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RESEARCH ARTICLES

In vitro high frequency multiplication and assessment of genetic fidelity of *Corallocarpus epigaeus*: an endangered medicinal plant

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Abstract

Corallocarpus epigaeus (Rottler) Hook.f. is an endangered tuberous medicinal climber of family Cucurbitaceae. Despite high medicinal value, over-exploitation made it threatened. In vitro propagation has been adopted for conserving this endangered medicinal plant. Direct shoots induction was achieved from nodal explants on MS medium fortified with various concentrations of BAP and TDZ individually and BAP + IAA, TDZ + IAA, BAP + L-glutamic acid and TDZ + L-glutamic acid combinations. The highest frequency of multiple shoots (43.33 ± 0.53) was achieved on MS medium fortified with 1.5 mg/l TDZ + 1.5 mg/l IAA from nodal explants but shoot length (12.9 ± 0.15 cm) was high on MS medium supplemented with 1.0 mg/l TDZ and 2.0 mg/l L-Glutamic acid. The highest percentage (78%) of rooting was achieved on half strength MS medium augmented with 1.0 mg/l IBA with a mean number of roots 10.76 ± 0.30 cm, an average root length is 1.69 ± 0.07 cm. Rooted plantlets were acclimatized in the greenhouse and successfully transplanted to natural conditions with a 68% survival rate. ISSR markers were used to check the genetic fidelity between in vivo and in vitro developed plantlets. The results indicated that the micropropagated plants are monomorphic and true type when compare with mother plant.

Keywords *Corallocarpus epigaeus* · Nodal explants · Micropropagation · ISSR · Genetic fidelity

Abbreviations

BAP	6-benzylaminopurine
TDZ	Thidiazuron
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
GA3	Gibberellic acid
CTAB	Cetyl trimethyl ammonium bromide
MS	Murashige and Skoog's (1962) medium
PCR	Polymerase chain reaction
ISSR	Inter simple sequence repeats
MNS/E	Mean number of shoots/explant
MLS/E	Mean length of the shoot/explant
S.E	Standard error

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Introduction

Corallocarpus epigaeus (Rottler) Hook.f. is an important medicinal tuberous plant, commonly called Nagadonda in Telugu and Paataala garuda in ayurvedic medicine and belongs to the family Cucurbitaceae. The genus *Corallocarpus* incorporates approximately 43 species distributed in Tropical Africa, Persian Gulf region and India (Sivkumar et al. 2009). Out of which *Corallocarpus epigaeus* plant labelled as a rare, threatened in its natural habitats (Oldfield 1997; Sharma 2009; Palni 2012) and endangered (Choudhary et al. 2008; Bhardwaj et al. 2011; Wagh and Jain 2013; Anil et al. 2014). It is a monoecious perennial tendrillar climber growing from tuberous roots, flowers small in size, greenish yellow in color, fruit berry. The tuber is employed in the treatment of snakebite (Nadkarni 1982; Murthy et al. 2013) and it is used to cure anti-respiratory, anti-cancer, anti-malarial, chronic venereal complaints and external application in conjunctivitis (Atal and Kapur 1982). Due to the medicinal value, it is widely used in traditional and pharmaceutical formulations. In situ environmental conditions are becoming unfavorable for its existence, so there is a need for an urgent conservation strategy for avoiding its extirpation.

Micropropagation is used for the clonal propagation of genetically superior threatened or endangered species. The importance of germplasm conservation of indigenous plants for the prevention of extinction was reported (Dhir and Shekhawat 2012; Jana and Shekhawat 2011). Plant tissue culture techniques of medicinal plants have paved new paradigms to meet their industrial demands (Dhir et al. 2014). The commercial application of in vitro techniques in cucurbitaceous taxa has been well demonstrated and the regeneration of plants has been reported from excised cotyledons, leaf explants (Stipp et al. 2001) and anther culture (Kumar et al. 2003). There has been progressing in tissue culture studies in many Cucurbitaceae members such as *Momordica dioica* (Mustafa et al. 2013). But no such in vitro micropropagation protocols have been developed in this rare and threatened medicinal tuberous plant *Corallocarpus epigaeus*.

Lack of suitable method for natural regeneration and overexploitation of *C. epigaeus*, drastically reduced the species thus listing as an endangered species. Hence, in vitro micropropagation has been attempted in *C. epigaeus* for the conservation of the species using nodal and shoot tip cultures during the present investigation.

For genetic integrity, DNA-based molecular markers have been proposed as an excellent tool for identifying geographical variation, genetic diversity, phylogenetic relationship and authentication of plant species, pharmacognostic characterization, species characterization and genetic mapping in medicinal plants (Joshi et al. 2004). Molecular marker techniques such as inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) analysis have been used to assess variability or similarity within or between the plants derived from plant tissue culture and the donor mother plant (Rout et al. 2009; Jeong et al. 2009; Jain et al. 2011).

Materials and methods

Plant material, explant preparation and surface sterilization

Corallocarpus epigaeus plant materials (Tubers) were carefully collected from distinctive geographical areas of Warangal and Nalgonda districts of Telangana state and planted these tubers in the research field area, Department of Botany, Kakatiya University, Warangal. The tubers began to sprout 20–25 days after potting. Tubers slowly developed into plants after 1 month period. The arisen tender shoots were used as a source of explants for the experiment (Fig. 1a). The plant specimen was authenticated and deposited in the museum of Department of Botany, Kakatiya University, Warangal. These shoots were surface sterilized to get rid of the surface borne microorganisms, for this, explants were thoroughly washed under running tap water for 10 min accompanied with 2–3 drops of tween20 and then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 3–4 min. After that these explants were washed with double sterile distilled water to remove $HgCl_2$ completely. These shoots were then placed on a sterilized filter paper to remove moisture and then aseptically cut into approximately 1.0 cm nodal explant and inoculated on MS medium. All the surface sterilization steps were carried out under laminar air flow chamber.

Media preparation, in vitro shoots induction and maintenance of culture conditions

For all experiments, Murashige and Skoog medium (1962) was used with 3% sucrose as carbon source then the pH was adjusted to 5.6 ± 0.2 before adding 0.8% of agar. The medium was then autoclaved at 121 °C for 15–20 min. All growth regulators were added before autoclaving for shoot induction, surface sterilized nodal explants of *Corallocarpus epigaeus* were inoculated aseptically on MS medium added with different concentrations of phytohormones like BAP (0.5–3.0 mg/l), TDZ (0.2–2.5 mg/l) either alone or in combination with IAA (0.2–2.5 mg/l), 2.0 mg/l L-glutamic acid. Cultures were incubated at 25 ± 2 °C in 16/8 h photoperiod provided by cool and white fluorescent tubes and 55 ± 5% RH.

Shoot elongation and multiplication

The in vitro raised shoots were individually subcultured on fresh MS medium fortified with GA_3 (0.5–3.0 mg/l) either alone or in combination with 2.0 mg/l BAP, 1.5 mg/l TDZ. The MNS/E and MLS/E of shoots were recorded after 4 weeks of each subculture.

In vitro root induction and acclimatization of cloned plantlets

The in vitro raised shootlets (about 5–6 cm in length) were excised and transferred on half strength and full strength MS medium with 3% sucrose, 0.8% agar and supplemented with different concentrations of IBA (0.2–2.5 mg/l) NAA (0.2–2.5 mg/l) for 3 weeks. In vitro raised plantlets were hardened in polycups containing a mixture of red soil + sieved sand + vermicompost (1:1:1). These plants were acclimatized in a culture room at 25 ± 2 °C in 16/8 h photoperiod provided by cool and white fluorescent tubes and 55 ± 5% RH for 2 weeks. These plantlets were then kept in the greenhouse at 80–90% RH 28 ± 2 °C before subsequent transfer to the field.

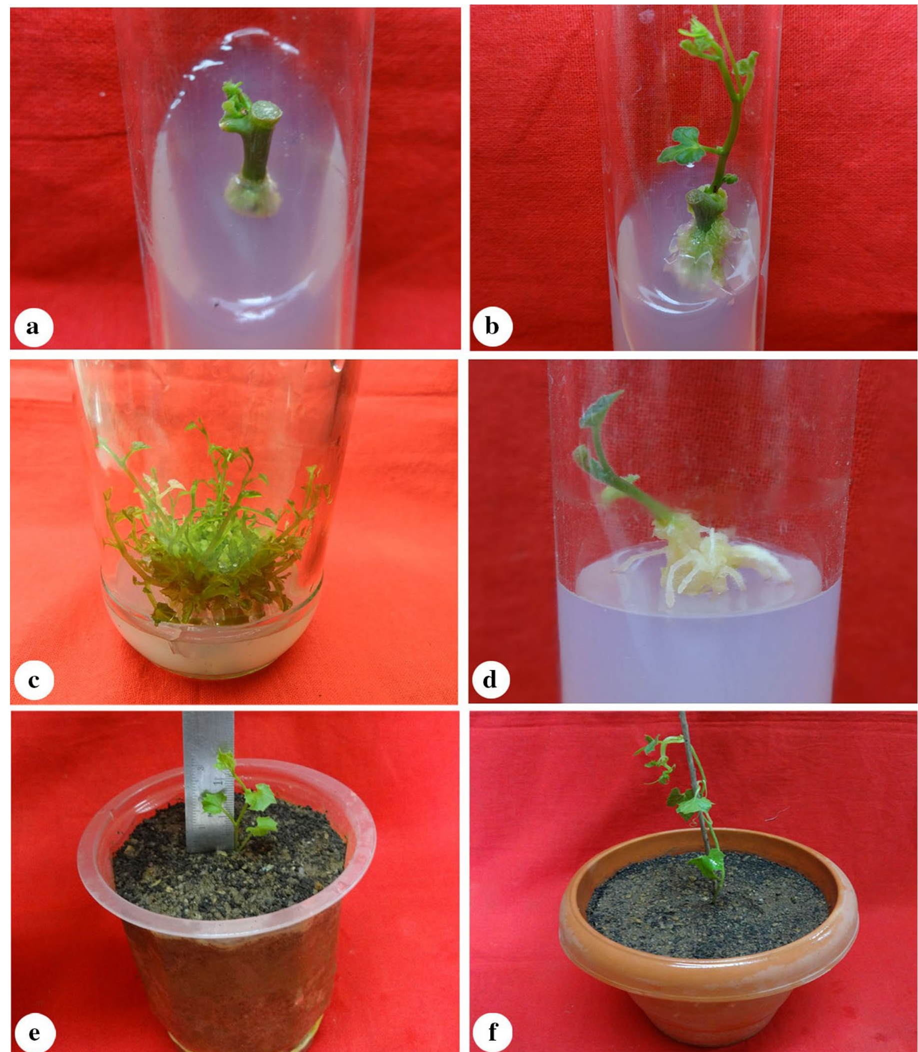

Fig. 1 Plant regeneration of *Corallocarpus epigaeus* using nodal explants. **a** Shoot bud induction after 2 weeks of inoculation on MS medium supplemented with 1.5 mg/l BAP from nodal explants; **b** shoot bud proliferation after 4 weeks of inoculation on MS medium supplemented with 1.5 mg/l BAP from nodal explants; **c** multiple shoot induction on MS medium fortified with 1.5 mg/l TDZ in combination with 1.0 mg/l IAA; **d** in vitro roots formation on half strength MS medium fortified with 1.0 mg/l IBA; **e** primary hardening in glass containing a mixture of red soil + sieved sand + vermicompost (1:1:1); **f** plantlet acclimatized and established in earthen pot containing natural soil

Statistical analysis

Each experiment was carried out in a completely randomized design with at least ten replicates for each treatment. Data from all experiments were subjected to ANOVA (analysis of variance) using SPSS statistical software and means were compared using Duncan's multiple range tests at a 5% probability level consistent with Gomez and Gomez (1976).

DNA isolation

The total genomic DNA was isolated from leaf tissue of both mother plant and in vitro regenerated plants using modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990).

Genetic fidelity analysis using ISSR Primers

The genetic fidelity is one of the main prospects to determine the genetic homogeneity between in vitro raised plantlets with in vivo mother plant. Here ISSR primers were used for genetic homogeneity studies. The PCR analysis was performed with 10 primers of ISSR. PCR amplification was performed using in a total volume of 25 µl containing 50 ng/µl DNA and 10 p mole of ISSR primer, 1X PCR master mix (GCC Biotech). The amplification reaction was carried out in a thermocycler (Eppendorf) for 30 cycles with an initial denaturation of DNA at 94 °C for 5 min, followed by 30 s denaturation at 94 °C, 45 s annealing at 48 °C, 2 min extension at 72 °C, final extension of 7 min at 72 °C and cool down holding to 4 °C. The PCR products were subjected to electrophoresis on 1.0% agarose gel. The size of amplicons was estimated using 1 kb DNA ladder (Thermo scientific). All amplification reactions were repeated twice to check the reproductivity.

Results and discussion

Effect of phytohormones on shoot induction

MS medium fortified with different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) for shoots multiplication from nodal explants was studied. MS medium supplemented with 2.0 mg/l BAP showed significantly maximum percent response (85%) with 28.95 ± 0.47 mean a number of shoots and attaining the shoot length 2.19 ± 0.07 (cm) after 4 weeks of culture (Table 1). MS medium augmented with 1.5 mg/I TDZ alone induced 33.40 ± 0.97 mean number of shoots and 3.53 ± 0.06 (cm) mean shoot length with 86% percentage of response after 4 weeks of culture. When the nodal explants

Table 1 Effect of different concentrations of phytohormones on shoot induction from nodal explants of *Corallocarpus epigaeus*

Phytohormones				Response of nodal explants		
BAP	TDZ	IAA	L-Glutamic acid	% shoot induction	Mean no. of shoots/explant (MNS/E) (mean ± SE*)	Mean length of the shoot/explant (cm) (MLS/E) (mean ± SE*)
0.5	–	–	–	NR	NR	NR
1.0	–	–	–	60	10.87 ± 0.54[d]	1.23 ± 0.08[c]
1.5	–	–	–	65	12.70 ± 0.52[c]	1.96 ± 0.08[a]
2.0	–	–	–	**85**	**28.95 ± 0.47[a]**	**2.19 ± 0.07[a]**
2.5	–	–	–	56	16.78 ± 0.57[b]	1.72 ± 0.08[b]
3.0	–	–	–	NR	NR	NR
–	0.2	–	–	NR	NR	NR
–	0.5	–	–	70	27.22 ± 1.07[b]	2.61 ± 0.05[b]
–	1.0	–	–	68	22.41 ± 1.10[c]	2.20 ± 0.06[c]
–	**1.5**	–	–	**86**	**33.40 ± 0.97[a]**	**3.53 ± 0.06[a]**
–	2.0	–	–	42	13.09 ± 1.37[d]	2.35 ± 0.07[c]
–	2.5	–	–	NR	NR	NR
2.0	–	0.2	–	NR	NR	NR
2.0	–	0.5	–	52	13.07 ± 0.41[b]	4.46 ± 1.28[a]
2.0	–	1.0	–	62	9.66 ± 0.38[c]	5.20 ± 1.19[a]
2.0	–	**1.5**	–	**68**	**17.35 ± 0.36[a]**	**7.11 ± 1.12[a]**
2.0	–	2.0	–	56	9.21 ± 0.39[c]	7.05 ± 1.23[a]
2.0	–	2.5	–	NR	NR	NR
–	1.5	0.2	–	NR	NR	NR
–	1.5	0.5	–	74	27.05 ± 0.56[c]	2.64 ± 0.05[b]
–	1.5	1.0	–	88	35.00 ± 0.52[b]	2.44 ± 0.05[c]
–	**1.5**	**1.5**	**–**	**82**	**43.33 ± 0.53[a]**	**3.45 ± 0.05[a]**
–	1.5	2.0	–	80	27.15 ± 0.54[c]	2.67 ± 0.05[b]
–	1.5	2.5	–	NR	NR	NR
0.5	–	–	2.0	NR	NR	NR
1.0	–	–	2.0	55	4.85 ± 0.26[c]	5.57 ± 0.10[bc]
1.5	–	–	2.0	58	5.00 ± 0.25[c]	5.58 ± 0.09[bc]
2.0	**–**	**–**	**2.0**	**68**	**6.94 ± 0.23[a]**	**8.42 ± 0.09[a]**
2.5	–	–	2.0	50	4.76 ± 0.27[c]	5.79 ± 0.10[b]
3.0	–	–	2.0	NR	NR	NR
–	0.2	–	2.0	NR	NR	NR
–	0.5	–	2.0	68	6.11 ± 0.23[c]	8.96 ± 0.17[b]
–	**1.0**	**–**	**2.0**	**86**	**8.77 ± 0.20[a]**	**12.91 ± 0.15[a]**
–	1.5	–	2.0	74	4.94 ± 0.21[d]	6.91 ± 0.16[c]
–	2.0	–	2.0	60	5.86 ± 0.24[c]	5.97 ± 0.18[d]
–	2.5	–	2.0	NR	NR	NR

*Data represents average of ten replicates. Data was collected after 4 weeks. Mean ± standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05. Bold letters indicates highest response

were cultured on MS medium supplemented with 2.0 mg/l BAP and 1.5 mg/l IAA induced 17.35 ± 0.36 mean number of shoots and 7.11 ± 1.12 (cm) mean shoot length with 68% of response. But the response was maximum (88%) on MS medium supplemented with 1.5 mg/l TDZ + 1.0 mg/l IAA with 35.00 ± 0.52 mean number of shoots, which is highly significant (Fig. 1b). Other combinations like 2.0 mg/l BAP + 2.0 mg/l L-Glutamic acid and 2.0 mg/l L-Glutamic acid + 1.0 mg/l TDZ were not so significant but, the latter combination promoted higher percentage of response and shoot length 12.91 ± 0.15 (cm) after 4 weeks of culture. The growth and perpetuation of the plantlets were continued after the first subculture on the respective MS medium supplemented with the same hormonal combinations. MS medium supplemented with 1.0 mg/l TDZ along with 2.0 mg/l L-glutamic acid contributed maximum response (86%) with 33.40 ± 0.97 mean number of shoots and 3.53 ± 0.6 (cm) mean shoot length after 4 weeks of culture (Table 1).

MS medium supplemented with 2.0 mg/l GA_3 and 1.5 mg/l TDZ resulted in significant response (89%) with the attaining of maximum shoot length (10.61 ± 0.25) (Fig. 1c). MS medium fortified with GA_3 (1.5 mg/l) alone and GA_3 in combination with BAP (2.0 mg/l) did not favor significant shoot elongation (Table 2).

In vitro rooting and acclimatization of cloned plantlets

Microshootlets were cultured on half strength MS medium fortified with different concentrations of IBA and NAA individually (Table 3). Maximum rooting responses 78% and 52% were observed at optimal levels of IBA (1.0 mg/l) and NAA (1.0 mg/l) respectively. A higher concentration of (2.0 mg/l) IBA and (2.0 mg/l) NAA favored callusing at the basal portion of shoots and produced less response in terms of a number of roots and average root length also. But Efficient rooting (64%) was observed on full strength MS medium fortified with 1.0 mg/l IBA with 6.42 ± 1.49 mean number of roots with an average root length 3.35 ± 0.12 (cm) after 4 weeks of culture. Whereas IBA (1.0 mg/l and 1.5 mg/l) on full strength MS medium has been proved the best for the induction of maximum mean number of roots 39.77 + 1.31 and 35.12 + 1.39 with and an average root lengths 1.77 + 0.10 (cm) and 1.41 + 0.11 (cm) respectively (Fig. 1d).

Table 2 Effect of different concentrations of phytohormones on shoot elongation in in vitro raised shoots from Nodal explants of *Corallocarpus epigaeus*

Phytohormones			Response of in vitro raised shoots		
GA3	BAP	TDZ	% shoot elongation	Mean no. of shoots/explant (MNS/E) (mean ± SE*)	Mean length of the shoot/explant (cm) (MLS/E) (mean ± SE*)
0.5	–	–	NR	NR	NR
1.0	–	–	72	1.00 ± 0.00	7.75 ± 0.16[b]
1.5	–	–	**82**	**1.00 ± 0.00**	**9.92 ± 0.14[a]**
2.0	–	–	70	1.00 ± 0.00	6.70 ± 0.17[c]
2.5	–	–	65	1.00 ± 0.00	5.78 ± 0.17[d]
3.0	–	–	NR	NR	NR
0.5	2.0	–	NR	NR	NR
1.0	2.0	–	56	1.00 ± 0.00	4.99 ± 1.47[a]
1.5	2.0	–	52	1.00 ± 0.00	6.18 ± 1.33[a]
2.0	**2.0**	–	**66**	**1.00 ± 0.00**	**6.83 ± 1.53[a]**
2.5	2.0	–	48	1.00 ± 0.00	4.97 ± 1.42[a]
3.0	2.0	–	NR	NR	NR
0.5	–	1.5	66	1.00 ± 0.00	7.03 ± 0.30[c]
1.0	–	1.5	84	1.00 ± 0.00	8.91 ± 0.26[b]
1.5	–	1.5	72	1.00 ± 0.00	6.85 ± 0.28[c]
2.0	–	**1.5**	**89**	**1.00 ± 0.00**	**10.61 ± 0.25[a]**
2.5	–	1.5	61	1.00 ± 0.00	7.13 ± 0.31[c]
3.0	–	1.5	NR	NR	NR

*Data represents average of ten replicates. Data was collected after 4 weeks. Mean ± standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05. Bold letters indicates highest response

Table 3 Effect of different concentrations of phytohormones on root induction in in vitro raised shootlets of *Corallocarpus epigaeus*

Phytohormones		Response of in vitro raised shoots for rooting			
Half strength MS medium with IBA	Full strength MS medium with IBA	% root induction	Nature of response	Mean no. of rootlets (mean ± SE*)	Mean length of rootlet (cm) (mean ± SE*)
0.2	–	18	C + R	2.88 ± 0.26^{c}	1.88 ± 0.11^{b}
0.5	–	28	C + R	3.60 ± 0.49^{b}	3.58 ± 0.12^{a}
1.0	–	**78**	**R**	$\mathbf{10.76 \pm 0.30^{a}}$	$\mathbf{1.69 \pm 0.07^{b}}$
1.5	–	68	R	4.00 ± 0.34^{b}	0.82 ± 0.89^{c}
2.0	–	32	C + R	4.12 ± 0.38^{b}	0.67 ± 0.10^{c}
2.5	–	NR	NR	NR	NR
–	0.2	NR	NR	NR	NR
–	0.5	25	C + R	19.83 ± 1.61^{c}	1.43 ± 0.13^{d}
–	**1.0**	**35**	**C + R**	$\mathbf{39.77 \pm 1.31^{a}}$	$\mathbf{1.77 \pm 0.10^{c}}$
–	1.5	30	C + R	35.12 ± 1.39^{b}	1.41 ± 0.11^{d}
–	2.0	22	C + R	8.70 ± 1.24^{c}	2.51 ± 0.10^{b}
–	2.5	18	C + R	6.42 ± 1.49^{c}	3.35 ± 0.12^{a}
–	0.2	NR	NR	NR	NR
NAA	**NAA**				
0.2	–	NR	NR	NR	NR
0.5	–	24	C + R	2.27 ± 0.14^{b}	1.94 ± 0.29^{b}
1.0	**–**	**52**	**C + R**	$\mathbf{3.12 \pm 0.02^{a}}$	$\mathbf{1.82 \pm 0.25^{b}}$
1.5	–	45	C + R	1.98 ± 0.01^{b}	2.12 ± 0.28^{a}
2.0	–	NR	NR	NR	NR
2.5	–	NR	NR	NR	NR
–	0.2	NR	NR	NR	NR
–	0.5	NR	NR	NR	NR
–	**1.0**	**64**	**C + R**	$\mathbf{6.42 \pm 1.49^{a}}$	$\mathbf{3.35 \pm 0.12^{a}}$
–	1.5	49	C + R	2.19 ± 0.16^{b}	2.41 ± 0.10^{b}
–	2.0	NR	NR	NR	NR
–	2.5	NR	NR	NR	NR

*Data represents average of ten replicates. Data was collected after 3 weeks. Mean ± standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05. Bold letters indicates highest response

The well-developed plantlets of *C. epigaeus* were successfully transferred into the polycups. The polycups were filled with red soil + sieved sand + vermicompost in 1:1:1 ratio. Then they were transferred into polyhouse and irrigated regularly for 3–4 weeks. The high survival rate of in vitro plants in the present study indicates that this procedure may be easily adapted for large scale multiplication and cultivation which facilitate to meet the medicinal demand in the market.

The in vitro rooted plantlets from nodal cultures were hardened on three different types of soil mixtures viz., red soil, red soil + sieved sand (1: 1), and red soil + sieved sand + vermicompost (1:1:1) (Fig. 1e). In the present investigation, the maximum survival percentage (68%) was found in red soil + sieved sand + vermicompost, when compared to all other substrates used. New leaves were formed after 14 days after transplantation in the same soil mix. Low survival percentage (28%) was recorded in red soil and the new leaf appeared after 18 days and a moderate percentage of response (46%) was observed in remaining substrates used. After acclimatization, the plantlets were shifted from greenhouse to earthenware pots containing garden soil and maintained in the research field (Fig. 1f). These acclimatized plants were normal, healthy and morphologically similar to parental plants.

The percentage of response for shoots induction, the mean number of shoot per explants and mean shoot lengths were affected by the various concentrations of BAP and TDZ either alone and is combinations with IAA and L-glutamic acid. Highest percentage of response (85%) was achieved on MS medium with 2.0 mg/l BAP exhibited 28.95 ± 0.47 mean number of shoots and 2.19 ± 0.07 (cm) mean shoot length. Similar results were found in *Lagerstroemia indica* (Niranjan et al. 2010) and *Salvadora persica* (Mathur et al. 2002). Effect of BAP on shoot formation and elongation in the establishment of the *Momordica dioica* were

reported by Nabi et al. (2002). However, a lower and higher concentration of BAP has decreased in terms of percentage of response, mean number of shoots and mean shoot lengths. MS medium fortified with both (2.0 mg/l) BAP together with (1.5 mg/l) IAA showed appreciable moderate response (68%) with a mean number of shoots 17.35 ± 0.36 and attaining mean shoot length 7.11 ± 1.12 (cm). Moreover, MS medium with BAP alone induced maximum response when compared to in combination with IAA but, the shoot length was enhanced in conjunction with IAA. Anand and Jeyachandran (2004) reported that high frequency of multiple shoots induction was achieved in *Zehneria scabra* through nodal explants with 5.0 mg/l BAP and combination of 0.5 mg/l IAA. The effect of BAP in bud breaking has been observed in many medicinal plants, such as *Vitex trifolia* (Arulanandam et al. 2011a, b, c), *Wattakaka volubilis* (Arulanandam et al. 2011a, b, c). The emergence of induction of shoot buds was seen after 5 days of inoculation and shoot elongation was observed after 10 days. MS medium with optimal levels of 2.0 mg/l BAP in conjunction with 2.0 mg/l L-glutamic acid resulted 68% of response with 6.94 ± 0.23 mean a number of shoots and attaining the shoot length 8.42 ± 0.09 (cm) after 4 weeks of culture. By increasing the concentration of BAP together with constant 2.0 mg/l L-glutamic acid reported less in terms of percentage of response, mean number of shoots and mean shoot lengths. However, 2.0 mg/l L-glutamic acid in conjunction with BAP favored in shoot elongation than L-glutamic acid in combination with IAA. Similarly, Mustafa et al. (2013) reported that a maximum number of shoots (12.1 ± 0.25), with an average shoot length (1.8 ± 0.08) cm were on MS medium supplemented with 2.0 mg/l BAP in combination with 2.0 mg/l L-glutamic acid in nodal explants of *Momordica dioica*. Similar investigations were also reported by Hoque et al. (1995). Moreno et al. (1985) reported that multiple shoots were regenerated on MS medium supplemented with 1.0 mg/l BAP in combination with 2.5 mg/l L-glutamic acid in nodal explants of *Cucumis melo*. The amino acid L-glutamic acid in combination with BAP had a synergistic effect for the production of multiple shoots via direct organogenesis in *Momordica charantia* and *Citrullus lanatus*, swelling of shoot bases were also accompanied by the formation of adventitious shoot bud (Sultana and Bari Miah 2003; Sultana et al. 2004). Shekhawat et al. (2009) obtained somatic embryos from leaf derived callus of *Azadirachta indica* by using the additional nitrogen source in combination with kinetin and IAA.

MS medium with 1.5 mg/l TDZ has proven significantly with a maximum response (86%) with 33.40 ± 0.97 mean number of shoots with an average shoot length 3.53 ± 0.06 (cm). TDZ was found to be more effective in medium for shoots induction compared to BAP. The effectiveness of TDZ over other cytokinins reported in many cucurbits like *Momordica charantia* (Thiruvengadam et al. 2010) and *Cucurbita pepo* (Pal et al. 2007). MS medium supplemented with 1.5 mg/l TDZ in combination with 1.0 mg/l IAA resulted 88% of response with 35.00 ± 0.52 mean number of shoots and an attaining shoot length 2.44 ± 0.05 (cm) from nodal explants after 4 weeks of culture. But the maximum mean no. of shoots (43.33 ± 0.53) were achieved on MS medium supplemented with 1.5 mg/l TDZ in combination with 1.5 mg/l IAA and the percentage response was limited to 82% with an average shoot length 3.45 ± 0.05 (cm) after 4 weeks of culture. Narayan (2016) reported only 8.41 ± 0.29 mean no. of shoots on MS medium augmented with 0.5 mg/l BA in combination with 2.0 mg/l IAA. Similar results were also reported in *Capsicum annum* (Raghu et al. 2016). MS medium with 1.0 mg/l TDZ in conjunction with 2.0 mg/l L-glutamic acid showed 86% of response with 8.77 ± 0.20 mean number of shoots and attaining the shoot length 12.91 ± 0.15 (cm) after 4 weeks of culture.

Appreciable highest percent response (89%) was obtained in terms of shoot elongation on MS medium with 2.0 mg/l GA_3 and 1.5 mg/l TDZ with 10.61 ± 0.25 (cm) an average shoot length. TDZ was most favorable in combination with GA_3 for shoot elongation. MS medium supplemented with 2.0 mg/l GA_3 and 1.5 mg/l TDZ supported more for shoot elongation than BAP + GA_3 combination and GA_3 alone. Likewise, GA_3 favored a better response for shoot elongation in many cucurbits such as *Cucumis sativus* (Thiruvengadam et al. 2010) and *Trichosanthes anguina* (Ambethkar et al. 2012).

Half strength MS medium supplemented with the optimal level of IBA (1.0 mg/l and 1.5 mg/l) favoured 78% and 68% of response with 10.76 + 0.30 and 4.00 + 0.34 mean number of roots attained root lengths 1.69 + 0.07 (cm) and 0.82 + 0.89 (cm) after 4 weeks of culture respectively. Root initiation was started after 7 days of incubation from the basal end of the micro shootlets. Increased (2.0 mg/l) and decreased (0.2 mg/l and 0.5 mg/l) concentrations of IBA favored in the induction of callus at the basal portion besides induction of roots. Callus production during the rooting process was also reported in *Eucalyptus tereticornis* (Das and Mitra 1990). This attributes in a lower concentration of IBA reduction of the mean number of roots (2.88 + 0.26 and 3.60 + 0.49) and root lengths 1.88 + 0.11 (cm) and 3.58 + 0.12 (cm) respectively. Similar observations have been reported in *Trichosanthes cucumerina* (Devendra et al. 2008) and *Citrullus lanatus* (Khatun et al. 2010). MS full strength medium supplemented with 1.0 mg/l IBA induced maximum number of roots compared to IBA on half strength MS medium. However the percentage response is higher (78%) in half strength MS medium supplemented with 1.0 mg/l IBA, but the mean number of roots were moderate (10.76 ± 0.30). Half strength MS medium supplemented with IBA influence effective rooting in *Ruta graveolens*

(Bohidar et al. 2008) and *Stevia rebaudiana* (Sivaram and Mukundan 2003) In full strength MS medium supplemented with 1.0 mg/l IBA promoted higher mean number of roots (39.77 ± 1.31) with lower percentage (35%) of response. IBA is a widely used plant growth regulator for root induction in cucurbits (Sarowar et al. 2003; Thomas and Sreejesh, 2004 and Krug et al. 2005). IBA alone has been reported in rooting after 8–10 days in *Acacia mangium* (Nanda et al. 2004) and *Acacia nilotica* (Dhabhai et al. 2010). Similar results were also observed in *Withania somnifera* (Sharma and Batra 2006), *Prosopis cineraria* (Kumar and Singh 2009). However, IBA favored maximum induction of roots compared to NAA (Benelli et al. 2001; Tanimoto 2005).

Hardening is a crucial step prior to transplantation of plants to the soil. The in vitro plantlets live in 100% relative humidity and they also depend on the medium for the supply of sugar and other nutrients (Ahuja 1993). Plants are, therefore, allowed to grow on rooting media for about 1 month after root initiation. During this phase, the nutrients in the culture medium getting depleted gradually and plants become strong and easy to acclimatize in the greenhouse.

In the present investigations, the hardening of in vitro rooted plantlets of *C. epigaeus* has been developed with 68% survival. The protocol developed for acclimatization can be used for rapid in vitro multiplication and conservation of *Corallocarpus epigaeus*.

Assessment of genetic stability

Somaclonal variation has been reported in many micropropagated plants (Larkin and Scowcroft 1981). The genetic fidelity among the regenerated plantlets is essential for micropropagation studies (Dhir and Shekhawat 2013). ISSR markers based analysis is the simple and cost-effective methods for the analysis of genetic homogeneity among the plants. In this study 10 randomly selected in vitro generated plantlets from nodal explants as well as the mother; the plant was subjected to ISSR analysis to the genetic fidelity. For this analysis, ten primers were selected for DNA amplification as they produce distinct reproducible scorable bands (Table 4). The number and size range of amplified scorable bands for each ISSR primer has been presented in Table 4 (Fig. 2). Recently, molecular markers have been shown to be the most desirable too for establishing genetic similarity or dissimilarity of in vitro propagated plants (Rani et al. 1995; Govind et al. 2012; Tiwari et al. 2013; Slazak et al. 2015). Furthermore, numerous studies on somaclonal variation of regenerated plants have been developed using PCR based techniques such as Random Amplification of Polymorphic DNA (RAPD), Simple sequence repeats (SSR), Inter-simple sequence repeats (ISSR) and Amplified fragment length polymorphism (AFLP) (Yang et al. 2008; Xing et al. 2010; Pandey et al. 2012; Ramakrishnan et al. 2014; Saha et al. 2014). As per the study, there is no genetic variation was observed in the randomly tested regenerated plants from the mother plant of *C. epigaeus*, hence somaclonal variations are not reported. Similar type of results in genetic fidelity

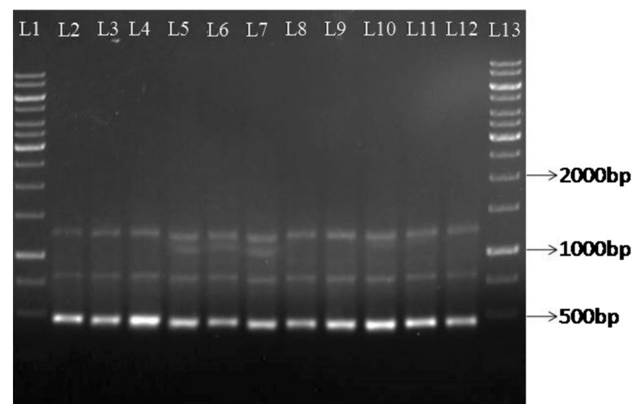

Fig. 2 DNA banding pattern of ISSR based genetic fidelity analysis of *C. epigaeus* amplified with ISSR-6 primer. Lane 1 and 13—1 kb DNA ladder, lane 2—DNA banding pattern of the mother plant, lane 3–12—DNA banding pattern of randomly selected in vitro regenerated plant

Table 4 List of primers, their sequence, number of bands and size of the amplified fragments generated by ISSR markers

S. no.	Primer code	Primer sequence (5′–3′)	Number of bands	Band length (bp)
1	ISSR1	AGAGAGAGAGAGAGAGG	5	350–1200
2	ISSR2	GAGAGAGAGAGAGAGAT	4	300–950
3	ISSR3	TCTCTCTCTCTCTCTCG	2	500–1500
4	ISSR4	AGAGAGAGAGAGAGAGYT	5	750–3000
5	ISSR5	AGAGAGAGAGAGAGAGTC	3	900–1500
6	ISSR6	GAGAGAGAGAGAGAGAYG	3	450–1350
7	ISSR7	CACACACACACACACARC	4	300–1300
8	ISSR8	TGTGTGTGTGTGTGTGRA	4	500–2000
9	ISSR9	ACACACACACACACACT	5	600–2000
10	ISSR10	CTCCTCCTCCTCCTCCTC	4	500–1500

analysis by using ISSR markers were reported in *Rauvolfia tetraphylla* (Rohela et al. 2019), *Artemisia absinthium* (Kour et al. 2015), *Sphagneticola calendulacea* (Kundu et al. 2017), *Withania coagulans* (Rathore et al. 2015), *Withania somnifera* (Nayak et al. 2013), *Gymnema sylvestre* (Saeed et al. 2018), *Morus* sp. (Rohela et al. 2018).

Conclusion

The present study provides the first report on genetic homogeneity of in vitro clonal propagated plants through nodal explants of *C. epigaeus*. This study also reveals a high frequency multiplication protocol for conservation of medicinally important endangered plant of *C. epigaeus*. This standardized clonal propagation protocol could be utilized for large scale mass propagation true-to-type genotype of *C. epigaeus.*

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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