British Biotechnology Journal
14(3): 1-10, 2016, Article no.BBJ.27229
ISSN: 2231–2927, NLM ID: 101616695

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Ex-situ Conservation of the Rare and Threatened Medicinal Climber *Corallocarpus epigaeus* Rottler through *In vitro* Regeneration Method

Jai Prakash Narayan[1*]

[1]*Department of Education in Science and Mathematics, Regional Institute of Education (NCERT), Pushkar road, Ajmer 305 004, India.*

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/BBJ/2016/27229
Editor(s):
(1) Standardi Alvaro, Department of Agricultural and Environmental Sciences, University of Perugia, Italy.
Reviewers:
(1) Aysun Cavusoglu, Arslanbey Agricultural Vocational School, Kocaeli University, Kocaeli, Turkey.
(2) Edgar Iván Sánchez Bernal, Institute of Ecology, Universidad del Mar Campus Puerto Escondido, Oaxaca, Mexico.
(3) T. Pullaiah, Sri Krishnadevaraya University, India.
Complete Peer review History: http://sciencedomain.org/review-history/15334

Original Research Article

Received 25th May 2016
Accepted 27th June 2016
Published 9th July 2016

ABSTRACT

Aims: To develop *in vitro* propagation and callus induction protocol for regeneration and conservation of the rare and threatened medicinal climber, *Corallocarpus epigaeus*, which is widely acclaimed for high medicinal values.
Place and Duration of Study: The plant was spotted on Nag Pahar hill forest of Ajmer district of Rajasthan, India. Its natural regenerative potential and exploitative practices by human beings were studied during November, 2012 to December, 2013. *In vitro* regeneration experiments were conducted during January, 2014 and January, 2016 at Regional Institute of Education, Ajmer.
Methodology: The organogenic and callus induction potential of various parts of the plant *C. epigaeus* were assessed through *in vitro* cultures. Murashige and Skoog`s (MS) media supplemented with various concentrations and combinations of 6-Benzyladenine (BA), Indole-3-acetic acid (IAA), α-Naphthaleneaceticacid (NAA), and Indole-3-butyric acid (IBA) were used for enhanced axillary branching, callus induction, and rooting of microshoots. Acclimatization of rooted plantlets and their successful establishment to the soil conditions were also studied.
Results: An efficient plant regeneration system has been developed through enhanced axillary branching which was initiated from nodal segments cultured on MS medium supplemented with

**Corresponding author: E-mail: jpnbiotech@gmail.com;*

various concentrations of BA, and IAA in combination. The maximum number of shoots 8.41±0.29 with average length of 6.29±0.32 cm per explant was achieved in MS medium containing 0.5 mg/L BA and 2.0 mg/L IAA. The best callus induction from stem and leaf explants was obtained on MS medium containing 2.0 mg/L (BA) and 0.5 mg/L NAA. Rooting of microshoots was achieved on half-strength MS medium supplemented with 1.0 mg/L IBA. Well developed plantlets transferred to the soil conditions showed 67% survival.

Conclusion: The methodology is reliable and highly beneficial for *ex situ* conservation, maintenance of *in vitro* biodiversity and multiplication of the Great Root for commercial production of tubers of diverse pharmacological properties.

Keywords: Great root; in vitro propagation; nodal explants; in vitro biodiversity; 6-Benzyladenine; Indole-3-acetic acid; α-Naphthaleneacetic acid; Indole-3-butyric acid; Murashige and Skoog's.

1. INTRODUCTION

Corallocarpus epigaeus (Rottler) Benth. & Hook. F. ex C.B. Clarke belongs to the family Cucurbitaceae. It is a monoecious, deciduous and perennial tendril climber with a large turnip-shaped root, popularly known as the Great Root; distributed in tropical Africa, Persian Gulf region and India [1]. Approximately eighty percent (4 billion people) of the world population use herbal medicine for some aspect of primary health care [2]. *C. epigaeus* is a widely used herb for effective and safe treatment of various human diseases in folklore, Ayurveda and Siddha systems of complementary and alternative medicines. Seeds contain amino acid, N-methylasparagine [3]; and the foliage, stem and leaves have been shown to possess antimicrobial and hepatoprotective activities [4,5]. The Great Root is nature's reservoir of therapeutic substances viz., a glycoside- bryonin [6], sesquiterpene, lactone-corallocarpenoyl ester and an aliphatic C_{32} keto diol [7]; Ishwarane and Ishwarone isolated by Gupta et al. [8], from root oil now considered as rare sesquiterpene of plant kingdom. Phytochemical screening of tubers reveals the presence of phytonutrients, carbohydrates, proteins, amino acids, vitamin C & E; and medicinally valuable secondary metabolites including flavonoids, alkaloids, phenolics, tannins, triterpenoides and steroids [9-12]. These phytochemicals perhaps impart potent pharmacological properties to the Great Root that include laxative, hypoglycemic, antioxidant, anti-inflammatory, anticoagulant, analgesic, antiallergic, anthelmintic, antitoxic, antipyretic, antibacterial, antifungal and immunomodulatory [10,12,13-20]. In folklore, Ayurveda, Siddha and modern pharmacopoeia Great Root is being widely used to cure common cold, cough, asthma, bronchitis, constipation, indigestion, abdominal pain, dysentery, chronic mucous enteritis, sunstroke, typhoid, wounds, tumors, boils, sunburn, swellings, eczema, leprosy, allergic dermatitis, jaundice, piles, glandular enlargement, cancer, AIDS, diabetes, arthritis, venereal complaints, chronic rheumatism, poisonous stings, and snake-bites [6,10,11,13,21-30]. In addition, *C. epigaeus* has also been used as a popular ethno veterinary medicine since time immemorial [31]. Due to exploitative harvesting of tubers for trade and denudation of forests, the natural population of *C. epigaeus* has declined to such an extent that it is now considered rare and threatened in its natural habitats [32-35]. Some authors [24,36-39] put it under endangered category since natural regeneration of this species is quite poor on account of long seed dormancy, abortive seeds, extremely poor germination, and inadequate availability of tuberous roots as propagules owing to exploitative harvesting. Hence, they explicitly predict apprehension of its extinction in the near future if adequate conservation measures are not adopted urgently. Conventional horticultural technique for propagation of *C. epigaeus* largely depends on tuberous roots and seeds. Due to inadequate availability of tuberous roots, damage caused to the seeds by insect pests, long dormancy period of seeds, significant number of abortive seed set, poor seed germination and high rate of seedling mortality; conventional commercial multiplication method is critically limited. Solution lies in interventions of biotechnological approaches for *in vitro* regeneration which offers rapid and unlimited availability of planting materials throughout the year. Biotechnological interventions for regeneration using *in vitro* culture technique have been successfully employed for many crops and some reports are also available for similar wild Cucurbitaceae taxa including *Zehneria scabra* [40], *Trichosanthes cucumerina* [41], *Trichosanthes dioica* [42], *Momordica dioica* [43],

Citrullus colocynthis [44], and *Coccinia indica* [45]. There has been no report available so far for *in vitro* propagation and maintenance of *in vitro* biodiversity for conservation of the germplasm for the taxon *Corallocarpus.* Therefore, in view of the emerging ecological imperative for rejuvenation of vastly eroded natural population of *C. epigaeus* for rendering essential goods and services to the mankind and ecosystem in a sustainable manner; an attempt has been made to develop *in vitro* regeneration protocol through enhanced axillary branching from nodal segments and callus induction methods for stem and leaf explants from scarcely available naturally growing representatives. Callus induction and multiplication in laboratory itself can pave the way for isolating pharmacologically valuable phytochemicals from calli in a sustainable manner. Moreover, such routine practice will avoid overexploitation of plant materials from natural sources.

2. MATERIALS AND METHODS

2.1 Plant Material and Surface Sterilization

The plant was spotted on Nag-Pahar hills of Aravalli Range surrounding Ajmer city, India. The species was taxonomically identified by Dr. I. B. Maurya, Agricultural University, Kota. The voucher specimens were deposited in the herbarium of Botany Department of R.I.E., Ajmer. Healthy and juvenile shoots of 5 to 20 cm were collected from the first flush of growth of the donor *C. epigaeus* plants (approximately 10-15 years old). Vine cuttings were immediately dip into water and brought to the laboratory for study. Leafy shoots were cut into small pieces of 4-5 cm length single nodes along with few leaves with a sharp knife. The single node segments were thoroughly cleaned by washing for 30 min. in running tap water. Nodal segments were then immersed in a solution containing 1% (v/v) labolene (liquid detergent) and 1% (v/v) savlon for 8-10 minutes for disinfection, followed by three times washing with distilled water. Leaves of the single node segments were separated by trimming and then surface sterilization of both parts was performed by dipping them in 70% ethyl alcohol for 30 seconds and subsequent rinsing with sterile distilled water, finally continuous shaking in 0.1% (w/v) mercuric chloride solution for 3-4 minutes followed by five times rinsing with sterile double distilled water under laminar flow hood. Nodal segments / leaves then blotted dry and nodal parts got trimmed from both sides to about 3.0 cm for culturing. Nodal segments and stem parts/ leaves were used as explants for enhanced axillary branching and callus induction respectively.

2.2 Media Preparation and Conditions for Maintenance of Cultures

The explants were inoculated on MS [46] media supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Himedia); pH of the medium was adjusted to 5.8 and 20 ml medium was dispensed into 25x150 mm culture tubes. The control was basal medium free from growth regulators, while others were fortified with various concentrations of BA in combination with IAA or NAA plant growth regulators for shoot proliferation and callus induction respectively. Half strength MS media fortified with various concentrations of IBA, IAA and NAA alone were prepared for rooting of microshoots. Culture vessels along with media were plugged with non absorbent cotton, then autoclaved at 121°C and 1.05 kg cm^{-2} of pressure for 20 min. for sterilization. Cultures were kept in a growth chamber maintained at 25±2°C with a photoperiod of 16/8 hour light and dark under an irradiance of 40-50 μ mol m^{-2} s^{-2} provided by cool white fluorescence tubes. Responsive explants with adequate morphological features were subcultured at four weeks intervals.

2.3 Shoot Induction, Multiplication and Callusing

Various concentrations of a cytokinin (BA) and auxins (IAA, NAA and IBA) either singly or in combination (cytokinins and auxins) were used to fortify MS basal medium to elicit morphogenic responses from nodal segments, stem/leaves, and microshoots for enhanced axillary branching, callus and root induction respectively. Efficaciously plausible concentration range of cytokinins and auxins were used singly or in combination to establish the cultures. Basal MS medium without growth regulator was used as control for each experiment. Some cultures were subcultured to the fresh medium of same composition every four weeks.

2.4 Root Induction

The microshoots of approximately 3-5 cm length were excised from the cultures obtained on optimal concentrations of IAA (2.0 mg/L) in

combination with BA (0.5 mg/L) and transferred to root induction medium comprising MS full and half strength fortified with various concentrations (0.5, 1.0, 1.5, 2.0 mg/L) of IBA.

2.5 Acclimatization

Prior to acclimatization a booster dose of 2MS along with optimal concentration of IBA was given to rooted plantlets for rapid expansion of leaves within a week. Well developed plantlets were removed from the booster rooting medium, washed gently under running tap water to remove any adherent gel and transplanted into thermocol cups containing sterile sand. Transplanted plants were kept in culture room and irrigated every alternate day with ¼ strength MS salt solution without vitamins for 15 days. After three weeks, plantlets were transferred to the soil.

2.6 Data Collection and Statistical Analysis

Twenty and ten culture tubes were used for each treatment for multiple shoot production and root induction respectively, and the entire experiment was repeated thrice. The data for percentage response, number of shoots per explant, shoot length, root length, and numbers of roots per plantlet were recorded for enhanced axillary branching and root induction experiment respectively. For callus induction, intensity of callus formation based on observable size, color and texture were recorded for stem and leaf explants. Data were statistically presented as the mean ± standard error.

3. RESULTS AND DISCUSSION

3.1 Synergistic Effect of BA and IAA on Multiple Shoot Production

Nodal segments cultured on MS basal medium without growth regulator did not show any response. Whereas, in MS basal medium supplemented with various concentrations of BA in combination with IAA, explants swelled in their size after 1 to 2 weeks of culture, formed small calli at and around their cut ends and differentiated shoots from nodes. The maximum percentage responding explants (68.33%), and the highest No. of shoots 8.41 with 6.29 cm average shoot length were observed at 0.5 mg/L BA in combination with 2.0 mg/L IAA per explant after seven weeks of culture (Table 1; Fig. 1-A, B, C). The second highest shoot proliferation with respect to No. of shoots per explant was observed in the MS medium containing BA (0.5 mg/L) + IAA (1.5 mg/L) in which 6.20 shootlets per explant with average shoot length of 3.20 cm were recorded. While the second highest shoot length growth of 4.14 cm was recorded at MS along with BA (0.5 mg/L) + IAA (2.5 mg/L) though No. of shoots per explant (3.70) was lower as compared to BA (0.5 mg/L) + IAA (1.5 mg/L) and BA (0.2 mg/L) + IAA (2.0 mg/L) MS media. Similar observations have been reported by Chaturvedi & Sharma; and Das et al. [47-48] in *Simmondsia chinensis* and *Dioscorea alata* respectively, where the best shoot proliferation occurred at higher concentration of auxins as compared to cytokinins on contrary to most of the micropropagation studies pertaining to shoot proliferation.

Table 1. *In vitro* shoot multiplication from nodal explants of *C. epigaeus* on MS medium fortified with various concentrations of BA and IAA

IAA (mg/L)	BA (mg/L)	% responding explants	No. of shoots/ explant (Mean±SE)	Shoot length (cm±SE)	Extent of callus
0	0	0	0	0	-
0.5	0.5	50	1.20±0.07	2.53±0.14	++
1.0	0.5	55	3.06±0.12	2.09±0.13	+
1.5	0.5	58.33	6.20±0.22	3.20±0.18	+
2.0	0.2	40	4.12±0.30	3.12±0.17	_
2.0	0.5	68.33	8.41±0.29	6.29±0.32	+
2.0	1.0	45	3.55±0.15	3.33±0.18	_
2.5	0.5	45	3.70±0.22	4.14±0.20	++
3.0	0.5	40	1.12±0.13	1.33±0.13	+++

SE: Standard error

Extent of callus formation around cut ends of explants: Profuse: +++; Moderate: ++; Poor: +; No response: -

Mean is based on three replicates, each of which consisted of twenty culture vessels; Data were recorded after seven weeks

Fig. 1. *In vitro* regeneration through nodal explants of *Corallocarpus epigaeus* Rottler. A- Induction of shoots on MS+BA (0.5 mg/L) and IAA (2.0 mg/L) from nodal explant after two weeks of culture; B,C- Proliferation and multiplication of axillary shoots upon subculturing on the same medium after four and seven weeks respectively; D,E- The microshoot with well-developed roots on half strength MS+IBA (1.0 mg/L) after four weeks of culture; F- Five weeks old acclimatized plant after transfer to the soil

Table 2. *In vitro* root induction in microshoots of *C. epigaeus* on half strength MS medium fortified with various concentrations of IBA

IBA (mg/L)	% response	No. of roots/plantlet (Mean±SE)	Root length (cm±SE)	Callus formation
0	0	0	0	_
0.2	76.66	2.78±0.25	1.86±0.13	+
0.5	100	11.23±0.34	3.03±0.14	+
1.0	100	14.16±0.22	5.3±0.16	+++
1.5	100	12.03±0.26	4.3±0.18	+++
2.0	86.66	5.19±0.13	3.0±0.14	+++
2.5	80	2.33±0.21	1.29±0.09	++
3.0	76.66	2.26±0.14	1.43±0.12	++

SE: Standard error, Extent of callus formation around media dip parts of microshoots: Profuse: +++; Moderate: ++; Poor: +; No response: - Mean is based on three replicates, each of which consisted of ten culture tubes. Data were recorded after 28 days

Table 3. *In vitro* callus induction from stem and leaf segments of *C. epigaeus* on MS medium fortified with various concentrations of BA and NAA

BA+NAA (mg/L)	Stem explant				Leaf explant			
	No. of days after callus induced	Intensity of callus formation	Color	Texture	No. of days after callus induced	Intensity of callus formation	Color	Texture
0+0	0	_	_	_	0	_	_	_
0.5+0.5	11	+	Off-white	Friable	12	+	White	Friable
1.0+0.5	10	+	Off-white	Friable	11	+	White	Friable
1.5+0.5	11	++	Off-white	Friable	10	++	Off-white	Friable
2.0+0.5	7	+++	Off-white	Friable	10	+++	Off-white	Friable
2.0+1.0	6	+	Brown	Nodular	15	++	Off-white	Friable
2.5+0.5	7	++	Brown	Nodular	15	+	Off-white	Friable
3.0+0.5	10	+	Brown	Nodular	-	-	-	-

Observation: Profuse callus: +++; Moderate callus: ++; Poor callus: +; No response: -

3.2 Callus Induction

Quick callus growth was obtained on MS medium supplemented with BA (2.0 mg/ L) and NAA (0.5 mg/L) from stem and leaf explants after 12- days of inoculation (Fig. 2-B, C, D; Table 3). Callus, thus obtained was friable and off-white in color. With further increase in concentration of NAA to 1.0 mg/L, brown compact calli were formed from stem explants. In subsequent subcultures of calli at same composition of medium nodular and brown calli were formed (Fig. 2- E, F). These calli could serve as a source material for extraction of pharmaceuticals and valuable therapeutic substances [49]. Nonetheless, the paste made of *in vitro* raised calli showed wound healing properties in cattle upon external application.

3.3 Rooting and Acclimatization

The well grown shoots were transferred to half strength MS medium containing IBA. Microshoots transferred to half strength MS media without any hormonal supplementation were unable to produce roots; in some shoots rooting was observed but in a very negligible amount. Media supplemented with IBA (1.0 mg/L) produced maximum number of 14.16 roots with average root length of 5.3 cm (Table 2). Similar observations have been reported in *Trichosanthes cucumerina* [41] and *Citrullus lanatus* Thumb. [50]. Within a week of inoculation of microshoots, a callus like brownish mass analogous to tubers first appear at the media dip cut end of microshoot and then minute thread like roots appear from the callus (Fig. 1-D, E); above and below the IBA (1.0 mg/ L), numbers and length of roots gradually decreased. Half strength MS showed more impressive results as compared to the full strength MS media. In terms of response of explants, two concentrations of 0.5 and 1.5 mg/L showed more or less similar results. A medium of 2 MS along with optimal concentrations of IBA was used to boost up the growth of plantlets within a week. It resulted in rapid expansion of leaves almost double in size. Rooted plantlets with well developed leaves were removed from culture tube and washed gently to remove adhering gel. They were transplanted to plastic cups containing sterile sand and kept inside the growth chamber. During 3-4 weeks period plants were irrigated regularly with ¼ strength MS salt solution and then transferred to the soil with 67% success rate (Fig. 1-F). One

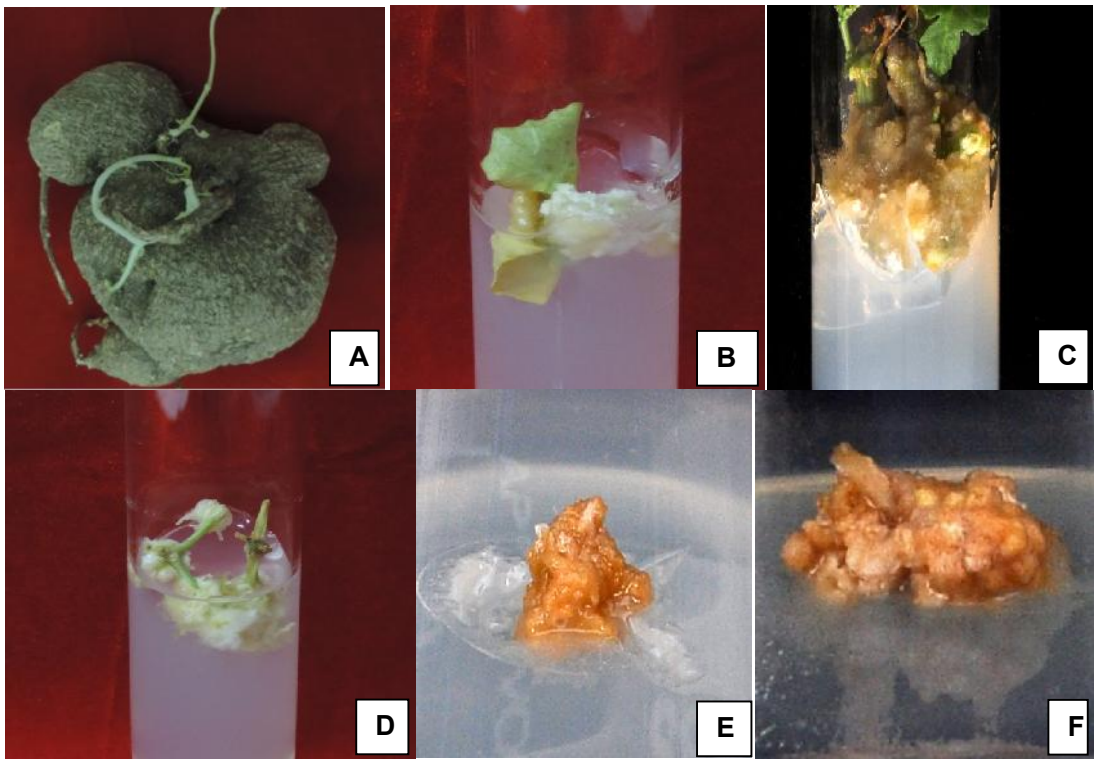

Fig. 2. Callus induction from nodal and leaf segments of the *C. epigaeus* A- External morphology of the Great Root; B, C, D- Off-white friable callus induction on MS+BA (2.0 mg/L) and NAA (0.5 mg/L) after 12-days from leaf and nodal segments respectively E, F- Upon subculture on MS+BA (2.0 mg/L) +NAA (1.0 mg/L), friable calli grew into brownish compact and nodular structures after four and six weeks respectively

hundred and fifty rooted plantlets have been reintroduced to the natural habitats. Morphological characteristics of the plantlets established in field conditions were identical to that of donor plant. However, the juvenile phase of tissue culture raised plantlets may be considered more robust as compared to seed derived seedlings since tissue cultured plantlets have several branches at the beginning whereas seedlings have single branch juvenile phase.

4. CONCLUSION

In conclusion, sterilized nodal explants were inoculated on MS medium containing different concentrations of cytokinins in combination with auxins. Among various treatments BA (0.5 mg/L) along with IAA (2.0 mg/L) were the best for multiple shoot production BA (2.0 mg/L) and NAA (0.5 mg/L) combination was found to be the optimum for callus induction from stem and leaf explants. The best rooting was achieved on half strength MS supplemented with IBA (1.0 mg/L). The rooted plants were transplanted to pots with 67% of success rate and about one hundred and fifty plants have been reintroduced to the natural habitat. Hence, this method is highly beneficial for *ex situ* conservation and propagation for commercial production of tubers of diverse pharmaceutical properties. Nevertheless, in *vitro* induced calli could serve as a source material for commercial isolation of pharmacologically valuable phytochemicals that will avoid overexploitation of plant materials from natural sources. The present paper is the first report that describes a protocol for large scale production of microshoots from nodal segment culture of *C. epigaeus*, callus induction from stem and leaf explants, *in vitro* rooting and acclimatization of plantlets for their successful establishment in the soil.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the Head of the institution, and staff of R.I.E., Ajmer for their direct or indirect cooperation and encouragement. The author also wishes to thank

some herbal practitioners for sharing their knowledge on medicinal formulations of the Great Root and testing the efficacy of wound healing properties of *in vitro* raised calli on animals.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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