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RESEARCH ARTICLES

In vitro regeneration of *Caralluma stalagmifera* var. *stalagmifera* through LCT and ex vitro rooting: a cost effective approach for conservation of succulents

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Abstract

In vitro regeneration of *Caralluma stalagmifera* var. *stalagmifera* has been standardized through liquid culture technique; *C. stalagmifera* var. *stalagmifera* is an important succulent plant species with several medicinal and nutraceutical properties. The present study focuses on in vitro micropropagation through liquid culture technique (LCT) and ex vitro rooting of *C. stalagmifera*. Nodal shoot segments (3–4 cm) of mature 06 months old *C. stalagmifera* var. *stalagmifera* were taken as explant. Murashige and Skoog's liquid medium (without agar) augmented with 0.5 mg L^{-1} BAP(6-benzylaminopurine) was found optimal for bud break; 3.4 ± 0.60 shoots with 2.33 ± 0.49 cm shoot length (SL) was obtained from each node. Shoots were multiplied by sub culturing on various combinations of BAP, indole-3-acetic acid (IAA) and kinetin. Maximum number (5.05 ± 1.60) of shoots obtained on liquid MS media supplemented with 0.1 mg L^{-1} each of BAP, Kinetin and IAA with SL of 3.60 ± 0.75 cm. Ex vitro rooting method was used to initiate rooting of in vitro generated shoots as it provides additional advantage in acclimatization and better adaptation to the newly formed shoots. Shoot bases were treated with various root inducing hormones thereafter transferred to sterilized soilrite and placed in the greenhouse. 90% in vitro regenerated plantlets were rooted successfully. Liquid culture medium is found to be better for micropropagation of plants; it is easy to prepare, less time consuming and require less manual handling and provides early response of cultures. Moreover, liquid culture technique provides fast and easy nutrients absorption and better aeration; results in faster shoot growth. An effective ex vitro rooting method was developed which is better over other methods of rooting as it provides hardening climate to the newly formed plantlets earlier to acclimatization. The liquid culture technique and ex vitro rooting both are helpful in reducing the cost of in vitro regeneration protocol of medicinal succulents.

Keywords Ex vitro rooting · In vitro conservation · Liquid culture technique · Medicinal plant

Abbreviations

MS	Murashige and Skoog's medium
IBA	Indole-3-butyric acid
NOA	2-Naphthoxy acetic acid
IAA	Indole-3-acetic acid
Kn	Kinetin (6-furfuryl amino Purine)
BAP	6-Benzylaminopurine
rpm	Rotation per minute
LCT	Liquid culture technique
SL	Shoot length
PPFD	Photosynthetic photon flux density

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Introduction

Caralluma stalagmifera var. *stalagmifera* is a thick, succulent perennial herb belongs to the family Apocynaceae, growing wild in hilly areas of southern part of India specifically in the states of Tamil Nadu, Karnataka and Andhra Pradesh (Fig. 1). Young shoots of the plant are edible and cooked as vegetable (Parihar 2016). It is a medicinal plant that contains many steroidal glycosides, carumbelloside III, lasianthoside A and B, etc. (Kunert et al. 2006). The aqueous and butanol extracts of whole plant have significant antiarthritic and anti-inflammatory properties when experimented on carrageenin induced rat paw edema and kaolin induced arthritis in rats (Reddy et al. 1996). Thirteen species of *Caralluma* are found in India and eleven in Southern India alone. Only one species of *Caralluma* i.e. *C. edulis* is endemic plant species of the Thar Desert of Rajasthan

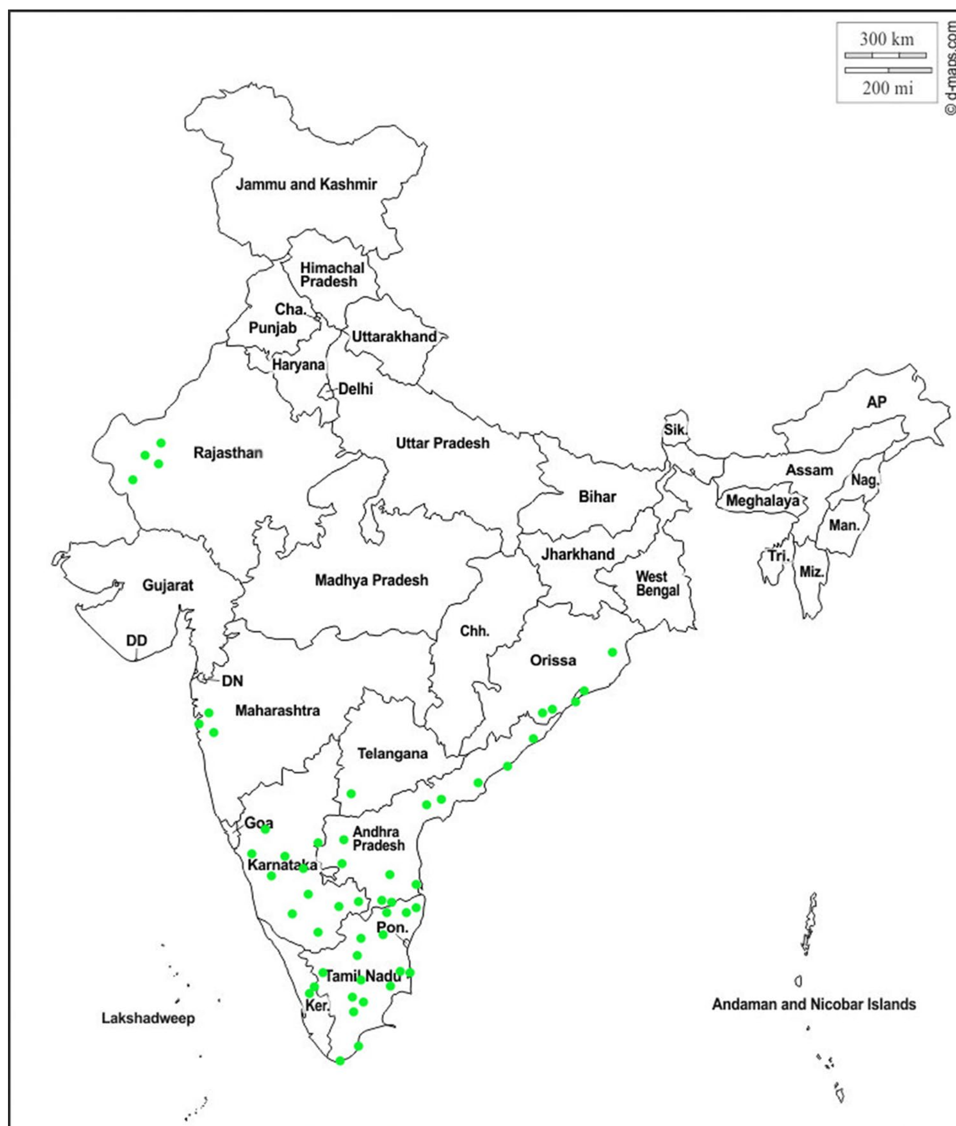

Fig. 1 Distribution of *Caralluma* species in India

(Jagtap and Singh 1999; Parihar 2020) (Table 1). *Caralluma* is generally found in dry habitats and distributed to many countries viz. Saudi Arabia, Ethiopia, Sudan, Jordan, Pakistan, Sri Lanka, Myanmar etc. (Fig. 2). All the *Caralluma* species available in India are used as traditional medicine (Aruna et al. 2009; Parihar 2018).

Caralluma contains many phytochemicals viz. saponins, glycosides, hydrocarbons, and flavonoids which are reported to used in various disorders like diabetes, leprosy, and rheumatism (Aslam et al. 2019; Chandran et al. 2014) (Table 2). Moreover, it has several other medicinal properties viz; antipyretic, antihelminthic, antinociceptive and antiobesity (Venkatesh et al. 2003; Lawrence et al. 2004; Kalimuthu et al. 2013). At the same time, medicinal products are also available in the market containing *Caralluma* extract in powdered/capsulated form for reducing body weight. *Caralluma* species acts as an effective appetite suppressant and weight loss promoter (Dutt et al. 2012). Extract of *Caralluma fimbriata* is available as GENASLIM (trade name) to reduce weight (Sreelatha et al. 2009; Ugraiah et al. 2011). Few patents have been issued for the preparation and usage of the pregnane glycoside for obesity associated problems along with inhibition of citrate lyase which is responsible for weight loss (Kunert et al. 2008; Heuer et al. 2010). Immunomodulatory agent, stigmasterols have been isolated from *Caralluma lasiantha* by Malladi et al. (2017).

The present study focuses on in vitro establishment of *Caralluma stalagmifera* var. *stalagmifera* using liquid culture technique (LCT) and its ex vitro rooting. In vitro liquid culture propagation protocol is successfully used in some other plant species like *Acacia nilotica* (Rathore et al. 2014), *Typhonium flagelliforme* (Rezali et al. 2017), *Caralluma edulis* (Parihar and Dwivedi 2019), *Anethum graveolens* (Bulchandani and Shekhawat 2020), *Stevia*

Table 1 Distribution of Caralluma species in India

S. no.	Plant name	Flowering and fruiting	Distribution
1	*Caralluma adscendens var adscendens*	July–October	AP: Kurnool, Kadapa, West Godavari, Srikakulam, Vishakhapatnam, Guntur Telangana: Mehboobnagar Kerala: Palakkad Odisha: Gajapati, Cuttak, Ganjam, TVM
2	*Caralluma adscendens var attenuata*	Throughout the year	AP: Anantapur, Kadapa, Chittoor, Srikakulam, East Godavari Karnataka: Ballari Kerala: Idukki Odisha: Ganjam Tamil Nadu: Namakkal, Dharmapuri, Vellore, Kanchipuram (Changalpattu-CGP)
3	*Caralluma adscendens var carinata*	July–December	Tamil Nadu: Dindigul, Eastern Ghats
4	*Caralluma adscendens var fimbriata*	Throughout the year	AP: Anantapur, Kurnool Telangana: Mehboobnagar Karnataka: Ballari Tamil Nadu: Salem
5	*Caralluma adscendens var gracilis*	Throughout the year	Tamil Nadu: Dindigul
6	*Caralluma bhupinderana*	–	Tamil Nadu: Thoothkudi
7	*Caralluma bicolor*	August–October	Tamil Nadu: Coimbatore
8	*Caralluma diffusa*	April–September	Tamil Nadu: Coimbatore, Tiruvannamalai
9	*Caralluma edulis*	February–September	Rajasthan: Jaisalmer
10	*Caralluma indica*	October–July	AP: Nellore Kerala: Idukki Tamil Nadu: Coimbatore, Cuddalore, Kanchipuram, Tiruvallur, Villipuram
11	*Caralluma nilagiriana*	–	Tamil Nadu: Sirumalai
12	*Caralluma pauciflora*	–	Kerala: Palakkad
13	*Caralluma procumbens*	September–November	Kerala: Idukki Tamil Nadu: Kanyakumari
14	*Caralluma sarkariae*	September–November	Tamil Nadu: Dindigul
15	*Caralluma sarkariae var longipedicellata*	Throughout the year	Tamil Nadu: Madurai
16	*Caralluma stalagmifera var intermedia*	July–August	AP: Anantapur Tamil Nadu: Dindigul
17	*Caralluma stalagmifera var longipetala*	December–June	Karnataka: Chitradurga, Hassan, Shivamogga Tamil Nadu: Madurai
18	*Caralluma stalagmifera var stalagmifera*	June–July	AP: Chittoor Karnataka: Davanagare, Uttara Kannada Tamil Nadu: Dindigul
19	*Caralluma truncatocoronata*	September	Karnataka: Dharwad
20	*Caralluma umbellata*	March–October	AP: Anantapur, Chittoor, Nellore Karnataka: Ballari, Kolar, Bengaluru, Hassan, Chitradurga, Davanagare, Mysuru, Tamakuru Kerala: Palakkad, Idukki Odisha: Puri, Ganjam, Gajapati Tamil Nadu: Salem, Tiruchirapalli, Villipuram, Tiruvannamalai, Dharmapuri
21	*Caralluma lasiantha*	March–July	AP: Anantapur, Chittoor Tamil Nadu: Dharmapuri

rebaudiana (Bulchandani et al. 2020) and *Vanilla planifolia* (Manokari et al.2021a). On the basis of literature study, present research work is the first report on liquid culture protocol and ex vitro rooting of *C. stalagmifera* var. *stalagmifera*.

Materials and methods

Explant collection and surface sterilization

The plant of *C. stalagmifera* var. *stalagmifera* was brought

Fig. 2 World distribution of *Caralluma* species (Afghanistan, Arabia, Canary Island, Eastern Ethiopia, Eritrea, India, Iran, Jordan, Mauritania, Myanmar, Pakistan, Somalia, South shores of Mediterranean, Sri Lanka, Sudan, Western Europe)

Table 2 Caralluma species containing different phytochemicals

S. no.	Plant name	Metabolite extracted	References
1	*Caralluma adscendens* (Roxb.) Haw. Var. *attenuata*	Flavone glycoside, Luteoline-4′-O-neohesperiodoside, Pentacyclic triterpenoid	Ramesh et al. (1998) Jayalakshmi et al. (2016)
2	*Caralluma adscendens* (Roxb.) Haw. Var. *gracilis*	β-Sitosterol, lupeol, Pregnane glycoside	Kunert et al. (2008) Reddy et al. (2011)
3	*Caralluma adscnedens* (Roxb.) Haw. Var. fimbriata	Pregnane glycosides	Kunert et al. (2008)
4	*Caralluma bicolor* Ramach et al	Alkaloids, flavonoids, glycosides, saponins, triterpens etc.	Vanitha et al. (2019)
5	*Caralluma diffusa* (Wight) N.E.Br	Phenols	Chandran et al. (2014)
6	*Caralluma edulis* (Edgew.) Benth. ex Hook.f	Phenols and flavonoids	Aslam et al. (2019)
7	*Caralluma indica* (Wight & Arn.) N.E. Br	Indicoside I and II	Kunert et al. (2006)
8	*Caralluma nilagiriana* Kumari & Subba Rao	Rutin	Renuka et al. (2014)
9	*Caralluma pauciflora* (Wight) N.E. Br	Pregnane steroids/pregnanes	Reddy et al. (2011)
10	*Caralluma stalagmifera* E.C.E.Fisch	Stalagmoside I–V	Kunert et al. (2006)
11	*Caralluma truncato-coronata* (Sedgw.) Gravely & Mayur	β-Sitosterol, lupeol	Kalimuthu et al. (2013)
12	*Caralluma umbellata* Haw. Syn *C. lasiantha*, *C. campanulata*	Flavones glycoside, luteoline-4′-O-neohesperiodoside, Stigmasterol and C_{21} Pregnane Steroid, steroidal glycoside	Qiu et al. (1999) Ramesh et al. (1999) Malladi et al. (2017)

from Rameshwaram, Tamilnadu, India. It was transported to Jodhpur, Rajasthan and established in earthen pots with soil and manure in the greenhouse of the Department of Botany, J N V. University, Jodhpur (Fig. 3A–C). Fresh shoots (10–12 cm long) were collected and harvested throughout the year. Nodal shoots (3–4 cm) were treated with 0.1% (w/v) Bavistin (a systemic fungicide) for 18–20 min thereafter 5–6 times washed with disinfected water. Consequently, shoots were surface sterilized with 0.1% (w/v) $HgCl_2$ for 3–4 min and washed 7–8 times with distilled water in laminar air flow bench.

Medium and culture conditions

Murashige and Skoog's medium (1962) supplemented with all macro and micro salts, sucrose (3% w/v) with agar (0.8% w/v) and liquid culture medium (LCM) was prepared (all chemicals were procured from HI-MEDIA, Mumbai, India). Before autoclaving the medium, pH was adjusted to 5.8 ± 0.02 using 1 N KOH or 0.1 N HCl. Test tubes (20 mL; Borosil, India) were capped using plugs made up of non-absorbent cotton. In conical flasks (250 mL) cotton cushions and Whatman filter

Fig. 3 Shoot bud induction, multiple shoot production, ex vitro rooting and hardening of *C. stalagmifera*. **A** A plant of *C. stalagmifera*. **B**, **C** A twig of *C. stalagmifera* showing flower bud and flower. **D** Bud breaking from nodal shoot segment in liquid culture medium containing BAP (0.5 mg L^{-1}). **E**–**G** In vitro generated multiple swollen buds in liquid medium. **H**, **I** Multiple shoots in liquid medium containing 0.1mg L^{-1} each of BAP, Kn and IAA. **J** Inoculation of in vitro generated shoots in glass bottles containing soilrite. **K** Ex vitro rooted plantlets during acclimatization process in greenhouse. **L** Ex vitro rooted plantlet of *C. stalagmifera* after pulse treatment of 250 mg L^{-1} IBA. **M** Ex vitro rooted plantlet of *C. stalagmifera* after pulse treatment of 500 mg L^{-1} NOA. **N** Successfully hardened plant of *C. stalagmifera* after 2 months in polybag

paper bridges were used to support the cultures in liquid medium. The medium was autoclaved at 121 °C temperature and 1.06 kg cm^{-2} pressure for 15 min. All the cultures were incubated at 26 ± 2 °C temperature, 12 hd^{-1} photoperiod at irradiance of 40–50 µmolm^{-2} s^{-1} PPFD (Photosynthetic photon flux density; given by cool and white fluorescent tubes [Philips, Mumbai, India]) and 55–60% RH.

Culture establishment, shoots induction and multiplication

Nodal shoots of *C. stalagmifera* var. *stalagmifera* were inoculated in both agar gelled and liquid MS medium containing 3% sucrose and different concentrations (0.1–1.0 mg L^{-1}) of 6-benzylaminopurine (BAP) or Kinetin (Kn) along with 25 mg L^{-1} each of citric acid, arginine, adenine sulphate, and 50 mg L^{-1} of ascorbic acid. The cultures were maintained in the same conditions described earlier on gyratory shaker (Infors HT) with 45 rpm (Rotation per minute). The in vitro generated shoots were amplified by subculture in liquid MS medium augmented with different combinations of BAP (0.1–1.0 mg L^{-1}), Kn (0.1–0.5 mg L^{-1}) and IAA (0.1 mg L^{-1}). Subculture was done regularly with a gap of 15–20 days.

Ex vitro rooting

The cloned and amplified shoots of *C. stalagmifera* var. *stalagmifera* obtained from nodal explants were rooted by ex vitro rooting technique. The micro cloned shoots were excised individually and given pulse-treatment of different concentrations (100, 250, 500, 750 or 1000 mg L^{-1}) of Indole-3-butyric acid (IBA) or 2-Naphthoxy acetic acid (NOA) for 3–4 min and subsequently transferred in glassware containing soilrite (a mixture of perlite (horticulture grade), exfoliated vermiculite and Irish peat supplied by Kel Perlite, Bangalore, India) conditioned with 1/4th concentration of MS salt solution. These were incubated in the greenhouse of Department of Botany, Jai Narain Vyas University, Jodhpur, Rajasthan. Initially, the glass bottles containing the in vitro generated shoots were placed close to the cooling pad unit (RH 75–80% with 26-28 °C temperature) in the greenhouse.

Hardening and acclimatization

After the initiation of roots, lids of the bottles were slightly loosened and after 2 weeks lids were completely removed allowing the in vitro generated plants to acclimatize in the greenhouse atmosphere for 20–25 days. The bottles containing rooted plants undergoing acclimatization were slowly

and gradually shifted from cooling pad section where humidity is high and temperature is low towards the fan segment (low moisture and high temperature, i.e., 55–65% RH and 32 ± 2 °C temp.) of the greenhouse allowing gradual rise in temperature and concurrent reduction in relative humidity.

Transplantation to field conditions

The hardened tissue culture-raised plants which became photoautotrophic and exhibited good growth with height/length of 12–14 cm were transferred to polybags containing a mixture of vermi-compost, soil and sand in equal amounts. After 20–25 days in the greenhouse, the plants were then transfered and maintained in nursery.

The experiments were conducted with 20 replicates per treatment and repeated thrice. The experiments were planned according to randomised block design (Compton and Mize 1999) and data were recorded as mean ± standard deviation.

Results

Green nodal shoot segments collected in the months of February–March were found to be more responsive for induction of shoot buds (Fig. 4). Both agar gelled MS medium and liquid MS medium were experimented for initiation of culture but explants showed bud breaking in vitro only through LCT. No bud break was achieved in MS gelled medium. From each node 3.4 ± 0.60 shoots with shoot length of 2.33 ± 0.49 cm was observed on BAP 0.5mg L^{-1} (Fig. 3D) (Data not shown). The explants did not respond to any concentration of BAP/Kn in gelled MS medium. The shoots performed better with BAP as compared to Kn in this experiment.

Shoots were multiplied through subculture technique on liquid MS medium. As a result of subculturing secondary multiple buds appeared on the surface of primary buds and 3.0 ± 0.64 shoots with 1.3 ± 0.47 cm length was achieved (Fig. 3E–H). The maximum number (5.05 ± 1.60) of shoots were obtained on liquid MS medium augmented with 0.1mg L^{-1} each of BAP, Kn and IAA with average SL of 3.60 ± 0.75 cm, in 30–35 days (Figs. 3I, 5).

Different auxins were tested for ex vitro root induction, out of which IBA was found most suitable for initiation of roots. The maximum rate of rooting (90%) was achieved when the shoot bases were given pulse treatment of IBA (250 mg L^{-1}) for 3 min and produced 12.20 ± 0.94 roots with an average length of 8.85 ± 0.67 cm (Table 3) (Fig. 3L). Higher concentration of IBA (1000 mg L^{-1}) proved harmful for shoots. Plant growth regulator free medium was served as control and no root formation was observed in this medium. More than 85% in vitro raised plants of *C. stalagmifera* were acclimatized after a month (35–40 days) of ex vitro rooting (Fig. 3J–M). Afterwards, the hardened plants were shifted to earthen pots having vermi compost, sand and soil (Fig. 3N). The in vitro generated plants of *C. stalagmifera* were moved to nursery after proper acclimatization in the greenhouse.

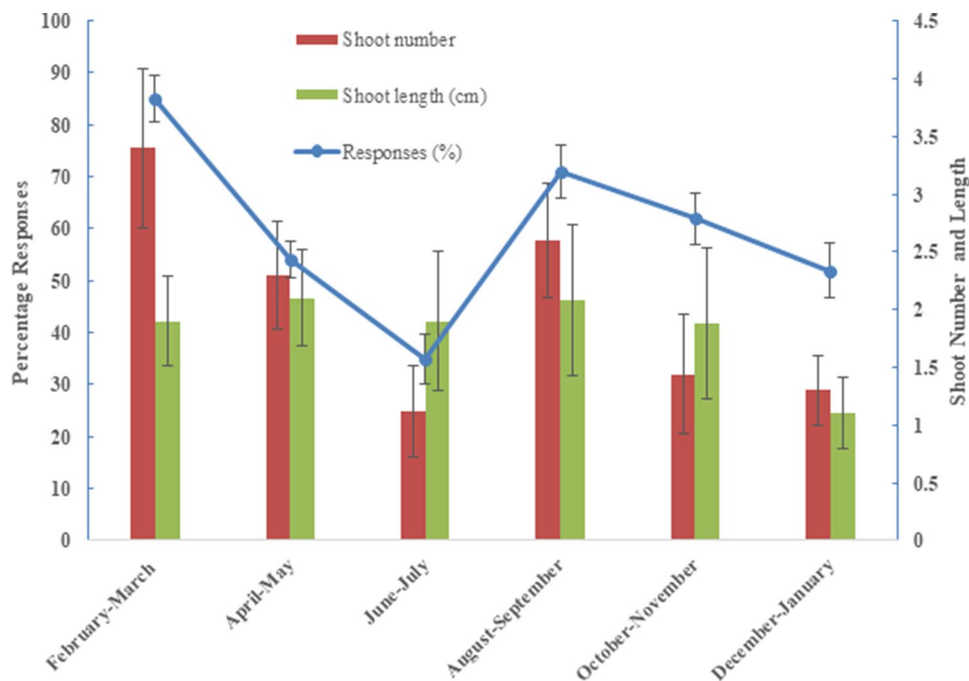

Fig. 4 Effect of different collection season on culture establishment in terms of percentage response, number of shoots and shoots length in liquid culture medium containing 0.5mg L^{-1} BAP

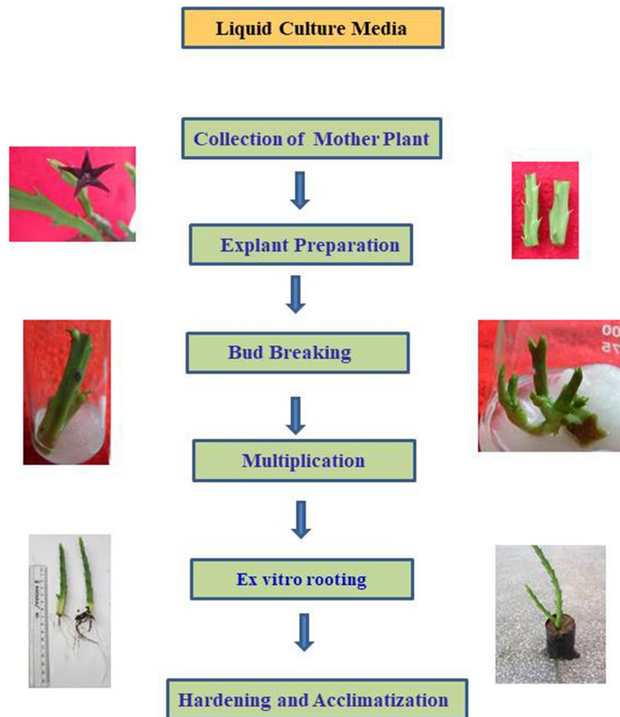

Fig. 5 Flow chart showing diagrammatic representation of the micropropagation protocol of *C. stalagmifera* through liquid culture technique (LCT)

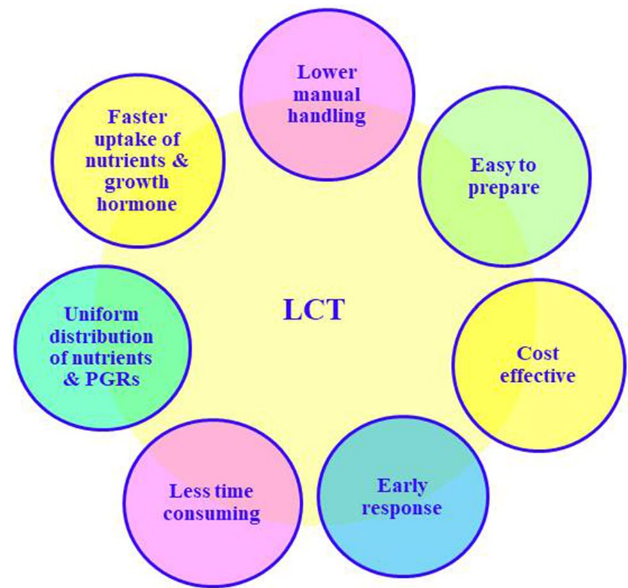

Fig. 6 Diagrammatic representation of advantages of liquid culture technique (LCT)

Discussion

Liquid culture technique provides several advantages over traditional gelled MS medium. The preparation of MS semisolid medium is time consuming and it requires a lot of manual labour; moreover the agar in the MS semisolid medium constitutes about 75–80% of the total cost of medium. In contrast, liquid medium is easy to prepare, less time consuming and it's cost effective also. Using liquid medium for micropropagation of plants reduces the total cost of the protocol. It was observed that explant responded faster in the liquid medium in comparison to semisolid medium. In LCT the explant is in closer contact to the medium than in semisolid medium which enables easy uptake of nutrients and hormone and results in improved in vitro shoot growth (Fig. 6). These all advantages make LCT very popular now in these days for in vitro conservation of important plant species (Pati et al. 2011; Mbiyu et al. 2012; Parihar 2017). BAP proved to be better cytokinin as compared to Kn for bud activation (Shekhawat et al. 2009, 2015; Mathur et al. 2017; Revathi

Table 3 Effect of different concentrations of auxins on in vitro generated shoots of *C. stalagmifera* for ex vitro rooting

Concentration of IBA (mg L^{-1})	Concentration of NOA (mg L^{-1})	Responses (%)	Root number mean ± SD	Root length (cm) mean ± SD
100		70	5.50 ± 0.51	5.75 ± 0.85
250		90	12.20 ± 0.94	8.85 ± 0.67
500		60	6.90 ± 0.96	5.10 ± 0.55
750		50	4.30 ± 0.47	4.50 ± 0.76
1000		10	1.60 ± 0.50	2.95 ± 0.76
	100	25	1.36 ± 0.49	1.60 ± 0.60
	250	35	1.65 ± 0.49	1.90 ± 0.79
	500	45	2.75 ± 0.78	2.55 ± 0.82
	750	35	1.85 ± 0.37	1.10 ± 0.30
	1000	10	1.75 ± 0.55	0.90 ± 0.26

Results are mean ± SD of three independent experiments

et al. 2018; Parihar and Dwivedi 2020; Manokari et al. 2021b).

Ex vitro rooting showed better results than in vitro rooting method. It supported the reducing of cost of the micropropagation protocol; moreover less time is required as both rooting and hardening occurs simultaneously. Ex vitro rooting provides supplementary acclimatization before field transfer (Yan et al. 2010; Ranaweeraa 2013; Manokari et al. 2023) and increases the rate of successful establishment and survival of in vitro generated plants in the natural environment which is actually the main obstacle of the traditional micropropagation protocol. Henceforth, ex vitro rooting is preferred for enhanced root system and faster adaptation in comparison to in vitro rooting.

Conclusions

Caralluma stalagmifera var. *stalagmifera* is an important edible and medicinal succulent member of family apocynaceae. In vitro LCT propagation protocol of *C. stalagmifera* var. *stalagmifera* has been standardized and it was found that a combination of plant growth regulators (BAP with Kn and IAA) is better for shoot multiplication. The study presents micropropagation of the plant species in liquid culture media which is found better for propagation as it is easy to prepare, less time consuming, provides faster growth of cultures and cost effective also. Liquid culture medium proves better for growth of cultures; uniform distribution of plant growth regulators and proper aeration enables the explant to grow at a faster rate. The study shows establishment of an improved ex vitro rooting method which provides prehardening environment to the in vitro generated shoots before acclimatization which helps in better survival of the plants in the natural condition. Present study is the first in vitro regeneration protocol of *C. stalagmifera* var. *stalagmifera* through liquid culture technique.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42535-023-00567-8.

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Author contributions SP conceptualized the study; conducted all the experiments; and prepared the manuscript.

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Data availability Data sharing is not applicable to this article as no datasets were generated during the current study.

Declarations

Conflict of interest Author declares that there is no competing interest.

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