Current Applied Science and Technology Vol. 23 No. 3 (May-June 2023)

Research article

Effects of Coconut Water, Benzylaminopurine, and Naphthalene Acetic Acid on Seed Germination and Rhizome Food Reserve: *In Vitro* Culture of *Eulophia flava* (Lindl.) Hook.f.

Ekarat Vasupen, Weenun Bundithya and Nuttha Potapohn*

Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand

Received: 13 December 2021, Revised: 5 April 2022, Accepted: 21 October 2022

DOI: 10.55003/cast.2022.03.23.006

Keywords

Eulophia flava;
coconut water;
plant growth regulators;
food reserve;
TNC

Abstract

Eulophia flava (Lindl.) Hook.f. in Thailand has been threatened by agricultural deforestation and city expansion. Efficient seed propagation via tissue culture technique is necessary to enhance the conservation and utilization of this orchid. Thus, the effects of coconut water (CW), benzylaminopurine (BA), and naphthalene acetic acid (NAA) on seed germination and rhizome food reserve were studied. Capsules were collected at 180 days old following hand-pollination. Ten media; MS, MS + 10% CW, MS + 2.69 µM NAA, MS + 5.38 µM NAA, MS + 2.22 µM BA, MS + 4.44 µM BA, MS + 2.69 µM NAA + 2.22 µM BA, MS + 2.69 µM NAA + 4.44 µM BA, MS + 5.38 µM NAA + 2.22 µM BA, and MS + 5.38 µM NAA + 4.44 µM BA, were used for seed germination and seedling development. MS without supplements was found to be suitable for *E. flava* seed culture, with seed germination of 26.39%, survival of 100%, and seedling development in the post-protocorm stage of 11.50%. After that, seedlings in early stage 4 were selected from MS medium treatment and transferred to five media: MS, 1/2 MS, 1/2 MS macronutrient, MS + 10% CW and MS + 2.69 µM NAA, for formation and food reserves of rhizomes. Four months after being subcultured, seedlings in early stage 4 developed to late stage 4. Rhizome size and cuticle thickness showed no significant differences in all media. Seedlings in 1/2 MS macronutrient showed the highest rhizome food reserve (201.21 mg/g DW of TNC).

*Corresponding author: Tel.: (+66) 813876776
E-mail: natorchid@gmail.com

1. Introduction

Eulophia flava (Lindl.) Hook.f. is a terrestrial orchid with underground rhizomes. It has been found in India, Nepal, China, Myanmar, Thailand, Laos, Vietnam, and Cambodia [1]. Its inflorescence is erect, 73-170 cm long, and consists of 9-41 yellow flowers per inflorescence (Figure 1A), and mild fragrant [2, 3] which makes it a conspicuous flower in the wild. *Eulophia flava* has been found in dry deciduous forests, 650-800 m above mean sea level, in the open sun [4]. It has been reported that its rhizomes can be used as a remedy for spider poison as well as food for wild boars [1]. Plants naturally propagated from seed have a low survival rate, while the rhizomes grow slowly [2]. Moreover, in the area we studied, deforestation occurred in order to expand agricultural areas as well as city expansion resulted in declining in habitat and population of this orchid. Without conservation, this plant is at risk of extinction.

In vitro culture is an effective method for propagating many plant species, especially orchid seeds. They do not have sufficient reserves of nutrients for germination and seedling growth [5]. Variability in germination *in vitro* may be due to a number of factors including maternal effects, media, culture conditions, and seed dormancy [6]. Many orchid seeds have low germination rates due to lack of supplements or plant growth regulators in media. Coconut water (CW) contains several vitamins, sugars, sugar alcohols, plant hormones, minerals, amino acids, organic acids, nucleic acids, and other unidentified substances [7]. It has commonly been used to promote seed germination and seedling development of various orchids including *Paphiopedilum liemianum* [8]. Numerous studies have been done on plant growth regulators such as benzylaminopurine (BA) that can support orchid seed germination. It could promote the germination of *Cypripedium candidum* [9], *Calanthe tricarinata* [10], and *Habenaria macroceratitis* [11]. Naphthalene acetic acid (NAA) was used to promote seed germination of *Dendrobium chrysotoxum* [12] and *Paphiopedilum spicerianum* [13]. However, research on *E. flava* seed germination is rare. In addition, studies on rhizome development and food reserve content in small rhizomes have not yet been reported.

Plants store non-structural carbohydrates (NSC), starch, and soluble sugars, in their vegetative organs as an energy reserve for survival and support of growth initiation [14-16]. In general, NSC of plants vary with the seasons, plant organs, plant function, and ecosystem [15]. Zimmerman and Whigham [16] found that the corms of *Tipularia discolor* contained a high concentration of NSC, which supported this orchid in the initiation of growth, reproduction, and response to artificial defoliation. NSC percentage in corms were unchanged during growth initiation. However, it declined during the reproductive period. Plant food reserves can be measured as the concentration of total NSC (TNC).

This research aimed to study the influence of CW, BA, and NAA on *in vitro E. flava* seed germination, seedling development, cuticle thickness, and food reserve in rhizomes in order to enhance the conservation and utilization of this orchid.

2. Materials and Methods

2.1 Seed source and sterilization

Eulophia flava flowers were pollinated in mid-May by hand, and capsules were collected from an orchard site near their habitat, Chiang Rai province, Thailand, at 180-days old (Figures 1B and 1C). Three capsules were harvested and prepared for germination studies. Then, the capsules were washed with dishwashing liquid and tap water before surface disinfected with 1.24% sodium hypochlorite for 15 min, followed by rinsing two times for 5 min each time in sterile distilled water. After that, capsules were dipped in 70% ethanol and burned for 5 s, and allowed to cool down in laminar airflow.

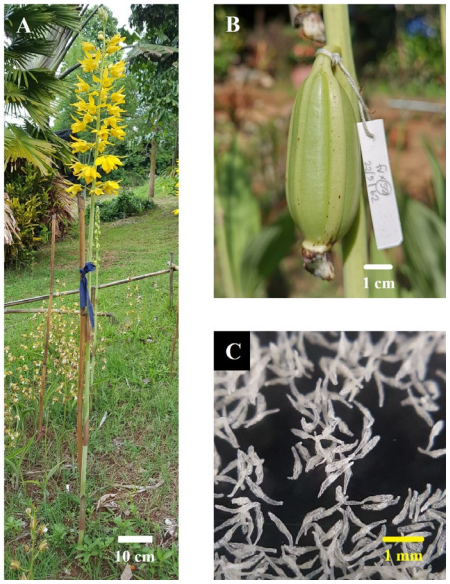

Figure 1. *Eulophia flava*: (A) whole plant during flowering period, (B) capsule, and (C) seeds

2.2 Effect of seed culture media

Asymbiotic seed culture based on Murashige and Skoog 1962 (MS) [17] with 3% sucrose and 7 $g.L^{-1}$ agar was employed. The supplements, coconut water (CW), benzylaminopurine (BA), and naphthalene acetic acid (NAA), were added. Seeds from the sterilized capsules were cultured on ten media, containing in 6 oz bottles. The media were MS, MS + 10% CW, MS + 2.69 µM NAA, MS + 5.38 µM NAA, MS + 2.22 µM BA, MS + 4.44 µM BA, MS + 2.69 µM NAA + 2.22 µM BA, MS + 2.69 µM NAA + 4.44 µM BA, MS + 5.38 µM NAA + 2.22 µM BA, and MS + 5.38 µM NAA + 4.44 µM BA. All media were adjusted to pH 5.6-5.8 and autoclaved for 20 min at 121°C. Culture bottles were incubated in dark condition for 4 months under 8/16 h L/D (40 µmol m^{-2} s^{-1}), 25±2° C. Ten replicates were used in each treatment, and each replication consisted of one bottle. Seed germination percentage, protocorm survival percentage, pre-protocorm to post-protocorm stage percentage were recorded at four months after culturing. Percentage of germination was calculated by counting the number of germinated seeds per total seeds sown x 100. Percentage of protocorm survival was calculated by counting the number of white/yellow or clear protocorms per total germinated seeds x 100. Brown or dark brown protocorms were counted as dead protocorms. The numbers of pre-protocorm to post-protocorm percentages were calculated by counting the number of those stages per average sown seeds (302.3 seeds) x 100.

2.3 Effect of media on formation and food reserves of rhizome

Five media were evaluated for promoting rhizome formation and food reserves. Plantlets at early stage 4 (Table 1 and Figure 2) from MS medium without supplements were transplanted to new solid media, MS, half-strength MS (1/2 MS), half-strength MS macronutrient (1/2 MS macronutrient), MS + 10% CW and MS + 2.69 µM NAA, to culture for 4 months under 8/16 h L/D (40 µmol m^{-2} s^{-1}), 25±2° C. Ten rhizomes from each medium were randomly sampled, measured for size and cross-sectioned by hand for assessing cuticle thickness under a light microscope. The free

hand section method was described as follows: several sections were cut and transferred to distilled water, the thinnest pieces were selected and placed onto a glass slide and flooded with 5% acetic acid for 4 min. The sectioned pieces were washed by flooding them with water several times. Then, they were flooded with 5% sodium hydroxide (NaOH) for 4 min and washed several times. A drop of 0.5% Congo red was applied on the sectioned pieces for 2 min and rinsed with water until there was no excess stain. Then, a drop of 0.5% fast green was applied for 30 min and washed until there was no excess stain. Finally, a drop of distilled water was added over the sectioned pieces, and a cover glass was put on top for examination under the microscope. Ten pieces per treatment and ten spots per section were measured for cuticle thickness.

Furthermore, ten rhizomes from each medium were placed in a hot air oven at 60° C for 72 h, after that each sample was ground thoroughly and sifted with a 40 mesh sieve. Samples were kept in clean bags and placed in a desiccator for TNC analysis. TNC extraction by acid extraction method from Smith *et al*. [14] was used and modified by Chaitrakulsup [18]. Nelson's reducing procedure was used for TNC content analysis [19]. The standard solution was determined using D-glucose at 0-0.5 $mg.ml^{-1}$ concentration, and distilled water was used as blank.

2.4 Statistical analysis

Completely Randomized Design (CRD), with 10 replications each, was applied in all experiments. The data of seed germination were transformed with arcsine transformation before analysis. One-way analysis of variance was used, and means were compared with Duncan's multiple range test (DMRT) at $p \leq 0.05$ [20].

3. Results and Discussion

In this study, the seed germination of *E. flava* on MS medium was observed and divided into 4 stages (Table 1 and Figure 2).

Table 1. Developmental stages of *E. flava* seed germination

Stage	Description
1	Ruptured seed coat, and embryo emerges (= pre-protocorm stage)
2	Round shape protocorm, presence of rhizoids, later enlargement and elongation of protocorm (= protocorm stage)
3	Protocorm proliferate to form rhizome-like body, shoots and roots develop and continue to elongate
4	Rhizome formation Early-stage; swollen shoot base, leaf emerges Middle-stage; rhizomes form round and proliferate, 1 or 2 leaves Late-stage; rhizome proliferate longitudinally more than crosswise (stage 3 and 4 = post-protocorm stage)

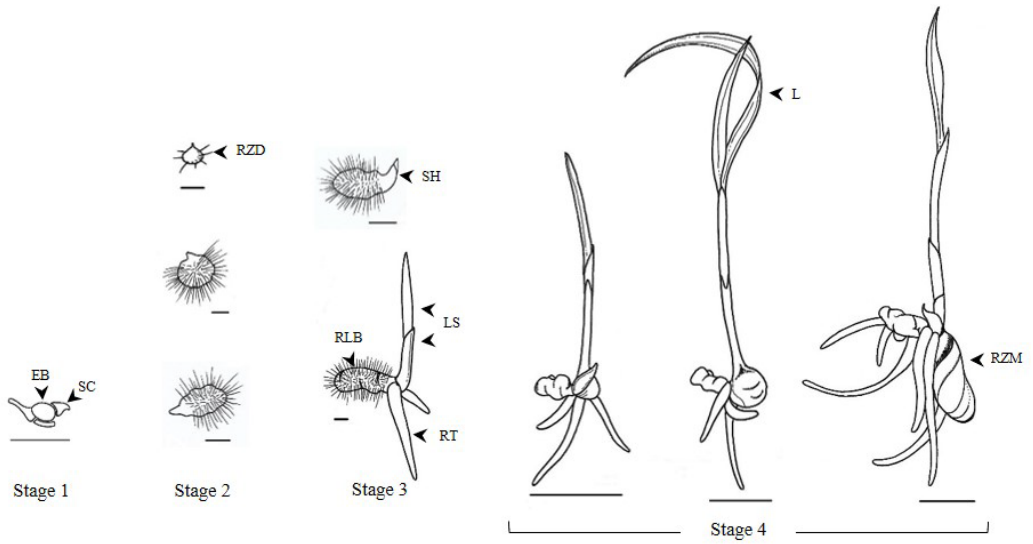

Figure 2. Development stages of *Eulophia flava* seed germination: Stage 1 = pre-protocorm stage, Stage 2 = protocorm stage, Stage 3 and 4 = post-protocorm stage, EB = embryo; SC = seed coat; RZD = rhizoid, SH = shoot; RT = root, RLB = rhizome-like body; LS = leaf sheath; L = leaf; RZM = rhizome; Stage 1-3: scale bar = 1 mm; Stage 4: scale bar = 1 cm

3.1 Effect of seed culture media

Seed germination could be seen on 21-36 days after sowing. The MS medium gave the same germination percentage at 26.39%, as MS + 4.44 µM BA (25.02%), MS + 5.38 µM NAA (23.33%), and MS + 10% CW (22.95%). These four media gave better seed germination percentage than MS + 2.69 µM NAA, MS + 2.22 µM BA, MS + 2.69 µM NAA + 2.22 µM BA, MS + 2.69 µM NAA + 4.44 µM BA, MS + 5.38 µM NAA + 2.22 µM BA, and MS + 5.38 µM NAA + 4.44 µM BA. There were significant differences in seed germination percentage, protocorm survival percentage, and pre-protocorm to post-protocorm stage percentages (Table 2). After germination, medium supplemented with 2.69 µM NAA, 5.38 µM NAA and 5.38 µM NAA + 2.22 µM BA showed approximately 40% brown protocorms.

Four months after culturing, seeds cultured on both MS + 10% CW and MS without supplements developed to the post-protocorm stage at 13.44% and 11.50%, respectively. Plants on these two media grew significantly faster than those on other media. Seeds cultured on a medium with added 4.44 µM BA had the highest percentage of pre-protocorm (5.42%) and protocorm stage (19.02%); however, it showed the lowest post-protocorm stage percentage (0.37%, Table 2). Seed development on ten different media are shown in Figure 3.

Seven months after culturing, seeds cultured in all media had developed to the post-protocorm. Seedlings grown in MS + 2.69 µM NAA developed to late stage 4 whereas seedling grown in MS, MS + 10% CW, and MS + 5.38 µM NAA developed to middle stage 4. Seedlings grown in MS + 2.69 µM NAA + 4.44 µM BA developed to early stage 4. Seedlings grown in MS + 4.44 µM BA had the lowest development (Figure 4).

Table 2. Effect of media on seed germination of *E. flava* at 4 months after culturing

Media	Germination (%)	Survival (%)	Pre-protocorm (%)	Protocorm (%)	Post-protocorm (%)
MS	26.39^{a}	100^{a}	2.48^{c}	12.41^{b}	11.50^{a}
MS + 10 % CW	22.95^{a}	100^{a}	2.36^{c}	7.15^{c}	13.44^{a}
MS + 2.69 µM NAA	18.36^{b}	61.05^{c}	1.99^{cd}	6.91^{c}	2.32^{b}
MS + 5.38 µM NAA	23.33^{a}	56.58^{c}	1.32^{d}	11.04^{b}	0.66^{de}
MS + 2.22 µM BA	14.39^{bc}	100^{a}	4.14^{b}	9.59^{bc}	0.66^{de}
MS + 4.44 µM BA	25.02^{a}	99.33^{a}	5.42^{a}	19.02^{a}	0.37^{e}
MS + 2.69 µM NAA + 2.22 µM BA	17.16^{bc}	80.01^{b}	1.61^{cd}	11.04^{b}	0.66^{de}
MS + 2.69 µM NAA + 4.44 µM BA	14.51^{bc}	83.85^{b}	1.74^{cd}	9.35^{bc}	1.07^{cd}
MS + 5.38 µM NAA + 2.22 µM BA	13.69^{c}	63.70^{c}	2.15^{cd}	4.63^{d}	2.19^{b}
MS + 5.38 µM NAA + 4.44 µM BA	15.72^{bc}	76.42^{b}	2.04^{cd}	8.54^{bc}	1.70^{bc}

Means within the same column followed by different letters showed significant difference ($p < 0.05$).

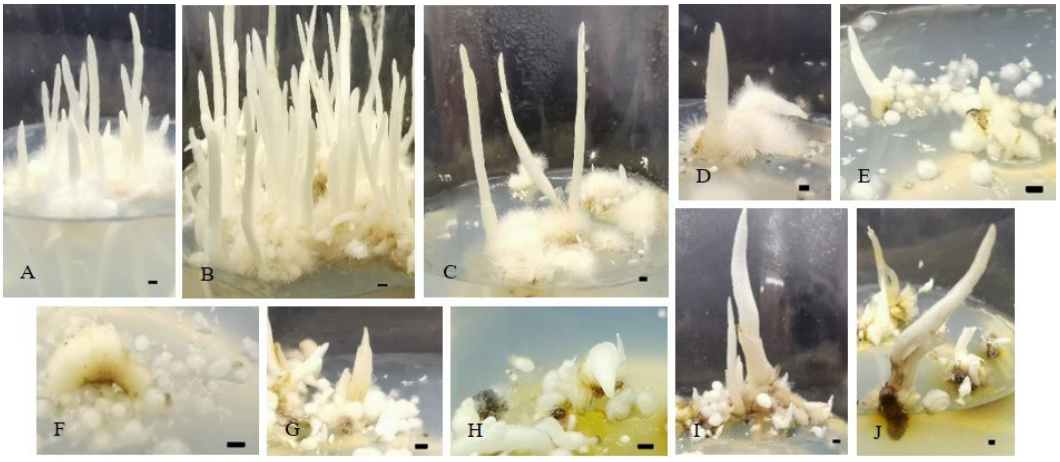

Figure 3. Seed development on ten different media at 4 months after culturing; (A) MS, (B) MS + 10% CW, (C) MS + 2.69 µM NAA, (D) MS + 5.38 µM NAA, (E) MS + 2.22 µM BA, (F) MS + 4.44 µM BA, (G) MS + 2.69 µM NAA + 2.22 µM BA, (H) MS + 2.69 µM NAA + 4.44 µM BA, (I) MS + 5.38 µM NAA + 2.22 µM BA, (J) MS + 5.38 µM NAA + 4.44 µM BA; scale bar = 1 mm

Figure 4. Seedlings on ten different media at seven months after culturing; (A) MS, (B) MS + 10% CW, (C) MS + 2.69 µM NAA, (D) MS + 5.38 µM NAA, (E) MS + 2.22 µM BA, (F) MS + 4.44 µM BA, (G) MS + 2.69 µM NAA + 2.22 µM BA, (H) MS + 2.69 µM NAA + 4.44 µM BA, (I) MS + 5.38 µM NAA + 2.22 µM BA, (J) MS + 5.38 µM NAA + 4.44 µM BA; scale bar = 1 cm

Plant growth regulators are generally used in plant tissue culture. Auxins such as NAA, and cytokinins such as BA, zeatin, thidiazuron and kinetin, were used to promote orchid seed germination [21]. De Pauw *et al*. [9] reported that BA and 6-(γ, γ-dimethylallylamino) purine (2iP) could increase germination of *Cypripedium candidum* significantly when compared with kinetin and medium without cytokinin. Types and concentration of cytokinin affected the morphological development of protocorm. In *Calanthe tricarinata*, BA could promote better seed germination than NAA [10]. Stewart and Kane [11] used different cytokinins in various concentrations for increasing germination of *Habenaria macroceratitis* seeds. They found that zeatin, kinetin, and BA increased germination percentage. Kinetin and zeatin gave better results than BA, and low concentrations gave better results than high concentrations. In *Cymbidium lowianum*, medium containing a combination of 4.44 µM BA with 2.69 µM NAA in 1/2 MS + 0.3% activated charcoal was optimum for germination, and resulted in 95% germination in 90 days, whereas medium without plant growth regulators had a germination percentage of only 7.33% [22]. However, the role of auxin and cytokinin in asymbiotic orchid germination is uncertain, and responses to growth regulators are often specific in each species [16]. In this study, although 4.44 µM BA promoted *E. flava* seed germination as same as MS without supplements, protocorms developed slowly. At 4 months, no root and shoot emerged (Figure 3F). And at 7 months, seedlings were small and had short roots and shoots (Figure 4F). Meanwhile, 5.38 µM NAA promoted seed germination but low survival percentage of protocorms (Table 2). However, a low concentration of NAA, 2.69 µM, promoted seedling development and rhizome formation in 7 months (Figure 4C).

CW was used as a supplement in *in vitro* orchid culture medium. It is a source of sugar, vitamins, and hormones that promote germination and growth. Bustam *et al*. [23] found that the best germination medium for some temperate Australian terrestrial orchids seeds was 1/2 MS + 5% CW, and the CW-added medium had higher percentage of germination than that adding with plant growth regulators. Shekarriz *et al*. [24] showed that CW increased germination of hybrid *Phalaenopsis* seed but decreased seed survival percentage. Utami *et al*. [8] reported that Vacin and Went medium (VW) added with 10% CW could enhance *Paphiopedilum liemianum* protocorm development. Consistent

with this study, coconut water could promote seed germination and development of seed to post protocorm stage. However, the results were not statistically different from MS without supplements.

3.2 Effect of media on formation and food reserves of rhizome

Four months after subculturing on five media, seedlings in early stage 4 developed to late stage 4 (Figure 5). Rhizome size (length and width) and cuticle thickness showed no significant difference in all media. TNC content in rhizomes cultured on 1/2 MS macronutrient was significantly higher than it was in the others (Table 3).

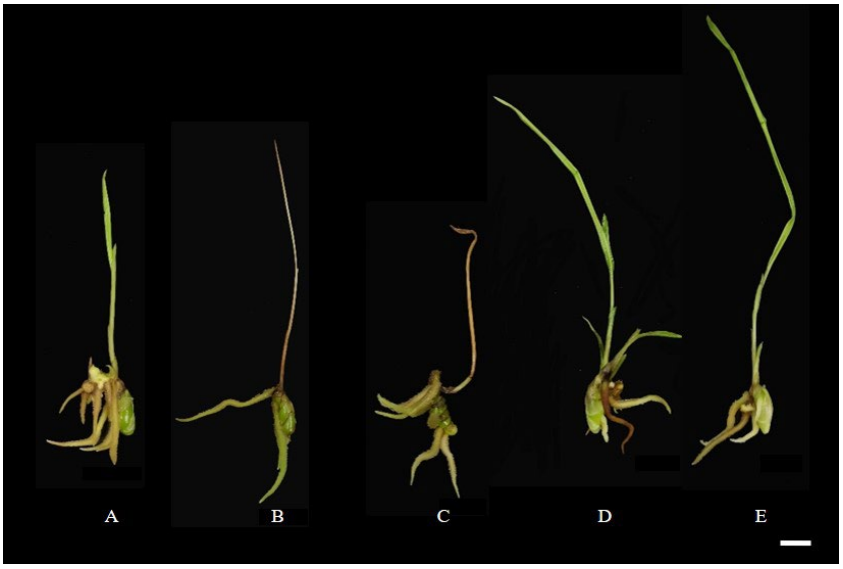

Figure 5. Seedling in late-stage 4 after subculturing from MS medium to five media for four months; (A) MS, (B) 1/2 MS, (C) 1/2 MS macronutrient, (D) MS + 10% CW, (E) MS + 2.69 µM NAA; scale bar = 1 cm

Table 3. Length and width of rhizome, cuticle thickness, and TNC analysis from rhizome of *E. flava* cultured on different media at four months after culturing

Treatment	Rhizome length (mm)	Rhizome width (mm)	Cuticle thickness (µm)	TNC (mg/g DW)
MS	19.75^{a}	5.37^{a}	3.17^{bc}	106.78^{c}
1/2 MS	15.97^{ab}	4.58^{bc}	3.38^{ab}	159.31^{b}
1/2 MS macronutrient	15.39^{ab}	5.07^{ab}	3.43^{a}	201.21^{a}
MS+10% CW	19.17^{a}	5.78^{a}	3.04^{c}	97.66^{d}
MS + 2.69 µM NAA	12.15^{b}	4.02^{c}	3.03^{c}	98.66^{d}

Means within the same column followed by different letters showed significant difference ($p < 0.05$).

Plant cuticles generally constitute of waxes, cutin and/or cutan, polysaccharides, phenolics, and mineral elements [25]. The functions of the plant cuticle are to prevent excessive transpiration and water loss, allow gas exchange, defend against pests and pathogens, help form a self-cleaning surface, prevent dust and other debris from blocking sunlight, screen excessive UV light, and develop organ boundaries. A thick cuticle is implicated with a lower water permeability and increased tolerance to water stress [26].

Plant cuticles change thickness during plant growth and development and thickness of each plant part responds differently to abiotic factors and hormone stresses [27]. In *Epidendrum radicans*, the stem cuticle thickness was 2-6 µm, and stem cuticles in regions covered by leaf sheaths were thinner than in regions not covered by leaf sheaths [28]. The rhizome of *Bulbophyllum sterile* displayed a thick cuticle (4-5 µm) that was similar to its pseudobulb [29]. In this study, the cuticle thickness of *in vitro* rhizomes was 3.03-3.43 µm (Figure 6) and there were no significant difference among treatments, which indicated that culture media had no effect on the thickness of the cuticle.

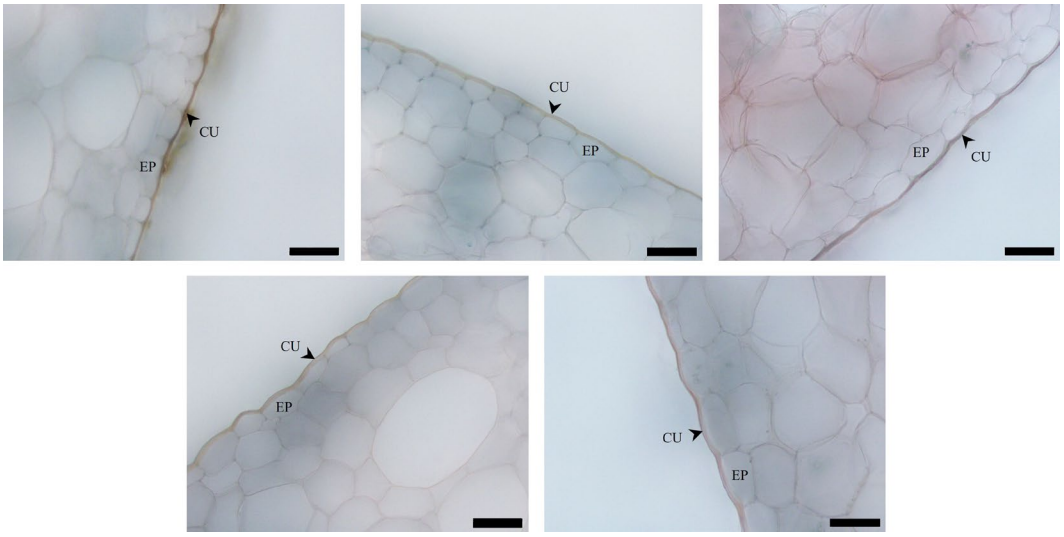

Figure 6. Cuticle thickness of rhizome in five different media; (A) MS, (B) 1/2 MS, (C) 1/2 MS macronutrient, (D) MS + 10% CW, (E) MS + 2.69 µM NAA; CU = cuticle, EP = epidermis; scale bar = 50 µm

Non-structural carbohydrates such as starch, sugars, fructans, and glucomannans are the main reserves in geophytes. These reserve carbohydrates are responsible for supporting sprouting and growth and protecting plants against abiotic stresses [30]. Growth initiation caused a reduction in corm mass but no change in percentage of TNC [16]. This study showed that the concentrations of the media, supplements, and plant growth regulators affected the TNC content in rhizomes. High TNC content appeared in low concentration of media, and low TNC content was measured in media added with CW or NAA. TNC content in rhizome was greatest in 1/2 MS macronutrients. In contrast, the highest TNC content of *Dendrobium* Anna protocorms was obtained in media supplemented with 0.5 $mg.L^{-1}$ NAA. Anuchai and Sasiangdee [31] concluded that auxin affected TNC content. Moreover, photoperiod and difference in day and night temperatures also affected TNC [32, 33].

The success of propagation via plant tissue culture does not stop at *in vitro* culture stage but must be at transplanting stage as well; this is a crucial problem in *E. flava* culture. We expect

that high TNC concentrations in rhizomes may benefit seedling survival after transplanting, which needs to be further studied.

4. Conclusions

The results suggest that a suitable medium for *E. flava* seed germination and protocorm development was MS without supplements. The germination percentage, survival rate, and post-protocorm stage percentage were 26.39%, 100%, and 11.50%, respectively. Different results were produced with MS + 4.44 µM BA, MS + 5.38 µM NAA and MS + 10% CW. Therefore, supplement is not required at this stage in order to save cost. After post-protocorm, seedlings should be transplanted to MS + 1/2 macronutrients. The rhizomes grown in this medium have a lot of food reserves (201.21 mg/g DW of TNC). This indicates that having strong rhizomes might increase success in transplantation. The results of this study should be able of assistance in conserving *E. flava* in the future.

5. Acknowledgements

This research fund was granted by the Rajamangala University of Technology Isan, Nakhon Ratchasima. The authors are very thankful for the plant materials and kindness by Mr. Samai Khok-kruat and Mrs. Jamjan Nuksit.

References

[1] Shrivastava, R.J., 2004. Range extension of *Eulophia flava* (Lindl.) J.D. Hook. (Orchidaceae) in India. *Selbyana*, 25(1), 23-26.

[2] Rungruang, C. and Sangtongpraow, S., 2001. Population variation of *Eulophia flava* (Lindl.) Hook.f. in Mae Ping National Park, Lumphun province. *Abstracts Research and Thesis 2001: 5th BRT Annual Conference, Biodiversity Research and Training Program*, Bangkok, Thailand, October 8-11, 2001, pp. 119.

[3] Kumar, P., Tewari, R. and Rawat, G.S., 2011. Assisted pollination and seed formation for conservation of *Eulophia flava* (Orchidaceae): a case study from Himalayan foothills, India. *Richardiana*, 12(1), 25-38.

[4] Rao, B.R.P., Sadasivaiah, B., Prasad, K., Basha, S.K., Miria, A., Khan, A.B. and Babu, M.V.S., 2010. *Eulophia flava* (Lindley) Hook.f. (Orchidaceae), in Eastern Ghats, India. *Indian Journal of Forestry*, 33(3), 403-404.

[5] Rasmussen, H.N., 1995. *Terrestrial Orchids from Seed to Mycotrophic Plant*. New York: Cambridge University.

[6] Johnson, T.R, 2011. *Developing a Model of Orchid Seed Germination: In Vitro Studies of the Threatened Florida species Bletia purpurea*. Ph.D. University of Florida, USA.

[7] Molnár, Z., Virág, E. and Ördög, V., 2011. Natural substances in tissue culture media of higher plants. *Acta Biologica Szegediensis*, 55(1), 123-127.

[8] Utami, E.S.W., Purnobasuki, H., Soedarti, T. and Hariyanto, S., 2015. Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum liemianum* Fowlie, an endangered terrestrial orchid in Northern Sumatra, Indonesia. *Journal of Plant Sciences*, 10(1), 25-34.

[9] De Pauw, M.A., Remphrey, W.R. and Palmer, C.E., 1995. The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. *Annals of Botany*, 75, 267-275.
[10] Godo, T., Komori, M., Nakaoki, E., Yukawa, T. and Miyoshi, K., 2010. Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture *in vitro*. *In Vitro Cellular and Developmental Biology*, 46, 323-328.
[11] Stewart, S.L. and Kane, M.E., 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture*, 86, 147-158.
[12] Kaur, S. and Bhutani, K.K., 2011. *In vitro* propagation of *Dendrobium chrysotoxum* (Lindl.). *Floriculture and Ornamental Biotechnology*, 5(1), 50-56.
[13] Chen, Y., Goodale, U.M., Fan, X.L. and Gao, J.Y., 2015. Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum spicerianum*: an orchid with an extremely small population in China. *Global Ecology and Conservation*, 3, 367-378.
[14] Smith, D., Paulsen, G.M. and Raguse, C.A., 1964. Extraction of total available carbohydrates from grass and legume tissue. *Plant Physiology*, 39, 960-962.
[15] Martínez-Vilalta, J., Sala, A., Asensio, D., Galiano, L., Hoch, G., Palacio, S., Piper, F.I. and Lloret, F., 2016. Dynamics of non-structural carbohydrates in terrestrial plants: a global synthesis. *Ecological Monographs*, 86(4), 495-516.
[16] Zimmerman, J.K. and Whigham, D.F., 1992. Ecological functions of carbohydrates stored in corms of *Tipularia discolor* (Orchidaceae). *Functional Ecology*, 6, 575-581.
[17] Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497.
[18] Chaitrakulsup, T., 1981. *Seasonal Changes in Total Nitrogen and Total Nonstructural Carbohydrates Contents in Leaves and Stem Apexes of Litchi chinensis Sonn. var. Hong-Huay*. MSc. Kasetsart University, Thailand.
[19] Hodge, J.E. and Hofreiter, B.T., 1962. Determination of reducing sugars and carbohydrates. In: R.L. Whistler and M.L. Wolfrom, eds. *Methods in Carbohydrates Chemistry*. New York: Academic Press, pp. 380-394.
[20] Duncan, D.B., 1955. Multiple range and multiple F test. *Biometrics*, 11, 1-42.
[21] Kauth, P.J., Dutra, D., Johnson, T.R., Stewart, S.L., Kane, M.E. and Vendrame, W., 2008. Techniques and applications of *in vitro* orchid seed germination. In: J.A. Teixeira da Silva, ed. *Floriculture, Ornamental and Plant Biotechnology. Volume V*. Middlesex: Global Science Books, pp. 375-390.
[22] Wang, Y., Li, Z., Huang, L. and Su, J., 2013. *In vitro* mass scale propagation of wild *Cymbidium lowianum* with a rare and endangered plant. *American Journal of Plant Sciences*, 4, 1500-1507.
[23] Bustam, B.M., Dixon, K.W. and Bunn, E., 2014. *In vitro* propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared with symbiotic germination. *Botanical Journal of the Linnean Society*, 176, 556-566.
[24] Shekarriz, P., Kafi, M., Deilamy, S.D. and Mirmasoumi, M., 2014. Coconut water and peptone improve seed germination and protocorm like body formation of hybrid *Phalaenopsis*. *Agriculture Science Developments*, 3(10), 317-322.
[25] Fernández, V., Guzmán-Delgado, P., Graça, J., Santos, S. and Gil, L., 2016. Cuticle structure in relation to chemical composition: re-assessing the prevailing model. *Frontiers in Plant Science*, 7, DOI: 10.3389/fpls.2016.00427.
[26] Yeats, T.H. and Rose, J.K.C., 2013. The formation and function of plant cuticles. *Plant Physiology*, 163, 5-20.
[27] Domínguez, E., Heredia-Guerrero, J.A. and Heredia, A., 2011. The biophysical design of plant cuticles: an overview. *New Phytologist*, 189, 938-949.

[28] Thangavelu, M. and Muthu, S., 2017. Vegetative anatomical adaptations of *Epidendrum radicans* (Epidendroideae, Orchidaceae) to epiphytic conditions of growth. *Modern Phytomorphology*, 11, 117-130.
[29] Muthukumar, T. and Shenbagam, M., 2018. Vegetative anatomy of the orchid *Bulbophyllum sterile* (orchidaceae: epidendroideae). *Lankesteriana*, 18(1), 13-22.
[30] Almeida, V.O., Carneiro, R.V., Carvalho, M.A.M., Figueiredo-Ribeiro, R.C.L. and Moraes, M.G., 2015. Diversity of non-structural carbohydrates in the underground organs of five Iridaceae species from the Cerrado (Brazil). *South African Journal of Botany*, 96, 105-111.
[31] Anuchai, J. and Sasiangdee, A., 2020. Effect of growth regulator on shoot induction from protocorm of *Dendrobium* Anna. *International Journal of Agricultural Technology*, 16(6), 1319-1330.
[32] Chidburee, A., Suwanthada, C., Bundithya, W., Ohyama, T. and Ruamrungsri, S., 2007. Effect of day length on growth, photosynthesis and food reserves in *Curcuma alismatifolia* Gagnep. *Journal of Agriculture*, 23(2), 105-113.
[33] Changjeraja, R. and Intanon, S., 2016. Differences in day and night temperature on growth and non-structural carbohydrate of *Eurycle amboinensis* Lindl. *Songklanakarin Journal of Plant Science*, 3(suppl. II), 64-69.