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IN VITRO PROPAGATION OF *CURCUMA SPAGANIFOLIA* GAGNEP., A RARE PLANT SPECIES FROM THAILAND

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ABSTRACT

Curcuma spaganifolia Gagnep., belonging to the family Zingiberaceae, has attracted widespread attention due to its considerable economic importance. The conventional propagation method for ginger family takes a long time for multiplication due to the low propagation rate and poor seed germination, therefore, tissue culture is an important method for the manipulation of plants for higher propagation. The present study deals with the *in vitro* and *ex vitro* conservation of *C. spaganifolia* Gagnep., a rare plant species from Thailand. Murashige and Skoog (MS) media with different concentrations of plant growth regulators, i.e., cytokinins (benzyladenine (BA) 6-furfurylamino purine (Kinetin), N, phenyl-N'-1,2,3 thidiazole-5-yl urea (TDZ), auxins (indole-3-acetic acid (IAA)), indole butyric acid (IBA) and naphthaleneacetic acid (NAA) were used to study the effect of plant growth regulators for eight weeks. Good development of shoot multiplication was observed in the MS media supplemented with 2 mgL^{-1} Kinetin, which produced the highest number of shoots per explant (3 shoots/explant) and roots per explant (9.57 roots/explant). Long divided microshoots of *C. spaganifolia* Gagnep. cultured on liquid MS media supplemented with 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA, showed significantly higher numbers of shoots and roots than the undivided microshoot explants. Acclimatization was successful when transplanted into soil, burned rice husk, sand and soil:burned rice husk (1/1 w/w) with 100% survival rate.

Key words: *Curcuma spaganifolia* Gagnep., Plant Tissue Culture, Propagation, Rare Plant, Zingiberaceae

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INTRODUCTION

Curcuma spaganifolia Gagnep. (Figure 1) is an important ornamental plant belonging to the family Zingiberaceae. It is known as Krachiao-Bua in Thailand. Its distribution includes several provinces in Thailand (Ubon Ratchathani, Nakhon Ratchasima, Si Sa Ket, Trat and Kanchanaburi) and in Cambodia. Due to the striking appearance of the small spikes with pink bracts, it has great potential to be developed as an economically important ornamental plant.

The market demand for *C. spaganifolia* Gagnep. is continuously increasing due to its multiple uses. The conventional method of propagation cannot support the increasing market demand. Conventional propagation of *C. spaganifolia* Gagnep. is via rhizomes, but this has a low proliferation rate and is easily infected by soil pathogens. Many ginger habitats have been eliminated by human activities like land clearance for agriculture and deforestation. Furthermore, this plant depends on the season and requires a long time to be built up to commercial quantities. The *in vitro* culture technique could be used as an alternative method for the multiplication of *C. spaganifolia* Gagnep. Micropropagation protocols have been successfully developed for many species of the *Curcuma*, such as *C. aeruginosa* (Theanphong *et al*., 2010; Alizah *et al*., 2019), *C. amada* (Prakash *et al.*, 2004), *C. angustifolia* (Shukla *et al.,* 2007), *C. aromatica* (Nayak, 2000), *C. attenuata* (Kou *et al.*, 2013), *C. caesia* (Bharalee *et al.,* 2005; Shahinozzaman *et al.*, 2013; Zuraida, 2013; Singh *et al.*, 2015; Chowdhury *et al*., 2020), *C. domestica* (Keng *et al.,*

2004), *C. kwangsiiensis* (Zhang *et al.,* 2011), *C. longa* (Nasirujjaman *et al.*, 2005; Rahman *et al.,* 2004; Prathanturarug *et al.*, 2005; Srirat *et al.,* 2009; Behera *et al.*, 2010; Goyal *et al.,* 2010; Jala, 2012; Jala, 2013; Ghosh *et al.,* 2013; Ugochukwu *et al.*, 2013), *C. manga* (Raihana *et al.,* 2011), *C. redoaria* (Stanly and Keng, 2007), *C.soloensis* (Zhang *et al.,* 2011), *C. xanthorrhiza* (Rahayu and Adil, 2012) and *C. zedoaria* (Keng *et al.*, 2004; Bharalee *et al.*, 2005; Loc *et al*., 2005; Stanly *et al.*, 2010). There has been previous research about the *in vitro* propagation of members of the ginger family when using liquid medium, but this has been limited in certain species. For *C. aeruginosa* (Alizah *et al.*, 2019), *C. longa* (Prathanturarug *et al.*, 2005), *C. zedoaria (*Stanly and Keng, 2007; Stanly *et al.*, 2010; Chong *et al*., 2012*)*, *Globba globulifera* (Yaowachai *et al.*, 2020) and *Zingiber zerumbet* (Stanly and Keng, 2007; Stanly *et al.*, 2010), which are members of this family, they cultured using liquid media for its microprogagation. When liquid rather than solid media is used, it is possible to increase the propagation rate while the frequencies for subculturing are decreased. There have been no reports of *in vitro* propagation of *C. spaganifolia* Gagnep., a rare plant species from Thailand, when using the plant tissue culture technique. This present study was carried out to optimize *in vitro* propagation protocol. Therefore, the objectives of this study were to investigate the effects of media type, explant type and plant growth regulators on shoot and root proliferation and to examine the effects of various potting media on hardening after transplantation under greenhouse conditions of *C. spaganifolia* Gagnep.

MATERIALS AND METHODS

Plant Materials and Surface Sterilization: Clean rhizomes of *Curcuma spaganifolia* Gagnep. collected from Ubonratchathani Province, Thailand and used in the experiment. Rhizome buds were rinsed with running tap water for two hours, then surface sterilized using 20% (v/v) sodium hypochlorite with two drops of tween 20 for 15 minutes and five minutes respectively and then rinsed three times in sterilized distilled water. Then, the rhizome buds were initially cultured on basal MS (Murashige and Skoog, 1962). After two months of bud culture, excised microshoots (1 cm long) were used as explants.

Culture Media and Conditions: All explants were transferred to MS media supplemented with 3% (w/v) sucrose and different concentrations of plant growth regulators. The plant growth regulators were BA, Kinetin or TDZ (0, 0.1, 0.5, 1.0, 2.0, 4.0. 8.0, and 16.0 mgL^{-1}), IAA, IBA or NAA (0, 0.1, 0.5, 1.0, 1.5, and 2.0 mgL^{-1}). Solid and liquid MS media supplemented with 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA or without Kinetin and IAA were used to study the effect of the type of media on the shoot and root formation. For the solid medium, 0.8% (w/v) agar was supplemented into the MS medium with plant growth regulators added. Each treatment consisted of 20 culture bottles and each culture bottle contained a single explant. The same composition was used for the liquid media except agar. For the liquid media, three aseptic shoots were cultured in a 250 ml Erlenmeyer flask containing 100 ml of the liquid MS medium. A rotary shaker was used for agitation at 120 rpm. Each treatment consisted of 10 flasks and each flask contained three explants. The experiments were replicated three times. The pH of the medium was adjusted to 5.7-5.8 with 1 N NaOH or 1 N HCl. The cultures were kept at a temperature of 25±2 °C with a florescent light intensity of 40-42 $mmolm^{-2}s^{-1}$ for a 16 hour photoperiod/day for eight weeks**.**

***Ex vitro* Transplantation:** Plantlets, 5 cm tall, with well-developed roots were removed from the culture medium. The roots were washed gently under running tap water and transferred into plastic pots containing soil, burned rice husk, sand, soil:burned rice husk (1:1 w/w), soil:sand (1:1 w/w), burned rice husk:sand (1:1 w/w) or soil:burned rice husk:sand (1:1:1 w/w) for eight weeks. Twenty plantlets were used for each treatment for *ex vitro* transplantation. The potted plants were maintained under greenhouse conditions at the Department of Biology, Faculty of Science, Mahasarakham University, Thailand and regularly irrigated with tap water.

Statistical Analysis: The experiments were set up in a completely randomized design (CRD) with 20 plantlets for each treatment in solid and liquid media using 30 plantlets for each treatment. The data were analyzed using Statistical Package for Social Sciences (SPSS) Version 11.5 on the mean number of shoots, roots, leaves and length of shoots and roots after eight weeks. Analysis of variance (ANOVA) was used to test the statistical significance, and the significance of differences among means was carried out using Duncan's Multiple Range Test (DMRT) at $p = 0.05$.

Figure 1. *Curcuma spaganifolia* Gagnep. (A) rhizome and (B) shoot with inflorescence.

RESULTS AND DISCUSSION

Microshoots (1 cm long) of *Curcuma spaganifolia* Gagnep.were cultured on MS media with various concentrations of cytokinins (BA, Kinetin and TDZ) and auxins (IAA, IBA and NAA) for shoot and root formation after eight weeks. The average number of shoots was maximum in MS media with 2 mgL^{-1} Kinetin. These result differ from previous reports, which demonstrated that BA alone or BA in combination with auxin supported shoot and root formation (Nayak, 2000; Salvi *et al*., 2000; Shirgurkar *et al*., 2001; Prakash *et al.*, 2004; Bharalee *et al.*, 2005; Loc *et al*., 2005). Salvi *et al.* (2002) found that *Zingiber zerumbet* cultured on BAP and NAA produced the highest number of shoots (5.6 shoots/explant). We observed from our study that Kinetin was the most effective cytokinin and promoted shoot and root multiplication in *C. spaganifolia* Gagnep. at a low concentration. However, shoot formation was observed in all MS medium with various concentrations of BA, Kinetin and TDZ added, but the number of shoots was low at all concentrations. The MS media supplemented with TDZ did not support root formation. Rooting was observed in MS media supplemented with BA or Kinetin. This is in agreement with the study performed by Prathanturarug *et al.* (2005) who found that TDZ induced shoots more effectively from *in vitro* cultured *Curcuma longa* than other cytokinins (BA, Kinetin and 2iP). According to Salvi *et al.* (2000), TDZ induced a high average number of shoots (8.9 shoots/explant) in turmeric. A concentration of cytokinin more than 8 mgL^{-1} was less effective for *in vitro* shoot initiation. MS media with 2 mgL^{-1} Kinetin was the optimum for *in vitro* shoot multiplication of *C. spaganifolia* Gagnep. with 3 shoots and 9.57 roots (Tables 1-2 and Figures 2-3).

The *in vitro* propagation of *C. spaganifolia* Gagnep. was assessed via solid and liquid MS culture media. Depending on the type of culture system implemented, when culturing on MS media with or without 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA, after eight weeks, there were a varied number of roots produced. The liquid and solid medium produced 4.51 and 2.79 shoots/explant, respectively (Table 3 and Figure 4), which means that there were significantly more shoots/explant produced by *C. spaganifolia* Gagnep. in the liquid rather than the solid media. The agar had significant effects on the *in vitro* growth and development due to the solid media giving less oxygen to the cultured plants. In contrast, when the plant was cultured on a rotary shaker in a liquid media it was better able to take up nutrients and plant hormones resulting in better shoot and root development (Ziv, 1989; Scholten and Pierik, 1998; Smith and Spomer, 1994; Sandal *et al*., 2001). It has been shown that continual shaking can enhance the growth and multiplication of the shoots of the explants due to better aeration as there is a better oxygen supply (Mehrotra *et al*., 2007). The liquid media also has the ability to increase the nutrient and plant growth regulator uptake as the partly submerged shoots had a larger surface area (Arshad *et al*., 2005). When plants are cultured in a liquid medium, so more plants can be produced. There was no hyperhydricity seen in the present study when they were grown in liquid medium (Figure 4).

During the present study, the microshoots of *C. spaganifolia* Gagnep. were cultured on solid or liquid MS media supplemented with 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA and they showed a significantly higher number of shoots and roots when compared to those cultured on the media without plant hormones. These results indicated that Kinetin and IAA have positive effects for shoot and root formation. The result showed that diving the microshoots longitudinally into halves could enhance the formation of multiple shoots. After eight weeks of culture, the long divided microshoots gave an average number of shoots and roots for each half explant that was more than from the undivided microshoots on both solid and liquid media. The relative growth rate of *C. spaganifolia* Gagnep. from liquid culture was higher than the rate from solid culture in all treatments. The beneficial effects of liquid culture were due to better aeration and by allowing the close contact of plant tissue with the nutrients and plant growth regulators in the media. Regarding the effects of the types of media, liquid media provided the maximum number of shoots and roots. These results indicated that liquid media has positive effects for shoot and root formation, which are in agreement with some studies. Similar to our observation, Stanly and Keng (2007) reported the micropropagation of *C. zedoaria* Roscoe and *Zingiber zerumbet* Smith in liquid and solid media. The present observation showed that the *in vitro* plantlets of both species when cultured in liquid media produced twice the number of shoots than those cultured in solid media of the same composition. They also found that long divided shoots of *C. zedoaria* Roscoe produced significantly higher numbers of shoots than undivided or whole shoots after culturing in liquid media for four weeks. These results are agreement with those of Chong *et al.* (2012) who studied three types of culture system, solid medium system, liquid shake flask system and temporary immersion system (TIS), for their efficiency when propagating *C. zedoria* Roscoe plantlets. The result showed that among the three systems used, the liquid shake flask system significantly induced more shoot formation and larger shoots from the shoot explants of *C. zedoria* Roscoe after eight weeks. The result indicated that the shoot culture on the liquid MS medium produced the highest number of shoots with an average of 7 shoots/explant when cultured on the liquid media containing 0.5 mgL^{-1} BA and 0.5 mgL^{-1} IBA. Alizah *et al.* (2019) investigated the *in vitro* propagation of *C. aeruginosa* Roxb. in liquid MS media containing 4 mgL^{-1} BA. Yaowachai *et al.* (2020) reported that a liquid MS media supplemented with 2 mgL^{-1} of TDZ show the highest number of shoots (12.25 shoots/explant) of *Globba globulifera* Gagnep. when cultured for eight weeks.

The long divided and undivided plantlets of *C. spaganifolia* Gagnep. were used to examine their effect on the explant type. After eight weeks of culture on liquid MS media supplemented with 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA, the number of shoots (4.51 shoots/explant) and the number of roots (8.53 roots/explant) were observed on the long divided plantlets when compared to the undivided shoots that produced 2.89 shoots/explant and 5.86 roots/explant within the same duration. Nevertheless, when the undivided plantlets were cultured on the solid or liquid MS media with or without Kinetin and IAA added, the plantlets did not grow well. These results showed that the long divided plantlets of *C. spaganifolia* Gagnep., when cultured on MS media with 2 mgL^{-1} Kinetin and 1.5 mgL^{-1} IAA added produced a significantly higher number of shoots and roots when compared to the undivided plantlets. The results indicated that when the plantlets were divided into halves, it could promote the formation of multiple shoots because it let direct access to the nutrients, plant growth regulators, water and oxygen. The MS medium supplemented with Kinetin and IAA showed greater potential for *C. spaganifolia* Gagnep. as compared to the MS media without Kinetin and IAA with a significantly higher number of shoots and roots.

Plantlets with four to five leaves and a well-developed root system were removed from the media and washed under running tap water carefully and transferred to pots containing soil, burned rice husk, sand, soil:burned rice husk (1:1 w/w), soil:sand (1:1 w/w), burned rice husk:sand (1:1 w/w) or soil:burned rice husk:sand (1:1:1 w/w) without a hardening process for eight weeks under greenhouse conditions at the Department of Biology, Faculty of Science, Mahasakham University, Mahasarakham, Thailand. After two weeks, plantlets of *C. spaganifolia* Gagnep. were transplanted into all various potting medium and produced new leaves, indicating that they can adapt well to the conditions outside the culture environment and can be used as planting material. The complete plantlets of *C. spaganifolia* Gagnep. were transplanted into soil, burned rice husk, sand and soil:burned rice husk:sand (1:1:1 w/w) showed excellent survival rates of 100%, average number of leaves per shoot of 7-9 leaves and average shoot height of 10-12 cm (Table 4 and Figure 5). The regenerated plants did not show any detectable variation in the morphological characteristics compared to the mother plant. Our planting media results are in agreement with Chowdhury *et al*. (2020), who noted that healthy and complete plants of *Curcuma caesia* Roxb. with well-developed roots were hardened, acclimatized and planted in the field successfully with a survival rate of 70% when transplanted to plastic pots containing sterilized soil and kept under a 50% shaded net house.

Table 1. Effects of cytokinins (BA, Kinetin and TDZ) on shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks.

Cytokinins	Average no. of shoots/explant mean±S.E.	Length of shoots/explant (cm) mean±S.E.	Average no. of roots/shoot mean±S.E.	Length of roots/shoot (cm) mean±S.E.
MS (control)	1.33±0.12^{bc}	7.83±0.66^{a}	3.53±0.30^{bcdef}	5.06±0.31^{a}
BA (mgL^{-1})				
0.1	2.13±0.40^{b}	7.25±0.76^{abc}	4.93±0.81^{bc}	4.56±0.28^{ab}
0.5	1.60±0.23^{bc}	7.25±0.40^{abc}	4.53±0.58^{bc}	4.30±0.24^{abc}
1	1.66±0.21^{bc}	7.67±0.67^{a}	3.93±0.50^{bcde}	3.99±0.30^{abcd}
2	1.86±0.19^{bc}	6.53±0.56^{abcd}	3.53±0.42^{bcdef}	3.05±0.22^{defg}
4	1.40±0.16^{bc}	6.55±0.37^{abcd}	3.40±0.42^{bcdef}	2.53±0.19^{efgh}
8	1.53±0.19^{bc}	5.27±0.31^{cde}	1.92±0.37^{defg}	1.81±0.20^{hi}
16	1.33±0.12^{bc}	5.48±0.43^{bcde}	2.00±0.27^{defg}	1.18±0.08^{i}
Kinetin (mgL^{-1})				
0.1	1.50±0.19^{bc}	7.32±0.67^{ab}	1.77±0.36^{defg}	1.15±0.36^{i}
0.5	1.80±0.24^{bc}	6.63±1.18^{abcd}	4.10±0.64^{bcd}	3.66±0.31^{bcde}
1	1.60±0.30^{bc}	4.67±0.61^{defg}	5.81±0.99^{b}	3.87±0.44^{bcd}
2	3.00±0.97^{a}	5.31±0.78^{bcde}	9.57±2.09^{a}	3.37±0.42^{cdef}
4	1.00±0.00^{c}	2.18±0.00^{h}	5.94±0.67^{g}	3.26±0.48^{j}
8	1.91±0.31^{bc}	4.77±0.39^{defgh}	4.66±1.21^{bc}	2.36±0.27^{fgh}
16	1.25±0.25^{bc}	4.71±0.48^{defg}	2.60±0.81^{cdef}	1.81±0.59^{hi}
TDZ (mgL^{-1})				
0.1	1.91±0.35^{bc}	5.34±0.52^{bcde}	5.00±0.77^{bc}	3.28±0.57^{cdef}
0.5	1.06±0.06^{c}	5.40±0.84^{bcde}	1.00±0.00^{fg}	4.10±0.44^{abcd}
1	1.72±0.30^{bc}	3.78±0.58^{efgh}	1.16±0.16^{fg}	2.97±0.69^{defgh}
2	1.81±0.29^{bc}	3.22±0.34^{fgh}	1.25±0.25^{fg}	2.63±0.70^{efgh}
4	1.86±0.29^{bc}	2.77±0.23^{gh}	1.57±0.29^{efg}	2.50±0.49^{efgh}
8	1.69±0.36^{bc}	3.73±0.54^{efgh}	1.00±0.00^{fg}	2.35±0.91^{fgh}
16	1.42±0.20^{bc}	3.43±0.43^{efgh}	1.00±0.00^{fg}	1.87±0.22^{ghi}

Means followed by the same latter are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test (DMRT).

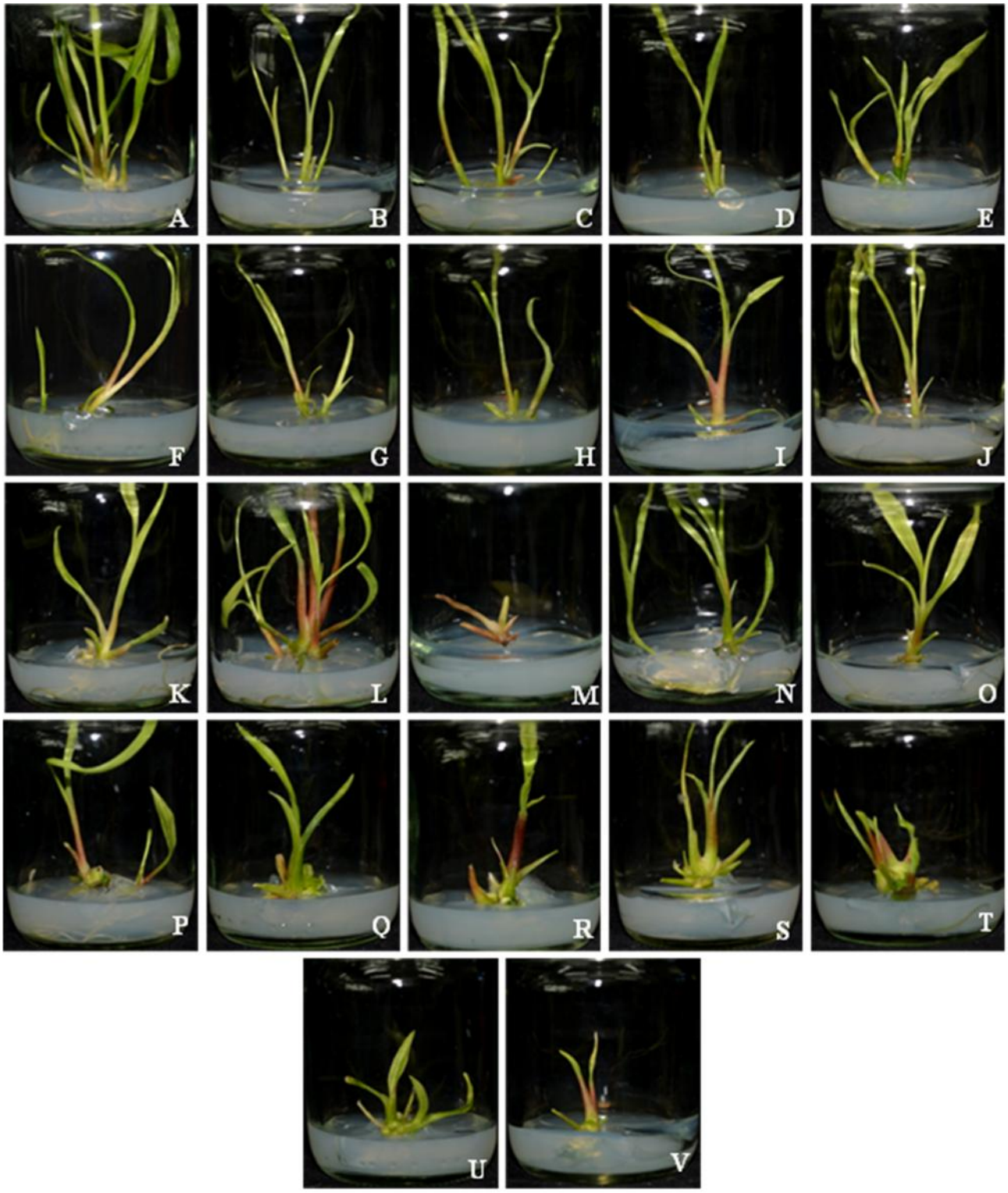

Figure 2. Shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks under *in vitro* conditions (A) hormone free, (B) BA 0.1 mgL^{-1}, (C) BA 0.5 mgL^{-1}, (D) BA 1.0 mgL^{-1}, (E) BA 2.0 mgL^{-1}, (F) BA 4.0 mgL^{-1}, (G) BA 8.0 mgL^{-1}, (H) BA 16.0 mgL^{-1}, (I) Kinetin 0.1 mgL^{-1}, (J) Kinetin 0.5 mgL^{-1}, (K) Kinetin 1.0 mgL^{-1}, (L) Kinetin 2.0 mgL^{-1}, (M) Kinetin 4.0 mgL^{-1}, (N) Kinetin 8.0 mgL^{-1}, (O) Kinetin 16.0 mgL^{-1}, (P) TDZ 0.1 mgL^{-1}, (Q) TDZ 0.5 mgL^{-1}, (R) TDZ 1.0 mgL^{-1}, (S) TDZ 2.0 mgL^{-1}, (T) TDZ 4.0 mgL^{-1}, (U) TDZ 8.0 mgL^{-1} and (V) TDZ 16.0 mgL^{-1}.

Table 2. Effects of auxins (IAA, IBA and NAA) on shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks.

Auxins	Average no. of shoots/ explant mean±S.E.	Length of shoots/ explant (cm) mean±S.E.	Average no. of roots/shoot mean±S.E.	Length of roots/shoot (cm) mean±S.E.
MS (control)	1.13±0.09^{c}	11.07±0.38^{a}	4.00±0.37^{c}	5.03±0.20^{a}
IAA (mgL^{-1})				
0.1	1.73±0.22^{abc}	7.99±0.45^{bc}	5.13±0.37^{abc}	4.74±0.23^{a}
0.5	1.46±0.16^{bc}	7.54±0.51^{c}	5.06±0.35^{abc}	4.53±0.17^{a}
1	2.20±0.27^{abc}	7.22±0.64^{c}	6.26±0.66^{ab}	3.70±0.17^{c}
1.5	2.40±0.36^{ab}	6.99±0.51^{c}	7.06±0.93^{a}	3.56±0.16^{c}
2	1.60±0.25^{bc}	7.62±0.59^{c}	4.66±0.38^{bc}	3.62±0.20^{c}
IBA (mgL^{-1})				
0.1	2.40±0.38^{ab}	7.58±0.60^{c}	6.00±0.70^{abc}	3.74±0.23^{c}
0.5	2.33±0.39^{ab}	8.43±0.68^{bc}	5.33±0.87^{abc}	4.46±0.21^{ab}
1	1.16±0.11^{c}	10.68±0.60^{a}	4.76±0.53^{bc}	3.59±0.18^{c}
1.5	2.06±0.52^{abc}	9.66±0.71^{ab}	5.66±0.92^{abc}	3.84±0.25^{c}
2	1.46±0.21^{bc}	9.69±0.32^{ab}	5.40±0.70^{abc}	3.87±0.20^{bc}
NAA (mgL^{-1})				
0.1	1.46±0.29^{bc}	9.62±0.76^{ab}	4.13±0.35^{bc}	3.89±0.17^{bc}
0.5	2.20±0.69^{abc}	8.09±0.63^{bc}	5.13±0.79^{abc}	3.63±0.27^{c}
1	1.40±0.23^{bc}	9.69±0.48^{ab}	5.66±0.75^{abc}	3.28±0.13^{cd}
1.5	1.80±0.20^{abc}	8.21±0.58^{bc}	5.86±0.59^{abc}	2.67±0.14^{e}
2	2.73±0.35^{a}	6.93±0.73^{c}	5.86±0.46^{abc}	2.81±0.19^{de}

Means followed by the same latter are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test (DMRT).

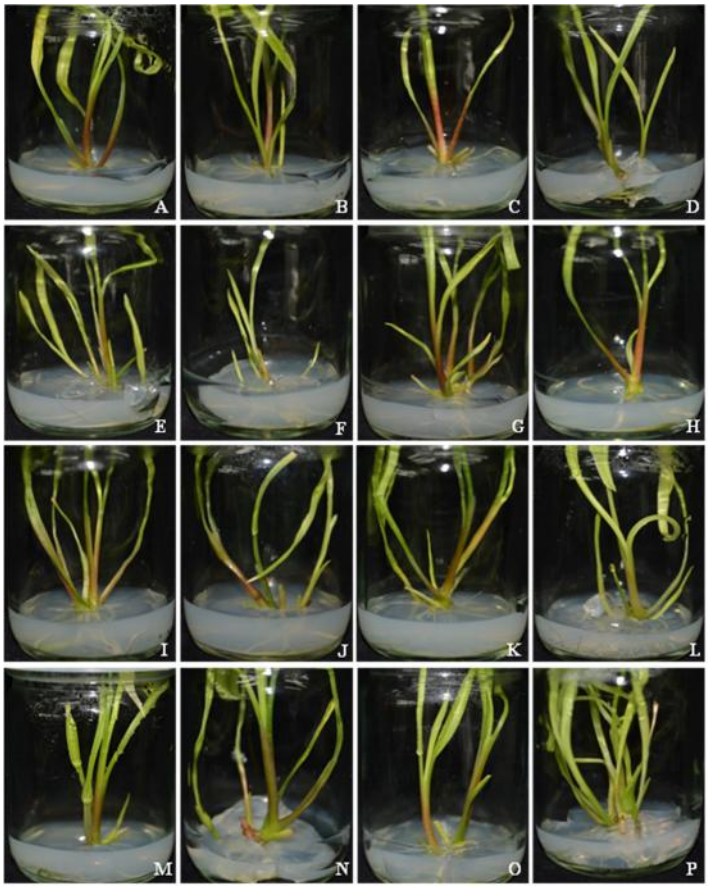

Figure 3. Shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks of cultivation under *in vitro* conditions (A) hormone free, (B) IAA 0.1 mgL^{-1}, (C) IAA 0.5 mgL^{-1}, (D) IAA 1.0 mgL^{-1}, (E) IAA 1.5 mgL^{-1}, (F) IAA 2.0 mgL^{-1}, (G) IBA 0.1 mgL^{-1}, (H) IBA 0.5 mgL^{-1}, (I) IBA 1.0 mgL^{-1}, (J) IBA 1.5 mgL^{-1}, (K) IBA 2.0 mgL^{-1}, (L) NAA 0.1 mgL^{-1}, (M) NAA 0.5 mgL^{-1}, (N) NAA 1.0 mgL^{-1}, (O) NAA 1.5 mgL^{-1} and (P) NAA 2.0 mgL^{-1}.

Table 3. Types of media, plant growth regulators, explant type on shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks.

Type of media	Plant growth regulators	Explant type	No. of shoots/ explant mean±S.E.	Length of shoots/ explant (cm) mean±S.E.	No. of roots/shoot mean±S.E.	Length of roots/shoot (cm) mean±S.E.
Solid media	MS	Undivided	1.33±0.2^{d}	8.47±0.59^{ab}	3.20±0.14^{d}	4.43±0.27^{ab}
		Long divided	2.42±0.34^{bc}	7.18±0.85^{bc}	4.22±0.29^{c}	3.39±0.47^{bc}
	MS+2 mgL^{-1} Kinetin +1.5 mgL^{-1} IAA	Undivided	2.20±0.40^{bc}	5.89±0.48^{bc}	4.18±0.20^{c}	2.69±0.23^{cd}
		Long divided	2.79±0.12^{b}	3.85±0.59^{cd}	5.38±0.28^{b}	2.56±0.34^{cd}
Liquid media	MS	Undivided	1.87±0.13^{c}	12.26±1.10^{a}	4.48±0.21^{c}	4.96±0.41^{a}
		Long divided	2.43±0.12^{bc}	8.82±1.63^{ab}	5.95±0.19^{b}	4.98±0.46^{a}
	MS+2 mgL^{-1} Kinetin +1.5 mgL^{-1} IAA	Undivided	2.89±0.09^{b}	5.82±0.85^{bc}	5.86±0.13^{b}	2.86±0.33^{cd}
		Long divided	4.51±0.14^{a}	6.12±1.22^{bc}	8.53±0.21^{a}	2.66±0.30^{cd}

Means followed by the same latter are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test (DMRT).

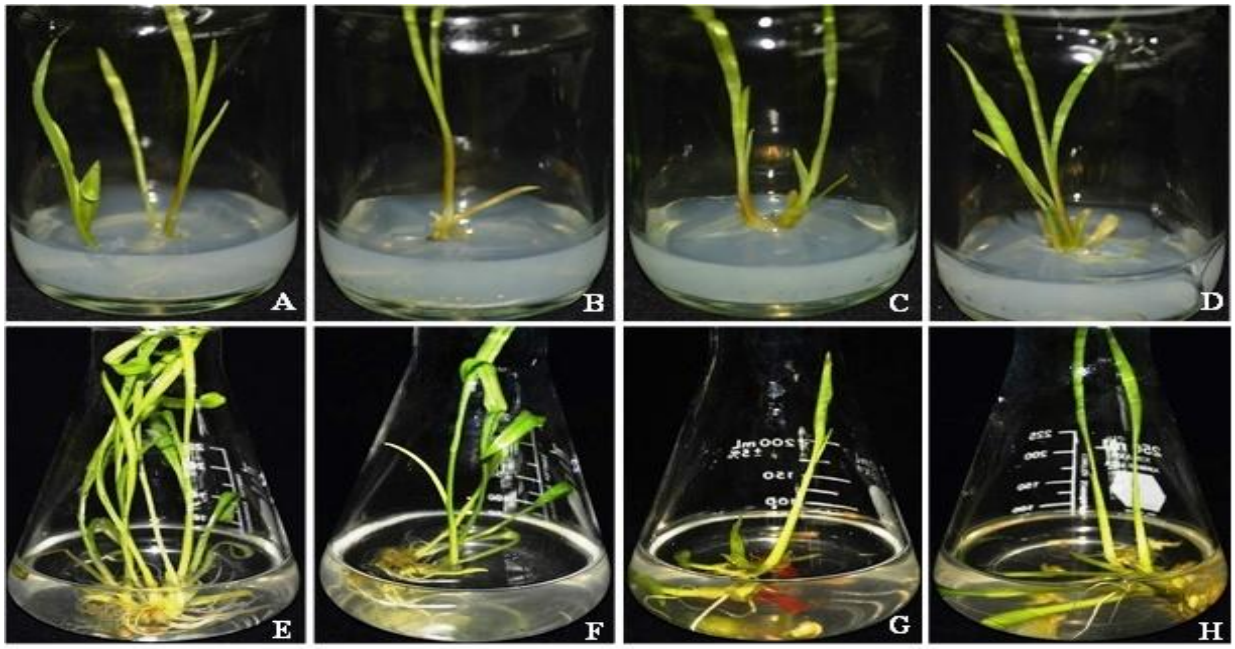

Figure 4. Shoot and root formation of *C. spaganifolia* Gagnep. after eight weeks of cultivation undivided and divided microshoots on solid and liquid MS media supplemented with (A-D) solid media, (E-H) liquid media, (A, E) undivided microshoots on MS media, (B, F) long divided microshoots on MS media, (C, G) undivided microshoots on MS media with Kinetin and IAA added and (D, H) long divided microshoots on MS media with Kinetin and IAA added.

Table 4. Effects of various potting media on hardening of *C. spaganifolia* Gagnep. after eight weeks.

Potting media	Percentage of surviving plantlets	Average no. of leaves/shoot mean±S.E.	Average shoot height (cm) mean±S.E.
Soil	100	9.23±0.13^{a}	12.62±0.80^{a}
Burned rice husk	100	7.14±0.12^{b}	12.52±0.98^{a}
Sand	100	7.56±0.10^{b}	11.80±0.57^{a}
Soil:Burned rice husk (1:1 w/w)	100	7.19±0.07^{b}	10.88±0.79^{a}
Soil:Sand (1:1 w/w)	86.66	6.87±0.06^{bc}	12.57±0.78^{a}
Burned rice husk:Sand (1:1 w/w)	73.33	5.88±0.08^{c}	12.77±1.59^{a}
Soil:Burned rice husk:Sand (1:1:1 w/w)	93.33	7.08±0.14^{b}	11.62±0.74^{a}

Means followed by the same latter are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test (DMRT).

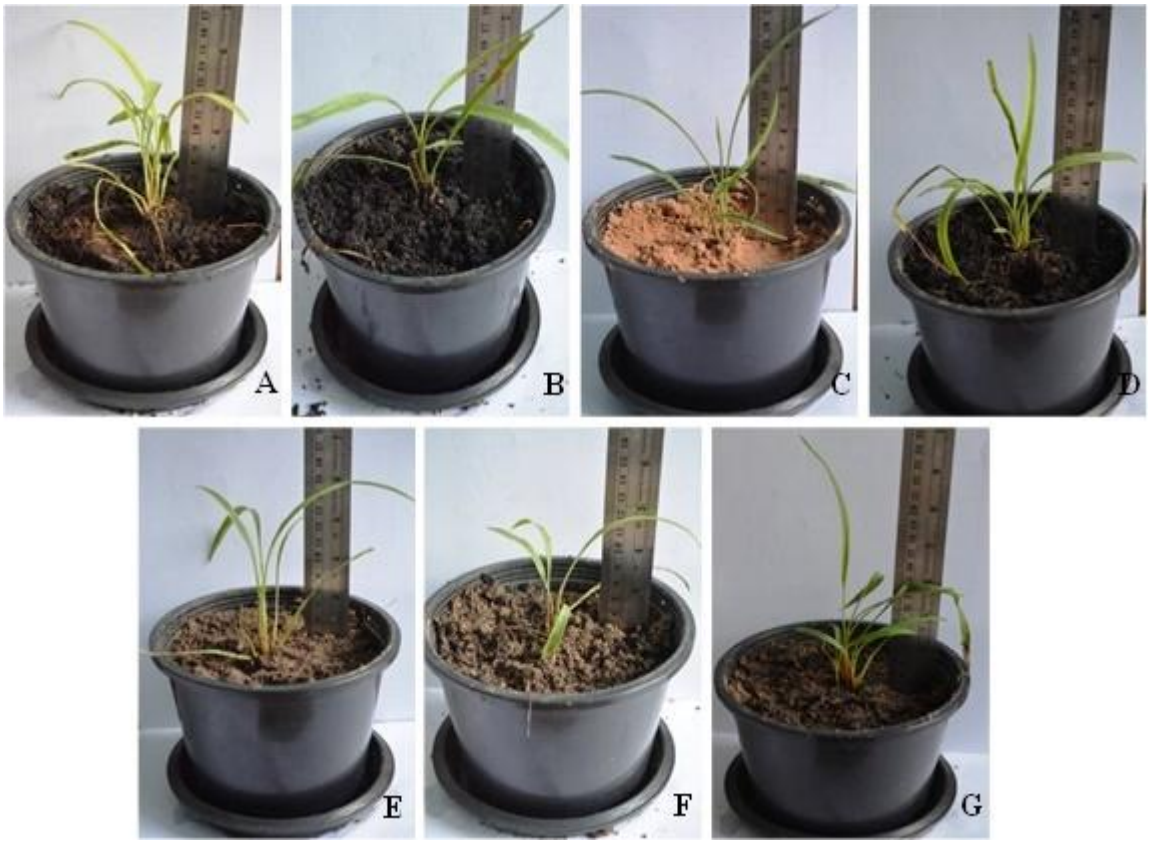

Figure 5. Acclimatized plantlets of *C. spaganifolia* Gagnep. after transplanting to plastic pots containing different potting media for eight weeks (A) soil, (B) burned rice husk, (C) sand, (D) soil:burned rice husk (1:1 w/w), (E) soil:sand (1:1 w/w), (F) burned rice husk:sand (1:1 w/w) and (G) soil:burned rice husk:sand (1:1:1 w/w).

Conclusion: In summary, this work has successfully established a protocol for the micropropagation of *C. spaganifolia* Gagnep., a rare plant species from Thailand. We have reported that the *in vitro* shoot propagation of *C. spaganifolia* Gagnep. from rhizome bud explants in MS media containing 2 mgL^{-1} Kinetin could regenerate multiple shoots (3.00 shoots/explant) after eight weeks of culture. The liquid MS media with Kinetin and IAA added significantly induced more shoot formation and larger shoots in *C. spaganifolia* Gagnep. Moreover, long divided microshoots produced significantly higher numbers of shoots than the undivided microshoots. The survival rate of the plantlets under *ex vitro* conditions was 100% when they were transferred into pots containing soil, burned rice husk, sand and soil:burned rice husk (1:1 w/w). Therefore, this protocol can be extended to other economically valuable, rare and endangered gingers for mass propagation and germplasm conservation and for other research and commercial applications.

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