African Journal of Biotechnology Vol. 11(89), pp. 15523-15528, 6 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.032
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Effect of medium, explants, cytokinins and node position on *in vitro* shoot multiplication of *Caralluma lasiantha* (Wight) N.E.Br., an endemic and medicinally important plant

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Accepted 3 August, 2012

An efficient shoot multiplication protocol was developed for *Caralluma lasiantha*, an endemic medicinal plant belonging to the family Asclepiadaceae. Mature explants were cultured on Murashige and Skoog (MS), (1962) medium, Gamborg's B_5 (B_5) and woody plant medium (WPM) supplemented with 6-benzyladenine (BA), 8.87 μM, for shoot induction. The nodal explants exhibited maximum shoot sprouting frequency when cultured on MS medium supplemented with BA (8.87 μM). Nodal explants cultured on MS medium supplemented with different concentrations of cytokinins with 3% sucrose exhibited 100% sprouting frequency. Maximum number of three to four shoots was induced from mature second nodal explants on MS medium containing BA (8.87 μM). The isolated microshoots were rooted on half strength MS medium supplemented with naphthalene acetic acid (NAA), 0.54 μM. The plantlets thus developed were hardened and successfully established in soil.

Key words: 6-Benzyl adenine, nodal explant, naphthalene acetic acid, *Caralluma lasiantha,* micro propagation.

INTRODUCTION

Caralluma lasiantha (Wight) N.E.Br. (Asclepiadaceae) is a succulent, perennial herb with a sour taste, occurring wild in rocky and dry southern parts of south India. Locally, it is known as 'Kundaetikommulu' in Telugu. Two bisdesmosidic C-21 steroidal pregnanes, flavonoid glycoside, flavone glycosides and luteolin-4'-O-neohesperidoside have been reported by Qiu et al. (1999) and Ramesh et al. (1999).

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Abbreviations: BA, 6-Benzyladenine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **MS,** Murashige and Skoog (1962) medium; **NAA,** naphthalene acetic acid; **2-iP,** 2-isopentyladenine; **B5,** Gamborg's B_5; **WPM,** woody plant medium.

Luteolin-4'-O-neohesperidoside is more potent than ibuprofen, and has significant anti-inflammatory action and antinociceptive activity (Ramesh et al., 1998).

The compounds which are present in *Caralluma* are ingredients used in the preparation of drugs. Natural stands are fast disappearing and are threatened with extinction due to indiscriminate collection and over exploitation for commercial purposes and to meet the requirements of the pharmaceutical industry. Therefore, the present study was conducted to develop a rapid *in vitro* propagation system for wild medicinal *Caralluma.* Tissue culture studies on *Caralluma edulis* (Kaur et al., 1992; Rathore et al., 2008), *Caralluma adscendens* (Aruna et al., 2009), *Caralluma sarkariae* (Sreelatha et al., 2009) and *Caralluma bhupenderiana* (Ugraiah et al., 2011) have been reported. However, there is no report on *C. lasiantha* on *in vitro* propagation methods. In the present paper, we report a prime protocol for the

micropropagation of *C. lasiantha.*

MATERIALS AND METHODS

Plant

C. lasiantha was collected from Gooty hills, Anantapur district, Andhra Pradesh, India, and was potted in 15 cm diameter pots and maintained at Sri Krishnadevaraya University garden, Anantapur. The plants were grown at 30 to 35°C with natural day light and irrigated with water as required.

Surface sterilization of explants

Actively growing shoots with seven nodes were used as the source of explants. The shoots were washed under running tap water for 5 min, followed by 1% Tween-20 for 10 min. Shoots were thoroughly washed under running tap water until the traces of Tween-20 was removed. Remaining steps of surface sterilization was carried out under aseptic conditions in laminar air flow chamber. After washing with sterilized double distilled water, surface sterilization was done with mercuric chloride (0.1% w/v) solution for 5 min and rinsed four to five times with sterilized double distilled water. Explants were then subjected to 70% ethanol treatment for 1 min and again washed with sterilized double distilled water at least three to four times. Explants were dissected, damaged ends were removed and blotted on a sterile filter paper disk and inoculated on medium for shoot induction.

Sterilization of media

Explants were cultured on Murashige and Skoog (MS), (1962) medium containing 3% sucrose and solidified with 0.8% agar after adjusting the pH to 5.8, and poured at approximately 15 ml per test tube (150 × 25 mm Borosil, India). The tubes were sealed with aluminum foil and sterilized at 121°C and 1.06 kg cm^{-2} pressure for 15 min.

Shoot induction

Explants were cultured on various media, namely MS, Gamborg's B_5 (B_5) (Gamborg et al., 1968) and woody plant medium (WPM) (Lloyd and McCown, 1981). The morphogenic response of different explant types (node, shoot tip, root and internodal segments) were investigated for efficient and effective production of shoots with all cytokinins. The following concentrations of cytokinins were tested: 6-benzyladenine (BA), 0.44, 4.44, 8.87, 13.31, 22.19 and 35.50 μM; kinetin (Kn), 0.46, 4.65. 9.29, 13.94, 23.23, 37.17 μM; 6-(y-y dimethyl-allylamino)-purine (2iP), 0.49, 4.92, 9.84, 14.7, 24.61 and 39.40 μM; zeatin, 0.46, 4.56, 9.12, 13.68, 22.81, 36.49 μM. Then, the MS medium fortified with various concentrations of cytokinins BA, Kn, zeatin and 2iP individually were investigated to optimize salt and hormonal requirements for shoot induction. Control experiments were carried out in hormone free medium. The shoot regeneration ability of the nodal segments (about 6 cm length) was also studied by using 1, 2, 3, 4, 5, 6 and 7th nodal segments from tip on MS medium fortified with BA (8.87 μM). The cultures were incubated at 25 ± 2°C, with irradiance of 50 μmol m^{-2} s^{-1} for 16 h photoperiod.

Shoot multiplication

After four weeks of incubation, the microshoots were sub cultured for further multiplication to culture bottles containing 50 ml culture media. Culturing of shoots in culture bottles makes the multiplication process cost effective and provides required space for the growth.

Rooting of *in vitro* regenerated shoots

Shoots with 4 to 5 nodes, excised from proliferating shoot were used for root initiation in 0.6% agar gelled medium containing half strength MS salts with 1% sucrose, incorporated with different concentrations of auxins (naphthalene acetic acid (NAA), 0.54, 2.69, 5.37, 10.74, 16.11 μM, indole-3-acetic acid (IAA), 0.57, 2.85, 5.71, 11.42, 17.13 μM and indole-3-butyric acid (IBA), 0.49, 2.46, 4.90, 9.80, 14.70 μM).

Acclimatization

After four weeks of culture, the shoots with well developed roots were removed from culture medium and washed with sterile distilled water. The plants were transplanted in 5 × 5 cm plastic pots containing sand, farmyard manure and peat moss in 1:1:1 ratio and pots were irrigated with half strength basal liquid medium without sucrose. The plastic pots were covered with a polythene bag to maintain humidity and small holes were made. The width of the holes was slowly increased until the humidity inside and outside the polythene bags became equal. Later on, the polythene bag was removed and well developed plant was further transferred into soil.

Data analysis

The experiments were randomized and repeated thrice. Each treatment consisted of 15 replicates. Data were statistically analyzed by analysis of variance (ANOVA) and means were compared by Tukey's test at 0.05% probability level.

RESULTS AND DISCUSSION

Selection of medium

The highest rate of micropropagation often depends not only on the selection of the most suitable explant, but also on the discovery of the correct basal medium for that organ or tissue. Among the three different media used and all the cytokinins tested, MS medium fortified with BA 8.87 μM was found to be the best basal medium for shoot sprouting, number and length without callus formation, followed by B_5 and WPM. All the media were tested with all the cytokinins. The shoot buds sprouted on B_5 and WPM showed only limited growth even if they were maintained for longer period. Thus, need for salts for shoot sprouting and proliferation showed the high salt requirement for the growth of *Caralluma.* MS medium has also been found to be more effective than other media for *in vitro* propagation of plants belonging to Asclepiadaceae by other investigators in *Ceropegia candelabrum* (Beena et al., 2003), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Leptadenia reticulata* (Arya et al., 2003), *Caralluma adscendens* (Aruna et al., 2009) and *C. bhupenderiana* (Ugraiah et al., 2011).

Selection of explants

Among the various explants tested, only those of node and shoot tip showed positive morphogenic response. Root and internodal explants did not respond even after one month of incubation on the medium. Nodal explants responded with 80% bud break and emergence of bud within a week on MS medium supplemented with BA (8.87μM) with 3% sucrose. The highest number of shoots (2.6 shoots/explant) with mean length of shoot (2.83 cm) was obtained from nodal explants of *C. lasiantha* cultured on MS medium fortified with BA (8.87 μM), whereas from shoot tip explants, 1.4 shoots/explant with mean length of 2.12 cm shoot was obtained on MS medium fortified with BA (8.87 μM). There was no response in hormone free medium (not shown in the table).

Shoot induction by nodal explants

Since the shoot multiplication rate was very effective with nodal explants than the shoot tip explants, shoot induction experiments were carried out with nodal explants in *C. lasiantha*. There are numerous examples in literature showing that nodal explants are most effective in Asclepiadaceae, these include *C. adscendens* (Aruna et al., 2009), *C. candelabrum* (Beena et al., 2003), *Cryptolepis buchanani* (Prasad et al, 2004), *Decalepis arayalpathra* (Gangaprasad et al., 2005; Sudha et al., 2005), *G. sylvestre* (Komalavalli and Rao, 2000), *Leptadenia reticulata* (Arya et al., 2003), *Wattakaka volubilis* (Chakaradhar and Pullaiah, 2006) and *C. adscendens* (Aruna et al., 2009).

Nodal explants of *C. lasiantha* cultured on media supplemented with various concentrations of BA showed different response during primary establishment. The response of nodal explants treatment results are presented in Table 1. Out of these treatments, medium fortified with BA (8.87 μM) had a better shoot sprouting frequency of 80% with 2.6 shoots/explants, and attained a length of 2.83 ± 0.12 cm without basal callus. High concentration of BA (13.31 μM) resulted in reduced number of 2.13 shoots/explant with a shoot length of 2.26 cm. Various concentrations of Kn containing cultures produced less than two shoots per explant. Kn at the concentration of 9.29 μM induced 1.93 shoots with shoot length of 1.95 cm. The response of shoot induction was investigated on MS medium containing various concentration of 2iP. 2iP (9.84 μM) resulted with 75% shoot sprouting frequency and with highest shoot length of 3.03 ± 0.04 cm. Similarly, various concentrations of Zeatin were used for shoot induction. Better shoot sprouting frequency (73%) with shoot number of 2.13 ± 0.14 shoots/explant was induced on zeatin (9.12 μM). When the concentration of zeatin (13.68 μM) was increased, the number of shoots reduced to 2.00 shoots/explant.

Among different hormonal concentrations tried in the present study, BA (8.87 μM) produced maximum number of shoots with minimum time for bud break. In *C. lasiantha*, maximum shoot length (3.03 cm) resulted with 2iP 9.84 μM. Sprouting of axillary bud was observed within a week of culture. Initially, one to three shoots were formed from axillary bud but multiple shoot formation was observed only after one subculture in the same medium.

Shoot multiplication

Another important factor that determines the morphogenetic response of the explant is physiological age. Seven nodes when cultured on MS medium fortified with BA 8.87 μM showed significantly different response. 2nd node position from above produced maximum number of 3 to 4 shoots with maximum shoot length when compared to shoot tip and other nodal position (Figures 1, 2A and B). Most mature seventh node showed least number of shoots and shoot length. Sreekumar et al. (2000) have also reported similar type of response in case of *Hemidesmus indicus*. After 30 days of incubation, cultures from test tubes were transferred to culture bottle for further multiplication of shoots. After 30 days of incubation of explants in test tube, maximum shoots were produced; this will become difficult for the further growth of shoots in test tube. Transfer of cultures to culture bottles enhanced growth of shoots by axillary branching within few days. The shoot multiplication at an enhanced pace by subsequent cultures observed in this study (Figure 2C) is in agreement with the reports of other Asclepiadaceae members such as *G. sylvestre* (Komalavalli and Rao, 2000) and *H. indicus* (Sreekumar et al., 2000).

Rooting of *in vitro* regenerated shoots

In *C. lasiantha*, half strength MS medium supplemented with three auxins such as NAA, IAA and IBA at different concentrations showed varied effect of rooting (Table 2). NAA 0.54 μM induced 8.73 ± 0.35 roots with 88% of rooting frequency (Figure 2D). However, increasing concentrations of NAA induced the formation of short, thick fleshy roots. Although, IAA did not induce callusing, the percentage of rooting and the number of roots formed were poor. Maximum of 4.80 ± 0.22 roots per shoot with 4.92 ± 0.04 cm root length was developed on IAA 2.85 μM. Among different treatments with IBA, IBA (9.80 μM) induced 4.53 ± 0.19 roots per shoot with 2.67 ± 0.02 cm root length. Root induction was possible with all auxins. However, survival of plantlets required strong, healthy, long roots, which were obtained from half strength MS medium supplemented with NAA 0.54 μM. The reason for the reduced survival in higher concentrations of NAA

Table 1. Individual effect of various concentrations of BA, Kn, 2iP and Zeatin on shoot formation in mature nodal explant of *Caralluma lasiantha* cultured on MS medium with 3% sucrose.

Plant growth regulator (µM)				Shoot sprouting frequency (%)	Mean shoot number per explant ± SE	Mean length of shoots (cm) ± SE
BA	Kn	2iP	Zeatin			
0.44	-	-	-	68	1.26±0.08^{c}	1.56±0.03^{ef}
4.44	-	-	-	75	1.66±0.10^{bc}	2.57±0.04^{b}
8.87	-	-	-	80	2.60±0.12^{a}	2.83±0.12^{ab}
13.31	-	-	-	71	2.13±0.15^{ab}	2.26±0.05^{cd}
22.19	-	-	-	66	1.86±0.14^{bc}	2.00±0.05^{d}
35.50	-	-	-	57	1.46±0.09^{bc}	1.62±0.03^{ef}
-	0.46	-	-	20	0.93±0.10^{c}	1.05±0.03^{g}
-	4.65	-	-	37	1.26±0.10^{c}	1.62±0.03^{ef}
-	9.29	-	-	60	1.93±0.14^{b}	1.95±0.04^{de}
-	13.94	-	-	57	1.73±0.10^{bc}	2.24±0.04^{cd}
-	23.23	-	-	46	1.33±0.10^{bc}	1.60±0.04^{ef}
-	37.17	-	-	28	1.06±0.06^{c}	1.07±0.04^{g}
-	-	0.49	-	55	1.26±0.10^{c}	1.62±0.03^{ef}
-	-	4.92	-	66	1.86±0.12^{bc}	2.56±0.03^{b}
-	-	9.84	-	75	2.33±0.27^{ab}	3.03±0.04^{a}
-	-	14.79	-	68	1.80±0.26^{bc}	2.72±0.03^{b}
-	-	24.61	-	60	1.53±0.12^{bc}	2.12±0.04^{cd}
-	-	39.40	-	53	1.26±0.20^{c}	1.76±0.05^{e}
-	-	-	0.46	48	1.13±0.10^{c}	1.02±0.03^{g}
-	-	-	4.56	55	1.66±0.14^{bc}	1.98±0.03^{de}
-	-	-	9.12	73	2.13±0.10^{ab}	2.30±0.04^{c}
-	-	-	13.68	64	2.00±0.10^{ab}	2.98±0.04^{a}
-	-	-	22.81	57	1.86±0.10^{bc}	2.02±0.03^{d}
-	-	-	36.49	51	1.53±0.12^{bc}	1.44±0.02^{f}

Means ± SE, n = 45. Means followed by the same letter are not significantly different by the Tukey test at 0.05% probability level.

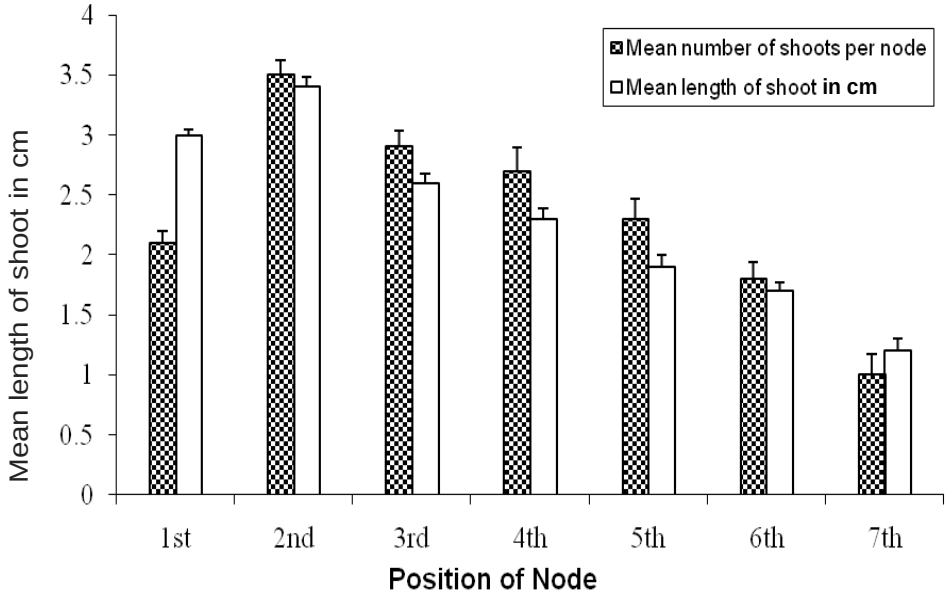

Figure 1. Effect of node position of *Caralluma lasiantha* on MS medium containing BA 8.87 µM after 30 days of culture.

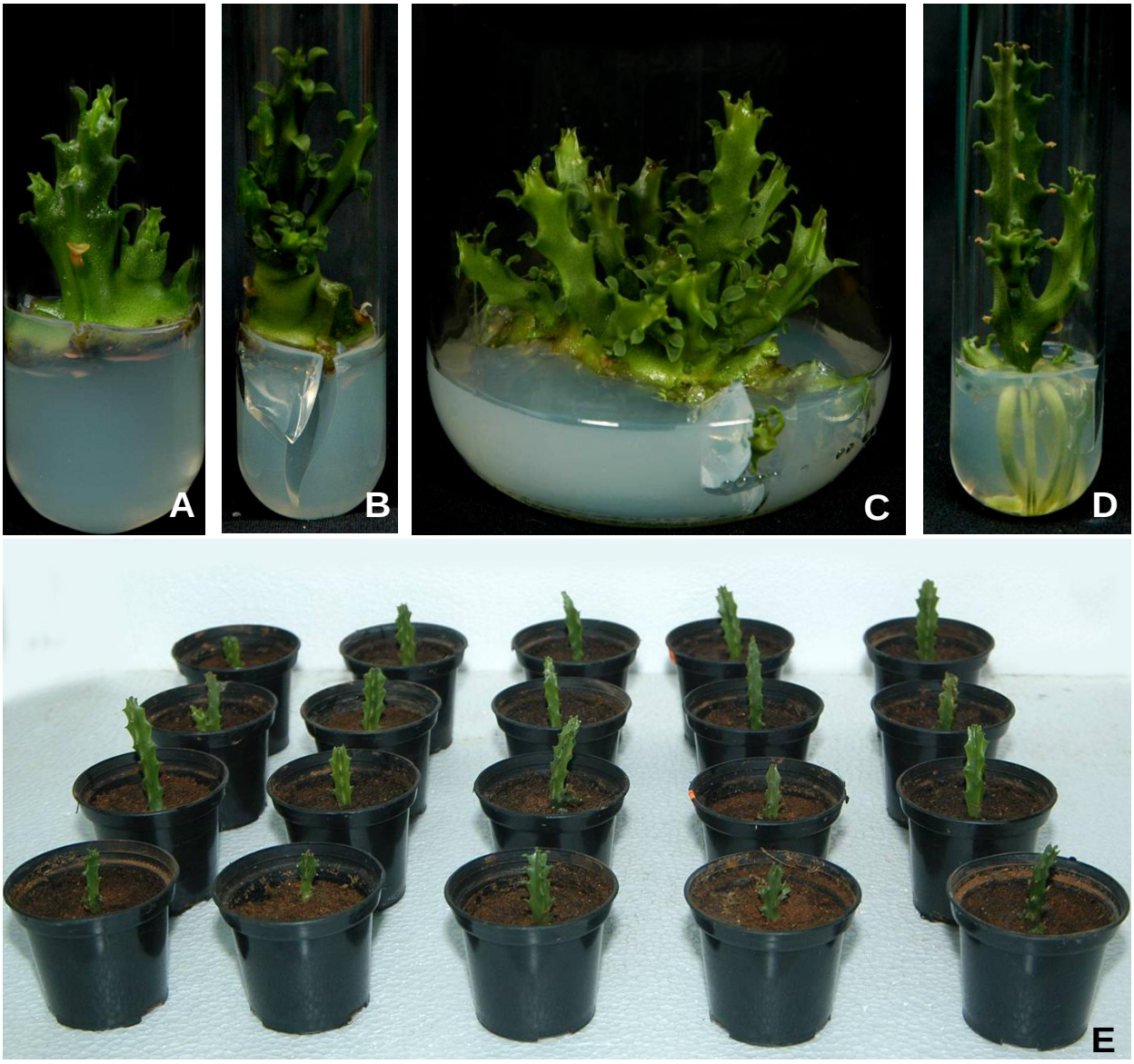

Figure 2. *In vitro* propagation of *C. lasiantha*. (A) Shoot regeneration from shoot tips explants cultured on MS medium fortified with BA 8.87 µM after 30 days; (B) shoot regeneration from second nodal explants cultured on MS medium fortified with BA 8.87 µM after 30 days; (C) shoot multiplication on MS medium fortified with BA 8.87 µM after 50 days; (D) *In vitro* rooting of in vitro raised shoots on ½ MS medium fortified with NAA 0.54 µM after 25 days; (E*) In vitro* rooted plants transferred to containing peat moss, farmyard manure and soil after two weeks.

treatments may be due to poor vascular connection of the root with the stem because on the intervention of callus. The positive response of rooting in the present study is similar to observations of other Asclepiadaceae member *D. arayalpathra* (Sudha et al., 2005). Similar observations are also reported in *Euphorbia tirucalli* (Uchida et al., 2004).

Acclimatization

In vitro regenerated plantlets with well developed shoots and roots were washed with water and were transferred to pots containing peat moss, farmyard manure and garden soil in 1:1:1 ratio. The potted plants were covered with polythene cover to ensure high humidity and irrigated every three days with half strength MS macro salt free of sucrose for two weeks (Figure 2E). After two weeks, the hardened plantlets were transferred to earthen pots and maintained under shade for one more week. Then, plants were exposed to sunlight for few hours for a week and then plants were transferred to soil and watered with tap water. The rooted plants were successfully established in soil with 75% survival rate in *C. lasiantha*.

Table 2. Rooting response of *in vitro* regenerated shoots of *C. lasiantha* in half strength MS containing NAA, IAA and IBA in various concentrations with 1% sucrose after 25 days.

Plant growth regulator (μM)			Response (%)	Mean number of roots per shoot ± SE	Mean length of roots (cm) ± SE	Degree of callusing
NAA	IAA	IBA				
0.54	-	-	88	8.27±0.31^{d}	5.47±0.04^{a}	-
2.69	-	-	66	14.07±0.31^{c}	3.22±0.03^{e}	-
5.37	-	-	60	14.87±0.23^{c}	1.33±0.02^{i}	-
10.74	-	-	55	17.07±0.26^{b}	1.21±0.03^{ij}	+
16.11	-	-	40	18.40±0.27^{a}	1.08±0.03^{j}	++
-	0.57	-	60	3.13±0.17^{fg}	4.47±0.02^{d}	-
-	2.85	-	75	4.80±0.22^{e}	4.92±0.04^{b}	-
-	5.71	-	66	4.47±0.21^{ef}	4.76±0.05^{c}	-
-	11.42	-	60	3.93±0.15^{ef}	4.55±0.04^{d}	-
-	17.13	-	55	2.87±0.14^{fg}	4.41±0.03^{d}	-
-	-	0.49	40	1.33±0.09^{h}	1.91±0.04^{h}	-
-	-	2.46	55	2.47±0.12^{g}	2.51±0.04^{g}	-
-	-	4.90	66	3.67±0.20^{f}	2.55±0.02^{fg}	-
-	-	9.80	71	4.53±0.19^{ef}	2.67±0.02^{f}	-
-	-	14.70	68	3.93±0.21^{ef}	2.46±0.03^{g}	-

Means ± SE, n = 45. Means followed by the same letter are not significantly different by the Tukey test at 0.05% probability level; -, no callusing, number of +, the intensity of callusing.

Conclusion

The outlined procedure offers a potential system for improvement, mass propagation and conservation of *C. lasiantha*.

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