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MICROPROPAGATION OF TWO ENDEMIC THREATENED *CRYPTOCORYNE* SPECIES OF SRI LANKA

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ABSTRACT

An efficient protocol was developed for the micropropagation of *Cryptocoryne beckettii* and *Cryptocoryne bogneri*, two endemic threatened aquatic herbs of Sri Lanka, highly valued as aquarium plants. Rhizomes of *C. beckettii* and *C. bogneri* were established on full strength Murashige and Skoog (MS) medium with 3% sucrose and regenerated shoots were cultured on MS medium supplemented with combinations of 6-benzylaminopurine (BAP) 2, 5, 8, 10 mg l^{-1} and 0.1 mg l^{-1} indole acetic acid (IAA) for multiple shoot regeneration. For root induction and shoot growth, agar solidified full and half strength MS medium and a soil-based medium were used. The presence of growth regulators in MS medium had a significantly high ($p<0.05$) effect on shoot multiplication in both species. Lowest mean number of shoots proliferated per shoot explant (*C. beckettii* 10.4 and *C. bogneri* 4.2) was observed in the absence of growth regulators. BAP alone or IAA alone in the medium also had no significant effect. Presence of both BAP and IAA in the medium significantly increased the shoot number. Highest mean number of shoots proliferated per single shoot explant (43.0) was observed in the combination of 5.0 mg l^{-1} BAP with 0.1 mg l^{-1} IAA for *C. beckettii* while the combination of 5.0 mg l^{-1} BAP with 0.1 mg l^{-1} IAA or 8.0 mg l^{-1} BAP with 0.1 mg l^{-1} IAA had highest mean number of shoots proliferation (51.8, 50.4) for *C. bogneri*. Mean number of roots induced per plant was highest (*C. beckettii* 8.4: *C. bogneri* 7.8) in the soil based medium consisting of a mixture of equal parts of 2 mm size ground brick fragments: sand: charcoal: top soil (medium A) with half strength MS and 0.1 mg l^{-1} NAA. Treatments of soil based medium had a significantly high effect on root and shoot development than the agar solidified media in both species. The plants were acclimatized with 90 % survival in *C. beckettii* and 100% survival in *C. bogneri*.

***Key words**: Cryptocoryne beckettii*, *Cryptocoryne bognerii*, Growth regulators, Micropropagation.

INTRODUCTION

Cryptocoryne (Vernacular name: Athi-udayan) species are evergreen herbs distributed in Asia from India to New Guinea in and along springs, streams and rivers in the lowland and midland rain forests and monsoon forests often forming large submerged or immerged growths. They are important as aquarium plants all over the world and several of the Sri Lankan species are the most widely used (Dasanayake and Fosberg, 1987). The genus *Cryptocoryne* is an amphibious member of Araceae family and having more than 50 different species distributed throughout Southeast Asia. More than 20 species are being used for aquarium decorations in the world (Wijesundara and Shantha Siri., 2004). Ten endemic *Cryptocoryne* species are found in and along the rivers, springs and streams of lowland and midland rain forests of Sri Lanka (Yapabandara and Ranasinghe, 2006).

Cryptocoryne are perennial plants and this genus has not been widely researched. Most of the easily kept and available *Cryptocoryne* species in aquaria come from Sri Lanka (Xema, 2005). *Cryptocoryne bogneri* is found in South West of Sri Lanka (Wijesundara and Shantha Siri, 2004). *Cryptocoryne beckettii* is also an endemic threatened aquatic herb of Sri Lanka (Rajathewa, 1999) and distributed in slow moving fresh water streams and shallow rivers of Sri Lanka (Wijesundara and Shantha Siri., 2004). Due to the reddish brown leaves, the plant has high value as an aquarium herb and has a very high export potential.

Aquatic plants are grown in aquaria for their beauty and to maintain the quality of water. High demand for aquatic plants mainly from the developed countries has created an aquatic plant industry in both developed and developing countries. This industry requires a continuous supply of high quality plants on a large scale. It is necessary to prevent wild collection and conserve aquatic eco-systems and develop mass propagation technique for aquatic plants for the export market (Yapabandara and Ranasinghe, 2006). Wild collection for export without artificial propagation leads to an extinction of endemic aquatic plant species and also affects the natural aquatic ecosystem. Therefore, research and development are needed for making the industry sustainable and conservation of the natural ecosystem (Wijesundara and Shantha Siri., 2004). In vitro propagation is the best technology for mass production to make the industry sustainable.

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In vitro propagation is a most efficient and cost effective method of propagating large number of clonal offspring. According to Alistock and Shafer (2006), the plants produced by in vitro propagation are genetically uniform, vigorous and free from associations with other organisms an attribute particularly useful for the culture of under water grasses where contaminating organisms can dominate other types of production systems. Many tissue cultured water plant species show a more bushy growth with more adventitious shoots, qualities that many aquarists appreciate (Christensen, 1996).

Agar is used as the gelling agent and it is one of the most expensive components in the culture media (Tyagi *et al.*, 2007). Of medium components the gelling agents such as agar contributes 70% of the costs. Proper choice of media can reduce the cost of micropropagation (Prakash *et al.*, 2004). Low cost tissue culture technology will stay a higher priority in agriculture, horticulture, floriculture of many developing countries for the production of suitably priced high quality planting material (Savangikar, 2004).

This experiment was conducted to develop an efficient *in vitro* propagation procedure for the micropropagation of both *Cryptocoryne bogneri* and *Cryptocoryne beckettii*. This study the objectives were to investigate the effect of (1) different growth regulators (2) type of culture media and (3) nutrient concentration of the media for the micropropagation of species to get adequate quantity of plantlets with good quality, especially with well developed root system. In this experiment we also wanted to develop a new low cost medium for large scale plant production.

Table 1: Effect of BAP (mg l-^{1}) and IAA (mg l-^{1}) during in vitro shoot multiplication of C.beckettii and C. bogneri two months after the culture establishment

Treatment			Mean number of shoots ± s.e	
Code	**BAP (mg l^{-1})**	**IAA (mg l^{-1})**	***C.beckettii***	***C. bogneri***
T1	0	0	$10.4^{e} \pm 3.88$	$4.2^{g} \pm 0.31$
T2	2.0	0	$13.6^{de} \pm 4.90$	$12.0^{ef} \pm 2.14$
T3	5.0	0	$11.6^{e} \pm 1.68$	$12.2^{ef} \pm 2.32$
T4	8.0	0	$16.0^{d} \pm 3.11$	$11.4^{f} \pm 1.56$
T5	10.0	0	$16.8^{d} \pm 5.55$	$13.4^{e} \pm 2.41$
T6	0	0.1	$23.6^{c} \pm 1.53$	$19.2^{d} \pm 1.31$
T7	2.0	0.1	$30.4^{b} \pm 1.95$	$38.8^{b} \pm 4.33$
T8	5.0	0.1	$43.0^{a} \pm 2.36$	$51.8^{a} \pm 5.41$
T9	8.0	0.1	$29.6^{b} \pm 1.57$	$50.4^{a} \pm 3.56$
T10	10.0	0.1	$30.0^{b} \pm 2.74$	$27.4^{c} \pm 3.72$

(T1 = without hormones; T2 – T5 = 2.0 -10.0 mg l-1 BAP alone ; T6 = 0.1 mg l-1 IAA alone; T7 – T10 ; 2.0 – 10.0 mg l-1 BAP in combination with 0.1 mg l-1 IAA)

MATERIALS AND METHODS

Materials and disinfection

Rhizomes were collected from healthy growing mother plants of *C.beckettii* and *C. bogneri* in the fresh water ponds of the Royal Botanic Gardens, Peradeniya, Sri Lanka. These rhizomes were washed well with soap and water and rinsed under running tap water for six hours. Next they were dipped in a 3% (v/v) 'thiophanate methyl 70%' solution (fungicide) overnight. The rhizomes were washed again under running tap water for one hour, disinfected in 70% (v/v) ethanol for one minute and 20% (v/v) sodium hypochlorite solution for 15 minutes. The materials were rinsed well with sterilized distilled water five times before culture.

Culture establishment

The rhizomes were cut into 1 cm length fragments and five explants were placed horizontally on full strength Murashige and Skoog (1962) medium with 3% sucrose in flasks with 40 ml medium per flask. Fifty culture flasks were established and they were kept under white fluorescent light (30 µE $m^{-2}s^{-1}$) with 16 hour photoperiod for shoot regeneration.

Shoot multiplication

To develop a suitable medium for shoot multiplication, MS medium was supplemented with 2, 5, 8, 10 mg l^{-1} 6 benzylaminopurine (BAP) with or without 0.1 mg l^{-1} indole acetic acid (IAA). Murashige and Skoog medium without growth regulators was used as the control (Table 1). After six weeks, the shoots regenerated from rhizome explants were excised and three regenerated shoots were cultured in each replicate culture bottle. Culture bottle with 40 ml medium was considered as a replicate and there were five replicates per each treatment. The experiment was repeated twice. After two months of culture the number of shoots regenerated from each shoot explant was counted.

Root induction and shoot growth

For the selection of a suitable media for root induction and further shoot growth, two types of basal media were tested. Agar solidified full or half strength MS medium supplemented with 0.1, 0.2 mg l^{-1} α- naphthaline acetic acid (NAA) or without hormones and a soil based medium consisting of a mixture of equal parts of 2 mm size ground brick fragments: sand: charcoal: top soil (medium A) saturated with full or half strength liquid MS medium with 0.1, 0.2 mg l^{-1} NAA or without hormones (Table 2). Shoots of 0.5 cm in height were

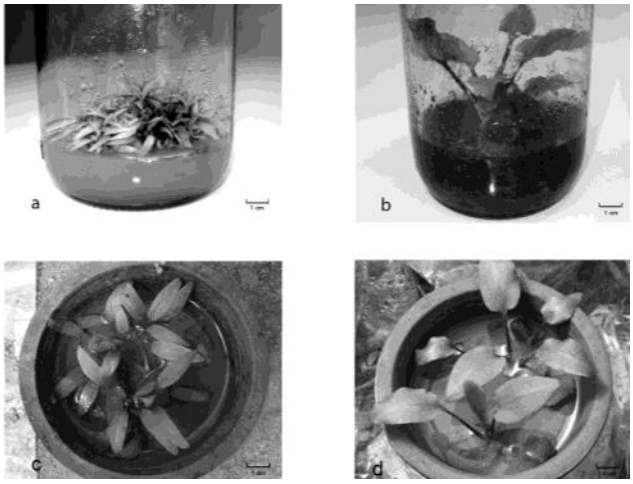

Figure 1: In vitro propagation of *C. beckettii* and *C. bogneri.* (a) Multiple shoots development in *C. beckettii* on MS medium supplemented with 5.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA. (b) *C. bogneri* plant grown on soil based medium with equal parts of 2 mm size ground brick: sand: charcoal: top soil soaked in half strength MS medium supplemented with 0.1 mg l^{-1} NAA. (c) *C. beckettii* plants acclimatized in clay pots with 2: 1 clay: sand mixture. (d) *C. bogneri* acclimatized in clay: sand mixture.

cultured in these media. Each treatment was replicated five times and each culture bottle had five shoots. After two months time, the number of roots induced in each plant, root length and shoot height were recorded.

Acclimatization

Plantlets that developed root system were planted in a mixture of two parts sterilized clay: one part sand (2 mm) in clay pots provided with sterilized distilled water up to 1 cm height.

Statistical analyses

The experiment was arranged in a Completely Randomized Design (CRD). Statistical analyses of data were performed using Statistical Analysis System (SAS Release 9.1). Data were subjected to analyses of variance and the mean comparison was done using Duncan Multiple Range Test (DMRT) at 5% significance level.

RESULTS

Shoot initiation and multiplication

During culture initiation, rhizomes of *C. beckettii* regenerated new shoots in MS medium in the absence of growth regulators. The presence of growth regulators (BAP and IAA) in the MS medium significantly ($p<0.05$) improved further multiplication of shoots (Table 1, Fig 1a). The interaction effect of BAP and IAA was also significant ($p<0.05$). The lowest mean number of shoots multiplied per single shoot explant (10.4: T1) developed in the absence of growth regulators. The highest mean number of shoots per single shoot explant (43.0: T8) developed in the combination of 5.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA. There was no significant difference in shoot development between the treatments with BAP at 2.0, 8.0 or10.0mg l^{-1} with IAA 0.1mg l^{-1} (T7, T9, T10). However, these treatments resulted in developing a significantly higher number of shoots than in treatments with BAP or IAA only.

The rhizome explants of *C. bogneri* initiated shoots in hormone free MS media after three weeks from culture establishment. The addition of growth regulators to the MS medium had significantly ($p<0.05$) influenced the shoot multiplication. The effect of BAP and IAA in the medium for the number of shoots proliferated was significantly higher at 5% significance level. Their interaction effect was also significant for shoot multiplication ($p<0.05$). Highest mean number of shoots (51.8) were proliferated in the medium supplemented with 0.1 mg l^{-1} IAA and 5.0 mg l^{-1} BAP (T8) (Table 1). Similarly, 8.0 mg l^{-1} BAP with 0.1 mg l^{-1} IAA (T9) had significantly high number of shoots development. Medium supplemented with combinations of IAA and BAP increased more shoot proliferation than the medium supplemented with BAP and IAA alone. However, high level of BAP (10 mg l^{-1}) with 0.1 mg l^{-1} IAA (T10) reduced the number of shoots proliferated. Lowest number of shoots (T1 : 4.2) development was observed in hormone free medium.

Root induction and shoot growth

Type of media, MS concentration and NAA level in the media significantly affected the root length and shoot height ($p<0.05$) of *C. beckettii*. Type of media (agar solidified/ soil based) had significant interaction ($p<0.05$) with MS concentration and NAA level of the media for the root length. The MS concentration and NAA level of media also had interaction effect on root length. This implies that, the effect of MS level on root induction varies among different levels of NAA. However, the three way interaction effect of type of media, MS concentration and level of NAA is not significantly different for the root length. The interaction effect of type of media with NAA level and MS concentration with NAA level was significantly different ($p<0.05$) on shoot growth of *C. beckettii*. The interaction effect of all the above three factors were also significant ($p<0.05$) for the shoot growth.

Root and shoot growth of *C. beckettii* was significantly high ($p<0.05$) in the soil based 'medium A' containing liquid MS than the agar solidified media (Table 2). The highest root length (18.2 cm) and shoot height (3.1 cm) observed in the 'medium A' containing half strength MS medium supplemented with 0.1 mg l^{-1} NAA (Fig 1b). The root length was comparatively higher in the 'medium A' provided with half strength liquid MS media.

The type of media, MS concentration and NAA level significantly ($p<0.05$) affected the root length and shoot height in *C. bogneri*. The interaction effects of type of media and MS concentration, type of media and NAA level, MS concentration and NAA level was significantly different ($p<0.05$) for the root length. The three way interaction effect of type of media, MS concentration and NAA level was also significantly different ($p<0.05$) for the root length. The two-way interaction effect of type of media and NAA level and the three way interaction effect of type of media, MS concentration and NAA level was significantly different for the shoot height.

Highest root length (28.8 cm) and shoot height (7.3 cm) were observed in the plantlets grown in 'medium A' (Table 2) provided with half strength liquid MS medium and NAA 0.1 mg l^{-1} level (T11). Treatment of 'medium A' added with half strength liquid MS medium provided with NAA 0.2 mg l^{-1} also had significantly high ($p<0.05$) root growth and shoot height than the other treatments (T12). 'Medium A' with half strength MS and no NAA (T10) had significantly high ($p<0.05$) root and shoot growth than the other treatments indicating that half strength MS is better for root and shoot growth. Plantlets grown in 'medium A' had significantly ($p<0.05$) higher root length and shoot growth than the agar solidified medium. The lowest root length and shoot height were observed with agar solidified full strength MS medium with no added growth regulators (T1).

Effect of the basal media, MS level and level of NAA significantly affected the number of roots of *C. beckettii* at $p<0.05$. Similarly the interaction of MS level and NAA level was also significant ($p<0.05$) for the root induction. The three way interaction was not significant for the number of roots. Highest mean number of roots (8.4) was observed in the 'medium A' soaked in half strength MS supplemented with 0.1 mg l^{-1} NAA (T4) (Table 2). Comparatively higher number of roots were observed in the plantlets grown in the 'medium A' than the agar solidified medium. Number of roots induced in the plantlets grown in 'medium A' added with half strength MS medium was significantly higher than the other treatments (T10 – T12).

The effect of media, MS concentration and level of NAA significantly ($p<0.05$) affected the number of roots developed in *C. bogneri* plantlets. However, the interaction effects of the above factors are not significant. When consider the effect of medium and MS concentration, highest number of roots were induced in 'medium A' provided with half strength MS solution. The root induction was highest in 'medium A' provided with NAA 0.1 mgl $^{-1}$ and 0.2 mg l^{-1}. Lowest mean number of roots was occurred in agar solidified hormone free medium. The root induction was significantly ($p<0.05$) higher in 'medium A' than the agar solidified medium. For the root induction, 'medium A' provided with half strength liquid MS medium and NAA 0.1 mgl^{-1} can be considered as the best other than high concentration of NAA.

Cryptocoryne becketiii, the plantlets developed in 'medium A' had a well-developed root system that resulted in 90% survival after acclimatization while the plantlets grown in agar solidified medium was acclimatized with 81% survival. Plantlets grown in 'medium A'of *Cryptocoryne bogneri* were acclimatized with a survival rate of

Table 2: Root development and shoot growth of C. beckettii and C. bogneri after two months of culture in root induction medium.(n = 5)

Treatment				Mean Root number ± s.e		Mean Root length(cm)± s.e		Mean Shoot height (cm)± s.e	
Code	Culture medium	MS concentration	NAA (mg l^{-1})	*C. beckettiii*	*C. bogneri*	*C. beckettiii*	*C. bogneri*	*C. beckettiii*	*C. bogneri*
T1	Agar + MS	1	0	$0.2^{e} \pm 0.10$	$0.2^{h} \pm 0.01$	$0.6^{i} \pm 0.31$	$0.6^{g} \pm 0.20$	$0.7^{e} \pm 0.42$	$2.6^{h} \pm 0.02$
T2	Agar + MS	1	0.1	$0.6^{de} \pm 0.21$	$0.6^{gh} \pm 0.20$	$1.3^{i} \pm 0.01$	$1.6^{fg} \pm 0.01$	$0.9^{de} \pm 0.32$	$3.7^{fg} \pm 0.34$
T3	Agar + MS	1	0.2	$1.0^{de} \pm 0.78$	$0.8^{g} \pm 0.10$	$2.4^{h} \pm 0.65$	$3.3^{ef} \pm 0.03$	$1.0^{d} \pm 0.70$	$3.4^{g} \pm 0.21$
T4	Agar + MS	0.5	0	$1.2^{de} \pm 0.72$	$0.8^{g} \pm 0.21$	$0.9^{i} \pm 0.41$	$3.1^{efg} \pm 0.21$	$0.9^{de} \pm 0.43$	$4.0^{f} \pm 0.10$
T5	Agar + MS	0.5	0.1	$1.6^{d} \pm 0.70$	$1.4^{f} \pm 0.33$	$3.5^{g} \pm 1.01$	$6.5^{d} \pm 0.12$	$0.7^{de} \pm 0.21$	$3.7^{fg} \pm 1.16$
T6	Agar + MS	0.5	0.2	$1.6^{d} \pm 0.70$	$0.9^{g} \pm 0.32$	$7.1^{f} \pm 2.92$	$1.1.^{fg} \pm 0.03$	$1.6^{c} \pm 0.42$	$4.4^{f} \pm 0.03$
T7	A + liq. MS	1	0	$3.2^{c} \pm 1.92$	$2.0^{e} \pm 0.22$	$11.3^{e} \pm 4.23$	$10.2^{c} \pm 1.22$	$1.9^{b} \pm 0.70$	$5.9^{cd} \pm 2.24$
T8	A + liq. MS	1	0.1	$3.3^{c} \pm 0.23$	$2.6^{d} \pm 0.11$	$12.0^{d} \pm 3.24$	$11.2^{c} \pm 2.12$	$1.9^{b} \pm 0.21$	$5.8^{cde} \pm 3.13$
T9	A + liq. MS	1	0.2	$3.0^{c} \pm 1.67$	$2.4^{cd} \pm 0.32$	$12.2^{d} \pm 2.23$	$9.8^{c} \pm 1.13$	$2.1^{b} \pm 0.31$	$5.5^{de} \pm 1.03$
T10	A + liq. MS	0.5	0	$5.2^{b} \pm 2.17$	$5.4^{b} \pm 0.41$	$13.4^{c} \pm 5.25$	$23.1^{b} \pm 3.42$	$2.0^{b} \pm 0.71$	$6.4^{b} \pm 5.31$
T11	A + liq. MS	0.5	0.1	$8.4^{a} \pm 2.56$	$7.8^{a} \pm 1.22$	$18.2^{a} \pm 5.21$	$28.8^{a} \pm 5.11$	$3.1^{a} \pm 0.32$	$7.3^{a} \pm 2.35$
T12	A + liq. MS	0.5	0.2	$3.0^{c} \pm 1.17$	$3.0^{c} \pm 0.71$	$14.6^{b} \pm 4.52$	$24.5^{b} \pm 2.11$	$2.0^{b} \pm 0.42$	$6.1^{bc} \pm 3.51$

Data indicate mean + standard error (s.e) and means followed by the same letter in a column are not significantly different ($p<0.05$). Five replicates were used per treatment and experiment was repeated twice.

100% and the plantlets grown in agar solidified medium were acclimatized with 86% survival rate.

DISCUSSION

The results of this study indicates that, the growth regulator supplementation is essential for the micropropagation of *Cryptocoryne* species. Both species had highest shoot proliferation in the combination of 5.0 mg l^{-1} BAP and 0.1 mg $^{-1}$ IAA. The effect of growth regulators in shoot multiplication has been reported for other *Cryptocoryne* species as well as other water plant species. According to Hongrat *et al* (2005), maximum number of shoots of *Cryptocoryne cordata* was regenerated in MS medium supplemented with BA at 1 mg l^{-1}. According to Zhou *et al* (2006), both MS and NAA significantly influenced the root number, total root length of *Myriophyllum spicatum* L. a submerged macrophyte. Koch and Durako (2005) have reported that, NAA addition to the medium has resulted in increased leaf and internodal lengths of submerged angiosperm *Ruppia maritima.* Wang *et al.* (2004) reported that, presence of BAP 3 mg l^{-1} in the medium effectively increased the number of regenerated shoots of *Scirpus robustus* an emergent hydrophyte.

Auxins commonly used in plant tissue culture media includes IAA and NAA. Auxins are particularly included in a culture medium to stimulate callus production and cell growth, to initiate shoots, particularly roots and to induce somatic embryogenesis. Cytokinines (BAP) have been reported to induce the development of axillary buds and adventitious buds through decreasing apical dominance (Taji *et al.*, 1995). When cytokinin level was higher than the auxin level, shoot development was promoted. In the present investigation too, the presence of cytokinin at a relatively high level with low level of auxin promoted the shoot multiplication. In this experiment without hormones the shoot multiplication and root induction were lowest. High level of shoot multiplication could observe when both auxin and cytokinin were present in the medium and their ratio was low.

Plantlets established in 'medium A' had significantly high shoot and root growth compared to the agar solidified medium. The charcoal in the 'medium A' may have adsorbed inhibitory compounds in the medium and it may have facilitated root growth. Eymar *et. al* (2000) have reported that, when root morphogenesis of *Lagerstroemia indica* was inhibited by addition of BA to the medium it can be overcome by the activated charcoal. Similarly, darkness of the 'medium A' compared to the agar solidified medium may have facilitated the root growth of the plantlets. The composition of above medium also may have facilitated the root penetration. Nutrient and hormone uptake from the liquid MS added to the 'medium A' may have been easier than the solid medium which may have facilitated the better shoot and root growth. Cao *et al* (2006) have observed the fresh weight and length of shoots of *Wasabia japonica* derived from MS liquid medium were significantly higher then those derived from MS semi solidified medium. Seneviratne *et al* (2004) have reported that, a mixture of equal parts of sand, charcoal, tile pieces and coir dust can be successfully used in orchid *in vitro* culture provided with liquid Knudson C medium. Composition of the 'medium A' is similar to the soil medium where the plantlets were acclimatized. The root system of plantlets grown in this medium was also well developed. These reasons may have resulted in a higher survival. According to Savangikar (2004), low cost options should lower the cost of production without compromising the quality of the plants. The generated plants must be vigorous and capable of being successfully transplanted in the field and must have high field survival. The plants established in soil based medium had highest root induction and shoot growth and were more vigorous with high field survival than plants grown in agar medium.

In conclusion, the present study showed that highest number of shoots proliferation in both *C. beckettii* and *C. bogneri* was with the presence of 5.0 BAP mg l^{-1} and 0.1 mg l^{-1} IAA in a basal MS medium. This medium could there be recommended for shoot proliferation of these species. Root induction and shoot growth were optimal in half strength MS medium supplemented with 0.1 mg l^{-1} NAA soaked mixture of equal parts of 2mm size ground brick: sand: charcoal: top soil. The results of this study establish an efficient low cost *in vitro* propagation protocol with use of a soil based medium for *C. beckettii* and *C. bogneri* . Using this protocol, starting with single shoot explant of *C. beckettii* produced 43-45 plants while *C. bogneri* produced 51-56 plants in four months time.

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