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Factors Affecting the *in vitro* Multiplication of the Endemic Zingiber *Curcuma haritha* Mangaly and Sabu

M. Bejoy, M. Dan and N.P. Anish
Commercial Tissue Culture Unit, Tropical Botanic Garden and Research Institute,
Palode, Thiruvananthapuram-695 562 Kerala, India

Abstract: *In vitro* shoot multiplication studies were carried out in *Curcuma haritha* using rhizome tip explants. The rate of multiplication depended mainly on plant growth regulator, its concentration and nutrient media. Optimal morphogenic responses were obtained from longitudinally split explants on Murashige and Skoog basal medium supplemented with N^6-benzyladenine and indole-3-acetic acid or α-naphthalene acetic acid. The best shoot multiplication of 11 shoots per explant was achieved on MS medium containing BA (4.4 μM) and IAA (2.9 μM). Rooting was also observed simultaneously in the multiplication medium. Rooted plantlets were successfully transferred to greenhouse conditions with 98% survival. Effect of plant growth regulators, nutrient formulation, pH and agar concentrations on various *in vitro* responses are discussed.

Key words: Micropropagation, Zingiberaceae, endemic species, Western Ghats

INTRODUCTION

Curcuma haritha Mangaly and Sabu, is an aromatic rhizomatous herb in the ginger family, Zingiberaceae. The species is endemic to Southern Western Ghats, especially Kerala (Jain and Prakash, 1995) and distributed in partially open fields or as undergrowth in plantations. The rhizomes of *C. haritha* yielded considerably good amount of essential oil (0.58%) which contained about 21.24% of camphor (Dan *et al*., 2002). Locally, the rhizomes are used for the extraction of Indian arrowroot (Sabu and Mangaly, 1996). Natives of Parambikulum forest region of Palakkad District (Kerala), apply the paste of the rhizome to repel blood sucking leeches (Dan, 2003). The antimicrobial properties of *C. haritha* were reported by Umesh *et al.* (2003). Its essential oil can be used to develop commercial herbal microbicides. This underexploited wild relative of turmeric is a potential candidate for further pharmacological investigations.

C. haritha, is propagated vegetatively from the rhizome. As an endemic species, its valuable germplasm deserves special conservation measures. Tissue culture technique can be utilised for the propagation and mass multiplication of rare and endemic plants and their subsequent *ex situ* conservation (Henshaw, 1981). *In vitro* plantlet regeneration and medium-term genotype conservation has been optimised in a few wild *Curcuma* sp. (Tyagi *et al*., 2004).

In vitro clonal multiplication methods have been reported for various species of the genus *Curcuma* viz., *C. zedoaria*, *C. aromatica* (Yasuda *et al*., 1987), *C. aeruginosa*, *C. caesia* (Balachandran *et al*., 1990) and *C. amada* (Barthakur and Bordoloi, 1992; Prakash *et al*., 2004). Rapid multiplication of *C. longa* has also been achieved from buds pre-treated with thidiazuron (Prathanturarug *et al*., 2005). In *Kaempferia galanga* and *K. rotunda* microrhizome formation were achieved from four months old *in vitro* shoots (Chirangini *et al*., 2005). Although a number of studies have been reported on *in vitro* regeneration, most dealt with evaluation of Plant Growth Regulators (PGRs) with a single nutrient formulation system. Evaluation of different nutrient media on new taxa is beneficial especially under conditions of poor multiplication rate. The optimum response of explant in culture is dependent on the basal medium and PGR, ideal nutrient condition is essential to allow explants to express their full potential. According to Preece (1995) the tissue culture medium is crucial and it can partially substitute PGRs. It is useful to study such important factors and optimise regeneration systems for *C. haritha*. Moreover, to our knowledge, there is no report on tissue culture studies in this endemic member of Zingiberaceae. Here in, we have studied and compared the effect of different PGRs, nutrients formulations, agar concentration and pH levels on *in vitro* morphogenic responses of *C. haritha*.

Corresponding Author: M. Bejoy, Commercial Tissue Culture Unit, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram-695 562 Kerala, India

MATERIALS AND METHODS

Plant material and explant preparation: Young finger shaped rhizomes of *C. haritha* were collected during the months July-October from the Zingiberaceae germplasm of Medicinal Garden of the Tropical Botanic Garden and Research Institute, Palode. About 3.0 cm apical buds from the rhizomes were excised and washed thoroughly in running tap water and soaked in 2% (v/v) commercial bleach and 0.2% (v/v) Teepol (Reckitt Benckiser Ltd., Kolkata, India) initially for 20 min. They were treated with 15% (v/v) bleach solution for 15 min and subsequently with 0.1% (w/v) $HgCl_2$ for 6 min. Then the buds were rinsed in sterile distilled water thrice and trimmed into 3.0-4.0 mm size sections with terminal bud which served as the primary explant. After 30-35 days of culture, the explants showing 1.0-2.0 cm shoots were subcultured in the same media. Initially, this was done in two ways ie., half of them were longitudinally split into two equal halves (LS explants) and the rest subcultured as whole explants. Considering the suitability, all further experiments were carried out using split explants.

Media and culture conditions: Basal MS medium (Murashige and Skoog, 1962), supplemented with different combinations and concentrations of PGRs (Table 1) and sucrose at a concentration of 30.0 g L^{-1} was used. Standard procedures were followed for media preparation and culture maintenance (Vincent *et al*., 1992). Appropriate changes were made to this basic composition in accordance with following different experiments.

Effect of plant growth regulators: As no protocol for tissue culture of *C. haritha* has yet been published, a broad range of experiments were designed to determine the most effective PGR condition for multiplication. The LS explants were inoculated onto basal media containing various combinations of N^6-benzyladenine (BA), indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA) and kinetin (KN) to study their effect (Table 1). Initially, individual cytokinins (BA 4.4-13.3 μM and KN 2.3-4.6 μM) were also tested. Based on the above treatments the PGR regime of 4.4 μM BA and 2.9 μM IAA was selected for all further experiments.

Effect of nutrient media: Four mineral salt media, viz. MS, White (1963), Nitsch (1969) and Mitra *et al.* (1976) formulations were evaluated.

Effect of pH: Basal media with five different pH levels (5.3, 5.5, 5.7, 5.9, 6.1) were also studied to find out the suitable pH of the medium for maximum production.

Table 1: Effect of growth regulators on shoot multiplication from rhizome tip explants of *C. haritha*

Treatment (μM)*				Mean No. of shoots**	Average shoot length (cm)**	Mean No. of roots**
BA	KN	IAA	NAA			
4.4	2.3	-	-	6.5±0.89^b	4.4±0.40^a	15.8±0.95^b
4.4	4.7	-	-	5.5±1.4^c	4.2±0.34^a	13.8±4.26^b
8.9	2.3	-	-	5.7±0.67^b	4.4±0.38^a	14.7±4.26^c
8.9	4.7	-	-	4.0±0.52^c	5.4±0.29^a	16.6±1.77^b
4.4	-	0.6	-	8.3±1.02^a	4.9±0.23^a	21.3±6.93^a
4.4	-	2.9	-	10.8±1.07^a	3.2±0.24^b	11.3±2.63^c
4.4	-	5.7	-	8.1±0.54^b	4.3±0.26^a	19.7±4.34^a
8.9	-	0.6	-	7.8±1.14^b	4.1±0.28^a	23.3±6.90^b
8.9	-	2.9	-	5.5±0.62^c	3.9±0.35^b	24.0±3.01^a
8.9	-	5.7	-	5.6±1.33^b	4.6±0.31^a	26.0±2.10^a
13.3	-	2.9	-	4.8±0.87^c	4.3±0.24^a	19.0±5.07^b
4.4	-	-	2.7	3.8±0.65^c	3.9±.28^b	27.3±4.30^a
4.4	-	-	5.3	3.0±0.68^c	3.6±0.52^b	28.0±3.33^b
8.9	-	-	2.7	5.5±0.62^c	4.3±0.29^a	27.2±3.43^a
8.9	-	-	5.4	3.8±0.87^c	3.9±0.35^b	25.0±5.38^b

*Basal medium: MS + 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar and pH 5.7. **All data after 6 weeks of culture. Means in a column with the same letter do not differ significantly ($p<0.05$, LSD test)

Effect of agar concentration: Multiplication medium fortified with various concentrations of agar (0, 4.0, 7.0 and 10.0 g L^{-1}) were studied to demonstrate the most effective level of the gelling agent.

Deflasking and greenhouse transfer: Healthy shoot clumps were washed thoroughly and then immersed in a commercial fungicide (Indofil M-45) for 5 min before planting in earthen pots containing a potting mixture of river sand and broken charcoal (3:1). The plantlets were maintained in a greenhouse under semi-shade (75%) and high humid (RH 75-85%) conditions for hardening. After 60 days of hardening individual plantlets were isolated carefully and re-potted in small poly bags for further establishment.

Statistical analysis: Each treatment contained 10 replicates and was repeated twice. All experiments were conducted in a completely randomised manner. Microsoft Excel 98 PC programme was used for statistical analysis. Data were analysed by ANOVA ($p< 0.05$) and means were compared using Least Significant Difference (LSD) test.

RESULTS

Effect of growth regulators: Two sets of experiments were conducted to find out the suitable inocula. In the first set, whole explants were utilised for the entire culture period. In the second, the explants were split longitudinally (LS) after the initiation of cultures (Fig. 1A). LS inocula always showed more multiplication response than whole explants. This procedure resulted in approximately 45% more shoots regardless of the media (data not shown). Therefore, all further experiments were conducted with LS

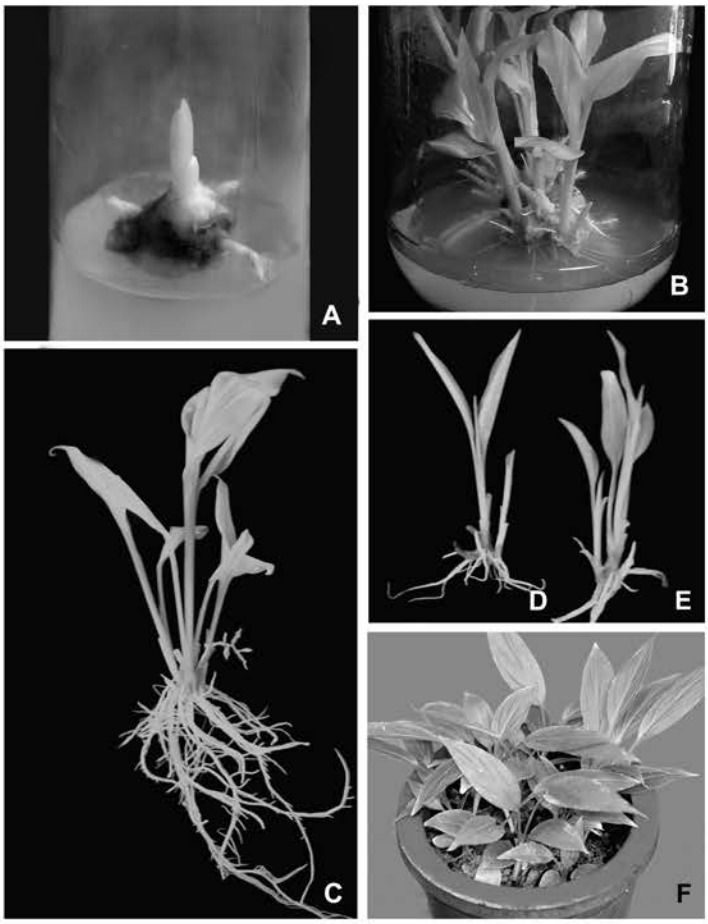

Fig. 1: Morphogenic response of rhizome tip explants of *C. haritha* after 6 wk of culture (A) *In vitro* proliferation of rhizome tip explant after 4 week (B) Induction of multiple shoots on MS + 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} IAA and pH 5.7 (C) Shoot multiplication on Mitra basal medium containing 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} IAA and pH 5.7 (D) Response of explants on White's basal medium (E) Shoot regeneration on Nitsch basal medium (F) Hardened microplants in clay pots after 10 week of transfer

explants. About 40% of the explants in PGR free MS basal medium produced single slender shoots with 4-5 roots. To increase the multiplication rate, the explants were inoculated on to MS medium supplemented with different PGRs, either alone or in combination. Single treatments of BA or KN showed only the growth of scale leaves and occasional development of 1-2 shoots after 35-40 days of culture (data not shown). Subsequently, another group of experiments employing various combinations of auxins and cytokinins was designed to find out the optimum PGR requirement.

The explants on different combinations and concentrations of PGRs showed a large variability in culture response. The shoot multiplication was found to occur by development of axillary buds from the axil of the scale leaves. About 92% of the explants initiated more than 2 shoots in all the PGRs tested (Table 1). The presence of BA along with IAA in the medium markedly increased the number of shoots produced per explant. The highest shoot induction was found in 4.4 μM BA and 2.9 μM IAA, which produced nearly 11 shoots in LS explants, compared with 4 shoots obtained on medium substituted with another auxin (Fig. 1B). However, this treatment resulted in reduced rhizogenic activity and shoot elongation response of the neo-formed shoots (Table 1). Higher concentrations of BA was found to be inhibitory to shoot multiplication irrespective of the IAA concentrations used. Rhizome tip explants cultured on

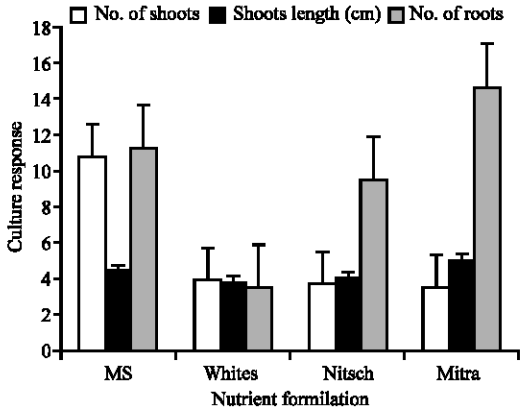

Fig. 2: Influence of nutrient formulations on *in vitro* multiplication rhizome tip explants of *C. haritha* on a basal medium of 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} IAA and pH adjusted to 5.7. Vertical bars are mean±SE

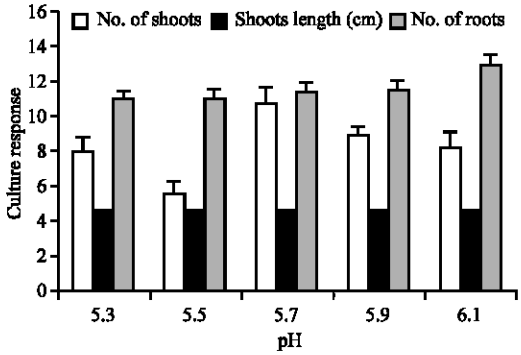

Fig. 3: Effect of pH on shoot multiplication of *C. haritha* explants on a basal medium of MS + 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar + 1.0 mg L^{-1} BA + 0.5 m g L^{-1} IAA. Vertical bars are mean±SE

medium containing 8.9 μM BA and 2.3 μM IAA produced an average of 5.5 shoots. Concentrations of IAA above 2.3 μM also showed a reduction in the rate of multiplication. When NAA was used instead of IAA, shoot bud induction and its growth were decreased, whereas the rhizogenic response was maximum compared to all other treatments. However, with this PGR combination, the shoots appeared to be healthier compared to other PGR treatments. Despite the promotive effect of cytokinins, shoot regeneration frequency was not improved significantly in BA and KN combinations. The rate of multiplication in treatments with BA and KN was intermediate between treatments with BA+IAA and BA+NAA.

Root initiation was observed after 25-30 days of incubation and the simultaneous formation of shoots and

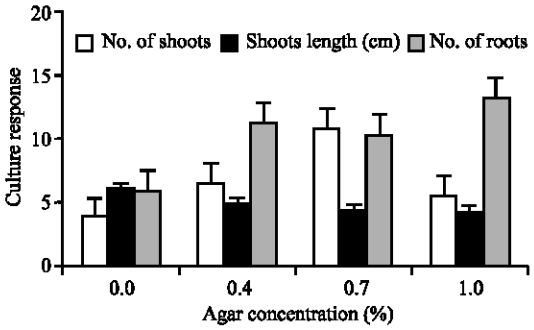

Fig. 4: Effect of gelling agent on morphogenic response of *C. haritha* after 6 wk of culture on MS + 30.0 g L^{-1} sucrose + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} IAA and pH 5.7. Vertical bars are mean±SE

roots were observed in all treatments regardless of the concentration of the PGRs used. As compared with IAA, the response to NAA treatment of explants was better (Table 1). The best rooting response with an average of 28 roots was observed when the explants were treated with BA (4.4 μM) and NAA (5.4 μM).

Effect of basal media: Experiments to ascertain the effect of selected nutrient formulations revealed visible variability on *in vitro* responses in *C. haritha.* The use of relatively low salt formulations especially White's medium showed least response with regard to shoot growth and rooting. The shoots were also weak with narrow leaves in these formulations compared to shoots obtained on MS medium. About 35 and 40% of the explants initiated more than 2 shoots in Mitra and White's basal media respectively while 80% developed 5 shoots or above on MS medium with the same PGR regime. The best shoot multiplication response of an average of 10.8 shoots and moderate rooting (11.3 roots) were recorded on MS basal medium supplemented with BA (4.4 μM), IAA (2.9 μM) and sucrose (30.0 g L^{-1}), proving its suitability as basal nutrient formulation for the *in vitro* multiplication of *C. haritha* system (Fig. 2). Though the shoot growth and rooting were better in Mitra medium than other formulations, shoot production was significantly reduced (Fig. 1C). The explants also showed reduced multiplication in both Nitsch (Fig. 1D) and White's (Fig. 1E) formulations.

Effect of pH: Of the different levels of pH tested, 5.5 was found best for the induction of multiple shoots, with a mean number of 11.1 shoots. An alteration from this optimum pH showed an unfavourable response and reduced the rate of multiplication (Fig. 3). The explants incubated at various pH levels did not show significant

difference in shoot elongation while slight incremental effect of rooting response was recorded when the pH was enhanced from 5.5 to 6.1. At lower pH level (5.3) the shoots were weak compared to the regenerants from 5.5 and above.

Effect of agar: In agar free media, sizeable difference in shoot multiplication, shoot elongation and rooting were detected in *C. haritha* (Fig. 4), besides, showing healthy shoot and root growth. The medium containing a low concentration of agar (4.0 g L^{-1}) supported shoot elongation, but exhibited a low frequency of axillary shoot formation. With an increase in the level of agar to 7.0 g L^{-1} in the medium, the number of adventitious shoots and roots also increased to 10.8 and 11.3 respectively, while shoot growth was reduced. With further increased level of agar (10.0 g L^{-1}), the explants showed reduced growth and development of shoots from rhizome explants. Furthermore, rhizogenic response from the neo formed shoots was also enhanced.

Greenhouse transfer: About 90% of the shoots survived in non-sterile potting mixture. But most of the shoots below 2.0 cm perished mostly due to necrosis or decay. The plantlets isolated from the clumps after 60 day of hardening showed a survival rate of 98% after 4 weeks of repotting. After 35-45 days of transfer, new leaves were also developed from the *in vitro* shoots (Fig. 1F).

DISCUSSION

Morphogenic responses vary with different combinations of PGRs. In *C. haritha*, the use of whole explants after shoot initiation always showed less response than LS explants, regardless of the culture media used. This is in agreement with the results reported in *Allium wallichii* Kunth. (Warwrosch *et al*., 2001), *Narcissus* sp. (Squires and Langton, 1990) or *Yucca glauca* (Bentz *et al*., 1988). In the present study, the combination of two PGRs was found to be more effective than when used singly. Similar results were observed in *Kaempferia galanga* (Vincent *et al*., 1992) and in *K. rotunda* (Anand *et al*., 1997). A combination of BA (4.4 μM) and IAA (2.9 μM) was found to be most effective in *C. haritha* for shoot multiplication. The effect of BA along with IAA on multiple shoot induction was also reported in *Alpinia calcarata* (Agretious *et al*., 1996) and *A. galanga* (Anand and Hariharan, 1997) whereas in *Curcuma amada* (Prakash et al, 2004) and in *C. longa* (Salvi *et al*., 2002) a combination of BA+NAA produced best results, while in *K. galanga* (Vincent *et al*., 1992) a combination of BA and KN gives increased shoot number. In the present investigation, higher concentrations of BA reduced the frequency of shoot induction. This was in agreement with the studies in *K. rotunda* (Anand *et al*., 1997). Copious root formation, obtained along with the shoots were observed in many zingibers like, turmeric and ginger (Balachandran *et al*., 1990) *Paracautleya bhatii* (Rai and Thoyajaksha, 2001) and *Alpinia galanga* (Barthakur *et al*., 1999). The present study also confirms the same results. According to Agretious *et al*. (1996) it may be due to the root inducing factors which are 'intrinsic' in the rhizomes of rhizomatous plants.

MS medium has been frequently used as nutrient medium for tissue culture programmes (George, 1996). This medium was also found suitable for the micropropagation of *C. haritha*. Zingiberaceae members generally prefer MS medium as in the case of *Curcuma* sp. (Yasuda *et al*., 1987) and *Alpinia calcarata* (Agretious *et al*., 1996). Where as in *C. amada*, B_5 media was found more effective than MS (Barthakur and Bordoloi, 1992). Similarly, foliar explants of *Juniperus oxycedrus* produced double the number of buds by using modified SH medium (Schenk and Hildebrandt, 1972) compared to MS (Gomez and Segura, 1994). The nutrient formulation is critical in determining the success of *in vitro* propagation. The mineral components in the culture medium have a swaying role in the rate of multiplication in *C. haritha*, as suggested by Ramage and Richard (2002). The extent of shoot multiplication in *C. haritha* could be increased to almost three-fold when MS represented the mineral formulation. It appears that the sensitivity of explanted tissues to PGRs could be changed with the change in the mineral salt nutrition in the culture medium. The regenerative capacity of the immature embryo-derived callus of indica rice differs when N_6 minerals (Chu *et al*., 1975) were substituted with MS under identical 2,4-D regime (Koetje *et al*., 1989).

The present study also revealed that an unfit nutrient medium reacted poorly, regardless of the shoot multiplication potential of the explant or optimum PGR levels. By simply replacing it with a favourable formulation, the shoot initiating ability of the explant was enhanced. According to Preece (1995) optimisation of nutrient salts in the medium could help to reduce the concentration of PGRs. Nutrient composition, like PGR, also plays similar and crucial role in achieving optimum explant response *in vitro* and changing to a suitable formulation can have a favourable influence on explant survival, growth and development.

In this study, the pH level of the culture medium did not show very significant differences in culture response compared to other treatments. However, pH 5.5 was found

to be most effective for achieving optimum shoot multiplication. Better rhizogenic response was observed at pH 6.1 than at lower pHs. These data were similar to those of Venus fly trap (Jang *et al*., 2003) that the number of shoots was highest on the medium at pH 5.5 while better rhizogenesis at pH 6.5.

The concentration of gelling agent affects the physical and chemical properties of the medium and it could modify the accessibility of soluble substances (Marga *et al*., 1997). In the present investigation, optimum shoot multiplication was obtained in media fortified with 7.0 g L^{-1} agar. Contrary to this, in *C. longa* (Salvi *et al*., 2002) liquid medium was found more favourable than semisolid medium for shoot multiplication and in solid media, 4.0 and 6.0 g L^{-1} agar produce best results. In the present study when the concentration was increased to 10.0 g L^{-1}, a reduction in shoot number was observed. Similarly, Igawa *et al*. (2002) reported reduced shoot differentiation in *Limonium sinuatum* at higher concentrations of agar. This may be due to the reduced uptake of PGRs, due to higher rigidity of the medium. Addition of agar reduces the shoot length in corn shoots (Mohamed-Yasseen, 2001). *C. haritha* also shows the same effect in liquid media, having elongated shoots and healthy roots.

The present study describes an efficient protocol for the micropropagation of *C. haritha*, which is endemic to Western Ghats of peninsular India. As this system produces shoots directly without the involvement of a callus phase, it is an efficient method for clonal multiplication and conservation of this endemic species.

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