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In Vitro Propagation of the *Dendrobium anosmum* Lindl. Collected in Vietnam

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Abstract: Hoa Binh province is one of the best places for orchids in Vietnam. The climate and environment of Hoa Binh province are favorable for the development of orchids, especially rare indigenous ones. *Dendrobium anosmum* Lindl., which stands out because of the unique fragrance and colors, is one of the most popular varieties in Hoa Binh province. To meet the increasing demands of the industrial market as well as to contribute to the preservation and development of genetic resources of *Dendrobium* sp. in Hoa Binh province, propagating *D. anosmum* Lindl. is a crucial step. Plant tissue culture, which has been applied to improve reproducibility of orchids for many years, is still an effective method, especially for large-scale propagation. Studies on in vitro propagation of *D. anosmum* Lindl. from Hoa Binh province showed that growth regulators (BA, kinetin, α-NAA) did not have a significant effect on protocorm initiation because *D. anosmum* Lind. from Hoa Binh province already has a high rate of regeneration. However, MS medium + 1.0 mg/L kinetin + 0.5 mg/L α-NAA + 30 g sucrose + 8.0 g agar per liter, pH 5.7–5.8 was the optimal medium to increase shoot length. The MS medium + 1.0 g activated charcoal + 30 g sucrose + 8.0 g agar per liter, pH 5.7–5.8 was the most suitable medium for shoot growth—after 6 weeks of culture, the average shoot length was 1.09 cm, the average number of leaves was 6.13, the average number of roots was 3.17, and the average root length was 1.11 cm—about 3.3, 4.17, 3.41, and 1.67 times higher, respectively, than in the control (without activated charcoal).

Keywords: in vitro; *Dendrobium anosmum*; Vietnam; micropropagation; protocorm

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1. Introduction

Dendrobium Swartz is known as the genus with the largest number of species in the family Orchidaceae. It includes mostly epiphytic and lithophyte orchids, containing more than 1800 species that are widely distributed in China, Japan, India, the Philippines, Indonesia, Australia, Papua New Guinea, Vietnam, and many of the islands of the Pacific [1]. In Vietnam, this genus has about 107 species and one variety, distributed in most of the country from north to south and is found in diverse habitats [2]. Many *Dendrobium* species, in addition to decorative effects for living spaces, have medicinal values. *D. officinale* Kimura & Migo, a precious medicinal plant is used to support the treatment of tuberculosis, fever, sore throat, back pain, stomach pain, enteritis, etc [3]; *D. anosmum* Lindl. and *D. nobile* Lindl. are used to treat physical weakness, nervousness, sore throat, and physiological weakness in men [4,5].

Dendrobium anosmum Lindl., an orchidaceous plant called "Lưỡng điểm hạc", "Phi điệp", or "Giả hạc tím" is widely distributed in Thai Nguyen, Hoa Binh, Bac Giang, Ha Noi, Ha Nam, Nghe An, and Lam Dong provinces of Vietnam [6]. In addition, it is found in India, Myanmar, Laos, Cambodia, Thailand, Malaysia, Sri Lanka, Indonesia, and the Philippines. *D. anosmum* Lindl. is a very beautiful forest orchid, the most popular on the market today. *D. anosmum* Lindl. distributed in Hoa Binh has large, beautiful flowers with a characteristic aroma and better growth ability than those distributed in other localities. In recent years, this species has been over-exploited and is at risk of being depleted in the wild. Uncontrolled exploitation of *D. anosmum* Lindl., in particular, and other wild orchid species, in general, is happening in many places such as the Central Highlands, Lam Dong, and the northern mountainous provinces. Most species of wild orchids in Vietnam have been listed in the Vietnam's Red Data Book and many of which are at a particularly endangered level [7]. Therefore, the conservation of natural genetic resources is essential. In the natural environment, orchids propagate mainly by asexual reproduction, but the propagation coefficient is low. Although the fruit of the orchids has many seeds, it is difficult to germinate in the wild because it does not contain endosperm; to germinate, it is necessary to have a symbiosis of some suitable fungi [8]. The application of propagation by tissue culture is a good way to solve the problem. Plant tissue culture is a technique that allows plant cells, tissues, and organs to grow on artificial medium under controlled conditions. The whole plant can be regenerated from any plant parts or cells, which means faster multiplication. Another advantage is that seedlings are genetically stable thus bringing about a practical effect on improving the quality of the seedlings and reducing the price.

There has been much written on the micropropagation of *Dendrobium* species, such as *D. candidum* Wall., *D. officinale* Kimura & Migo, *D. primulinum* Lindl., *D. lituiflorum* Lindl., *D. densiflorum* Lindl., *D. crumenatum* Sw., *D. chrysanthum* Wall., etc. [9]. In Vietnam, some *Dendrobium* species, such as *D. officinale* Kimura & Migo [10], *D. lituiflorum* Lindl. [11], and *D. heterocarpum* Wall. [12] have been studied for micropropagation. In order to contribute to the conservation and supply of seeds for the wild orchid market, we conducted in vitro propagation studies of the orchid species *D. anosmum* Lindl. collected in Hoa Binh province, Vietnam.

2. Materials and Methods

The seed pods of *D. anosmum* Lindl. orchid were collected at Dong Lai commune, Tan Lac district, Hoa Binh province. The seed pods were transported to the lab and kept in a paper bag at 5 °C in the refrigerator for two weeks before sterilization for initial material culture.

2.1. Creation of Initial Material

Since mature orchid seeds have hard seed coats, chemicals substances must be used to sterilize *D. anosmum* Lindl. orchid seeds [13]. Protocol for sterilization of *D. anosmum* Lindl. seed included four steps and all steps were conducted in a laminar cabinet. The first step started with the washing of intact *D. anosmum* Lindl. seed capsules by soap, followed by rinsing in sterilized water. The capsules were then immersed in 70% ethanol for 1 min then soaked in sterilized water for 1 min in the sterile box. This second step was then repeated three times. After that, the pods were flamed for about 5–7 s for heat sterilization. Finally, the capsules were carefully dissected and the seeds were taken out and transferred onto shoot-initiation medium. The shoot-initiation medium was comprised of hormone-free MS medium with 30 g/L sucrose and 8 g/L agar, pH 5.75–5.8 [14], 20–30 mL of medium per glass vessel.

2.2. Propagation of Protocorm

After 6–8 weeks of culture, tissue culture was applied to propagate the protocorms. Protocorms were subcultured on to a medium supplemented with growth regulators with different concentrations to determine the shooting ability.

2.3. Experimental Design and Statistical Analysis

The basal medium was MS supplemented with 8 g/Lagar, 30 g/L sucrose, pH was adjusted 5.7–5.8 before autoclaving for 20 min at 121 °C. Growth regulators (BA, kinetin, α-NAA), mashed potato (washed, boiled, finely mashed) and mashed banana (ripe, peeled, finely mashed), coconut water, and activated charcoal were added prior to autoclaving.

All the experiments followed a completely randomized design with three replications. The explants were cultured at 25 ± 2 °C, photoperiod (16/8 h light/dark), light intensity of 2300 lux.

Each experiment was performed in triplicate with 20 samples per treatment per replicate.

Data were analyzed by Microsoft Excel, IRRISTAT 5.0 and SPSS. Comparative formulae were processed through the approach of testing the difference between average values that were measured and used by the LSD standard (for a reliability of 95%) and ANOVA.

3. Results

3.1. Observation of Protocorm Development and the Effect of Growth Regulators on the Propagation Efficiency of D. anosmum Lindl. Orchid Protocorms

The orchid seeds were sterilized and implanted on MS medium. After 5 weeks, the seeds were transplanted into the new medium. The seed germination and protocorm developments can be summarized in three stages (Figure 1).

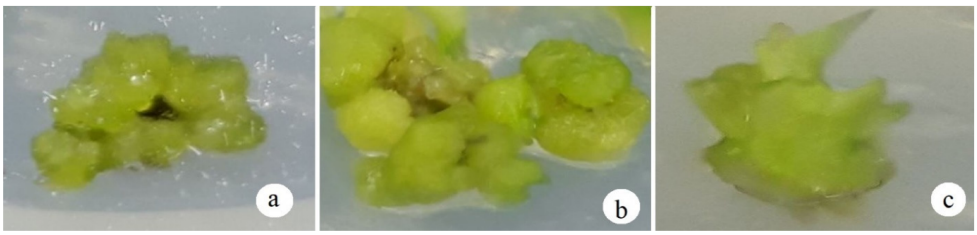

Figure 1. Protocorm development stages: (**a**) seeds started to increase in volume and size (4 weeks after implantation), (**b**) the initiation of protocorm (4–6 weeks after implantation), and (**c**) protocorm with one leaf primordium (6–8 weeks after implantation).

Many media have been used for axenic seed germination and protocorm formation of orchids; however, none is universally used. Different media contain growth regulators, at different scales, which affect the regeneration of plants [15]. Therefore, it is essential to determine the suitable medium as well as the concentration of growth regulators suitable for protocorm growth. The experiments were conducted with plant regulators that are popularly used for orchids including BA, kinetin, and α-NAA.

3.1.1. Effect of BA on the Regeneration Efficiency of *D. anosmum* Lindl. Orchid Protocorms

BA is commonly used to promote cell division and induce cell differentiation [16]. This experiment was conducted on MS medium supplemented with BA at different concentrations. Eight-week-old protocorms (after germination from seeds) were used for this experiment. The results were obtained after 10 weeks of culture and are shown in Table 1.

Table 1. Effect of BA on the regeneration efficiency of the *D. anosmum* Lindl. protocorms (10 weeks after culturing).

Treatment	BA (mg/L)	Protocorm Formation Rate (%)	Shoot Length (cm)	Leaves (Leaves/Plant)	Rooting Rate (%)
T1(Control)	0.0	100	0.33 ^{a}	1.47 ^{a}	53.33
T2	0.5	100	0.51 ^{b}	0.53 ^{b}	20.00
T3	1.0	100	0.69 ^{c}	1.93 ^{c}	45.00
T4	1.5	100	0.49 ^{be}	0.33 ^{be}	33.67
T5	2.0	100	0.37 ^{ad}	0.33 ^{be}	27.78
T6	2.5	100	0.42 ^{ab}	1.00 ^{d}	40.00
p-value (ANOVA)			$p < 0.001$	$p < 0.001$	
$LSD_{0.05}$			0.11	0.40	

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8. Means followed by the same letter are not significantly different at $p < 0.05$.

Table 1 shows that all seeds regenerated protocorms. The protocorm formation rate was 100% on medium with or without BA. BA concentration was not necessary for the regeneration of protocorms. This result indicates that the *D. anosmum* Lindl. protocorm has a high ability of regeneration. *D. anosmum* Lindl. protocorm can be easily formed in an environment that has sufficient nutrition, light, and humidity.

Shoot lengths on media supplemented at concentrations of 0.5 mg/L, 1 mg/L, and 1.5 mg/L were significantly longer than the control (Figure 2). The number of leaves on media with 1 mg/L and 2.5 mg/L BA was significantly greater than the control.

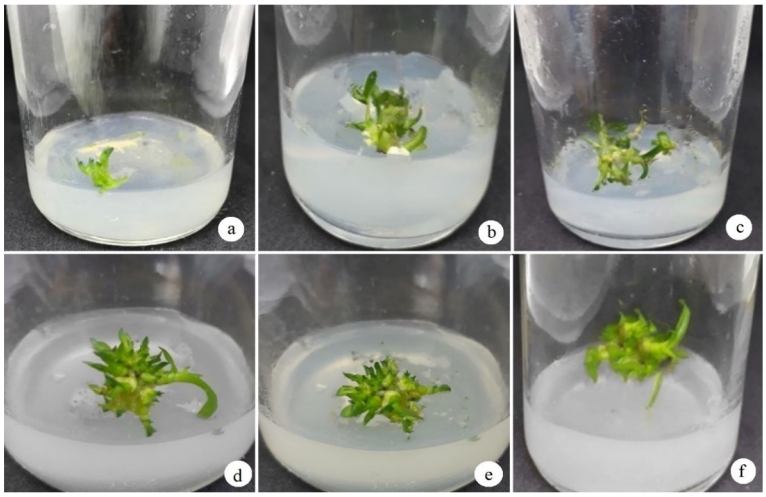

Figure 2. Effect of BA on the regeneration efficiency of the *D. anosmum* Lindl. protocorms (10 weeks after culturing): (**a**) MS, (**b**) MS + 0.5 mg/L BA, (**c**) MS + 1 mg/L BA, (**d**) MS + 1.5 mg/L BA, (**e**) MS + 2 mg/L BA, and (**f**) MS + 2.5 mg/L BA.

At 95% confidence, the addition of 1 mg/L BA was the most suitable for protocorm growth. A higher concentration of BA led to the inferior propagation of the protocorm.

3.1.2. Effect of Kinetin on the Regeneration Efficiency of the *D. anosmum* Lindl. Protocorms

Kinetin (6-furfurolaminopurrine), which is a member of cytokinin group, promotes cell division and activates the processes of cellular growth and differentiation [17]. Results are presented in Table 2.

Table 2. Effect of kinetin on the regeneration efficiency of the *D. anosmum* Lindl. protocorms (10 weeks after culturing).

Treatments	Kinetin (mg/L)	Protocorm Formation Rate (%)	Shoot Length (cm)	Leaves (Leaves/Plant)	Rooting Rate (%)
T1 (Control)	0.0	100	0.33 ^{a}	1.47 ^{ae}	53.33
T2	0.5	100	0.46 ^{ab}	0.93 ^{ab}	42.86
T3	1.0	100	1.15 ^{cb}	3.00 ^{c}	75.00
T4	1.5	100	0.50 ^{ac}	1.83 ^{a}	57.14
T5	2.0	100	0.44 ^{ac}	0.23 ^{b}	40.00
T6	2.5	100	0.40 ^{ac}	0.77 ^{be}	38.46
p-value (ANOVA)			$p < 0.001$	$p < 0.001$	
$LSD_{0.05}$			0.81	0.99	

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8. Means followed by the same letter are not significantly different at $p < 0.05$.

Table 2 shows that *D. anosmum* Lindl. protocorm had a high ability for regeneration. The protocorm formation rate was 100% in all media indicating that addition of kinetin was not necessary for protocorm formation.

The supplementation of kinetin resulted in increased shoot length and 1 mg/L kinetin significantly increased shoot length (1.15 cm), number of leaves (3.0 leaves/plant), and rooting rate (75%), with a 95% confidence. The increase of the concentration of kinetin to 1.5 mg/L, 2.0 mg/L, and 2.5 mg/L resulted in a decrease in terms of protocorm quality.

In summary, shoots were successfully propagated in the presence of BA and kinetin. Kinetin was more efficient than BA in promoting shoot and root formation from protocorms. MS medium with 1.0 mg/L kinetin added had the best efficiency.

3.1.3. Effect of BA and α-NAA on the Regeneration Efficiency of the *D. anosmum* Lindl. Protocorms

The work of Miller et al. (1957) [18] was the foundation for the use of auxin–cytokinin in tissue culture. Miller et al. (1957) [18] indicated that the auxin–cytokinin ratio in culture media determines the degree of the shoot and root formation. A high ratio of cytokinin to auxin favors shoot production, whereas a high auxin to cytokinin ratio favors root production. The concentration of each substance also plays an important role in plant growth.

Experiment 3 was conducted to investigate the effect of simultaneously adding two plant growth regulators, BA and α-NAA, on the regeneration efficiency of the *D. anosmum* Lindl. Eight-week-old protocorms (after germination from seeds) were used for this experiment. Results are shown in Table 3.

Table 3. Effect of BA and α-NAA on the regeneration efficiency of *D. anosmum* Lindl. protocorms (10 weeks after culturing).

Treatment	BA (mg/L)	α-NAA (mg/L)	Protocorm Formation Rate (%)	Shoot Length (cm)	Leaves (Leaves/Plant)	Rooting Rate (%)
T1(Control)	1.0	0.0	100	0.69 ^{a}	1.93 ^{a}	45.00
T2	1.0	0.1	100	0.64 ^{ae}	2.5 ^{ab}	7.69
T3	1.0	0.2	100	0.75 ^{ad}	4.23 ^{c}	40.91
T4	1.0	0.3	100	0.98 ^{b}	7.17 ^{d}	50.00
T5	1.0	0.5	100	0.52 ^{c}	3.97 ^{ce}	28.57
p-value (ANOVA)				$p < 0.001$	$p < 0.001$	
$LSD_{0.05}$				0.12	0.95	

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8. Means followed by the same letter are not significantly different at $p < 0.05$.

On MS medium supplemented with 1.0 mg/L BA and α-NAA at the different concentration, 100% of *D. anosmum* Lindl. protocorms regenerated shoots and some of them rooted. The combination of BA and α-NAA had a strong effect on shoot development. In the control without α-NAA, the shoot length was 0.69 cm; however, shoot length reached 0.98 cm in medium supplemented with 0.3 mg/L α-NAA, and at 0.5 mg/L it reached 0.52 cm. In addition, leaf number was also significantly more than in the control. However, when we added α-NAA at 0.5 mg/L into the medium, the shoot length and rooting rate were lower than the control. The addition of α-NAA stimulated leaf formation; the treatments of 0.2 mg/L, 0.3 mg/L, and 0.5 mg/L α-NAA had the significantly higher numbers of leaves than the control with 1.0 mg/L BA alone. In the current study, with a 95% confidence analysis, the concentration of 1 mg/L BA and 0.3 mg/L α-NAA had the best result.

3.1.4. Effect of Kinetin and α-NAA on the Regeneration Efficiency of the *D. anosmum* Lindl. Protocorms

Experiment 4 was conducted to investigate the effect of simultaneously adding two plant growth regulators, kinetin and α-NAA, into the medium. Eight-week-old protocorms (after germination from seeds) were used for this experiment. The results are presented in Table 4.

Table 4. Effect of kinetin and α-NAA on the regeneration efficiency of the *D. anosmum* Lindl. protocorms (10 weeks after culturing).

Treatment	Kinetin (mg/L)	α-NAA (mg/L)	Protocorm Formation Rate (%)	Shoot Length (cm)	Leaves (Leaves/Plant)	Rooting Rate (%)
T1(Control)	1.0	0.0	100	1.15 ^{a}	3.00 ^{a}	75.00
T2	1.0	0.1	100	1.28 ^{ab}	3.97 ^{b}	90.00
T3	1.0	0.2	100	1.32 ^{b}	4.03 ^{bc}	100.00
T4	1.0	0.3	100	1.38 ^{bc}	4.23 ^{be}	100.00
T5	1.0	0.5	100	1.49 ^{c}	4.40 ^{bd}	100.00
p-value (ANOVA)				$p < 0.001$	0.031	
$LSD_{0.05}$				0.14	0.92	

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8. Means followed by the same letter are not significantly different at $p < 0.05$.

Table 4 shows that the protocorm formation rate was 100% in all media. This shows, along with previous experiments, that *D. anosmum* Lindl. protocorm has a high ability for regeneration.

Protocorms cultured on the medium supplemented with kinetin in combination with α-NAA produced better results than the ones on the medium containing 1 mg/L kinetin and without α-NAA. MS medium supplemented with 1 mg/L kinetin and α-NAA (0.2 mg/L, 0.3 mg/L, and 0.5 mg/L) resulted in increasing shoot length (1.32 cm, 1.38 cm, and 1.49 cm) and number of leaves (4.03, 4.23, and 4.40) and rooting rates that all reached 90% and 100%. The presence of α-NAA stimulated the development and plant regeneration from protocorm of *D. anosmum* Lindl.

At 95% confidence, the optimal concentration of kinetin and α-NAA was 1 mg/L and 0.5 mg/L, respectively.

In conclusion, since *D. anosmum* Lindl. collected from Hoa Binh province has a high ability to form protocorm from seed, it is not necessary to supplement with growth regulators (BA, kinetin, or α-NAA) for regeneration of protocorms. However, supplementation with growth regulators can improve the quality of generated protocorms and shoots. The combination of 1 mg/L kinetin and 0.5 mg/L α-NAA resulted in the highest protocorm, shoot development, and plant regeneration from protocorms of the *D. anosmum* Lindl. orchid.

3.2. Effect of Additives on the Growth Efficiency of D. anosmum Lindl. Orchid Shoots

3.2.1. Effect of Mashed Potato on the Growth and Development of *D. anosmum* Lindl.

According to Table 5, MS medium supplemented with mashed potato influenced shoot development. Potato provided nutrition to the culture medium effectively, thus, shoot length, the number of leaves, and root length were improved, especially in the MS medium with 20 g/L (Figure 3). The control treatment had shoot length of 0.33 cm, the number of leaves was 1.47 leaves/plant, the number of roots was 0.93 roots/plant, and root length was 0.36 cm. Addition of 20 g/L mashed potato to the MS medium resulted in significantly more shoot length (1.1 cm), root length (0.57 cm), leaf number (3.4 leaves/plant), and root number (2.5 roots/plant). With a 95% confidence, MS medium with 20 g/L mashed potato added was the most suitable culture medium for growth and development of *D. anosmum* Lindl.

Table 5. Effect of mashed potato on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing).

Treatment	Potato (g/L)	Shoot Length (cm)	Leaves (Leaves/Plant)	Roots (Roots/Plant)	Root Length (cm)
T1 (Control)	0	0.33 ^{ab}	1.47 ^{a}	0.93 ^{ab}	0.36 ^{a}
T2	10	0.59 ^{ac}	3.30 ^{b}	1.33 ^{ae}	0.45 ^{ab}
T3	20	1.10 ^{c}	3.40 ^{bc}	2.50 ^{c}	0.57 ^{c}
T4	30	0.59 ^{ac}	2.17 ^{ad}	1.23 ^{ad}	0.55 ^{bc}
T5	40	0.61 ^{bc}	2.83 ^{bd}	1.50 ^{de}	0.52 ^{bc}
p-value (ANOVA)		$p < 0.001$	$p < 0.001$	$p < 0.001$	0.003
$LSD_{0.05}$		0.77	0.71	0.48	0.12

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8; Means followed by the same letter are not significantly different at $p < 0.05$.

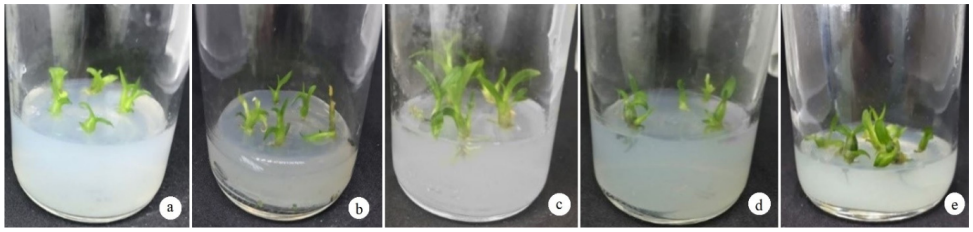

Figure 3. Effect of mashed potato on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing): (**a**) MS, (**b**) MS + 10 g/L potato, (**c**) MS + 20 g/L potato, (**d**) MS + 30 g/L potato, and (**e**) MS + 40 g/L potato.

3.2.2. Effect of Mashed Banana on the Growth and Development of *D. anosmum* Lindl.

Among the natural additives used for culturing orchid, mashed banana is widely used widely as an additive to MS medium because it is a simple, affordable medium recipe [19]. Experiment 7 used different amounts of mashed banana to determine the best medium for the growth and development of *D. anosmum* Lindl. The results obtained after 6 weeks of culture are shown in Table 6 and Figure 4.

Table 6 shows that the addition of mashed banana to MS medium resulted in a better quality of shoot than the control. All the treatments had significantly higher shoot length (0.78 cm, 0.82 cm, 0.72 cm, and 0.7 cm) than the control medium without the mashed banana. The number of leaves on media supplemented with 10 g/L or 20 g/L banana was significantly higher than the control. The number of roots and root length was significantly higher in medium with 20 g/L mashed banana. With a 95% confidence, MS medium with 20 g/L mashed banana added was the most suitable medium for shoot growth.

Table 6. Effect of mashed banana on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing).

Treatment	Banana (g/L)	Shoot Length (cm)	Leaves (Leaves/Plant)	Roots (Roots/Plant)	Root Length (cm)
T1(Control)	0.0	0.33 ^{a}	1.47 ^{a}	0.93 ^{a}	0.36 ^{a}
T2	10	0.78 ^{bd}	1.87 ^{bd}	1.00 ^{ab}	0.39 ^{ab}
T3	20	0.82 ^{bc}	2.07 ^{b}	1.40 ^{c}	0.55 ^{c}
T4	30	0.72 ^{be}	1.67 ^{ad}	1.00 ^{ab}	0.49 ^{ac}
T5	40	0.70 ^{de}	1.63 ^{ac}	1.00 ^{ab}	0.49 ^{ac}
p-value (ANOVA)		0.0017	0.024	0.008	0.017
$LSD_{0.05}$		0.11	0.38	0.35	0.13

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8; Means followed by the same letter are not significantly different at $p < 0.05$.

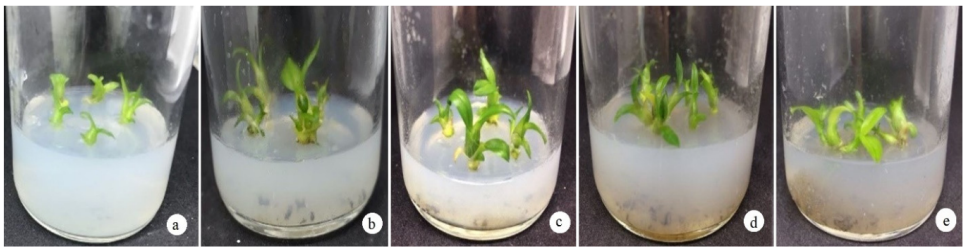

Figure 4. Effect of mashed banana on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing): (**a**) MS, (**b**) MS + 10 g/L banana, (**c**) MS + 20 g/L banana, (**d**) MS + 30 g/L banana, and (**e**) MS + 40 g/L banana.

3.2.3. Effect of Coconut Water on the Growth and Development of *D. anosmum* Lindl.

The addition of coconut water brought a positive result for *D. anosmum* Lindl. shoot growth. The control medium (without CW) had a shoot length of 0.33 cm, the number of leaves was 1.47 leaves/plant, the number of roots was 0.93 roots/plant, and root length was 0.36 cm. When CW was added to MS medium, improvement of the shoot and root quality were observed. At the concentration of 200 mL/L coconut water, orchid plants had a shoot length of 0.88 cm, a leaf number of 2.17 leaves/plant, a root number of 1.40 roots/plant, and root length of 0.56 cm (Table 7). Coconut water at the concentration of 200 mL/L was also found to be the best condition for early protocorm differentiation and rapid seedling growth [20].

Table 7. Effect of coconut water on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing).

Treatment	Coconut Water (mL/L)	Shoot Length (cm)	Leaves (Leaves/Plant)	Roots (Roots/Plant)	Root Length (cm)
T1 (Control)	0	0.33 ^{e}	1.47 ^{a}	0.93 ^{a}	0.36 ^{a}
T2	50	0.84 ^{ac}	1.53 ^{ab}	1.07 ^{ab}	0.38 ^{ab}
T3	100	0.86 ^{ab}	1.87 ^{ac}	1.30 ^{bc}	0.54 ^{cd}
T4	200	0.88 ^{a}	2.17 ^{c}	1.40 ^{b}	0.56 ^{c}
T5	300	0.71 ^{d}	1.80 ^{bc}	1.03 ^{ac}	0.44 ^{bd}
p-value (ANOVA)		$p < 0.001$	0.01	0.045	$p < 0.001$
$LSD_{0.05}$		0.13	0.42	0.34	0.11

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8; Means followed by the same letter are not significantly different at $p < 0.05$.

At 95% confidence, MS medium with 200 mL/L CW added was the most suitable medium for shoot growth.

3.2.4. Effect of Activated Charcoal on the Growth and Development of *D. anosmum* Lindl.

Activated charcoal (AC) is a form of carbon processed to have small, low-volume pores that increase the surface area available for adsorption or chemical reaction. These characteristics mean that activated charcoal has become a useful tool for micropropagation of orchids. AC adsorbs the inhibitory phenolic and carboxylic compounds produced by the tissues in culture [21].

Table 8 shows that the supplementation of activated charcoal at suitable concentration had a positive effect of in vitro *D. anosmum* Lindl. growth. Shoot length, root length, and the number of leaves and roots were higher compared to the control (Figure 5). The highest shoot length (1.09 cm), number of leaves (6.13 leaves/plant), and number of roots (3.17 roots/plant), were obtained in MS medium in addition with 1 g/L AC. The result observed in MS medium with 1.5 mg/L AC and 2 mg/L AC indicated a decline in plant growth. With a 95% confidence, MS medium with 1 mg/L AC added had the best effect on explants.

Table 8. Effect of activated charcoal on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing).

Treatment	Activated Charcoal (g/L)	Shoot Length (cm)	Leaves (Leaves/Plant)	Roots (Roots/Plant)	Root Length (cm)
T1(Control)	0.0	0.33 ^{a}	1.47 ^{a}	0.93 ^{a}	0.36 ^{a}
T2	0.5	0.93 ^{bd}	5.77 ^{bc}	3.13 ^{b}	0.48 ^{b}
T3	1.0	1.09 ^{c}	6.13 ^{b}	3.17 ^{bc}	0.60 ^{cd}
T4	1.5	0.97 ^{b}	5.97 ^{bd}	2.53 ^{bd}	0.58 ^{bc}
T5	2.0	0.84 ^{d}	4.23 ^{cd}	2.47 ^{be}	0.53 ^{bd}
p-value (ANOVA)		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
$LSD_{0.05}$		0.10	1.86	1.01	0.11

Background is the MS medium + 30 g/L sucrose + 8.0 g/L agar, pH = 5.7–5.8. Means followed by the same letter are not significantly different at $p < 0.05$.

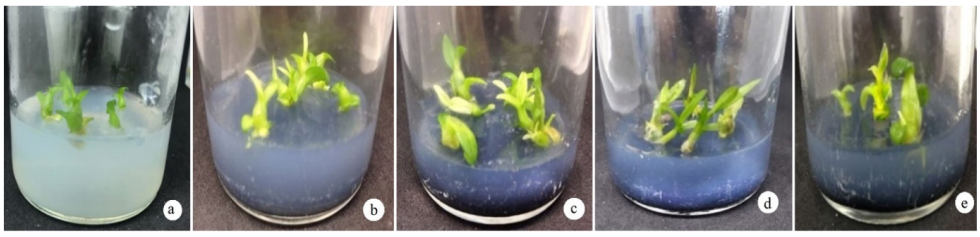

Figure 5. Effect of activated charcoal on the growth and development of *D. anosmum* Lindl. (6 weeks after culturing): (**a**) MS, (**b**) MS + 0.5 g/L AC, (**c**) MS + 1 g/L AC, (**d**) MS + 1.5 g/L AC, and (**e**) MS + 2.5 g/L AC.

Based on the experiments conducted with potato, banana, and coconut water, as well as activated charcoal, we concluded that the addition of additives increases the growth and quality of *D. anosmum* Lindl. shoots. The most suitable medium for growth and development of in vitro *D. anosmum* Lindl. was MS medium supplemented with activated charcoal at a concentration of 1 g/L.

4. Discussion

Its exotic beauty and extended longevity along with high economic efficiency have made *D. anosmum* Lind. a well known, popular flower, which translates into pent up demand for this orchid among growers and consumers in Vietnam. In order to produce orchids on a massive scale, in vitro propagation has proved to be a technique to obtain healthy and high-quality plantlets. However, each orchid has its own unique characteristics which leads to differences in the propagation procedure, especially in the concentration

of added growth regulators and additives. Being among the first investigations into propagation of *D. anosmum* Lindl. and the first conducted with the *D. anosmum* Lindl. collected in Hoa Binh province, Vietnam, our results are as follows.

With regard to the effect of growth regulators on the propagation efficiency of *D. anosmum* Lindl. orchid protocorms, in the present study, we found growth regulators have no impact on protocorm formation rate (always at 100%), which is not in line with previous observations on *D. chrysotoxum* Lindl. [22] and *Aerides ringens* Fisch. [23] that the rate at which germinated seeds develop into plantlets after 8 weeks treatment with growth regulator (BA, kinetin) differs from the rate in medium without BA and kinetin. In addition, Asghar et al. (2011) [24] and Kalimuthu et al. (2007) [25] were not in agreement with our finding, and both studies concluded that medium with 2 mg/L BA achieved the maximum multiplication of the protocorm. However, Asghar et al. (2011) [24] did not mention root formation and the conclusions of Kalimuthu et al. (2007) [25] were based on protocorm formation alone. The current experiment was based on protocorm formation rate, shoot length, the number of leaves, and rooting rate. It is thought that *D. anosmum* Lindl. has a high rate of regeneration.

The effects of single growth regulators (BA or kinetin) or a combination of growth regulators (BA and α-NAA or kinetin and α-NAA) on factors such as shoot length, leaves, and rooting rate were different. Firstly, the medium with 1 mg/L BA was suitable for generating the highest shoot length and leaves, while the one without BA was best for rooting rate. Among the five treatments of BA levels, the shoot length of orchids grown in media with 1 mg/L BA was highest, and nearly 2.09 times higher than in the control. Our result is in agreement with Acemi et al. (2018) [26] (in vitro propagation of *Ipomoea purpurea* Roth) although the fold change in shoot length was around 1.53 times, while other previous researchers such as Reddy et al. (2021) [27] concluded that media containing 1.5 mg/L generated the longest shoot of *D. primulinum* Lindl. Enhancing BA level increased the shoot length of *D. thyrsiflorum* Rchb.f as has been previously highlighted in Tikendra et al. (2018) [28] but this was not observed in our research. By contrast, we found the opposite trend with 0.5 mg/ L BA where the shoot length (0.51 cm) was higher than the shoot length in 2 mg/L BA (0.37 cm). In addition, the number of leaves in media with 1 mg/L BA was 1.31 times more than control. The previous study showed the leaf number of *B. papyrifera* L. was nearly 3.9 times higher in 0.5 mg/L BA compared to the control [29]. In terms of rooting rate, the highest rate was recorded in media without BA. Secondly, the highest shoot length, leaf number, and rooting rate were recorded in the media with 1 mg/L kinetin. The shoot length when treated with only 0.5 mg/L kinetin was also longer than the one with 2 mg/L. The same trend was observed by Nguyen et al. (2011) [30], in which a high concentration of kinetin inhibited shoot from elongating. In our research, 1 mg/L kinetin produced the greatest shoot length (1.15 cm). This was not corroborated by the research of Sana et al. (2011) [24] which showed that *(D. nobile)* var. *Emma white* exhibited the highest shoot length with 5 mg/L of kinetin. The shoot length, leaves, and rooting rate in medium with 1 mg/L added kinetin was approximately 3.48 times, 2.04 times, and 1.41 times, respectively, that of the control treatment. Thirdly, the effect of BA and α-NAA on the regeneration efficiency of *D. anosmum* Lindl. is worth considering. The highest shoot length was observed in the treatments of 1 mg/L BA and 0.3 mg/L α-NAA. The shoot length in the control treatment was 70% of the length of shoot in the 1 mg/L BA + 0.3 mg/L α-NAA treatment. In media containing both 1 mg/L BA and NAA (0.2/0.3/0.5 mg/L), our rooting rate were reduced from 40.91% to 28.57%, while Yao et al. (2021) [31] found that this rate went up from 2.5% to 10%. Fourthly, the best regeneration efficiency of *D. anosmum* Lindl. was found to be on the medium with 1 mg/L kinetin and 0.5 mg/L α-NAA. In addition, with 1 mg/ L kinetin, the higher the α-NAA, the longer the shoot length, and the highest (1.49 cm), 1.3 times higher than with the control level (1 mg/L kinetin and without BA), was achieved with 1 mg/L kinetin + 0.5 mg/L α-NAA. By contrast, the shoot length of *Moringa stenopetala* Cufod. in the same combination was nearly 2.87 times lower than the one with the control level (without both kinetin and α-NAA). The medium supplemented with 1 mg/L kinetin

+ 0.5 mg/L α-NAA was 1.47 times higher than control level, while about 1.57 times higher was found in the study of Adugna et al. (2020) [32] on in vitro propagation of *M. stenopetala* Cufod. from shoot explants. A combination of 0.2, 0.3, or 0.5 mg/L α-NAA with 1 mg/L kinetin in the medium all produced 100% rooting rate, which was significantly higher than rooting rate in other treatments. So, in our study, the medium with 1 mg/L kinetin + 0.5 mg/L α-NAA added gave the highest protocorm formation rate, the highest shoot length, the highest number of leaves/plant, and the highest rooting rate.

The effect of additives on the growth efficiency of orchids has been researched in *Vanda* Mme Rattana A/M × *Kasem's Delight* [33], *Vanda helvola* Blume [34], *Phalaenopsis* Hybrid 'Pink' [15], *Sedirea japonica* Garay & H.R. Sweet [35], and *Renanthera imschootiana* Rolfe [36]. Important additives such as mashed banana [37], mashed potato [38], coconut water [39], and activated charcoal [40] facilitated root development and shoot growth. Firstly, in terms of mashed potato and mashed banana, according to Nguyen et al. (2020) [41] in *D. nobile* Lindl., raising the mashed potato concentration and mashed banana concentration to 30 g/L resulted in higher shoot length and a higher number of leaves per plant, which is not in agreement with our findings. We found shoot length and leaf number/plant did drop when the concentration went up from 20 to 30 g/L. While Islam et al. (2011) [42] showed the number of roots/seedling and length of root of *Vanda roxburgii* orchid were highest in 100 mL/L and 200 mL/L, respectively, our study highlighted 20 g/L potato is best for both root number/plant and root length because at this concentration, the shoot length and leaf number were, significantly, 3.33 times, and 2.31 times, respectively, higher than the control level. Similarly, shoot length, leaf number, root number, and root length peaked higher with 20 g/L banana than with 0, 10, 30, or 40 g/L banana. Nicomrat and Anantasaran (2015) [19] also found that in terms of height, *D. farmeri* Paxton and *D. griffithianum* Lindl. grew better in MS medium supplemented with Namwa banana. Secondly, along with potato and banana, coconut water is also used in in vitro tissue culture, particularly orchid production [43] because it contains nutritional and hormonal substances [39]. According to Baque et al. (2011) [44], shoot length and the number of roots of 'Bukduseong' × 'Hyesung' increase when CW are raised from 0 to 30 mL/L, and reduce as the CW increase from 30 mL/L to 100 mL/L. In contrast, in our research, in the range of 0–100 mL/L of CW, the shoot length and the number of roots increase. Shoot length, leaves (leaves/plant), roots (roots/plant) and root length continued to go up until they reached the peak at 200 mL/L. Thirdly, 1 g/L activated charcoal was found to be the level that ensured better propagation compared to 0, 0.5, 1, 1.5, or 2 g/L activated charcoal. Increased concentration of activated charcoal (from 0 to 0.5 g/L) raised the length of the roots in *Cattleya* orchid plantlets [45] and in palm *Phenix Dactylifera* L. cv. *Sufedy* [46]. Likewise, our research also showed that root length increases as activated charcoal concentration goes from 0 to 0.5 g/L due to the ability to reduce the light intensity of the activated charcoal. However, as the activated charcoal was raised from 1 g/L to 2 g/L in the medium, shoot length, leaves, roots, and root length all went down. This trend was also obtained in Nguyen et al. (2012) [47]. Activated charcoal has characteristics that include adsorption and removal of phytotoxic metabolites. However, too high a concentration of AC produced the worst propagation efficiency of orchids because the carbon absorbed the nutrition of the medium and slowed down the development process of *D. anosmum* Lindl. With a 95% confidence, MS medium with 1 mg/L AC added had the best effect on explants. This result agrees with Nguyen et al. (2012) [47] when they also concluded the same for *D. fimbriatum* Hook, although Prizão et al. (2012) [48] found 6 g/L in *Cattleya bicolor* Lindl., in KC medium, and Abahmane et al. (2017) [49] indicated 0.75 g/L charcoal. For shoot length, while we and Mittal et al. (2016) [50] agreed that activated charcoal increases shoot length in propagating *D. anosmum* Lindl., Rodrigues et al. (2017) [51] showed the opposite trend in sugar cane in a previous study. In the assessment of four types of additives with different concentrations, 1 g/L activated charcoal proved to be suitable for propagation of *D. anosmum* Lindl.

5. Conclusions

It is possible to in vitro propagate *D. anosmum* Lindl. collected in Hoa Binh province. *D. anosmum* Lindl. seeds have strong ability to germinate and develop into healthy seedlings. Therefore, reproduction of protocorms from seedlings may not require the addition of growth regulators (BA, kinetin, or α-NAA). Nevertheless, the addition of growth regulators improves the quality of protocorms and shoots. The combination of 1 mg/L kinetin and 0.5 mg/L α-NAA resulted in the best growth and development of protocorms.

Supplementation of additives can increase the quality of *D. anosmum* Lindl. shoot in vitro. The most suitable medium for orchid growth was MS medium with 1.5 g/L activated charcoal.

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