures. Some studies have previously reported the significant role of TDZ in shoot proliferation like cytokinins in a number of

plant species (Lee, 2005; Shen *et al*., 2010; Janarthanam *et al*., 2012).

Rooting of *in vitro* derived shoots were carried out by transferring healthy shoots with 3 to 4 nodes to the root induction media which include various combinations of NAA, BAP and KN. The cumulative effect of BAP and NAA was clearly observed with the growth characteristics. Among all the combinations tested, NAA 0.1 mg/l in combination with BAP 0.05 mg/l was found optimum for *in vitro* rooting in *C. ensifolia* (Table – 3, Fig – 1G). The maximum root induction (84%) with mean number of roots (6.7±0.26) with mean root length (3.5±0.28 cm) was noticed in this medium after six weeks of transfer. Our data indicate that, combinations of NAA and KN did not assist in root induction of *C. ensifolia*. However when the medium was supplemented with NAA, a significant root induction was clearly noticed. Interestingly among all the combinations of NAA and KN treated, NAA 0.1 mg/l in combination with KN0.05 mg/l induced maximum rooting (66%) with mean number of roots (4.7±0.26) and with mean root length (2.8±0.46 cm). Previous publications have already addressed the similar feature that in most of the *Ceropegia* species viz. *C. spiralis* (Murthy *et al*., 2010), *C. fantastica* (Chandore *et al*., 2010), *C. attenuate* (Chavan *et al*., 2011); individual auxins played an effective role in root induction. The prominent role of IAA and NAA in combination with BAP on root induction in *C. bulbosa* was reported by Goyal and Bhadauria (2006). The plantlets with well developed shoot and root system were successfully hardened off inside the growth room in sterile soil, sand and vermi compost in 1:1:2 ratios for about 4 to 6 weeks (Fig – 1H). The primary hardened plantlets were eventually established in earthen pots with nearly 85 percent survival rate.

Table 1. Effect of sucrose in combination of BAP (3.0 mg/l) on *in vitro* shoot proliferation of *Ceropegia ensifolia.*

Sucrose (%)	% of explant response	No of shoots/explant mean ± standard error	Shoot length (cm) mean ± standard error
1.0	93	4.0±0.06 c	6.7±0.26 c
2.0	98	4.5±0.27 b	7.1±0.23 b
3.0	100	6.5±0.68 a	8.4±0.25 a
4.0	92	4.6±0.20 b	4.6±0.20 d
5.0	87	3.8±0.16 d	4.1±0.16 d

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

Table 2. Effect of TDZ on *in vitro* shoot proliferation of *Ceropegia ensifolia.*

TDZ	% of explant response	No of shoots/explant mean ± standard error	Shoot length (cm) mean ± standard error
0.1	70	2.1±0.20 b	6.1±0.25 c
0.2	90	3.6±0.18 a	10.1±0.81 a
0.3	82	3.2±0.18 a	7.2±0.94 b
0.4	80	2.6±0.32 b	7.1±0.75 b
0.5	83	2.5±0.28 b	6.4±0.93 c
1.0	75	2.1±0.20 b	6.1±1.01 c
1.5	75	2.0±0.06 **b**	4.7±0.48 d
2.0	78	1.6±0.35 c	4.4±0.11 d
2.5	74	1.4±0.30 c	4.8±0.22 d
3.0	71	1.1±0.21 c	3.8±0.33 e

Data indicate mean±standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

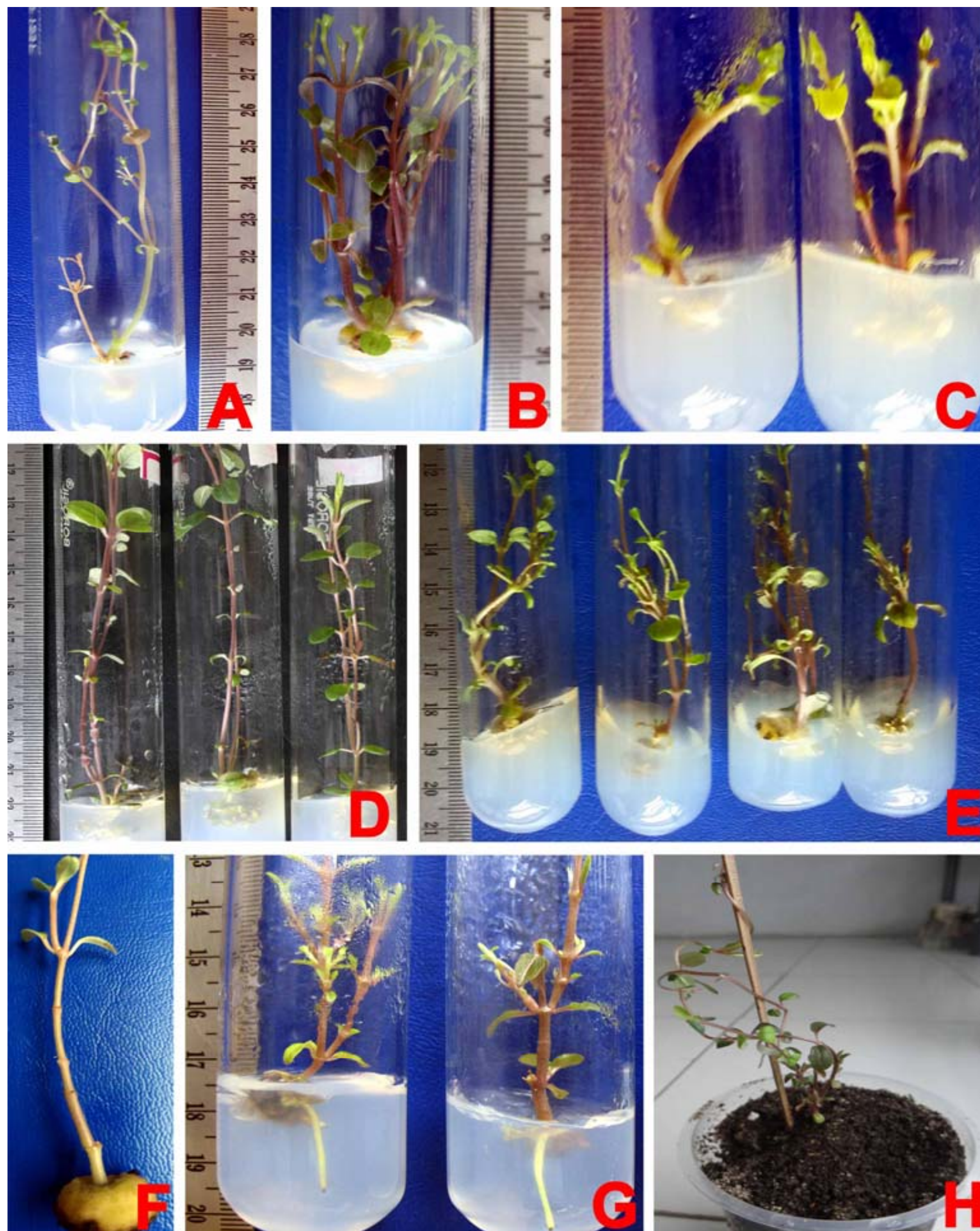

Figure 1: Effect of sucrose and TDZ on *in vitro* morphogenesis of *Ceropegia ensifolia*

A. Induction of thin linear shoots on MS medium supplemented with 1.0 % sucrose + 3.0 mg/l BAP
B. Healthy shoots developed on MS medium supplemented with 3.0 % sucrose + 3.0 mg/l BAP
C. Short stouter shoots that developed on MS medium supplemented with 5.0 % sucrose + 3.0 mg/l BAP
D. Induction of multiple shoots on MS medium supplemented with 0.2 mg/l TDZ
E. Induction of multiple shoots on MS medium supplemented with 2.0 mg/l TDZ
F. Tuberization on MS medium supplemented with 5.0 % sucrose + 3.0 mg/l BAP
G. Induction of rooting on MS medium supplemented with 0.1 mg/l NAA + 0.05 mg/l BAP
H. Primary hardened plant ready to transfer in to field conditions

Table 3. Effect of auxins in combinations with cytokinins on *in vitro* rooting of *Ceropegia ensifolia.*

NAA	BAP	KN	% of explant response	No of shoots/explant mean ± standard error	Shoot length (cm) mean ± standard error
0.1	-	-	72	5.6±0.47 [b]	2.97±0.15[b]
0.1	0.01	-	76	5.9±0.31 [b]	3.2±0.14 [a]
0.1	0.05	-	84	6.7±0.26 [a]	3.5±0.28 [a]
0.1	0.10	-	60	4.0±0.06 [c]	2.1±0.60 [c]
0.1	-	0.01	61	4.3±0.20 [c]	2.4±0.30 [b]
0.1	-	0.05	66	4.7±0.26 [c]	2.8±0.46 [b]
0.1	-	0.10	58	2.1±0.60 [d]	1.5±0.2 [c]

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

CONCLUSION

In the present study the effect of sucrose and TDZ on *in vitro* morphogenesis of *C. ensifolia* was investigated. It was observed that 3% sucrose was optimum for healthy shoot regeneration. Whereas amongst the concentrations of TDZ tested, 0.2 mg/l was good for inducing multiple shoots with broad elongated dark green leaves. This protocol can be helpful for large-scale multiplication of *C. ensifolia* plant species for effective conservation.

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