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In vitro regeneration of hybrid *Dendrobium* sect. Spatulata through pseudobulb segment culture

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Abstract

The low rate of *Dendrobium* regeneration through seed culture is a significant limitation of mass propagation development in new hybrids. An efficient in vitro regeneration protocol through pseudobulb segment culture has been established for *Dendrobium*'Dandy Dame'. Leaves and roots of seven-month-old seedlings were detached from the pseudobulb. Unsegmented pseudobulb and segments of apical, medial, and basal excised from seedlings. The four types of explants were cultured on Murashige and Skoog (MS) basal medium supplemented with different combinations of 1-naphthaleneacetic acid (NAA) and 6-benzyl amino purine (BAP). The highest number of shoots was observed in 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA using unsegmented pseudobulb explant after two months of culture. The unsegmented pseudobulb had a higher survivability rate and the number of shoots per explant than the segmented type. However, segmented pseudobulbs can produce 2-4 times more shoots than unsegmented when accumulated. The basal segment had the highest average number of shoots, and the two types observed were the single and multi-shoots. They were developed from protuberance and axillary bud. Additionally, the roots continuously grew on the basal part of unsegmented explants and basal segments.

Keywords: *Dendrobium*'Dandy Dame', *in vitro* regeneration, PGRs, pseudobulb segment

Introduction

Dendrobium is one of the most popular orchid genera in the floriculture market globally. The flower has a relatively long freshness, a wide variety of colors and shapes, and high productivity. The orchids were developed continuously into new hybrids to meet market demand. Meanwhile, mass propagation using *in vitro* techniques played an essential role for many years (Bhattacharyya et al., 2015). Regeneration from different explants such as the leaf, nodal, shoot tip, meristem, flower stalk, thin cross-sections (TCs), or pseudobulb segment has been successfully conducted in various *Dendrobium* (Silva et al., 2015).

Shoot induction is significantly affected by certain plant growth regulators (PGRs). Several studies have used a combination of 6-benzyl amino purine (BAP) and 1-naphthalene acetic acid (NAA) to induce shoots from pseudobulb or nodal segments in species such as *D. transparens* (Sunitibala & Khishor, 2009), *D. candidum* (Shiau et al., 2005), and *D. nobile* (Bhattacharyya et al., 2016). Hossain (2013) conducted a pseudobulb segment culture on *D. aggregatum* and reported that direct organogenesis produces multiple shoots. The differentiation of shoots arises from dormant buds without passing through the callus phase. Mata-Rosas et al. (2010) conducted pseudobulb culture on the *Lycaste aromatica* by dividing it into three basal, medial, and apical parts. The results show that shoots emerged in apical and basal segments.

D. transparens, *D. candidum,* and *D. nobile* are included in the *Dendrobium* section within the *Dendrobium* genus (McHatton, 2016), while *D. aggregatum* is included in the *Callista* section (Cootes, 2016). The two sections have different characters in the pseudobulb. *Callista* section has more or less erect pseudobulbs thickened upwards from a slender base. In contrast, pseudobulbs in

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the Dendrobium section are evenly fleshy or thickened at the nodes (Seidenfaden & Wood, 1992). In the *Spatulata* section, the plants have cane-like pseudobulbs, swollen at the base and often slightly dilated in the middle or lower half (Cribb, 1983). There are no reports on pseudobulb segment culture in the *Spatulata* section.

Twisted flower perianth-type within the *Spatulata* section is one of Indonesia's most popular Dendrobium hybrids. D. 'Dandy Dame' is an offspring of *D. helix* x *D. lasianthera*, where both parents are included in the *Spatulata* section (Harrison, 2007). Recently, this hybrid can be easily propagated using seed culture. This method can provide many seedlings with diverse somaclonal variations (Meilasari & Iriawati, 2016). The first selection using healthy seedlings as explants produces uniform characters in the next generations. Therefore, the pseudobulb segment of *D.* 'Dandy Dame' was experimented on basal medium supplemented with cytokinin and auxin in different combinations to achieve efficient regeneration with high frequencies.

Materials and Methods

Seven-month-old seedlings (2-3 times of subcultures) *D.* 'Dandy Dame' from *in vitro* seeds cultured on VW medium (Vacin & Went, 1949) with certain modifications were used as the source of explants. These modifications include two iron minerals with the same ferric tartrate and ferrous sulfate 26.29 mg L^{-1} in a micronutrient stock solution. The culture medium was supplemented with thiamin 2 ml L^{-1}, commercial fish oil 0.5 ml L^{-1}, and activated charcoal 1 g L^{-1} to strengthen the roots. Furthermore, the leaves and roots were detached from pseudobulb explants *in vitro* cultures without requiring sterilization.

The pseudobulb was cut at the internode, divided into apical, medial, and basal segments. Additionally, cutting was not required for the entire pseudobulbs treatment. The explants were inoculated into MS medium (Murashige & Skoog, 1962) supplemented with sucrose 3% (w/v), 0.2 g L^{-1} gellan gum, and 0.1 mg L^{-1} NAA combined with 0.0, 1.0, 2.0, 3.0 mg L^{-1} of BAP. The pH was adjusted to 5.8 with 0.1 N KOH or HCl before autoclaving at 121°C and 104 kPa, for 15 minutes.

Observations were conducted for eight weeks with subcultures every four weeks. Morphological analyses were carried out under a stereomicroscope by Eschenbach, and images were taken using OptiLab Viewer 2.2. by Miconos Transdata Nusantara. Furthermore, the data on survivability, the number of shoots, leaves, and induction time were recorded. Bud forming capacity (BFC) was calculated based on the study of Tandon et al. (2007).

Newly formed structures were sampled for histological analysis, and the sample was fixed in FAA solution (90 mL 70 % ethanol, 5 mL acetic acid, and 5 mL 36% formaldehyde). Furthermore, it was washed repeatedly in 70% ethanol and dehydrated with ethanol series at 80%, 90%, and 95%. Dealcoholization using 96% ethanol and xylene mixture, for instance, ethanol and xylene ratios of 3:1, 1:1, and 1:3 were followed by infiltration of combined liquid paraffin and xylene at 9:1 for 24 hours. Then the sample was immersed in pure liquid paraffin for one hour and embedded using the refined paraffin. The sample was sectioned into thin slices using a rotary microtome and stained with 1% safranin in 70% ethanol. Furthermore, the slices were observed under the light microscope Olympus CX22LED, and the photograph was taken using the OptiLab. The entire treatment was repeated five times, subsequently the data were subjected to a completely randomized design and analyzed using one-way ANOVA statistics, while the means were compared with the LSD at $p \leq 0.05$.

Results and Discussion

After eight weeks of planting, the highest survival percentage was found in the entire pseudobulb explants (84%), followed by basal segment (76%), apical segment (72%), and medial segment (36%) (**Figure 1**). Dead explants experienced a browning event, a condition where phenolic compounds are released as a defense mechanism against biotic and abiotic stresses (Ahmad et al., 2013). The polyphenol oxidase (PPO) caused darkening and toxicity to the explant (Klenotičová et al., 2013).

PPO activity occurs in damaged explants by excision that has lost cell compartmentalization (Wang et al., 2014; Chuanjun et al., 2015). The pseudobulb explant had the lowest browning percentage because cutting

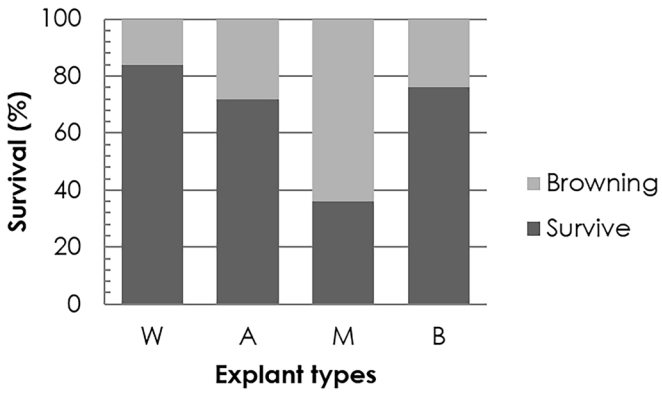

Figure 1. Survival rate (%) of explants of *D.*' Dandy Dame' after eight weeks of culture. W: whole pseudobulb, A: apical segment, M: medial segment, B: basal segment.

was not performed. Medial segments had the highest browning rate because they had two cutting sides, while basal and apical segments only had one cutting side.

Parthibban et al. (2015) reported that *in vitro* developmental stages in *Dendrobium* using vegetative explant can be divided into shoot proliferation, regeneration from callus, and direct embryogenesis somatic. The shoot was formed directly from the pseudobulb segment without passing through the callus phase or PLB (protocorm-like bodies). Protuberance formation (**Figure 2**a-d) and axillary bud induction are the two ways to develop shoots (Figure 2e-h). Multiple shoots (Figure 2i-l) and roots (Figure 2m-p) were observed in some treatments.

Shoot development from protuberance is characterized by swelling and the appearance of a greenish dome-like structure on the surface of the explants. Chen et al. (2004) also reported the development of white protuberance in the pseudobulb segment culture of *Paphiopedilum philipinense* hybrids. Meanwhile, the shoots from axillary buds are developed above the pseudobulb node under the leaf midrib. These two developmental ways produce single and multiple shoots.

Medium without the addition of PGRs resulted in a higher percentage of single shoot formation than other treatments (**Figure 3**A). Multi-shoot formation increases with an increased BAP concentration. The term 'multiple shoots' describes a shoot group that appears almost simultaneously at one growth point. A single BAP dose

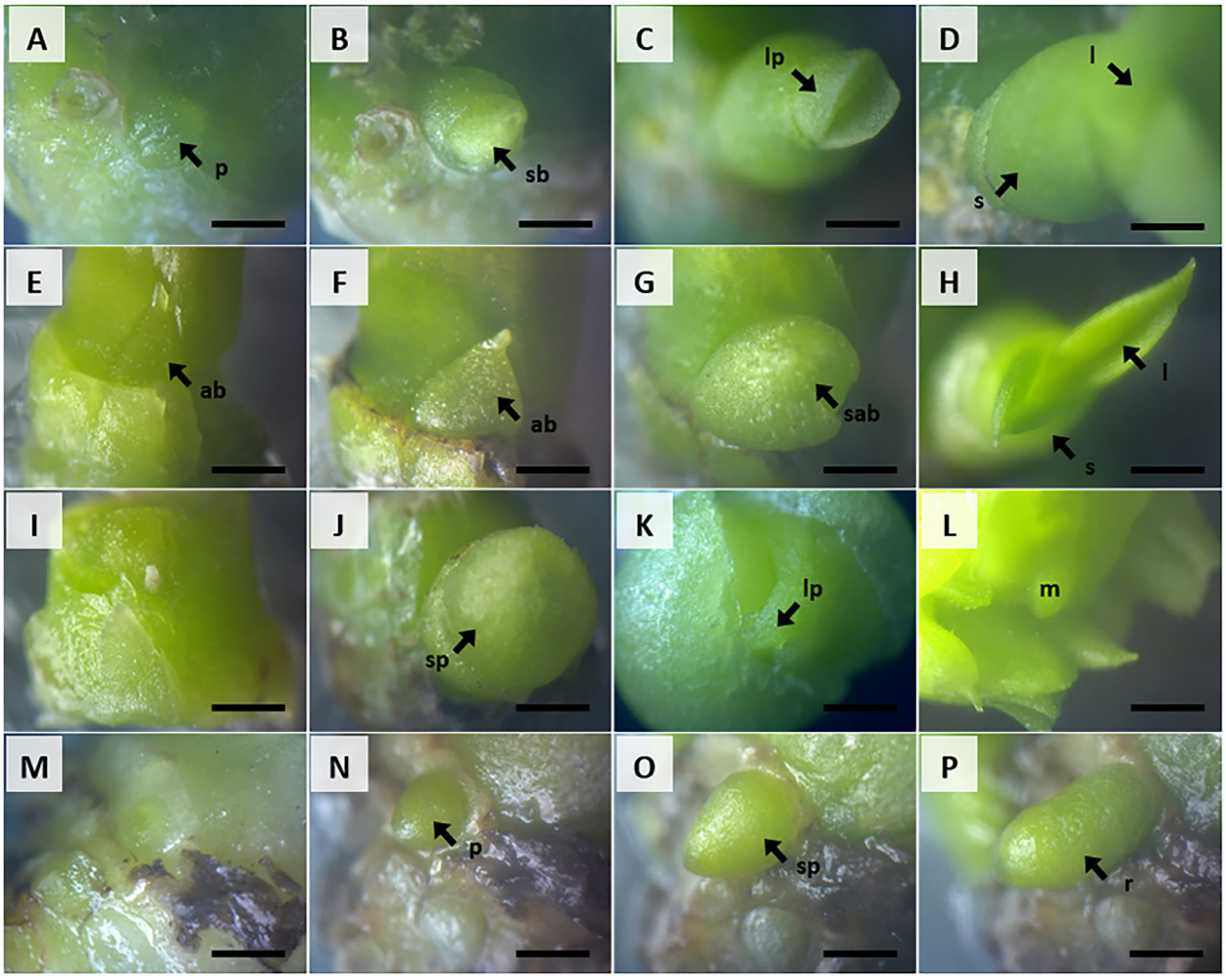

Figure 2. Shoot and root development of *D.*' Dandy Dame' through pseudobulb segment culture. A-D: Shoot development through protuberance of the basal segment in MS0 medium. (A) protuberance (p) emerged on the 3rd day. (B) swollen protuberance (sp) on the 7th day. (C) shoot (s) with leaf primordia (lp) appeared after the 14th day. (D) leaves (l) appeared after the 21st day. E-H: Shoot development through axillary bud induction of whole pseudobulb (medial node) in MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA. (E) axillary bud (ab) at the nodal segment on the 3rd day. (F) axillary bud after the 14th day. (G) swollen axillary bud (sab) on the 21st day. (H) shoot (s) with leaves (l) appears after the 28th day. I-L: Multishoot development of medial segment in MS + 3 mg L^{-1} BAP + 0.1 mg L^{-1} NAA. (I) the medial segment at 0 day; (J) swollen protuberance at 21st day. (K) swollen protuberance with leaf primordia (lp) on the 28th day. (L) multi shoot (m) starts to grow on the 35th day. M-P: root development of basal segment in MS0 medium. (M) the basal segment at 0 day. (N) protuberance (p) appeared on the 7th day. (O) swollen protuberance (sp) on the 14th day. (P) root (r) appear after the 14th day. Bar: 1 mm.

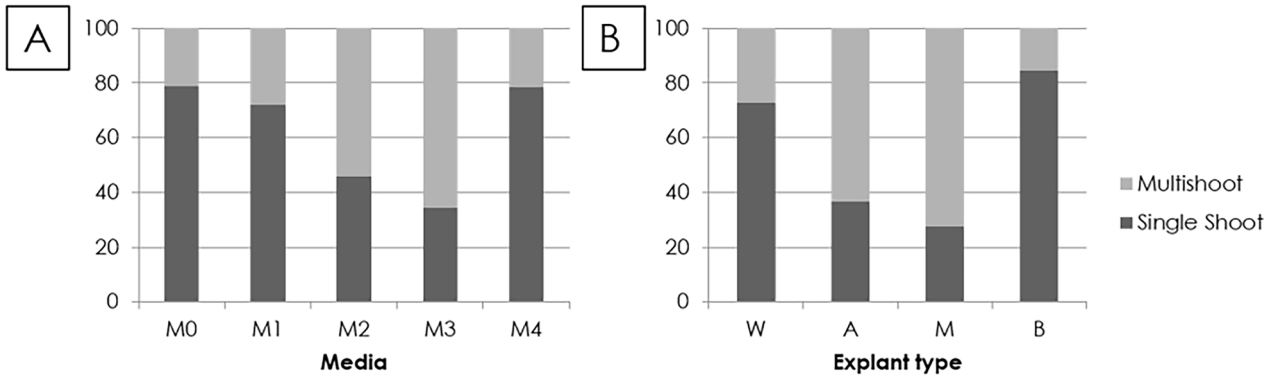

Figure 3. Effect of different media (A) and explant type (B) on shoot type formation. M0: MS basal medium; M1: MS + 1 mg L^{-1} BAP + 0,1 mg L^{-1} NAA; M2: MS + 2 mg L^{-1} BAP + 0,1 mg L^{-1} NAA, M3: MS + 3 mg L^{-1} BA + 0,1 mg L^{-1} NAA; M4: MS + 0,1 mg L^{-1} NAA.

was reported to induce multi-shoot from segment nodes explant in other orchid species, such as *D. longicornu* (Dohling et al., 2012), *Paph. philippinense* (Chen et al., 2004), and *Vanilla planifolia* (Gopi et al., 2006).

Based on the type of explant, the basal segment produced a higher percentage of single shoots than other treatments (Figure 3B). Similar results were reported by Mata-Rosas et al. (2010), where the basal part has the highest response to shoot formation than the apical and medial parts of *Lycaste aromatica*. Meanwhile, multi-shoots were mostly formed on the medial segment, and previous studies reported complex organic matter requirements, such as peptone, yeast extract, or urea for the induction in *Dendrobium* (Sunitibala & Kishor, 2009). This current study showed that multi-shoot induction in *D.* 'Dandy Dame' was not required organic supplements.

In this study, several new structure formation stages were observed: protuberance, swollen buds, single shoots, and multi-shoots or multiple shoots (**Table 1**). Each of the structures has a variation induction time. In general, protuberance appeared on the surface of the explants during the first to sixth weeks of planting. The fastest induction time was observed on medial explants in the medium without hormones, having a mean induction time of 6 days. Swelling of protuberance and axillary buds occur in the second to fourth week after planting.

There are two types of shoots observed in this study: single and multi-shoots. Single shoots are formed in the third to fourth weeks after planting, with the fastest time on explants without cutting in the medium by adding 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA for 18 days. In contrast, multi-shoot was formed after four weeks of planting, with the fastest time on explants medial in medium with the 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA.

The pseudobulb explants in medium with 1 mg L^{-1} BAP and 0.1 mg L-^{1} NAA produced the highest shoots (4-5 shoots per explant) and BFC index (3.40). BFC index shows

Table 1. Induction time of the explants forms a new structure in *D.*' Dandy Dame'. W whole pseudobulb, A apical segment, M medial segment, B basal segment

PGRs (mg L^{-1})		Explant type	Type of new structure			
BAP	NAA		Protuberance (days)	Swollen bud (days)	Single Shoot (days)	Multishoot (days)
0	0	W	30.89 ^{bc}	14.43 ^{ab}	20.23 ^{a}	0
		A	21.00 ^{abc}	24.60 ^{c}	47.60 ^{d}	0
		M	6.00 ^{a}	25.50 ^{c}	37.50 ^{cd}	42.00 ^{a}
		B	23.43 ^{abc}	14.57 ^{ab}	24.60 ^{abc}	0
1	0.1	W	21.75 ^{abc}	12.13 ^{a}	17.81 ^{a}	59.00 ^{b}
		A	0	12.33 ^{a}	18.00 ^{a}	0
		M	0	14.00 ^{ab}	21.00 ^{a}	33.00 ^{a}
		B	0	12.83 ^{ab}	25.17 ^{abc}	0
2	0.1	W	17.75 ^{abc}	15.00 ^{ab}	27.07 ^{abc}	33.00 ^{a}
		A	0	18.33 ^{abc}	21.88 ^{ab}	0
		M	9.00 ^{ab}	10.00 ^{a}	24.00 ^{abc}	33.00 ^{a}
		B	24.50 ^{abc}	12.00 ^{a}	18.67 ^{a}	0
3	0.1	W	19.50 ^{abc}	17.67 ^{abc}	28.00 ^{abc}	0
		A	36.00 ^{c}	17.33 ^{abc}	21.50 ^{a}	47.5 ^{ab}
		M	0	12.67 ^{ab}	21.00 ^{a}	39.00 ^{a}
		B	0	12.00 ^{a}	18.33 ^{a}	42.00 ^{a}
0	0.1	W	21.00 ^{abc}	22.14 ^{bc}	22.14 ^{ab}	0
		A	0	24.67 ^{c}	35.33 ^{bc}	0
		M	0	19.33 ^{abc}	30.33 ^{abc}	45.00 ^{ab}
		B	9.00 ^{ab}	18.64 ^{abc}	28.31 ^{abc}	0

Means followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.005$)

the ability to form shoots by comparing the average number formed per explant and the percentage (Kehie et al., 2012). Similar results were reported by Bhadra et al. (2002), where the addition of 1 mg L^{-1} BAP in MS medium induces five new shoots on *D. aphyllum* nodal segments. Kabir et al. (2013) also reported that the highest shoot number (4 shoots per explant) on *D. fimbriatum* nodal segment was observed on a medium containing 1 mg L^{-1} BAP after 60 days of planting.

Compared to segmented explants, the entire pseudobulb explants produce more new shoots. They naturally have more axillary buds than the segmented

explants. Furthermore, the metabolism in the whole pseudobulb is not disturbed, especially in terms of the distribution of endogenous hormones. Adding exogenous auxin or cytokinins to the culture medium increases hormones' concentration in the cells, making PGRs a trigger factor in tissue development (Cai et al., 2018). However, when accumulated, one pseudobulb segmented into numerous explants produces 2-4 times more shoots than one whole pseudobulb. The basal segments had the highest shoot number than the apical and medial segments (**Table 2**).

Table 2. Effect of BAP and NAA on shoot formation of *D.*' Dandy Dame' after eight weeks of culture. W whole pseudobulb, A apical segment, M medial segment, B basal segment

PGRs (mg L^{-1})		Explant type	Shoot no. per explant	BFC	Shoot length (mm)	No. of leaves
BAP	NAA					
0	0	W	3.20^{bcd}	3.20	13.61^{abc}	2.93^{abc}
		A	2.67^{abcd}	1.60	12.13^{abc}	2.67^{abc}
		M	1.00^{a}	0.20	14.51^{abc}	1.50^{a}
		B	2.40^{abcd}	2.40	21.76^{bc}	2.80^{abc}
1.0	0.1	W	4.25^{d}	3.40	16.53^{bc}	2.69^{abc}
		A	1.67^{abc}	1.00	21.69 ^{bc}	3.25^{bc}
		M	2.00^{abc}	0.40	13.03^{abc}	3.00^{bc}
		B	3.50^{cd}	1.40	14.29^{abc}	2.25^{abc}
2.0	0.1	W	2.60^{abcd}	2.60	19.51^{bc}	3.29^{bc}
		A	1.40^{ab}	1.40	19.08^{bc}	2.79^{abc}
		M	1.00^{a}	0.40	22.90^{c}	2.00^{ab}
		B	3.33^{bcd}	2.00	23.20^{c}	3.22^{bc}
3.0	0.1	W	1.50^{abc}	0.60	17.08^{bc}	3.67^{c}
		A	1.00^{a}	1.00	4.44^{a}	1.86^{ab}
		M	1.67^{abc}	1.00	10.05^{ab}	2.67^{abc}
		B	2.75^{abcd}	2.20	20.43^{bc}	3.00^{bc}
0.0	0.1	W	2.60^{abcd}	2.60	13.10^{abc}	2.00^{ab}
		A	1.5^{abc}	0.60	17.93^{bc}	2.67^{abc}
		M	1.50^{abc}	0.60	9.54^{ab}	1.80^{ab}
		B	2.80^{abcd}	2,80	15.09^{abc}	2.33^{abc}

Means followed by the same letter are not significantly different by Duncan's multiple range test (P ≥ 0.005)

Basal segments produced the highest shoot length (23.20 mm) on the medium with the addition of 2 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. The use of 1 mg L^{-1} BAP and without BAP showed a lower mean of shoot length. As a result, there is an elongation of the effect of BAP concentration on shoots. A similar result was reported by Sharma et al. (2007), where the addition of 2 mg L^{-1} BAP produces the longest mean shoots from *D. microbulbon* nodal segments.

In general, 2-3 leaves grew on new shoots after 60 days of induction. The highest number (3-4 leaves per shoot) was found in unsegmented explants in the medium supplemented with 3 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. Leaf formation without PGRS cytokinin indicates that endogenous cytokinin is sufficient morphogenesis.

In this study, roots only appeared in the basal part of the whole pseudobulb. The highest number was found in the entire pseudobulb explants (**Table 3**). Unsegmented explants form roots because the distribution of auxins is minimally disturbed. The average time to induce roots is 4-5 weeks after culture. The shortest induction time of 27 days is observed in unsegmented explant on the media with the addition of 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. In general, each explant produces 1-2 roots, with the highest number of 5 per explant observed in the whole pseudobulb on the medium without the addition of PGRs. The longest root range of 7 mm was observed on the entire pseudobulb in medium with 2 mg L^{-1} BAP and 0.1 mg L^{-1} NAA.

Table 3. Effect of BAP and NAA on root formation of Den. 'Dandy Dame' after eight weeks of culture. W whole pseudobulb, A apical segment, M medial segment, B basal segment

PGR (mg L-1)		Explant type	Root no. per explant	Root length (mm)
BAP	NAA			
0	0	W	5.00^{b}	3.04^{a}
		A	0	0
		M	0	0
		B	1.00^{a}	3.20^{a}
1.0	0.1	W	2.50^{ab}	3.48^{ab}
		A	0	0
		M	0	0
		B	0	0
2.0	0.1	W	1.00^{a}	7.25^{b}
		A	0	0
		M	0	0
		B	0	0
3.0	0.1	W	1.00^{a}	3.42^{ab}
		A	0	0
		M	0	0
		B	0	0
0.0	0.1	W	0	0
		A	0	0
		M	0	0
		B	2.00^{ab}	3.94^{ab}

Means followed by the same letter are not significantly different by Duncan's multiple range test (P ≥ 0.005)

BAP has a vital role in stimulating plant cell division and differentiation (He et al., 2018). As a PGR in the auxin group, NAA has an important role in promoting cell division and elongation (Yan et al., 2014). BAP acts synergistically with the NAA while controlling shoot meristems antagonistically for root stem-cell niche (Yang et al., 2017).

In this research, the anatomical examination of shoots and roots was conducted. The shoot formation begins with the protuberances, a dome-like structure, on the explant's surface (**Figure 4**A). In addition, a parenchymal cell layer protected shoot apical meristem (SAM). Protuberance grows larger, morphologically looking like a swell structure (Figure 4B) to form new

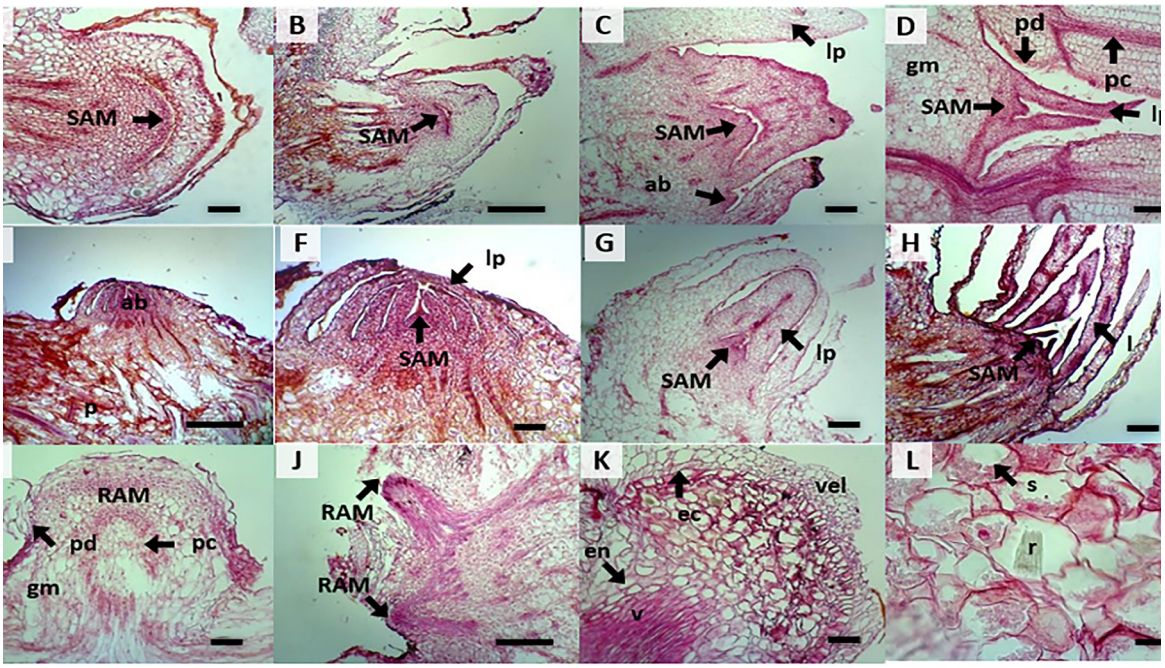

Figure 4. Histological development of shoot and root of *D.*' Dandy Dame' through pseudobulb segment culture. A-D: shoot development through protuberance. (A) Seven days old protuberance. (B) Fourteen days old swollen protuberance. (C) Twenty-one days old shoot apex appears after. (D) Fifty days old shoot apex. E-H: Shoot development through axillary bud induction. (E)-(F) axillary bud on pseudobulb node. (G) Twenty-one days old swollen axillary bud. (H) Thirty-five days old shoot apex. I-L: root anatomical development. (I) Seven days old protuberance. (J) Twenty-one days old swollen protuberance. (K) Forty-nine days old root apex. (L) root cortex. SAM: shoot apical meristem, lp: leaf primordia, ab: axillary bud, pd: protoderm, pc: procambium, gm: ground meristem, p: pseudobulb, l: leaf, RAM: root apical meristem, en: endoderm, ec: ectoderm, c: cortex, vel: velamen, s: statolites, r: rafida. Bar 1 mm.

shoots with leaf primordia (Figure 4C). Adult shoots have several observed meristematic tissues of protoderm, procambium, and ground meristem (Figure 4D). The initial meristem, called the promeristem, develops into protoderm, procambium, and ground meristem (Crang et al., 2018). Furthermore, protoderm, procambium, and ground meristem differentiate into epidermal, transport, and ground tissues.

Anatomically, the production of shoots through axillary buds was characterized as follows. The axillary bud is naturally present on the pseudobulb's side at the node (Figure 4E). They have SAM with 3 to 4 layers of leaf primordia for protection purposes (Figure 4F). There are leaf primordia attached to the outer part to cover and protect the SAM. The axillary buds grew more extensive to elongate the leaf primordia (Figure 4G), which morphologically appeared to be swelling. The fused leaf primordia separate and produce leaves on new shoots (Figure 4H).

In later observation, roots appeared to grow from protuberance. A group of meristematic cells at the tip is called the root apical meristem (RAM) (Figure 4I). The outer protoderm layer was observed with the procambium in the middle. The ground meristem contained parenchyma cells between them. Furthermore, meristematic cells perform anticlinal division, making protuberance grow extensively from the explants (Figure 4J). On day 21, the outermost part of the root tip was composed of 3-4 layers of velamen with one exodermis layer at the bottom (Figure 4k). Velamen is morphologically homologous to the epidermis but consists of 24 layers (Javelle et al., 2011). It is a white sheath on orchid roots commonly found in epiphytic orchids. The functions of absorbing water and nutrients have been discussed in previous studies. A well-matured velamen consists of dead cells with exodermis on the inside, a layer of long cells that become dead as adults. It has thickened walls and smaller absorbent cells to pass through water and nutrients from the velamen to the root cortex.

Statolites and raphide crystals are found in the parenchyma cells of the cortex (Figure 4L). Statolites are special plastids containing solid starch granules. They are located at the low point of the cell to differentiate up and down as a determinant of geotropic motion (Sato et al., 2015). Meanwhile, rafide crystals are a form of calcium oxalate crystals. The hypotheses regarding the function of calcium oxalate crystals in plants are calcium regulation, plant defense mechanisms, detoxification such as

heavy metals or oxalic acid, ion balance, tissue support components, or plant reinforcement with light collection and reflection (Franceschi & Nakata, 2005).

Plants from tissue culture propagation have a uniform character as the parent plant. Cell growth and development into a complete plant involve mitotic division (Bairu & Kane, 2011). However, random and spontaneous variations occur in the culture process, and the variations were found after the explants were six months. The many seedlings produced can be divided into several types of morphology. Type I has elongated shoots with leaves appearing on each segment. However, axillary buds were grown at nodes 3 to 5, making the shoots appear to be branched (**Figure 5**A). Type II has a morphology of short and clumping shoots (Figure 5B). Type III has characteristics like Type II, but the shoots do not grow lengthwise, and the leaves are wider (Figure 5C). In Type IV, the explants produce one main shoot from the base of the broad leaves (Figure 5D).

Jeong et al. (2009) divided the abnormal shoots into wide leaves and the Rosset type. Ramage & Williams (2004) explain that this abnormality was associated with the expression of a knotted1-type homeobox gene (TobH1) isolated from shoots with normal morphology. The cytokinins and homeobox genes have an important role in differentiating adventitious shoots *in vitro*. Overexpression of these genes interferes with normal shoot development. The two publications above perform molecular detection to compare normal and abnormal shoot characters. Meanwhile, further molecular studies need to be explored in this research.

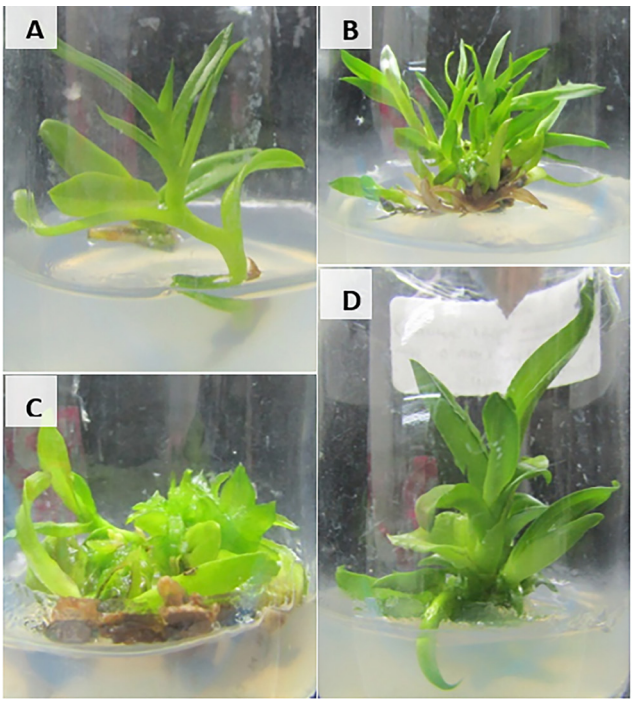

Figure 5. The appearance of morphological abnormalities of explants aged six months. (A) Type I. (B) Type II. (C) Type III. (D) Type IV.

Conclusions

Unsegmented explants of pseudobulb have higher survivability than the segmented. Furthermore, the basal segment is most responsive to shoot induction. The combination of 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA is an optimal concentration to form new shoots. The protocol presented is used for mass propagation of Den. 'Dandy Dame,' which is an important ornamental orchid hybrid.

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References

Ahmad, I., Hussain, T., Ashraf, I., Nafees, M., Maryam, R.M., Iqbal, M. 2013. Lethal effects of secondary metabolites on plant tissue culture. American-Eurasian Journal of Agricultural & Environmental Sciences13: 539-547.

Bairu, M.W., Kane, M.E. 2011. Physiological and developmental problems encountered by *in vitro* cultured plants. *Plant Growth Regulation* 63: 101-103.

Bhadra, S.K., Bhattacherjee, B., Barua, A.K., Hossain, M.M. 2002. Micropropagation of *Dendrobium aphyllum* (Roxb.) GEC Fisher. *Bangladesh Journal of Genetics and Biotechnology* 3: 47-50.

Bhattacharyya, P., Kumaria, S., Job, N. Tandon, P. 2015. Phyto-molecular profiling and assessment of antioxidant activity within micropropagated plants of *Dendrobium thyrsiflorum*: a threatened, medicinal orchid. *Plant Cell, Tissue and Organ Culture* 122: 535-550.

Bhattacharyya, P., Kumaria, S., Tandon, P. 2016. High frequency regeneration protocol for *Dendrobium nobile*: a model tissue culture approach for propagation of medicinally important orchid species. *South African Journal of Botany* 104: 232-243.

Cai, L., Zhang, L., Fu, Q., Xu, Z.F. 2018. Identification and expression analysis of cytokinin metabolic genes IPTs, CYP735A and CKXs in the biofuel plant *Jatropha curcas*. *PeerJ* 6: e4812.

Chen, T.Y., Chen, J.T., Chang, W.C. 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Plant Cell, Tissue and Organ Culture* 76: 11-15.

Chuanjun, X., Zhiwei, R., Ling, L., Biyu, Z., Junmei, H., Wen, H., Ou, H. 2015. The effects of polyphenol oxidase and cycloheximide on the early stage of browning in *Phalaenopsis* explants. *Horticultural Plant Journal* 1: 172-180.

Cootes, J. Den. Sec. Callista. 2016. https://aos.org/orchids/orchids-a-to-z/letter-d/den-sec-callista.aspx. <access on 5 Feb. 2020>.

Crang, R., Lyons-Sobaski, S., Wise, R. 2018. *Mitosis and Meristems*. In: Plant Anatomy. Springer, Cham. p. 123-154.

Cribb, P.J. 1983. *Dendrobium* sect. Ceratobium (Orchidaceae) in the Pacific Islands. *Kew Bulletin* 37: 577-590.

Dohling, S., Kumaria, S. Tandon, P. 2012. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. *AoB Plants* 2012: pls032.

Franceschi, V. R., Nakata, P.A. 2005. Calcium oxalate in plants: formation and function. Annual Review of Plant Biology 56: 41-71.

Gopi, C., Vatsala, T.M., Ponmurugan, P. 2006. *In vitro* multiple shoot proliferation and plant regeneration of *Vanilla planifolia* Andr. - a commercial spicy orchid. Journal of Plant Biotechnology 8: 37-41.

Harrison, M. Den. Sec. Spatulata (syn. sect. Ceratobium). 2007. https://aos.org/orchids/orchids-a-to-z/letter-d/den-sec-spatulata.aspx. <access on 3 Feb. 2020>.

He, H., Qin, J., Cheng, X., Xu, K., Teng, L., Zhang, D. 2018. Effects of exogenous 6-BA and NAA on growth and contents of medicinal ingredient of *Phellodendron chinense* seedlings. *Saudi Journal of Biological Sciences* 25: 1189-1195.

Hossain, M.M. 2013. *In vitro* embryo morphogenesis and micropropagation of *Dendrobium aggregatum* Roxb. Plant Tissue Culture and Biotechnology 23: 241-249.

Javelle, M., Vernoud, V., Rogowsky, P.M., Ingram, G.C. 2011. Epidermis: the formation and functions of a fundamental plant tissue. *New Phytologist* 189: 17-39.

Jeong, M.J., Song, H.J., Park, D.J., Min, J.Y., Jo, J.S., Kim, B.M., Kim, H.G., Kim, Y.D., Kim, R.M., Karigar, C.S., Choi, M.S. 2009 High frequency plant regeneration following abnormal shoot organogenesis in the medicinal tree *Hovenia dulcis*. *Plant Cell, Tissue and Organ Culture*. 98:59–65.

Kabir, M.F., Rahman, M.S., Jamal, A., Rahman, M., Khalekuzzaman, M. 2013. Multiple shoot regeneration in *Dendrobium fimbriatum* Hook an ornamental orchid. *Journal of Animal and Plant Sciences* 23: 1140-1145.

Kehie, M., Kumaria, S., Tandon, P. 2012. *In vitro* plantlet regeneration from nodal segments and shoot tips of *Capsicum chinense* Jacq. cv. Naga King Chili. *3 Biotech* 2: 31-35.

Klenoticová, H., Smýkalová, I., Švábová, L., Griga, M. 2013. Resolving browning during the establishment of explant cultures in *Vicia faba* L. for genetic transformation. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis* 61: 1279-1288.

Mata-Rosas, M., Baltazar-García, R.J., Moon, P., Hietz, P., Luna-Monterrojo, V.E. 2010. *In vitro* regeneration of *Lycaste aromatica* (Graham ex Hook) Lindl. (Orchidaceae) from pseudobulb sections. *Plant Biotechnology Reports* 4(2): 157-163.

McHatton, R. Den. Sec. *Dendrobium*. 2020. https://aos.org/orchids/orchids-a-to-z/letter-d/den-sec-dendrobium.aspx. <access on 3 Feb. 2020>.

Meilasari, D. Iriawati, I. 2016. Regeneration of plantlets through PLB (Protocorm-Like Body) formation in *Phalaenopsis* 'Join Angle X Sogo Musadian'. *Journal of Mathematical and Fundamental Sciences* 48: 204-212.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Parthibhan, S., Rao, M.V., Kumar, T.S. 2015. *In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley – An imperiled orchid. *Journal of Genetic Engineering and Biotechnology* 13: 227-233.

Ramage, C.M., Williams, R.R. 2004. Cytokinin-induced abnormal shoot organogenesis is associated with elevated Knotted1-type homeobox gene expression in tobacco. *Plant Cell Reports* 22: 919-924.

Sato, E.M., Hijazi, H., Bennett, M.J., Vissenberg, K., Swarup, R. 2015. New insights into root gravitropic signalling. *Journal of Experimental Botany* 66: 2155-2165.

Seidenfaden, G., Wood, J.J., Holttum, R.E. 1992. *The orchids of peninsular Malaysia and Singapore*. Olsen & Olsen, Fredensborg, Denmark. p. 779.

Sharma, U., Rao, V.R., Mohan, J.S.S. and Reddy, A.S., 2007. *In vitro* propagation of *Dendrobium microbulbon* A. Rich - A rare ethnomedicinal herb. *IJBT* 6: 381-384

Shiau, Y.J., Nalawade, S.M., Hsia, C.N., Mulabagal, V., Tsay, H.S. 2005. *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex Lindl., from axenic nodal segments. *In Vitro Cellular & Developmental Biology-Plant* 41: 666-670.

Sunitibala, H., Kishor, R. 2009. Micropropagation of *Dendrobium transparens* L. from axenic pseudobulb segments. *Indian Journal of Biotechnology* 8: 448-452.

Tandon, P., Kumaria, S. Choudhury, H. 2007. Plantlet regeneration of *Pinus kesiya* Royle ex Gord, from mature embryos. *Indian Journal Biotechnology* 6: 262-266.

Teixeira da Silva, J.A., Cardoso, J.C., Dobránszki, J., Zhang, S. 2015. *Dendrobium* micropropagation: a review. *Plant Cell Reports* 34: 671-704.

Vacin, E.F., Went, F.W. 1949. Use of tomato juice in the asymbiotic germination of orchid seeds. *Botanical Gazette* 111: 174–183.

Wang P, Hawkins, T.J., Richardson, C., Cummins, I., Deeks, M.J., Sparkes, I., Hawes, C., Hussey, P.J. 2014. The plant cytoskeleton, NET3C, and VAP27 mediate the link between the plasma membrane and endoplasmic reticulum. *Current Biology* 24: 1397–1405.

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