An efficient invitro plant regeneration of *Cryptocoryne wendtii* through shoot tip

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Abstract

An efficient protocol was developed for the micro propagation of *Cryptocoryne wendtii* an endemic threatened aquatic plant in Sri Lanka. Shoot tips of *C. wendtii* were established in Murashige and Skoog (MS) medium with 3% sucrose with 2 mg/L 6-benzyle aminopurine (BAP) and Indole Butyric Acid (IBA). Regenerated shoots were cultured on MS medium supplemented with different combinations of BAP, Naphthalene Acetic Acid (NAA) with IBA for multiple shoot generation. The control medium without growth hormones did not show any shoot generation. Most of the combinations showed the multiple shoots. However, the highest mean number of shoots 10.00±1.15 was showed in MS medium supplemented with 4 mg/L BAP and 1.0 mg/L IBA. The mean of the root length per plant was the highest in the MS liquid medium with 0.5 mg/L IBA (2.33±0.17 cm). Hundred percent survival of the plantlets observed during hardening. Maximum mean leaf length was observed in the hydroponic medium nutritious with 0.5 g/L Albert's Solution.

Keywords: *Cryptocoryne wendtii*, growth hormones, micropropagation, shoot tip culture

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Introduction

Cryptocoryne wendtii which belongs to family Araceae, is one of the most common and widely used aquatic plants in aquaria and water gardens. *Cryptocoryne* spp. mainly occurs in the South-western lowland ever-green rain forests, central midlands, and central western lowlands in the semi-deciduous monsoon forests having a seasonal change in precipitation and few are scattered in the dry zone riverine forests. Many of these species are restricted to the Kalutara and Ratnapura Districts in the low wetlands and the banks of the Mahaweli River in Gannoruwa-Hallolluwa area, Kandy (IUCN Red List, 2012). These species mostly thrive in slow running water or seasonally inundated soils. They occur both submerged or emerged depending on the growth stage, vegetative or reproductive.

This genus consists of more than 50 species in South East Asia. In Sri Lanka, ten endemic species are recorded, all of which are listed in the IUCN Red list; five *Cryptocoryn*e species are placed under the Critically Endangered (CE) category, three species are under Endangered (E) category and two are under vulnerable category. *Cryptocoryne* plants show polyploidy. *C. wendtii* plants show two chromosome numbers (2n), which consist 28, 36 or 42 chromosome numbers. The triploid plants were showing more variations (chromosome 42) than the diploid plants (Chromosome 28) (Jacobsen, 1976). The triploid plants are sterile and could not produce seeds; hence, the only way for the propagation is vegetative propagation (Dissanayake *et al*., 2007).

C. wendtii is very popular among the fresh water aquaculture and aqua scaping industry (Dissanayake *et al*., 2007). It is one of the easiest plants to grow and cultivate; and also could survive for longer periods (Wijesundara & Shatha Siri, 2004). *C. wendtii* can tolerate low or high light, and seems to respond with longer foliage in lower light conditions. The plant only requires stable conditions and sometime to adjust after being introduced into a new setting. Most important character of these species are the range of colors in the leaves such as reddish, reddish-brown, reddish-brown marble leaf coloration to the aquarium, hence these plants are popular in aqua scaping industry (Wijesundara & Siri, 2004).

Introduction of tissue culture to produce these valuable aquatic plants holds several advantages to the industry. It will provide mass production of good quality plants without pest or disease problems at a competitive price for the export market, while conserving aquatic plants in their natural habitats (Yapabandara & Ranasinghe, 2007). Production cycles can be planned according to the demand, without the effect of climatic and environmental condition. It is another big advantage in tissue culturing these plants. Thus, development of a proper protocol for the production of *C. wendtii* will help to supply continuous and mass production of this plant to cater the demand in the industry.

Materials and Methods

Materials

Basal shoot tip explants (2-5 mm long) of *C. wendtii* were taken from mother plants grown in plant house at National Aquatic Resources Research and Development Agency (NARA). Mother plants were cultured in hydroponic system.

Sterilization of explant

Explants were first thoroughly washed with liquid soap and kept under running tap water for one day. After that they were washed with 70% ethanol for one minute. Then different concentrations of Sodium Hypochlorite solutions 20% - 4% were applied with one drop of tween twenty per 100 mL. Exposure times used ranged from 5 to 15 minutes. There were five treatments with a control as 70% Ethanol (C), 20% Clorox (T1), 15% Clorox (T2), 10% Clorox (T3), 8% Clorox (T4) and Clorox 4% (T5). Each treatment had three replicates (10 samples/replicate). After sterilization procedure, all explants were thoroughly washed with sterilized distilled water for three times for three minutes. Washed explants were cultured in solid Murashige and Skoog medium (MS) for 60 days. Numbers of survived explants were counted and the percentage survival of plants was taken as a parameter to assess its effectiveness.

Explant culture

Sterilized explants were cultured in MS Medium with 3% Sucrose with 0.8% Agar. pH of the medium ranged from 5.6 to 5.8. Different concentration of 6-benzyleaminipurine (BAP), Naphthalene Acetic Acid (NAA) and Indole Butyric Acid (IBA) used as Growth promoting Hormones. Treatments comprised of hormone concentrations: BAP (1 mg/L-10 mg/L), NAA (1 mg/L-10 mg/L) and IBA (0.5 mg/L and 1 mg/L). Growth hormones were not added to the Control. In total, 24 types of treatments were used and each treatment had five replicates (10 sample / replicate). Samples were cultured *in vitro* with 16:8 light and dark hours condition for 60 days. Initiated shoots were counted and average percentage of shoot initiation was used as a parameter to assess surface sterilization.

Initiated shoots were subculture in MS Medium with different concentrations of BAP, NAA and IBA hormones. BAP (6 mg/L-1 mg/L), NAA (4 mg/L-1 mg/L), IBA (1 mg/L-0.5 mg/L). Control was free from growth hormones. There were 24 treatments and each treatment had 30 samples. Samples were cultured *in vitro* with 16:8 light and dark hours condition for 60 days.

Rooting

After shoot multiplication, shoots were cultured in three different culture Media with 2 mg/L IBA, 1 mg/L IBA or 0.5 mg/L IBA. MS medium with either 0.8% agar, 0.4% agar without agar were used as different culture media. There were nine treatments. Controls were free from growth hormones. Each treatment had five replicates (one replicate has 10 samples). Samples were cultured *in vitro* with 16:8 light and dark hours condition for 60 days. Length of roots was measured and average length of roots was selected to assess the growth.

Acclimatization

Plantlet's roots were washed with hot water to remove agar and transferred to a plant house at NARA. Plantlets were cultured in a hydroponic system with different concentrations of Albert's solutions. Control was free from fertilizers. T1 consisted

of 1.5 g/L Albert's solution, T2 1.0 g/L Albert's solution, T3 0.5 g/ L Albert's solution and T4 0.25 g/L. The number of leaves and leaf length of the selected leaves were measured for a period of one month.

Data Analysis

The cultures were arranged in a completely randomized manner and the experiments were conducted as three trials. One Way ANOVA was used to analysis of sterilization procedure, shoot initiation, multiplication and hardening treatments. Rooting treatments were analyzed by using Two Way ANOVA (SPSS 16 Software).

Results and Discussion

In this study, the treatment five (T5) showed the highest percentage of survival among the sterilization treatments (90.0±2.9% survival (Fig 1.)). Thus, it can be concluded that the best results obtained by sterilization using treatment 5 (10% Clorox for 5 minutes, 8% Clorox for 10 minutes and 4% Clorox for 15 minutes). Kane *et al.*, (1999) also mentioned that ethanol and Sodium Hypochlorite can be used for the surface sterilization of *C. wendtii*. Obtaining explants free of contamination was very difficult considering aquatic plants. Because aquatic plants grow submerged in water, and their wet surfaces highly colonized with microbes. In warm tropical environment a greater amount of microorganisms are available in the aquatic environment than in temperate conditions. The explant of this species, presence with an uneven and hairy surface, which prevents the proper contact of explant surface with the disinfectant. Therefore, microorganisms very easily could retain there and enter into the media leading to contamination.

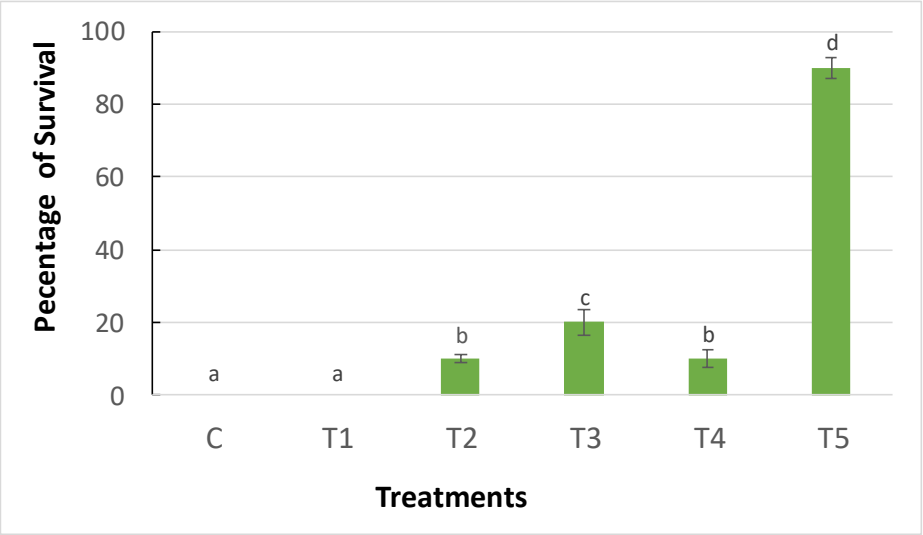

Fig. 1. The Percentage of survival of explants of *C. wendtii* by using different disinfectants for surface sterilization. Errors bars are standard error of mean.
C-Control (with 70% ethanol); T1 (20% Clorox), T2 (15% Clorox), T3 (10% Clorox), T4 (8% Clorox), T5 (4% Clorox) Different lowercase letters show significant differences between treatments.

Dissanayke *et al*., (2007) and Stanly *et al*., (2010) conducted research to determine a surface sterilization procedure for *C. wendtii*. Both of these studies showed that treating with ethanol followed by Sodium Hypochlorite was not sufficient to obtain best survival rate. Instead, they used 0.1% Mercuric Chloride ($HgCl_2$) to gain high survival rate. However, in our experiment, 90.0±2.9% survival was obtained without using $HgCl_2$. Developing a procedure without $HgCl_2$ is a good initiative as disposing this hazardous chemical into the environment is quite problematic.

Shoot initiation and multiplication in *C. wendtii*

In this study, various combinations of auxins and cytokinins were tested for better shoot initiation. The shoots were initiated with the combinations of BAP and NAA with IBA. In total, 24 combinations were tested (Fig 2.). Treatment five, containing

2 mg/L BAP with 0.5 mg/L IBA combination showed the maximum percentage (80.0±0.0%) of shoot initiation for the study species. The control did not show any shoot initiation (Fig 2.).

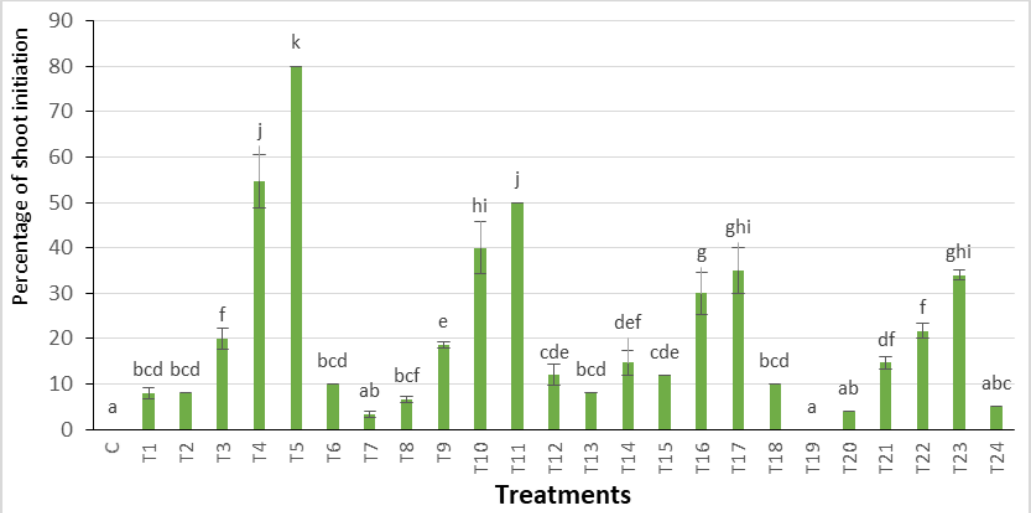

Fig. 2. Percentage shoot initiation in different treatments as different concentrations of BAP, NAA, and IBA. Error bars are standard error of mean. Different lowercase letters show significant differences between treatments.

The results of this study showed that the supplementation of growth hormones is essential in shoot initiation of *C. wendtii*. The effect of growth regulators was reported in various other studies. According to a study conducted by Herath *et al*., (2008) *C. backetii* initiated shoots without any growth regulators, but high number of shoots were obtained after the supplementation of growth hormones. The study done by Dissanayake *et al*., (2007) showed that shoot induction of *C. wendtii* occurred by supplementation of 44 µM BAP alone and the combination of 66 µM BAP with 13.4 µM NAA. Fig 3. shows the results of number of shoots per explant with the combination of growth regulators.

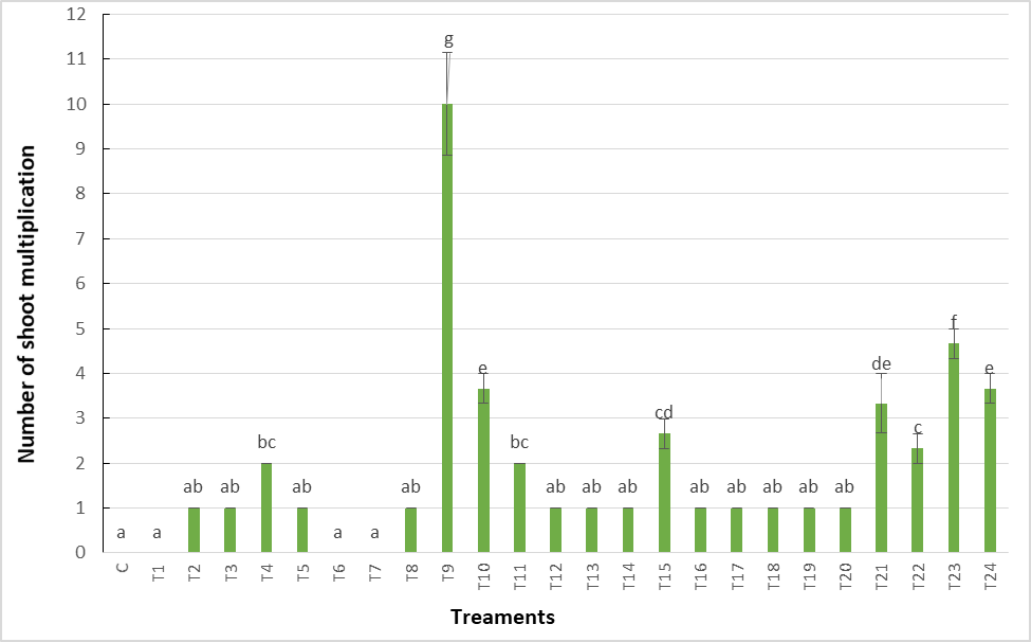

Fig. 3. The Percentage of shoot multiplication of *C.wendtii*. Errors bars are standard error of mean. Treatments are different concentrations of BAP, NAA, and IBA. Different lowercase letters show significant differences between treatments.

After 60 days of culture period the shoots were separated and sub culturing was conducted. During this study, different concentrations of BAP and NAA with IBA were tested. The number of shoots per explant was observed. In total, 24 treatments were conducted and treatment 9 (T9) showed a significantly high number of shoots with the combination of 4 mg/L BAP with 1 mg/L IBA (10.00±1.15). The study conducted by Herath *et al*., (2008) mentioned that the combination of BAP: IAA 5 mg/L: 0.1 mg/L showed the maximum number of shoots in multiplications of the same species. According to the results obtained by Dissanayake *et al.*, (2007), the maximum shoot number was obtained by using only BAP for the same species.

Rooting of *C. wendtii*

After the shoot multiplication, shoots were cultured in different culture medium (MS Solid, MS Semi Solid and MS Liquid in three different IBA concentrations) to select

the best medium. According to the results liquid medium was the most suitable for rooting (1.33±0.17). As agar is much expensive the cost for micro-propagation could be reduced by using a liquid medium. A gradient of IBA has been used in every medium to select the suitable IBA concentration. Treatment 9 in C3 showed the maximum average root length (2.33±0.17cm). Among all the medium 0.5 mg/L IBA showed highest average length of roots (2.33±0.17). The Fig 4. showed the average root length with different culture media and gradient of IBA.

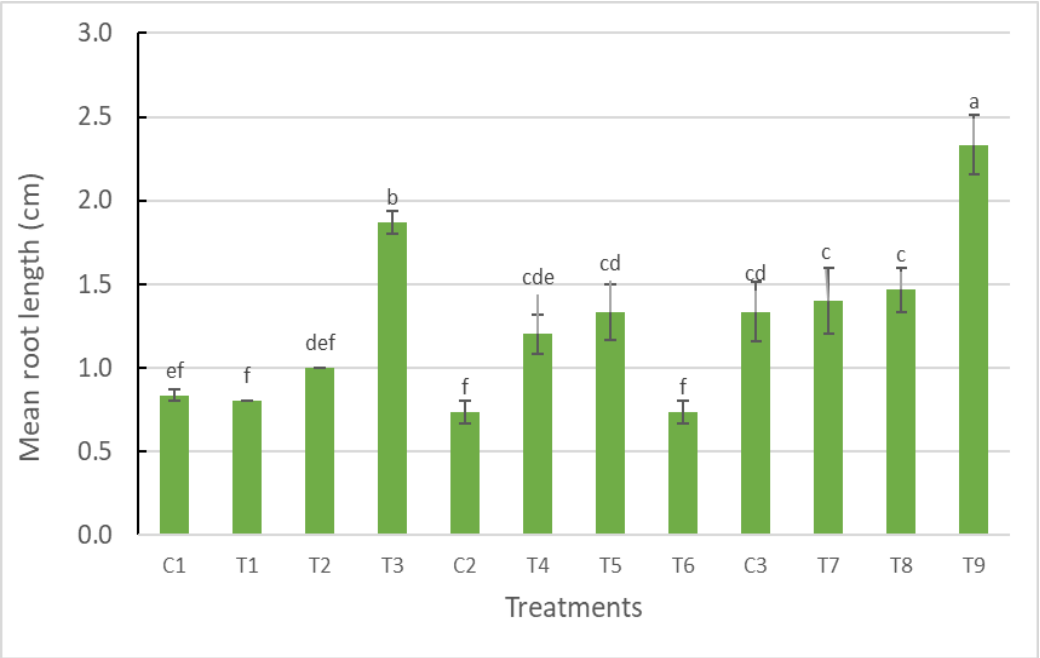

Fig. 4. The Percentage of root initiation of *C. wendtii* by using three different media as MS Solid, MS Semi Solid, and MS Liquid in three different IBA concentrations. Error bars are standard error of mean. Different lowercase letters show significant differences between treatments.

Ranasinghe *et al*., (2000) showed that the rooting could be obtained without growth regulators. Hence in our experiments, very low concentration of IBA was used for rooting. All the three controls showed rooting without any growth regulators. According to Dissanayake *et al*., (2007); control without any growth hormones has

given a very less amount of rooting and the medium with IBA 0.5µM showed the rooting capacity.

Hardening

Yapabandara & Ranasinghe (2007) mentioned that hardening of tissue cultured plants are the most important aspect that determines commercial feasibility in terrestrial plants. Even though aquatic plants grow in an aquatic environment they also require good care at this stage. Rooted plantlets were transferred into hydroponic tanks in plant house at NARA. Plants were treated with different concentrations of Albert's solution (inorganic fertilizer). The number of leaves and leaf length were recorded. Fig 5. showed the plants grown in the tanks.

Fig. 5. Hardening of *C. wendtii*

Albert's solution with 0.5 g/L (T3) showed the highest number of leaves (6.7±0.7 cm) and the maximum leaf length (16.3±0.4 cm) during hardening period (Fig 6.). Considering the leaf length T3 was significantly differed from other treatments. But considering the number of leaves T3 was not significantly different from T2 (5.3±0.7 cm). Harshani *et al*., (2020) showed similar results for the hardening of *C. wendtii.*

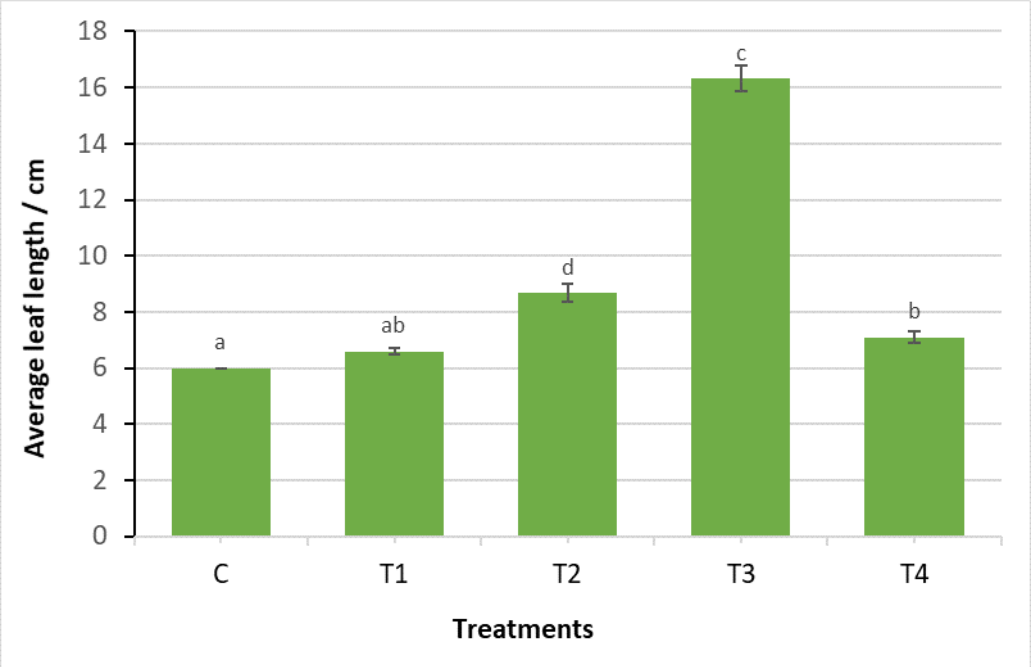

Fig. 6. The average leaf length of *C. wendtii* by using different concentrations of Albert's solution as 0 g/L (Control), 1.5 g/L (T1),1.0 g/L (T2), 0.5g/L (T3), 0.25g/L (T4). Errors bars are standard error of mean. Different lowercase letters show significant differences between treatments.

Conclusion

Most of the combinations showed the multiple shoot generations, but the highest mean number of shoots was showed in MS medium supplemented with 4 mg/L BAP and 1.0 mg/L IBA. During the acclimatization period, hundred percent survival was observed of all the plantlets and Albert's solution with 0.5 g/L showed the best results according to the assessed parameters.

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