Micropropagation of *Callisia Fragans* Lindl and Saponin Accumulation in Tissue Cultures

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ABSTRACT

Callisa fragans plant plant is one of the medicinal sources for treating diseases such as infections, osteoarthritis, burns.... Contains valuable active ingredients in inhibiting the growth of cancer cells, helping to increase cell resistance, and stimulating cell regeneration. *Callisa fragans* plant needs to be researched to preserve and develop precious medicinal resources in the tropics. Research on the development of in vitro micropropagation process of *Callisa fragans* plant and analysis of accumulated saponin (oleanolic acid). The shoots germinated from the stem nodes were used as culture explants. On MS medium supplemented with 2 mg/l BA, the shoot regeneration rate from the stem nodes was 82%. Shoot cluster culture on medium supplemented with 1 mg/l BA + 0.5 mg/l NAA produced 1.46 shoots/sample. Rapid shoot multiplication on medium supplemented with 1 mg/l BA + 0.5 mg/l IBA gave 5.87 shoots/sample after 4 weeks of culture. Shoots cultured to form roots on medium supplemented with 0.5 mg/l IBA had an average number of roots reaching 30 roots/shoot, average root length of 3 cm in 4 weeks of culture. Cultivating shoots on clean soil substrate has a high survival rate, good growth, and plant height reaches more than 15 cm after 2 months of care. The MS basal medium supplemented with 2 mg/l 2,4D + 1 mg/l kinetin gave the best callus formation results, reaching 76% after 18 days of culture. Callus cultured on MS medium supplemented with 1 mg/l 2,4D + 0.5 mg/l kinetin gave a growth index of 4.49. Analysis of active ingredients showed the accumulation of saponin in callus and in vitro shoots.

Key words: *Callisa fragans* L. plant, shoot tip culture, callus culture, oleanolic acid, saponin, micropropagation

INTRODUCTION

Callisia fragrans (Lindl.) belongs to the Commelinaceae family, also known as the *Callisia fragrans* plant. This is a perennial herb, with upright or horizontal stems, which can grow 20-50cm high. The Golden Comb stem is divided into many segments and branches, each segment is about 1-2cm long. The leaves are spongy and succulent; the leaves grow alternately and the large leaves are usually about 12-25cm in size and about 4-6cm wide. The *Callisia fragrans* plant is used as an ornamental plant and as a medicine.

The plant contains various bioactive components such as flavonoids, phenolic acids and anthraquinones [1]. The active ingredients in the plant are used to treat cardiovascular diseases, cancer, burns and osteoarthritis [2]. The two main flavonoids found in *C. fragrans* are quercetin (6.51%) and kaempferol (0.22%) [1]. Flavonoids with anti-inflammatory activity have been used to treat cancer and prevent cardiovascular mortality [3]. In addition, phenolic acid compounds are also anti-cancer components in pharmaceutical applications [4]. Polyphenols not only appear in cancer treatments but also prevent human skin from sunburn by absorbing UV rays [5]. Furthermore, ecdysteroids and amino acids can reduce blood cholesterol levels, treat cancer, and heal open wounds [6].

The main part used for medicinal purposes is the leaves, the growth of the stems and leaves in nature is only average due to damage by pests and diseases. Furthermore, the demand for medicinal plants is increasing rapidly, leading to continuous exploitation pressure on natural resources. The loss of biodiversity due to deforestation

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and monoculture is the main cause of the decline of the family Thai Lai in general. In Lam Dong province, this loss has led to warnings for many other herbs and plants, including the Commelinaceae family [7].

Callisia fragrans is a perennial herb and faces many obstacles from planting to harvesting over a long period of time. Difficulties in the cultivation process such as: diseases, biosynthesis, accumulation of active ingredients depend on ecological conditions, cultivation, and pesticide residues. Research on in vitro propagation of *Callisia fragrans* aims to preserve, develop and study the accumulation of saponin (oleanolic acid) in cells.

MATERIALS AND METHODS

Materials

The initial material is the stem segments taken from 1-year-old *Callisia fragrans* trees in Cu Chi District, Ho Chi Minh City (33).

Basic nutrient medium MS (Murashige and Skoog, 1962) [8] supplemented with 10% coconut water, 20 g/l sucrose, and 8 g/l agar adjusted to pH = 5.8-6 before autoclaving. Growth regulators (hormones) were added: NAA (Naphthaleneacetic acid), 2,4D (2,4-Dichloro-phenoxyacetic acid), IBA (Indol butyric acid), BA (6-Benzylaminopurine), kinetin (N6-furfuryladenine)

Culture conditions: In vitro culture conditions: 16 hours light/8 hours dark, light intensity 37.04 µmol/s/m^2, temperature 26 ± 2^{o}C

Methods

Sterilization of samples: The stems were washed with diluted soap and rinsed with clean water 2 to 3 times. The stem nodes were cut off and sterilized with 70% alcohol for 1 minute; further sterilized with 5% calcium hypochlorite Ca $(HClO)_2$ with 1-2 drops of tween 20 for 30 minutes; then washed with sterile distilled water 3 times, each time for 1 minute. Then, the stem nodes were cut at both ends; and inoculated into MS medium (Murashige & Skoog, 1962) [8] supplemented with BA (0-0.1-0.5-1-1.5-2-2.5-3 mg/l) to stimulate shoot germination.

In vitro shoot regeneration culture: Stem nodes were cultured on MS medium supplemented with BA (0-0.1-0.5-1-1.5-2-2.5-3 mg/l) to stimulate lateral shoot germination; culture time 4 weeks

In vitro shoot cluster culture: The shoots obtained after germination (height 2-2.5 cm) were cut and cultured on MS medium supplemented with BA (0-0.5-1-2-3 mg/l) combined with different concentrations of NAA (0-0.25-0.5 mg/l); culture time 4 weeks

In vitro shoot multiplication: Shoot clusters (2-3 shoots/shoot cluster) are placed on MS culture medium supplemented with BA (0.5-1-2-3 mg/l), NAA (0-0.1-0.25-0.5-0.75 mg/l) and IBA (0-0.1-0.25-0.5-0.75 mg/l) Shoot multiplication coefficient = Number of shoots (after 4 weeks) / Initial number of shoots

In vitro rooting culture: Shoots with a height of 3.5-4 cm, growing and developing well, were transferred to culture on MS medium supplemented with NAA (0-0.1-0.5-1 mg/l) and IBA (0-0.1-0.5-1 mg/l) to stimulate rooting; culture time 4 weeks

Acclimatization of shoots in the nursery: Complete in vitro plants, shoots 5 cm high, with 4 leaf layers, good root system were transferred to the environmental conditions outside the laboratory for 3 days to adapt to the outside temperature. Then, the plants were taken out of the jar, washed off the nutrient medium stuck on the roots and planted in plastic pots containing sterilized coconut fiber substrate and clean soil; data were recorded after 8 weeks.

Callus culture: In vitro leaves were cut into squares of 1 cm^2 length and cultured on MS medium supplemented with kinetin 1 mg/l and combined with NAA (1-2-3 mg/l) and 2,4D (1-2-3 mg/l) to create callus; culture time was 4 weeks.

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Callus culture on agar: White spongy callus was separated from the leaves. The initial mass of cultured callus was 0.1 g/cluster. Callus was cultured on MS medium supplemented with 10% coconut water, 2 g activated charcoal, kinetin (0.1-0.5-1 mg/l), 2,4D (0.1-0.5-1-2-3 mg/l) and a combination of kinetin and 2,4D; culture time was 4 weeks. Growth index was collected and calculated according to the formula GI = Wf / Wi (Wf is the cell mass at 4 weeks after culture and Wi is the cell mass at the beginning of culture – 0.1 g/cluster)

Saponin analysis: Callus tissue samples (after 4 weeks of culture), in vitro shoot samples (after 5 months), ex vitro shoots (after 2 months) were analyzed for saponin (oleanolic acid). Samples were dried to constant mass at 50 °C to determine dry mass. Total saponin content was determined by high-performance liquid chromatography (HPLC) according to Norhidayah et al. with appropriate adjustments to laboratory conditions.

HPLC running conditions: column temperature 30°C, Zorbax Eclipse-XDB C18 column (5 μm, 4.6 × 150 mm); flow rate: 1 ml/min; detector read at 220 nm; stationary phase was silica gel and mobile phase was channel A (H2O) and channel B (acetonitrile); mobile phase program: 95% B homogeneity. HPLC analysis was performed on an Agilent series 1200 system, with UV-VIS detector. Total saponin content was calculated based on the oleanolic acid (OA) standard curve. Results were expressed as μg (OA)/mg sample.

Experimental designs and data analysis

Laboratory experiments were arranged to be repeated 3 times per treatment, with 10 samples each time. The parameters were observed and data were collected after 4 weeks of culture.

The experiment of shoot adaptation in the external environment was repeated 3 times for each formula, 10 samples each time. The parameters were observed and data were collected after 8 weeks. Data were analyzed by anova using SPSS v.22 software.

RESULTS AND DISCUSSION

In vitro shoot regeneration culture

MS medium supplemented with different concentrations of BA was used to stimulate shoot emergence from the stem nodes of the cultured explants. In the BA concentration range of 0-3 ppm, the shoot formation rate increased gradually corresponding to the BA concentration in the medium, reaching the highest level at 2 mg/l BA and gradually decreasing at higher BA concentrations. After 4 weeks of culture, the shoot germination rate was high in the medium containing 2 mg/l BA (82%). The low germination rate in the culture medium without BA was 15% (Figure 1A). There was no difference between BA concentrations of 1 mg/l and 1.5 mg/l. The results of adding BA concentrations of 0.1, 0.5, 2 and 3 mg/l showed similarities, giving shoot emergence rates from 62.5 to 65%. Good germination must have a complete structure and new leaves developed. There were some callus formation samples on the cut surface of the stem (Figure 1B). MS medium supplemented with 2 mg/l BA was suitable for the process of stimulating shoot formation from stem nodes. In the study of Persian violet plant regeneration on MS medium supplemented with 2 mg/l BAP for 6 weeks, the number of shoots was the largest (Nisakorn Sarai et al., 2017) [9].

Cytokinins such as BA have many major functions in plant growth and development. BA is involved in the regulation of cell division, adventitious shoot formation, and reduction of apical dominance (Mangena, 2020) [10]. The efficiency of shoot formation depends on and is enhanced by BA supplementation in the medium compared to hormone-free medium. In this study, hormone-free medium had low shoot development efficiency. On the other hand, BA at a concentration of 2 mg/l was found to be effective in inducing shoots. This was similar to the report by Sachs et al. (1967) [11], where increasing BA concentration resulted in more shoots from the stem nodes. Similarly, BA has been shown to stimulate new shoots and plays an important role in plant growth and development (Ma and Wu, 2008; Paz et al., 2006; Islam et al., 2004) [12, 13, 14]. Furthermore, findings were made of improved shoot induction and regeneration, when the explants were transferred to MS medium with reduced BA content.

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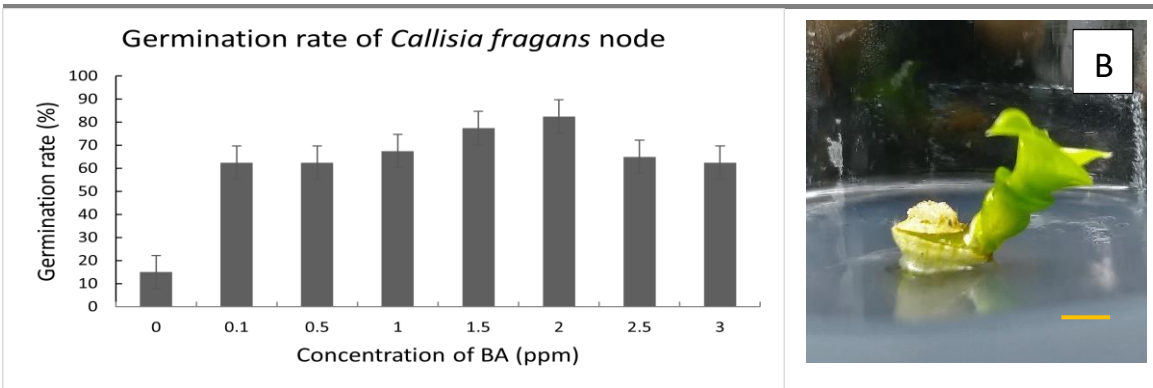

Figure 1. Effect of BA concentration on the germination rate of stem nodes after 4 weeks of culture (A); Germination morphology of shoots in culture medium containing BA. (Bar 0.5 cm (B))

Rapid multiplication of shoots in vitro

Effect of BA and NAA combination on in vitro shoot cluster formation

Shoots measuring 1 cm were used as explants and cultured on MS medium containing different concentrations of BA (0-1-2-3 mg/l) and NAA (0-0.25-0.5 mg/l). The combination of BA and NAA affected the shoot cluster formation as shown in Table 1. The MS basal medium without hormone supplementation had the lowest number of shoots per explant.

The effects of BA and NAA on shoot multiplication have been reported in many herbal plants such as *Rauvolfia tetraphylla* (Mohammad Faisal et al., 2012) [15]; *Psoralea corylifolia* (Anis and Faisal, 2005) [16*]; Asteracantha longifolia* (Panigrahi et al., 2007) [17]. As shown in Table 1, the shoot multiplication coefficient in the study reached the highest level of 1.2 shoots/explant in the treatment supplemented with 1 mg/l BA + 0.5 mg/l NAA. This is similar to the report of Al-Drisi et al. (2022) [18], MS medium containing 1 mg/L BA + 0.5 mg/L NAA gave the optimal rapid multiplication result for *Carica papaya* L.

The addition of increasing concentrations of BA to the treatments significantly reduced the shoot multiplication factor, shoot height as well as shoot quality (Bijaya Pant, 2012) [19]. Basically, the parameters decreased with increasing BA concentration from 1-3 mg/l; the multiplication factor gradually decreased from 1.06 shoots/explant in the treatment supplemented with 1 mg/l BA + 0.25 mg/l NAA to 0.73 shoots/explant in the treatment of 3 mg/l BA + 0.25 mg/l NAA.

The maximum shoot height was 0.63 cm on the medium supplemented with 1 ppm BA + 0.25 mg/l NAA. The new shoots were green and had developed leaves. The treatment with higher NAA concentration (0.5 mg/l) showed root development compared to the lower treatment (0.25 mg/l) (Figure 2).

Table 1. Effect of BA and NAA combination on in vitro shoot cluster formation (after 4 weeks of culture)

BA (ppm)	NAA (ppm)	shoots /cluster	Shoot height (cm)
0	0	0,11 ± 0,11 e	0,120 ± 0,080 e
0.5	0.25	1,06 ± 0,03 bc	0,420 ± 0,020 bc
1	0.25	1,27 ± 0,20 ab	0,630 ± 0,008 a
2	0.25	0,80 ± 0,05 cd	0,514 ± 0,030 ab
3	0.25	0,73 ± 0,03 cd	0,395 ± 0,007 bcd
0.5	0.5	1,20 ± 0,10 ab	0,420 ± 0,020 bcd

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1	0.5	1,46 ± 0,12 a	0,590 ± 0,008 a
2	0.5	0,60 ± 0,05 d	0,375 ± 0,007 cd
3	0.5	0,60 ± 0,05 d	0,280 ± 0,009 d

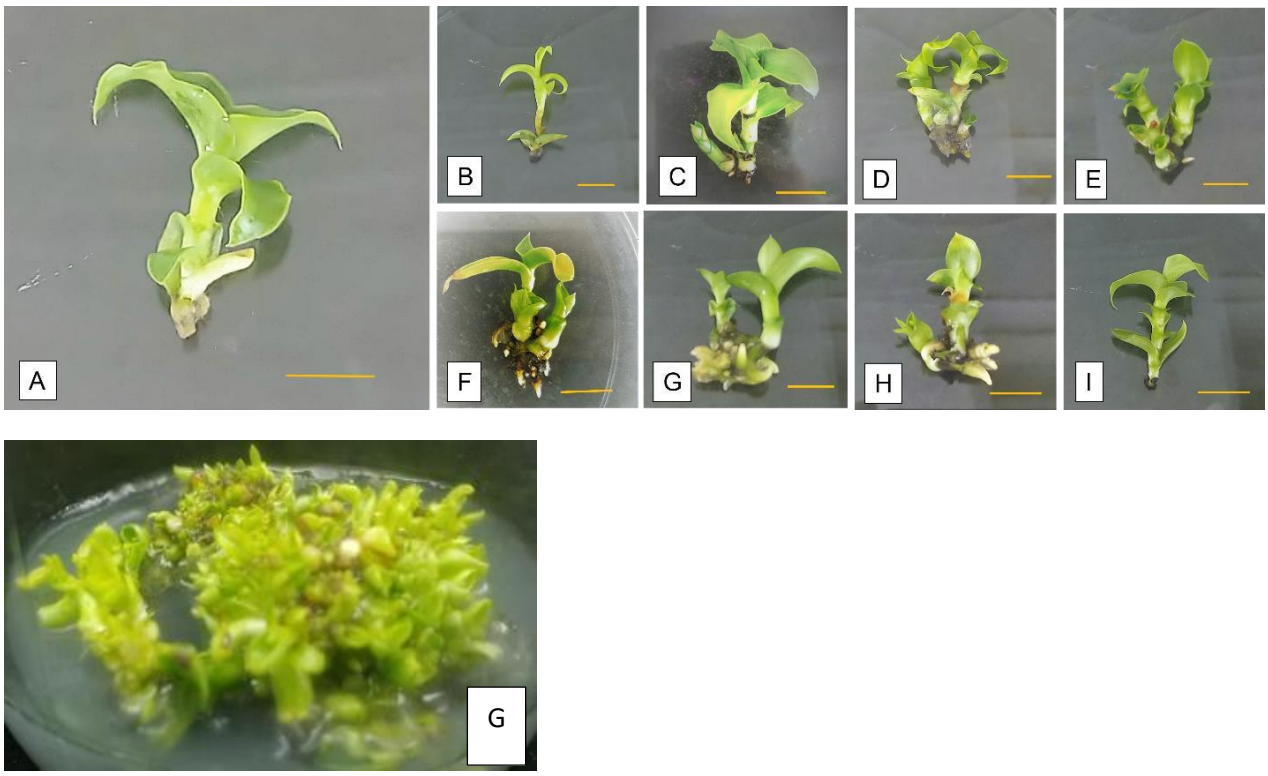

Figure 2. Shoot multiplication response of *Callisia fragrans* to different concentrations of BA and different combinations of auxin after 4 weeks of culture. Explants cultured on medium without growth regulators (A); 0.5 mg/l BA + 0.25 mg/l NAA (B); 1 mg/l BA + 0.25 mg/l NAA (C); 2 mg/l BA + 0.25 mg/l NAA (D); 3 mg/l BA + 0.25 mg/l NAA (E); 0.5 mg/l BA + 0.25 mg/l NAA (F); 1 mg/l BA + 0.5 mg/l NAA (G); 2 mg/l BA + 0.5 mg/l NAA (H); 3 mg/l BA + 0.5 mg/l NAA (I). (Bar A to I —1 cm)

Effect of BA + NAA and BA + IBA combinations on in vitro shoot multiplication

Shoot clusters (with 2-3 small shoots) were transferred to MS medium supplemented with 1 mg/l BA combined with NAA or IBA; after 4 weeks of culture, shoots 0.5 cm or higher were collected for data. The results in Table 2 show that NAA and IBA both have positive effects on the ability to multiply shoots in vitro. MS medium supplemented with 0.5 mg/l NAA achieved the highest shoot multiplication factor of 4.44 and the medium supplemented with 0.25 mg/l and 0.75 mg/l gave average shoot multiplication factors of 3.08 and 2.98, respectively (Figure 3). However, the effect of NAA on shoot multiplication factor was different when compared with IBA; IBA gave better results, with smaller shoots.

Adding IBA (0.1 - 0.5 mg/l) combined with 1 mg/l BA, the shoot multiplication coefficient gradually increased and was higher than that in the hormone-free medium (0.67 times) (Table 2). When increasing the IBA concentration from 0.5 to 0.75 mg/l, the shoot multiplication coefficient decreased, the shoots tended to form roots, the shoot quality was poor, the shoots were thin and short. The shoot multiplication coefficient reached the highest (5.87 times) after 4 weeks of culture on the medium supplemented with 1 mg/l BA and 0.5 mg/l IBA, the shoots grew well and many small shoot clusters were formed. The combination of BA and IBA has been proven effective in shoot multiplication in many species such as *Holostemma ada-kodien* Schult. (Martin, 2002) [20], [21] *Cryptolepis sanguinolenta* (Maame et al., 2016) [21], *Caladium bicolor* (Ahmed et al., 2023) [22].

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Table 2. Effect of BA + NAA and BA + IBA combinations on shoot cluster multiplication (after 4 weeks of culture)

BA (mg/l)	NAA (mg/l)	IBA (mg/l)	Multiplication rate
0	0		1,10 ± 0,10 d
1	0.10		2,71 ± 0,17 c
1	0.25		3,08 ± 0,19 c
1	0.50		**4,44 ± 0,24 ab**
1	0.75		2,98 ± 0,48 c
1		0.10	3,84 ± 0,18 bc
1		0.25	4,38 ± 0,82 abc
1		0.50	5,87 ± 0,86 a
1		0.75	3,65 ± 0,27 bc

Figure 3. Shoot multiplication response of *Callisia fragrans* to 1 mg/l BA concentration and different combinations of auxin (NAA and IBA) after 4 weeks of culture. Explants cultured on medium without growth regulators (A); 1.0 mg/l BA + 0.1 mg/l NAA (B); 1 mg/l BA + 0.25 mg/l NAA (C); 1 mg/l BA + 0.5 mg/l NAA (D); 1 mg/l BA + 0.75 mg/l NAA (E); 1 mg/l BA + 0.1 mg/l IBA (F); 1 mg/l BA + 0.25 mg/l IBA (G); 1 mg/l BA + 0.5 mg/l IBA (H); 1 mg/l BA + 0.75 mg/l IBA (I). (Bar A to I —1 cm)

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In vitro rooting culture for shoots

Single shoots, with a stem height of 2.5 - 3.5 cm, were separated from the shoot clusters. The samples cultured on hormone-free and hormone-supplemented media both developed roots. On the medium supplemented with IBA (Table 3), the number of roots reached 12.33 - 30 roots/shoot; the roots formed were shorter than those on the hormone-free MS medium (Table 3). The concentration of 1 ppm IBA resulted in the highest number of roots, about 30 roots/shoot. On the medium supplemented with NAA, the average number of roots increased but the average root length tended to decrease as the NAA concentration increased. The culture medium supplemented with 1 mg/l NAA gave a high average number of roots, corresponding to 27 roots/shoot, with the lowest average root length of 1.37 cm. Regarding the characteristics of the root system, on the medium supplemented with NAA, the roots formed relatively late, the roots were relatively short, and the shoots all had callus at the base (Figure 4). The root system formed on IBA-supplemented medium was 3 cm long, slender in shape and had many rootlets, which was also reported with the *D. trankimianum T. Yukawa* (H' Yon et al., 2020) [23].

Table 3. Effects of NAA and IBA on in vitro rooting (after 4 weeks of culture)

NAA (mg/l)	IBA (mg/l)	Roots	Root length (cm)
0	0	6,00 ± 0,57 c	4,56 ± 0,72 a
0.1		12,33 ± 1,45 c	2,46 ± 0,14 ab
0.5		25,00 ± 2,08 bc	3,56 ± 0,89 ab
1		27,00 ± 5,00 ab	1,26 ± 0,17 b
	0.1	12,33 ± 0,33 c	4,00 ± 0,76 ab
	0.5	17,00 ± 2,00 ab	3,00 ± 0,57 ab
	1	30,00 ± 3,60 a	3,03 ± 0,51 ab

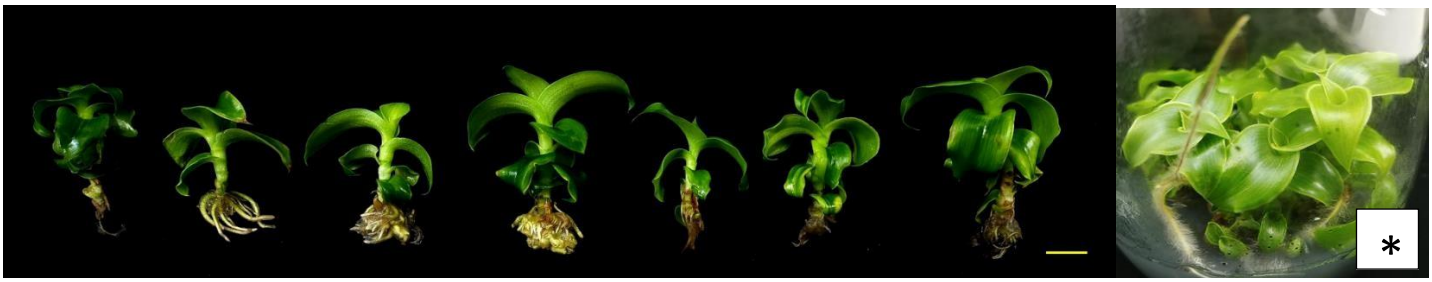

Figure 4. Rooting response of *Callisia fragrans* to combinations of NAA (1-2-3 mg/l) and IBA (1-2-3 mg/l) after 4 weeks of culture. From left to right, explants cultured on medium without growth regulators; 1 mg/l NAA; 2 mg/l NAA; 3 mg/l NAA; 1 mg/l IBA (*); 2 mg/l IBA; 3 mg/l IBA. (Bar—1 cm)

Cultivation of plantlets in the nursery

The growing medium (substrate) is a very important factor in creating conditions for plants to adapt outside the nursery. In vitro shoots reach a height of 3.0 - 3.5 cm, have 4 - 5 leaves, strong roots, grow well, and are transplanted outside the greenhouse. After being washed and planted in clean soil and clean coconut fiber, the shoots grow well on most growing media. On the coconut fiber substrate, the shoot height reaches 9.02 cm after 2 months (Table 4, Figure 5). Ara et al. (2013) [24] used soil substrate to acclimatize plants outside the greenhouse. Shoots grown in clean, nutritious soil have taller shoots, longer leaves, and a characteristic green color. Shoots grown in less nutritious coconut fiber have smaller shoots, light yellow leaves, but have more developed and longer roots because the coconut fiber is airy and light (Figure 5).

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Table 4. Domestication of Callisia fragrans (after 8 weeks)

Weeks	Clean coconut fiber		Clean soil	
	Plant height (cm)	Leaf length (cm)	Plant height (cm)	Leaf length (cm)
W1	3,5 ± 0,15	2,96 ± 0,13	3,00 ± 0,12	2,94 ± 0,11
W2	3,92 ± 0,13	3,09 ± 0,14	4,20 ± 0,12	3,50 ± 0,14
W3	5,37 ± 0.21	3,47 ± 0,13	6,61 ± 0,10	5,20 ± 0,19
W4	6,49 ± 0,26	4,37 ± 0,14	7,94 ± 0,15	6,11 ± 0,20
W5	7,31 ± 0,20	5,30 ± 0,13	11,22 ± 0,24	6,67 ± 0,21
W6	7,95 ± 0,17	5,31 ± 0,13	13,01 ± 0,20	6,95 ± 0,19
W7	8,43 ± 0,16	5,38 ± 0,13	14,49 ± 0,22	7,64 ± 0,21
W8	9,02 ± 0.,0	5,39 ± 0,11	15,09 ± 0,30	7,99 ± 0,26

Figure 5. *Callisia fragrans* shoots grown in coconut fiber tray (B) after 2 months. (Bar – 1 cm))

Callus culture

Callus induction culture

In vitro leaves were cut into square pieces of about 1 cm2. Leaf samples were cultured on MS medium supplemented with NAA and 2,4D at different concentrations. After 30 days of culture, callus formation on the medium supplemented with 2,4D gave better results than the medium supplemented with NAA. The medium with NAA stimulated callus to form roots better than callus growth. The medium supplemented with 1 - 3 mg/l NAA gave quite low callus formation results from 0.57 to 0.73. Compared to the medium supplemented with 2,4-D, the callus formed was more abundant and more spongy (Table 5). The ability to form callus depends on the concentration of the hormone 2,4D; at a concentration of 1 mg/l 2,4D, the callus ratio was lower than at a concentration of 2 mg/l, and at a concentration of 2 mg/l, the callus formation result was lower than at a concentration of 3 mg/l. However, when observing the combination of color and freshness of callus tissue, at high concentrations of 2,4D, the callus tissue turns brown. Therefore, a concentration of 1-2 mg/l 2,4D is suitable for producing better quality callus tissue (Table 5).

Callus culture of *Ruta Chalepensis* also showed that 1 mg/l 2,4D concentration was suitable (Jagadeesan et al., 2023) [25]. At appropriate concentrations, auxin is used to promote cell elongation, division and stimulate callus formation (Pierik, 1997) [26]. The addition of 2,4D and NAA to the culture medium increased the growth rate

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of callus. In this experiment, the medium containing 2,4D gave better results in both quantity and quality of callus compared to NAA. Using 2,4D gave superior callus formation results compared to NAA on *Moringa oleifera* Lam tissue samples (Al-Hamidi et al., 2023) [27] and *Pimpinella alpina* tissue samples (Faramayuda et al., 2022) [28].

Callus formation time on medium supplemented with 1 – 2 mg/l 2,4D and NAA was 21 – 18 days. The concentration of 3 mg/l NAA resulted in slower callus formation (16 days) compared to the same concentration of 3 mg/l 2,4D (15 days). Comparing the concentrations in each treatment, higher hormone concentrations resulted in faster callus formation. In the treatment supplemented with NAA, high NAA concentrations inhibited callus formation and lower concentrations allowed morphogenesis to occur; affecting both the quantity and quality of callus (Figure 6); and callus quickly formed roots on medium containing NAA. In the treatment supplemented with 2,4D, it was suitable for callus formation from Golden Comb leaves. Callus formed and developed better on 2,4D medium in *Moringa oleifera* (Asfandyar Chaudhary et al., 2019) [29]. Optimum callus formation can be achieved by adjusting the concentration of 2,4D, in this experiment the callus formation rate increased as the concentration of 2,4D increased (Table 5). The color of the callus was white, spongy, and opaque. However, after 1 month of culture, the callus turned yellow and brown when cultured on the medium with a concentration of 3 mg/l 2,4D (Figure 6). Therefore, through this experiment, the medium containing NAA was more suitable for root culture and the medium containing 2,4D was more suitable for callus culture.

Table 5. Effects of NAA and 2,4D on callus formation (after 4 weeks)

NAA (mg/l)	2,4D (mg/l)	Callus induction (%)	Time of callus induction (ngày)
0	0	-	-
1		7,30 ± 3,09	21,04 ± 2,09
2		7,30 ± 3,09	18,42 ± 1,30
3		5,70 ± 3,00	16,45 ± 1,27
	1	62,90 ± 6,94	21,58 ± 2,14
	2	76,80 ± 5,58	18,20 ± 1,58
	3	87,70 ± 5,15	15,43 ± 0,61

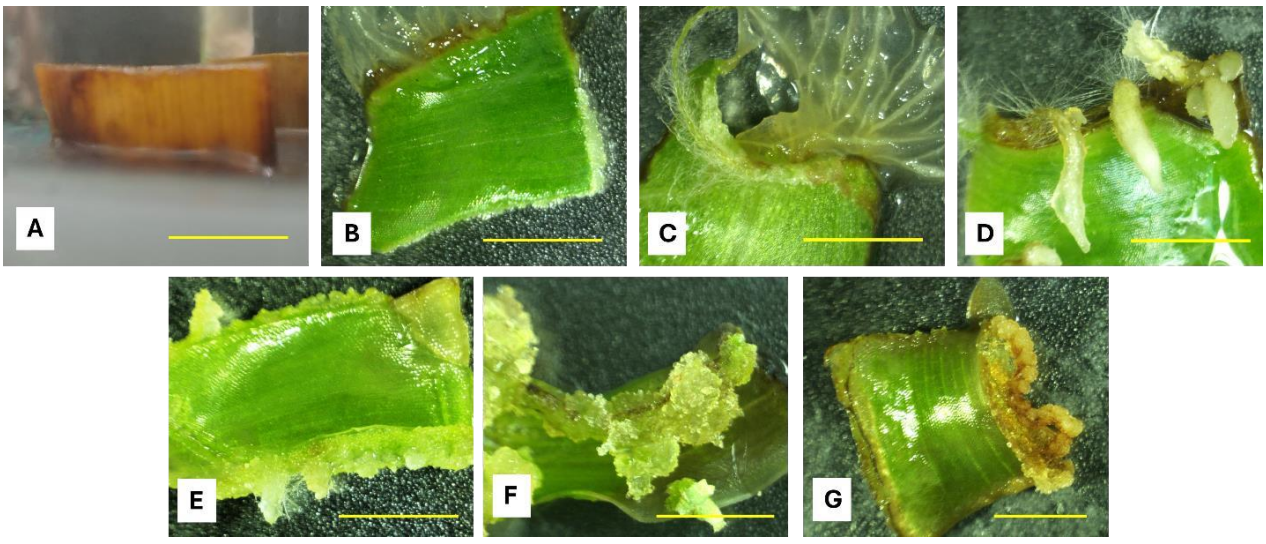

Figure 6. Callus formation from in vitro leaves of *Callisia fragrans* with 1ppm kinetin concentration and different auxin combinations (NAA + 2,4D) after 4 weeks of culture. Explants cultured on medium without growth regulators (A); 1 mg/l kinetin + 0.1 mg/l NAA (B); 1 mg/l kinetin + 2 mg/l NAA (C); 1 mg/l kinetin + 3 mg/l NAA (D); 1 mg/l kinetin + 1 mg/l 2,4D (E); 1 mg/l kinetin + 2 mg/l 2,4D (F); 1 mg/l kinetin + 3 mg/l 2,4D (G). Bar a to g —0.5 cm

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Callus subculture

Callus development depends on growth rate and culture medium composition. Accordingly, it is necessary to study suitable culture medium to maintain callus development to absorb active substances.

Callus was cultured on medium supplemented with kinetin and 2,4D at different concentrations. The results showed statistical differences between the treatments. MS medium supplemented with 1 mg/l 2,4D + 0.5 mg/l kinetin had a high callus growth rate of 4.49 after 4 weeks of culture (Table 6). This result was not different from the medium supplemented with 1 mg/l 2,4D + 0.5 mg/l Kinetin. When the concentration of 2,4D increased, the average weight of callus tended to decrease. The medium with a high concentration of 2 - 3 mg/l 2,4D (with callus proliferation of 3.94 and 3.22) both had lower results than the medium with 1 mg/l 2,4D (with proliferation of 4.37). The treatment with a concentration of 1 mg/l 2,4D had the highest callus formation in all treatments. With appropriate concentrations of auxin and cytokinin, callus proliferation is stimulated (Thomas et al., 1996) [30].

Differentiated callus formed roots on kinetin-containing medium. The medium with only kinetin resulted in non-proliferating callus; callus explants on kinetin-free and low kinetin media resulted in root formation. This result was also found in callus explants of *Chlorophytum borivilianum* (Jaafar et al., 2016) [31]. Culture media supplemented with 2,4D alone or in combination with kinetin were capable of stimulating cell proliferation (Table 6). Morphologically, all calluses were spongy and pale yellow in color. Toshio Murashige and Folke Skoog, 1962 [8] noted that 2,4D was suitable for callus explant culture. According to Narayanaswamy (1994) [32], root-forming callus will not differentiate into shoots.

Table 6. Effect of 2,4D and Kinetin on callus proliferation (after 4 weeks)

2,4D (ppm)	Kinetin (ppm)	Callus fresh weight (g)	Callus proliferation
0	0	Rooting	-
0	0.1	Browning	-
0	0.5	Browning	-
0	1	Browning	-
0	2	Browning	-
0.1	0	**0,150 ± 0,02 ef**	**1,50 ± 0,04 de**
0.5	0	0,137 ± 0,04 de	1,37 ± 0,46 cd
1	0	0,391 ± 0,07 bc	3,91 ± 0,58 ab
2	0	0,326 ± 0,08 ab	3,26 ± 0,47 abc
0.1	0.1	**0,145 ± 0,02 de**	**1,45 ± 0,05 cde**
0.5	0.5	0,286 ± 0,04 cd	2,86 ± 0,50 abcd
1	0.5	0,449 ± 0,02 de	4,49 ± 0,70 a
2	0.5	0,325 ± 0,03 de	3,25 ± 0,49 abc
3	0.5	0,299 ± 0,03 de	2,99 ± 0,44 abc
1	1	0,437 ± 0.06 a	4,37 ± 0,93 a
2	1	0,394 ± 0.03 a	3,94 ± 0,73 ab
3	1	0,322 ± 0.05 ab	3,22 ± 1,13 bcd

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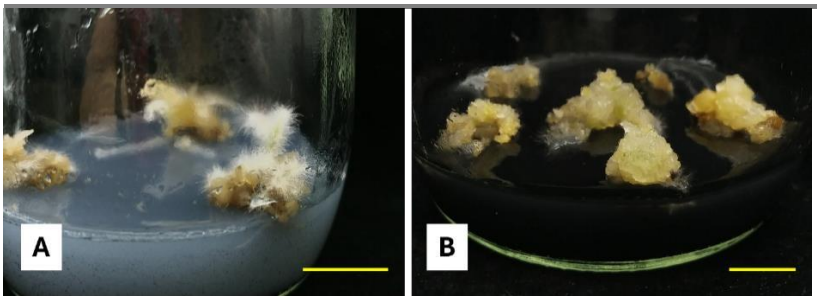


Figure 7. Callus culture after 4 weeks of culture on 2,4D medium combined with kinetin. Callus rooting on medium without added hormones (A); callus proliferation on 2,4D medium 1 mg/l combined with kinetin 0.5 mg/l (B). (Bar A, B – 1cm)

Active ingredient analysis

The results of HPLC analysis of the extract from 30-day-old callus on basic MS medium supplemented with 1 mg/l 2,4D + 1 mg/l kinetin, 5-month-old in vitro shoots and 2-month-old ex vitro shoots showed that on the chromatograms of all samples, there was a peak with the same retention time as the retention time of the standard substance oleanolic acid (saponin) (about 5 minutes) (Figure 9). The oleanolic acid content in the highest callus sample was 0.96 µg/mg, in vitro shoot sample was 0.23 µg/mg, ex vitro shoot sample was 0.01 µg/mg (Figure 8). Therefore, the callus sample is suitable for oleanolic acid extraction.

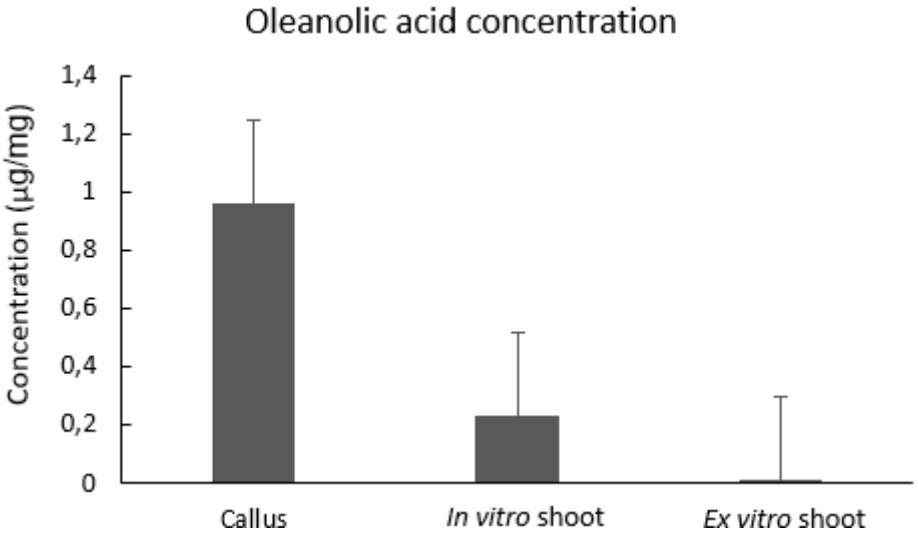
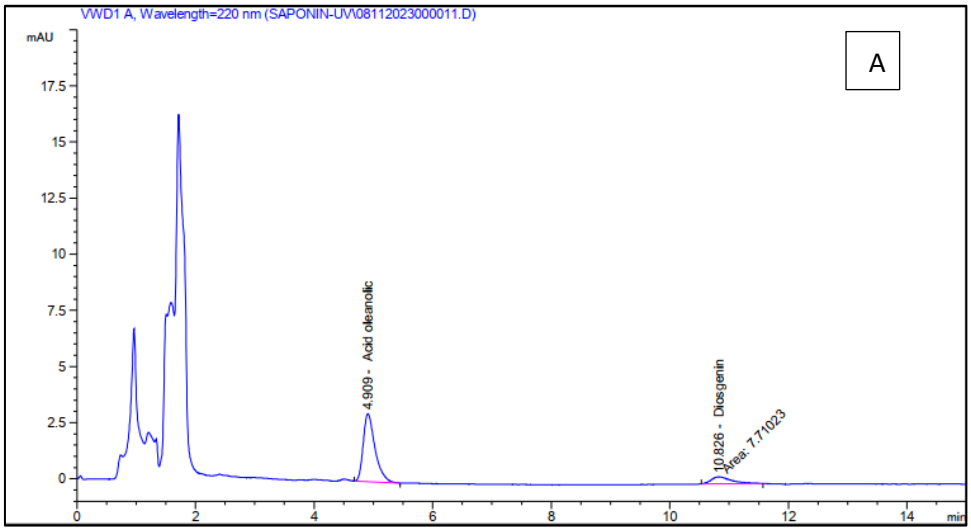


Figure 8. Results of saponin content analysis in 3 *Callisia fragrans* samples: callus, in vitro shoots, ex vitro shoots



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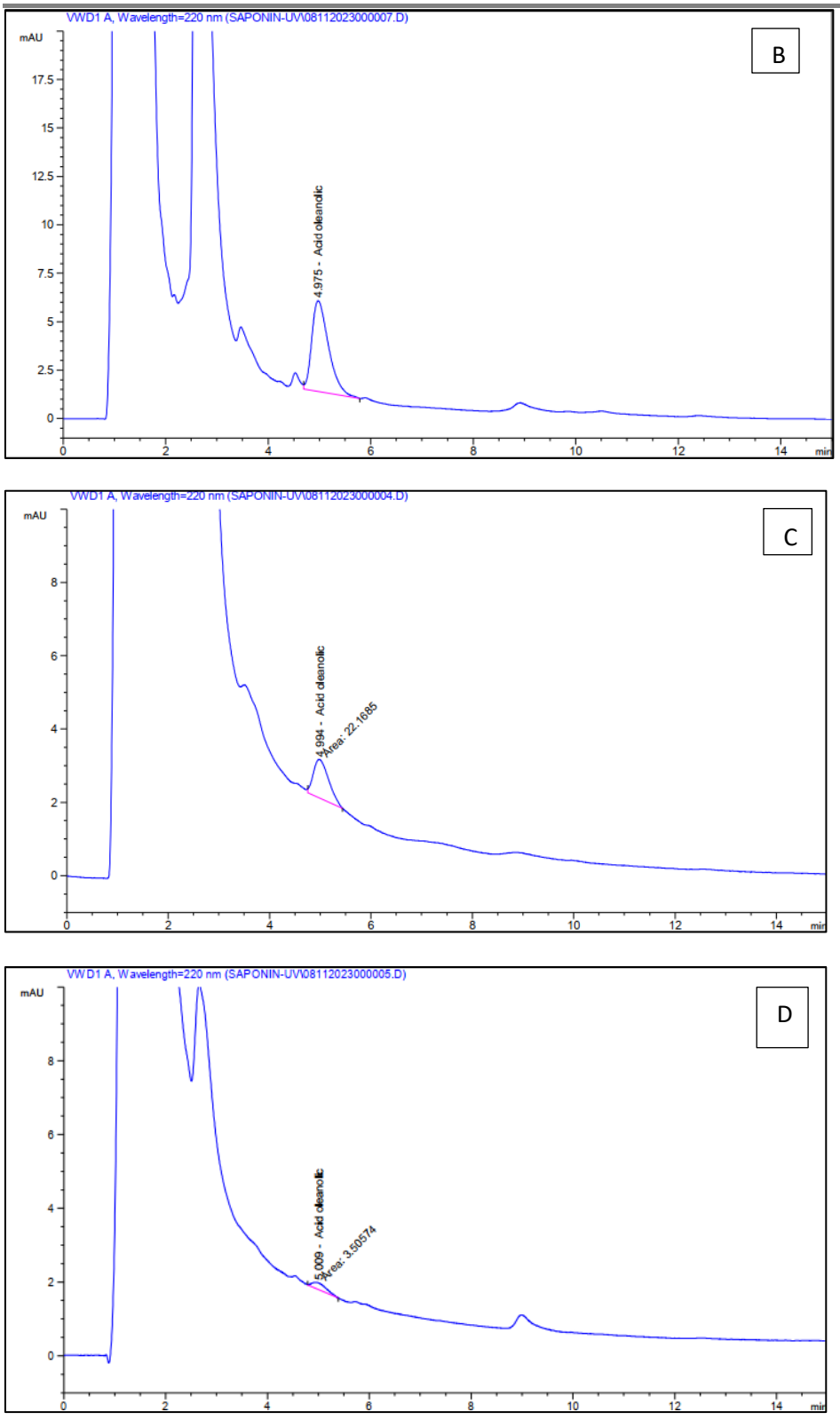

Figure 9. HPLC chromatogram of saponin (oleanolic acid) analysis. Standard saponin (A); Extract of golden comb plant callus (B); In vitro shoot extract (C); Ex vitro shoot extract (D).

CONCLUSION

The research results establish technical parameters for propagating Golden Comb through tissue culture method using stem nodes from 1-year-old mother plants. MS nutrient medium is suitable for culture. Plant samples are sterilized with 5% Ca (OHCl)2 for 30 minutes. Stimulate dormant buds from nodes on medium supplemented with 2 mg/l BA, create clusters of buds on medium supplemented with 1 mg/l BA + 0.5 mg/l NAA, quickly multiply clusters of buds on medium containing 0.5 mg/l IBA + 1 mg/l BA; culture to create roots on medium

0.5 mg/l NAA to create complete plants; shoots are acclimatized and grown on coconut fiber substrate when transferred to the greenhouse. During the process of callus formation, the medium supplemented with 1 - 2 mg/l 2,4D is suitable for creating quick and fresh callus; Callus inoculation was performed to increase callus proliferation on medium supplemented with 1 mg/l 2,4D + 1 mg/l kinetin. Analysis of saponin (oleanolic acid) showed accumulation in callus cells and in vitro shoots.

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