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Enhanced efficiency of *in vitro* plant regeneration of caladium (*Caladium bicolor* cv. 'Khum Thong') through young leaf culture

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ABSTRACT: Caladium cv. 'Khum Thong' is a popular and expensive ornamental caladium due to its attractive foliage. Although propagation of caladium by tuber separation is common, it takes a long time and the risk of pathogen infection is high. These challenges could be solved using plant tissue culture technique. Thus, the aim of this research was to investigate factors affecting plant regeneration and substrates on acclimatization. Sterile young leaves of caladium were cultured on Murashige and Skoog (MS) medium supplemented with 2 mg/l N^6-benzyladenine (BA) and 0.5 mg/l α-naphthaleneacetic acid (NAA) for four weeks. Calli were obtained and cultured on MS medium supplemented with 0.1% activated charcoal and different concentrations of BA for four weeks. The results showed that the medium containing 0.5 mg/l BA gave the highest shoot induction (100%), number of shoots (2.80 shoots/callus clump), and plant height (1.20 cm). For type of culture, the results revealed that liquid medium under shaking condition gave the best results in shoot proliferation at 100%, number of shoots at 12.20 shoots/clump, shoot height at 3.54 cm, and number of leaves at 2.60 leaves/plant. For acclimatization, all types of substrates gave 100% survival rate, while peat moss gave the best results in plant growth and development. Therefore, it can be concluded that shaking liquid medium with 0.5 mg/l BA and 0.1% activated charcoal looks to be the most suitable for mass propagation of caladium cv. 'Khum Thong'. Peat moss is suitable for acclimatization of this plant.

KEYWORDS: caladium, *in vitro* plant regeneration, young leaf culture, acclimatization

INTRODUCTION

Caladium bicolor, regarded as the queen of foliage plants with many improved hybrid varieties, is very popular in Thailand. It is a beautiful and attractive perennial herbaceous plant of the Araceae family that is grown as an ornamental for its large, arrowhead-shaped leaves marked by varying patterns of white, pink, and red [1]. The plant is considered a fancy-leaved cultivar characterized by cordate leaves held on elongated black petioles with green veins. It is very popular, but expensive, due to low production. In general, caladium plants are propagated by tuber separation which takes a long time, leading to the high price of tubers in commercial markets [2]. In addition, the tuber separation method poses a high risk of soft rot disease caused by *Fusarium* sp. [1, 3]. Caladium may also be propagated by seeds, but it is difficult because the seeds are very small and have very high mortality. Besides, plants grown from those seeds are very expensive [1].

Plant tissue culture technique is an alternative for mass propagation, germplasm conservation, and breeding of commercially important plants in a small space [4, 5]. Multiplication of a large number of plants is possible in a short period without seasonal and environmental limitations [6]. Although there are many reports of micropropagation of many caladium cultivars, there have been no reports of *in vitro* regeneration of caladium cv. 'Khum Thong'. Moreover, it is expensive, demand is high for commercial purposes, but supply is low. *In vitro* technique is a suitable method that can solve the aforementioned problems. Young leaves, or unexpanded leaves, are regarded as appropriate initial explant that have already been widely used for shoot induction in tissue culture of caladium.

The success of plant regeneration protocol depends on several factors, such as concentrations and combinations of plant growth regulators (PGRs), type of explant and culture medium, etc [7]. PGRs play a crucial role in plant regeneration in both direct and indirect organogeneses and somatic embryogeneses [5]. The use of cytokinin in combination with auxin was more efficient in caladium callus induction than the use of only cytokinin or auxin with shoots being induced from calli by reduction of PGRs. Buddharaksa et al [8] reported that sterilized young leaves of caladium cultured on solidified MS medium with 0.5 mg/l BA in combination with 2 mg/l NAA gave the best results in number of shoots at 12 shoots/explant. After transferring mature plantlets to soil for six weeks, the survival rate was 80%. Sterile young leaves cultured on MS medium supplemented with different concentrations of BA and NAA showed that

4 mg/l BA with 0.5 mg/l NAA gave the highest callus induction. The number of shoots, 6.43 shoots/explant, was obtained from 1 mg/l BA in combination with 0.5 mg/l NAA containing medium [1]. Varieties and explant types respond to different PGRs in terms of shoot induction as reported by many researchers [9–12]. For cytokinin, BA is the most effective for both shoot induction and multiplication. However, there is a lack of data regarding the concentrations of BA. Therefore, the objectives of this research were to investigate the effects of concentrations of BA and types of culture on plant regeneration and the effects of substrates on acclimatization of caladium cv. 'Khum Thong'.

MATERIALS AND METHODS

Explant preparation and sterilization

One-year-old leaves of caladium cv. 'Khum Thong' from field-grown plants were used in this study. The leaves were detached and wiped with 70% alcohol, then washed with Teepol solution, washed with running tap water for 20 min, dipped in 70% alcohol for 1 min, disinfected in 10% Clorox for 20 min, and finally washed with sterile distilled water three times in laminar air flow.

Culture medium and conditions

Culture medium used in all experiments was MS supplemented with 3% sucrose and 0.6% (w/v) agar. The medium was adjusted to pH 5.7 prior to autoclaving at 1.05 kg/cm^2 and 121 °C for 15 min. All cultures were kept at 26 ± 2 °C under 10-h photoperiods (15 μmol/m^2/s) provided by cool-white fluorescent lamps.

Methods

Callus induction

Sterile young leaves were cut into 5 × 5 mm squares and cultured on solidified MS medium supplemented with 2 mg/l BA and 0.5 mg/l NAA for four weeks. Meristematic nodular calli (Fig. 1) were obtained and used for proliferation and regeneration in subsequent experiments.

Effects of BA on shoot induction

Meristematic nodular calli induced from young leaves were collected at four weeks of culture, cut into squares of 5 × 5 mm, then cultured on solidified MS medium supplemented with 0.1% activated charcoal and different concentrations of BA (0–2 mg/l). The cultures were maintained under 10-h photoperiods for four weeks. The number of shoots and shoot height were investigated. Shoot induction rates were calculated from the number of calli that produced shoots, divided by the total number of callus clumps, then multiplied by 100. Completely randomized design (CRD) was performed with 5 replications, with each replication consisting of 5 bottles. The means among treatments were compared using Duncan's multiple range test (DMRT) at 1% or 5% probability.

Effects of types of culture on shoot proliferation

Shoot clumps were separated when they reached 5 mm and cultured in glass bottles containing 10 ml of MS medium with 0.5 mg/l BA, 0.1% activated charcoal, and 3% sucrose on three different types of culture: (1) solidified medium; (2) liquified medium without agitation; and (3) liquified medium with agitation on a rotary shaker at 100 rpm. Agar was added to the solid medium at a final concentration of 0.6%. The cultures were maintained for four weeks under 10-h photoperiods and 20 μmol/m^2/s. The number of shoots, shoot height, and shoot characteristics were recorded. Shoot proliferation rates were calculated from the number of explants that proliferated shoots, divided by the total number of shoot clumps, then multiplied by 100. CRD was performed with 5 replications, with each replication consisting of 5 bottles. The means among treatments were separated by least significant difference (LSD) at 1% or 5% probability.

Effects of substrate on acclimatization

Well-developed plantlets with roots were transplanted to plastic pots containing three different substrates: peat moss, soil, or soil + peat moss (1:1) (v/v) and transferred to closed plastic containers (50 × 35 × 8 cm) under high relative humidity (80–90%) and 70% shade light at 28–32 °C for four weeks. The plant height, number of leaves, leaf area, and leaf pigment formation were measured. The survival rate was calculated from the number of surviving plants, divided by the total number of plants, and multiplied by 100. Leaf pigment formation was calculated from the number of plants that produced leaf pigment, divided by the total number of plants, and multiplied by 100. CRD was performed with 5 replications, with each replication consisting of 5 pots. The means among treatments were separated by LSD at 1 or 5% probability.

Statistical analysis

The experimental data were analyzed using R 2.14.0 software. The data were subjected to one-way analysis of variance (ANOVA).

RESULTS

The experiment results show significant differences, depending on the concentration of BA, in three parameters: shoot induction rate, number of shoots, and shoot height. The low concentration of BA gave better results in shoot induction than the high concentration. BA at 0.5 and 1 mg/l gave the highest shoot induction rate of 100%, significantly different from other concentrations. The 0.5 mg/l BA medium gave the highest

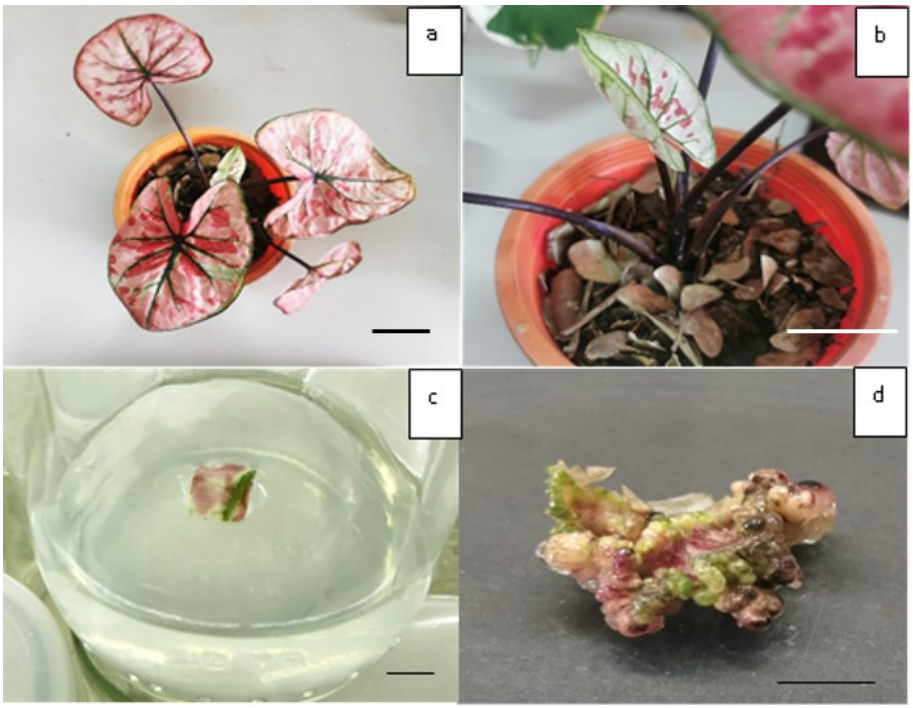

Fig. 1 Mother plant and callus from young leaves of caladium cv. 'Khum Thong' used as plant material in this study: (a) mother plant (bar = 5 cm); (b) young leaves used for sterilization (bar = 5 cm); (c) sterile leaves as initial explant cultured on callus induction medium (bar = 0.5 cm); (d) meristematic nodular callus obtained from leaf culture (bar = 0.5 cm).

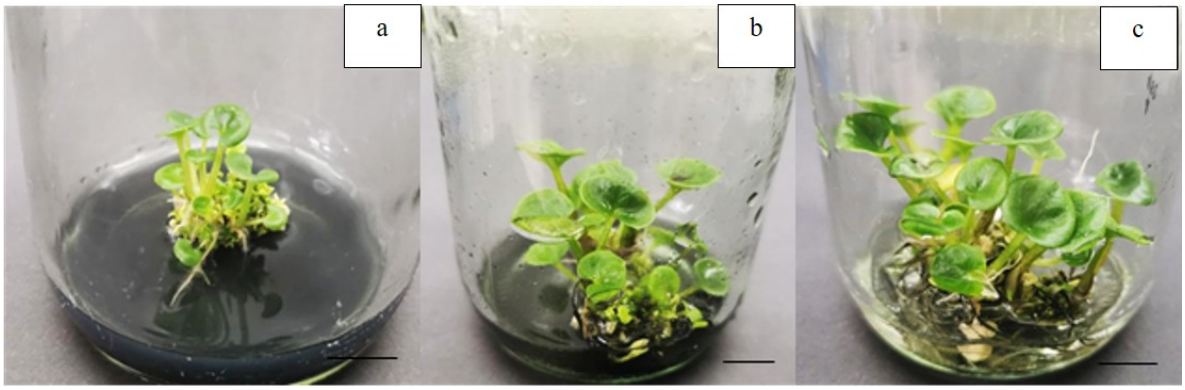

Fig. 2 Multiple shoots of caladium cv. 'Khum Thong' cultured on MS medium with 0.5 mg/l BA and 0.1% activated charcoal in different types of culture for four weeks (bar = 1 cm): (a) solid medium; (b) liquid medium without shaking; (c) liquid medium with shaking.

number of shoots, 2.8 shoots/clump, not significantly different from the higher concentrations but significantly different from the control treatment (0 mg/l BA). For shoot height, the 0.5 mg/l BA medium gave the highest result, at 1.2 cm, significantly different from other concentrations. Increased concentration of BA led to decreased shoot height (Table 1). High concentration of BA in the medium produced small shoots. Hence, low concentration (0.5 mg/l BA) was superior for shoot induction.

Shoot proliferation was 100% from all culture types. Similarly, there was no significant difference among the three types of culture on the number of leaves per plant. However, liquified medium with shaking gave the highest result at 2.60 leaves/-plant, followed by liquified medium without shaking (2.50 leaves/plant), and solidified medium gave the lowest number of leaves of 2.38 leaves/plant. Cultures in liquified medium with shaking gave the highest number of shoots of 12.20 shoots/callus clump, and

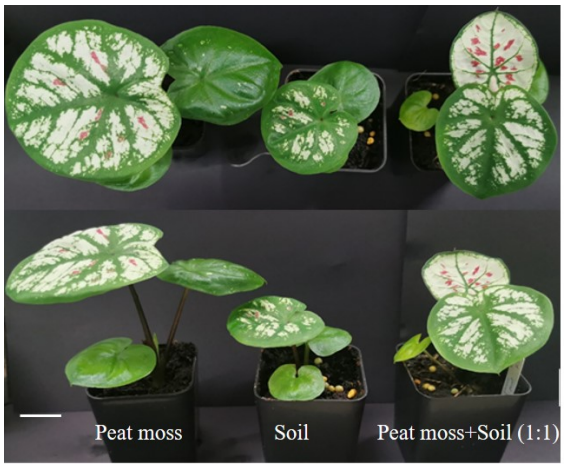

Fig. 3 Acclimatized caladium cv. 'Khum Thong' plants four weeks after transferring to different substrates: peat moss, soil, and peat moss + soil (bar = 3 cm).

Table 1 Effects of BA concentrations on shoot induction of caladium cv. 'Khum Thong' cultured on solidified MS medium with 0.1% activated charcoal for four weeks.

Conc. of BA (mg/l)	Shoot induction (%)	No. of shoots (shoots/clump)	Shoot height (cm)
0.0	25 ± 2.04^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
0.5	100 ± 0.00^{a}	2.80 ± 0.20^{a}	1.20 ± 0.20^{a}
1.0	100 ± 0.00^{a}	2.50 ± 0.50^{a}	0.66 ± 0.09^{b}
1.5	60 ± 6.12^{b}	2.33 ± 0.33^{a}	0.33 ± 0.08^{bc}
2.0	40 ± 4.18^{c}	2.00 ± 0.00^{a}	0.22 ± 0.02^{c}
F-test	**	**	**
C.V. (%)	11.66	19.78	39.19

**significantly different ($p < 0.01$). The total number of callus clumps used in the experiments was 25/treatment. Mean values followed by the same letter within a column are not significantly different, according to DMRT.

shoot height of 3.54 cm, significantly different from the other types (Table 2). Multiple shoots obtained from liquified medium with shaking grew larger, stronger, and more numerous than from the other types of culture (Fig. 2).

For plant acclimatization, a 100% survival rate was found on all types of substrates. At four weeks after transplanting, acclimatization with peat moss alone showed the highest efficiency of pigment formation in leaves of 100%, followed by peat moss with soil (72.22%) and soil alone (52.77%), In addition, peat moss alone gave the best results for plant height, number of leaves, leaf area, and leaf pigment formation, as shown in Table 3. Similarly, peat moss alone gave the highest plant height of 7.25 cm, significantly different from those of peat moss plus soil and soil alone which were 5.55 and 4.08 cm, respectively. The highest number of leaves of 3.50 leaves/plant and leaf area of 23.31 cm^2 were also obtained from peat moss alone, followed by peat moss with soil, and soil alone (Table 3; Fig. 3).

DISCUSSION

In vitro plant culture has been commonly used to propagate plants in large numbers [13], and unexpanded leaves are widely used for different varieties of caladium [4, 14]. Radomir [15] reported that 0.5 mg/l BA was more effective than other types and concentrations of cytokinins (namely, 2iP, TDZ, and kinetin) for shoot multiplication from apical buds of *Magnolia*. Seydi et al [1] reported that 1 mg/l BA with 0.5 mg/l NAA was suitable for shoot induction in *C. bicolor* from leaf explant. In Isah [16], shoot proliferation was obtained by optimizing PGRs and types of culture to reach their target numbers for commercial production within a short period of time. BA is a kind of PGRs in the cytokinin group and has been widely used to induce shoot formation of caladium as reported in previous studies. In our study, sterilized young caladium leaves were cultured on 2 mg/l BA in combination with 0.5 mg/l NAA for four weeks to induce calli and then were used for shoot induction with different concentrations of BA. The results showed that 0.5 mg/l BA had a significant beneficial effect on multiple shoot induction of *C. bicolor* cv. âĂŸKhum Thong'. A contrary result was obtained from Ali et al [17], who reported that 1 mg/l BA gave excellent results on shoot induction of *C. bicolor*, whereas 0.5 mg/l BA with 2 mg/l NAA gave the best results on shoot proliferation [8]. In vanilla, medium containing 2 mg/l BA was more effective in shoot formation than other concentrations [18]. These results indicate that BA is an important plant growth regulator for shoot multiplication and its proper concentration varies depending on plant species.

Regarding types of culture, liquid medium was more suitable than solid medium for shoot proliferation. Liquid medium with shaking had a more significant effect on shoot proliferation than solid medium and liquid medium without shaking, due to the extra nutrient content, PGRs, and sucrose accumulated in the liquid medium supported by rotary shaker, consistent with results obtained by Lin [19]. In previous studies, shaken liquid cultures were widely used to produce multiple shoots in many plant species: *Lindernia antipoda* (L.) Alston [20], *Scutellaria alpina* L. [21], *Curcuma zedoaria* Roscoe [22], *Vanilla planifolia* Andrews [23], *Musa acuminata* 'Cavendish' [24, 25]. Hung et al [26] carried out a research in which multiple shoots were totally submerged in the liquid medium, so they were able to uptake more nutrients and PGRs over the whole shoot surface compared with gelling agents on a large-scale *in vitro* propagation. In this study, *in vitro* shoots obtained from the shaken liquid culture absorbed activated charcoal from the medium into the plant root and petiole, as observed by the color of the culture medium clearly changing from black

Table 2 Effects of types of culture on shoot proliferation of caladium cv. 'Khum Thong' on solidified MS medium with 0.5 mg/l BA and 0.1% activated charcoal for four weeks.

Types of culture	Shoot proliferation (%)	No. of shoots (shoots/clump)	Shoot height (cm)	No. of leaves (leaves/plant)
Solid	100	8.87 ± 0.22^{b}	2.47 ± 0.13^{b}	2.38 ± 1.82
Liquid without shaking	100	6.16 ± 0.60^{c}	2.43 ± 0.12^{b}	2.50 ± 0.22
Liquid with shaking	100	12.20 ± 0.58^{a}	3.54 ± 0.19^{a}	2.60 ± 0.24
F-test	ns	**	**	ns
C.V. (%)	4.18	12.72	13.46	21.61

**significantly different ($p < 0.01$). ns = not significantly different. The total number of shoot clumps used in the experiments was 25/treatment. Mean values followed by the same letter within a column are not significantly different, according to LSD.

Table 3 Effects of growth media on acclimatization of caladium cv. 'Khum Thong' four weeks after transferring to greenhouse conditions.

Substrate	Survival rate (%)	Plant height (cm)	No. of leaves per plant	Leaf area (cm^2)	Leaf pigment formation (%)
Peat moss	100	7.25 ± 0.45^{a}	3.50 ± 0.17	23.31 ± 1.03^{a}	100.00 ± 0.00^{a}
Soil	100	4.08 ± 0.32^{b}	3.10 ± 0.27	10.82 ± 0.34^{c}	52.77 ± 4.33^{c}
Peat moss:Soil (1:1)	100	5.55 ± 0.46^{b}	3.20 ± 0.24	15.81 ± 0.92^{b}	72.22 ± 4.05^{b}
F-test		**	ns	**	**
C.V. (%)		23.60	22.82	15.67	7.87

**significantly different ($p < 0.01$). ns = not significantly different. The number of potting plants used in the experiments was 25/treatment. Mean values followed by the same letter within a column are not significantly different, according to LSD.

to white. The media containing activated charcoal in both solid and liquid cultures without shaking, on the other hand, were normal in color. It is possible that shaking can promote transportation of nutrients, PGRs, and some other chemicals into plants, resulting in vigorous development and proliferation of shoots. In this research, *in vitro* shoot regeneration of caladium in liquid culture under shaking condition was superior to other culture methods.

For acclimatization, the root environment in the substrate must be free of plant pathogens, and must have enough water, air and nutrients for plant growth [27]. Each of the three substrates (i.e., peat moss alone, soil, and peat moss with soil) gave a 100% survival rate. However, the highest values in plant height, number of leaves, and leaf area were obtained from peat moss alone. Peat moss also gave the best results in plant acclimatization of strawberries [28] which might have been due to favorable chemical, biological and physical properties, a low pH, and a unique combination of high water holding capacity and pathogen free substrates [29]. Many authors have reported efficient plant acclimatization using peat moss with aglaonema [30], miniature roses [27], strawberries [28], and tea [31]. Besides enhancing plant growth, peat moss also improved pigment formation in leaves.

CONCLUSION

It can be concluded that liquid medium culture containing 0.5 mg/l BA under shaking condition gave the best results in shoot proliferation (100%), number of shoots (12.20 shoots/callus clump), shoot height (3.54 cm), and number of leaves (2.60 leaves/plant). Peat moss was the most suitable type of substrate for plant acclimatization in terms of survival rate, plant height, number of leaves, and leaf pigment formation of caladium cv. 'Khum Thong'. These results can be useful for a large scale commercial production of caladium and for caladium breeder to produce new varieties.

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